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2nd Edition

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ECOPHYSIOLOGICAL ANALYSIS OF VULNERABILITY TO CLIMATE WARMING IN ECTOTHERMS,

2nd Edition

Topic Editors:
Lin Zhang, Hubei University of Chinese Medicine, China
Buddhi Dayananda, The University of Queensland, Australia
Bao-jun Sun, Chinese Academy of Sciences (CAS), China
Jigang Xia, Chongqing Normal University, China

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EDITED AND REVIEWED BY
Jonathon H. Stillman,
San Francisco State University,
United States

*CORRESPONDENCE
Lin Zhang
lzhangss@msn.com
Buddhi Dayananda
b.dayananda@uq.edu.au
Ji-Gang Xia
jigangxia@163.com
Bao-Jun Sun
sunbaojun@ioz.ac.cn

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Editorial: Ecophysiological analysis of vulnerability to climate warming in ectotherms

Lin Zhang[1]*, Buddhi Dayananda[2]*, Ji-Gang Xia[3]* and
Bao-Jun Sun[4]*

[1]School of Basic Medical Sciences, Hubei University of Chinese Medicine, Wuhan, China, [2]School of Agriculture and Food Sciences, The University of Queensland, Brisbane, QLD, Australia, [3]Laboratory of Evolutionary Physiology and Behavior, Chongqing Key Laboratory of Animal Biology, College of Life Sciences, Chongqing Normal University, Chongqing, China, [4]Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

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Editorial on the Research Topic
Ecophysiological analysis of vulnerability to climate warming in ectotherms

Identifying the species most vulnerable to extinction due to climate warming is the first step in their conservation and mitigating the impacts of climate change (Riddell et al., 2021; Song et al., 2021). However, organismal vulnerability to climate warming depends on the sensitivity of the organism to environmental changes, its exposure to those changes, its ability to recover from them, and its potential to adapt to the changes (Huey et al., 2012; van Heerwaarden and Sgro, 2021). The complexity of organismal response to temperature change makes predicting the effects of climate warming a great challenge for ecologists. Developing a deeper knowledge of the vulnerability of ectotherms to climate warming enhances our understanding of extinction processes, thereby aiding conservation efforts through the implementation of better policies and management strategies to prevent the extinction of remaining populations (Dayananda et al., 2016). The main objective of this interdisciplinary Research Topic is to collate research on how ectotherms respond to climate warming at various levels. This topic comprises investigations conducted at multiple research scales from meta-analyses to molecular determination, and focuses on insects, amphibians, and reptiles, combining some novel ecophysiological evidence with the considerations for evaluating the vulnerabilities of ectotherms to climate change.

Changes in environmental temperature can alter the body temperature of ectotherms and thus their physiological performance (Huey et al., 2012; Seebacher et al., 2015). Ectotherms have been found to shift their geographic range to higher latitudes or altitudes in response to climate change (Jacobsen, 2020). Body temperatures above the thermal optimum create physiological stress, reduced performance, and increased disease susceptibility, ultimately leading to population declines and extinction (Buckley et al., 2021).

The magnitude of the effects of climate warming on ectotherms depends on their physiological and/or behavioral plasticity (Paaijmans et al., 2013; Dayananda et al., 2016) as well as their evolutionary adaptations, which enhance the fitness of an organism and whose current beneficial characteristics reflect the selective advantage of the trait at the time of its origin (Hochachka and Somero, 2002). Most ectotherms can precisely control their body temperature *via* behavioral and postural adjustments; thus, plasticity is a significant factor in ectotherms' response to fluctuating environmental conditions (Huey et al., 2003). Phenotypic plasticity can occur faster than evolutionary genetic changes in ectotherms and is therefore likely to directly influence their responses to climate change (Chevin et al., 2010). However, if plasticity is inadequate in response to climate warming, or if the adaptive change is too slow, ectotherms face a greater risk of extinction (Lafuente and Beldade, 2019; Logan and Cox, 2020). Furthermore, species with a low capacity for plasticity are expected to be vulnerable to climate warming (Rohr et al., 2018; Norin and Metcalfe, 2019), particularly tropical ectotherms (Morley et al., 2019) which already live close to their physiological thermal optima (Deutsch et al., 2008).

The effects of increasing temperature on ectotherms occur on multiple dimensions, across life-history stages, and temporal and spatial clues (Dayananda et al., 2016). Incubation temperature strongly influences the development of ectotherms with important consequences for hatchling fitness (Noble et al., 2018). In oviparous species, the thermal environment for embryonic development depends on the location and depth of the nest, however, in viviparous species, the thermal environment for embryonic development depends on the mother's body temperature (Zhang et al., 2018). Incubation experiments suggest that incubation temperature affects the incubation length, embryo survival, size, shape, behavior, sex, and performance of hatchlings (Angilletta, 2009). Thus, assessing the oviparous embryonic responses to ongoing climate warming and understanding their effects will provide crucial information that can aid in their conservation (Mitchell et al., 2016; Sun et al., 2021).

The oviparous incubation process was interactively modulated by the warming and precipitation. For example, high soil temperature and low soil moisture had a significant negative effect on egg development, survival, and egg hatching of three dominant grasshopper species (*Dasyhippus barbipes*, *Oedaleus asiaticus*, and *Chorthippus fallax*) in the Inner Mongolian grasslands (Wu et al.).

Ectotherms are sensitive to seasonal variations in environmental temperature (Taylor et al., 2020; Liu et al., 2022). In particular, tropical ectotherms already live in temperatures close to their optimum physiological levels (Deutsch et al., 2008). Body temperatures higher than the optimum create physiological stress, reduce performance, and increase disease susceptibility, ultimately leading to population declines and extinction (Huey et al., 2010; Sinervo et al., 2010). For instance, reptiles in Sri Lanka are highly vulnerable to extinction due to the severity of anthropogenic disturbances, however, no research has been conducted thus far to assess how they are affected by climate warming (Dayananda et al.). Furthermore, climate change could have strong potential effects on amphibians in China. A recent analysis found that 54 species are moderately vulnerable, including *Echinotriton chinhaiensis* and *Hynobius chinensis*, and 14 species are highly vulnerable, including *Ichthyophis kohtaoensis* and *Zhangixalus prasinatus* (Zhao et al.).

Climate warming has increased the frequency, duration, and intensity of heat waves during summer, causing greater impacts on species than increased average temperatures (Breitenbach et al., 2020). The physiological and biochemical responses of ectotherms to heat waves remain poorly understood. However, a recent study on the oxidant physiology of ectotherms after exposure to a simulated heat wave found heat waves did not lead to oxidative damage in ectotherms with low metabolic rates. For example, *Mauremys mutica* (Li et al.) and *Nanorana pleskei* (He et al.) did not suffer any oxidative damage after exposure to heat waves. However, the physiological responses to heat waves differed between the two species.

Lizards from medium and high latitudes could respond to thermal variation through multiple levels of metabolic acclimation, whereas their congeners from low latitudes lacked any level of modification and are thus more vulnerable to global warming (Sun et al., 2022). Embryonic and hatchling development can be improved by moderate warming in *Lacerta agilis* from the low-latitude margin population of a high-altitude species (Cui et al.), and moderate warming benefits hatchling fitness in the cold-climate lizard, *Takydromus amurensis* (Liu et al., 2022). In tropical regions, species with limited dispersal abilities, small geographic ranges, and restrictions to high altitudes are particularly vulnerable to extinction (Huey et al., 2012). However, the thermal biology traits of a tropical lizard (*Takydromus kuehnei*) are not severely threatened by ongoing climate change, highlighting the importance of thermal biology traits in evaluating the vulnerability of a species to climate change (Tao et al.). Furthermore, a study on Asiatic toads (*Bufo gargarizans*) from two altitudinal zones found that low-altitude toads might enhance their hypothermic reaction if they shift their ranges to higher altitudes to survive the warming climate (Yao et al.). Two low-altitude lizards (the oviparous *Phrynocephalus axillaris* and the viviparous *P. forsythii*) may live at high altitudes by reducing behavioral activity and increasing energy efficiency (Qi et al.). Taken together, more studies are requited to understand how the species with limited dispersal abilities, small geographic ranges, and restriction to high altitudes shift their phenotypic plasticity to adapt to climate warming.

In the future, investigations of species' vulnerability to climate warming are likely to benefit from measurements of environmental conditions, taken at the scale experienced by the organisms (Williams et al., 2008). Moreover, a comparison of intra- and inter- species vulnerability provides a variety of adaptive strategies for global warming (Huey et al., 2009). However, predicting the effects of climate warming on species is extraordinarily difficult owing to the complex nature of ecosystems; thus, this remains a major challenge for ecologists.

Author contributions

LZ wrote the first draft. BD and B-JS edited the manuscript. J-GX advised the other authors. All authors reviewed and approved the manuscript.

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ORIGINAL RESEARCH
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Effects of Simulated Heat Wave on Oxidative Physiology and Immunity in Asian Yellow Pond Turtle (*Mauremys mutica*)

Shuran Li, Jinhuan Li, Wei Chen, Zhiwang Xu, Lei Xie and Yongpu Zhang*

College of Life and Environmental Science, Wenzhou University, Wenzhou, China

Global warming has led to an increase in the frequency, duration, and intensity of heat waves in the summer, which can cause frequent and acute heat stress on ectotherms. Thus, determining how ectothermic animals respond to heat waves has been attracting growing interest among ecologists. However, the physiological and biochemical responses to heat waves in reptiles, especially aquatic reptiles, are still poorly understood. The current study investigated the oxidant physiology, immunity, and expression levels of heat shock proteins (HSP) mRNA after exposure to a simulated heat wave (1 week, 35 ± 4°C), followed by a recovery period (1 week, 28 ± 4°C) in juvenile Asian yellow pond turtle (*Mauremys mutica*), a widely farmed aquatic turtle in East Asia. The contents of malondialdehyde (MDA) in the liver and muscle were not significantly affected by the heat wave or recovery. Of all antioxidant enzymes, only the activity of glutathione peroxidase (GSH-Px) in muscles increased after heat wave, while the total superoxide dismutase (T-SOD), catalase activity (CAT), and total antioxidant capacity (T-AOC) did not change during the study. The organo-somatic index for the liver and spleen of *M. mutica* decreased after the heat wave but increased to the initial level after recovery. In contrast, plasma lysozyme activity and serum complement C4 levels increased after the heat wave, returning to the control level after recovery. In addition, heat waves did not alter the relative expression of HSP60, HSP70, and HSP90 mRNA in the liver. Eventually, heat wave slightly increased the IBR/n index. Therefore, our results suggested that heat waves did not lead to oxidative damage to lipids in *M. mutica*, but deleteriously affected the turtles' immune organs. Meanwhile, the constitutive levels of most antioxidative enzyme activities, HSPs and enhanced blood immune functions might protect the turtles from the threat of heat waves under the current climate scenarios.

Keywords: climate warming, heat wave, turtle, oxidative stress, immune function, heat shock proteins

Edited by:
Lin Zhang,
Hubei University of Chinese Medicine,
China

Reviewed by:
Wenyi Zhang,
Nanjing Normal University, China
Carolina Madeira,
New University of Lisbon, Portugal

****Correspondence:***
Yongpu Zhang
zhangyp@wzu.edu.cn

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INTRODUCTION

The global average air temperature has increased by approximately 0.85°C over the past century and may increase by a further 0.3–4.8°C by the end of this century (IPCC, 2014). Such climate change has dramatically affected fauna, causing problems, such as advances in phenology, shifts in distribution, and increased risk of species extinction (Sinervo et al., 2010;

Hoegh-Guldberg et al., 2019). In addition to the mean temperature, the impacts of climate warming on animals are also associated with the increasing frequency, duration, and intensity of extreme temperature events, such as summer heat waves (Bozinovic et al., 2011; IPCC, 2014). Indeed, there is increasing awareness on heat wave events that may pose greater threats to animals than gradual warming (Thompson et al., 2013; Vasseur et al., 2014; Pansch et al., 2018), as heat waves can induce more severe impacts on animals than increasing mean temperatures, including on morphology, physiology, reproduction and even survival (Bauerfeind and Fischer, 2014; Zhang et al., 2018; Breitenbach et al., 2020). Therefore, investigating how animals respond to heat wave events is essential to enrich our understanding of the vulnerability of animals to climate warming.

For ectotherms, thermal environments can strongly influence their biological processes, including behavior, metabolic rates, growth, and reproduction (Angiletta, 2009; Adamo and Lovett, 2011; Goessling et al., 2019), as their body temperatures are highly dependent on the ambient temperature. The body temperatures of ectotherms usually fluctuate to some extent during the day or season, under natural conditions (Christian and Weavers, 1996; Angiletta, 2009). Thus, ectotherms are well adapted to daily and seasonal temperature variations. Nevertheless, dramatic temperature variation would have a negative impact on ectotherms at several levels, including molecular, biochemical, and physiological (Stahlschmidt et al., 2017; Ferreira-Rodríguez et al., 2018; Gao et al., 2021). For example, acute thermal stress can stimulate the formation of reactive oxygen species (ROS) and in turn induce oxidative damage in ectotherms (Ben Ameur et al., 2012; Zhang et al., 2019). To clear ROS and maintain the oxidative balance, antioxidant enzyme systems, including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT), are activated (Zhang et al., 2019; Baker et al., 2020). Additionally, immune functions in ectotherms are sensitive to acute temperature changes (Goessling et al., 2019; Gao et al., 2021). For example, innate immunity of common musk turtles (*Sternotherus odoratus*) compensated for new temperature when exposed to a 5 or 10°C temperature change for 48 h (Goessling et al., 2019). Moreover, acute heat exposure increases the expression of heat shock proteins (HSPs), which act as molecular chaperones to protect cells from heat damage and enhance thermal resistance in many ectotherms, including (Zhao and Jones, 2012), fish (Narum et al., 2013), and reptiles (Tedeschi et al., 2015).

Increasingly common heat waves may cause frequent and acute heat stress on ectotherms. However, many studies have exposed animals to different acute constant temperature heat waves, which unfortunately neglects the diurnal fluctuations in temperature under natural conditions (Dittmar et al., 2014; Leicht et al., 2019). This lack of ecological relevance may lead to misleading interpretations, as the performance of ectotherms is not always identical under constant and fluctuating thermal conditions (Bozinovic et al., 2011; Moore et al., 2021). Moreover, the biochemical and physiological responses to heat waves are still poorly understood in ectotherms, especially in non-avian reptiles, which are particularly vulnerable to climate warming (Bauerfeind and Fischer, 2014; Zhang et al., 2018). Only a few studies have detected how heat waves affect reptile biochemistry and physiology, and no consistent patterns have been identified. For example, a simulated heat wave reduced oxidative damage in the corn snake (*Pantherophis guttatus*) (Stahlschmidt et al., 2017) but did not affect antioxidant capacity in a desert lizard (*Phrynocephalus przewalskii*) (Zhang et al., 2018); heat waves reduced immunity in rattlesnakes (*Crotalus durissus*) (Fabrício-Neto et al., 2019) but not in *P. guttatus* (Stahlschmidt et al., 2017).

The Asian yellow pond turtle (*Mauremys mutica*) is an aquatic turtle distributed in China, Japan, and Vietnam. It has been classified as "endangered" on the IUCN red list but is commonly cultured in southern China due to its high economic value as food, pets, and in traditional Chinese medicine (Lu et al., 2020; Wei et al., 2020). Recent studies have shown that extreme heat stress could downregulate the expression of immunity-associated genes in *M. mutica*, which may be the main reason underlying the increased morbidity and mortality rates associated with extreme heat events in turtle farms (Wei et al., 2020; Gao et al., 2021). However, like other turtles, the response of *M. mutica* to heat waves has not yet been investigated. In the current study, we conducted an experiment in *M. mutica* to reveal the effects of heat waves on its antioxidant responses, immune function, and gene expression. We exposed *M. mutica* to a simulated heat wave with subsequent recovery and measured malondialdehyde (MDA), total superoxide dismutase (T-SOD), GSH-Px, CAT activity, and total antioxidant capacity (T-AOC) in the liver and muscle. We also calculated the organo-somatic index (OSI) for the liver and spleen; quantified serum complement protein (C3, C4) levels, and lysozyme activity in the plasma; and expression of HSPs (HSP60, HSP70, HSP90) in the liver. At last, we used the integrated biomarker response (IBR) approach to evaluate the comprehensive effects of heat wave on *M. mutica*. We predicted that heat waves would induce oxidative stress and depress immune function in *M. mutica*, whereas antioxidant capacity and expression of HSPs would be enhanced to protect *M. mutica* from heat waves.

MATERIALS AND METHODS

Ethics Statement

The animal study was reviewed and approved by Animal Ethical and Welfare Committee of Wenzhou University (Approval No. WZU-049).

Experimental Procedure

In late July 2018, 30 yearling *M. mutica* (carapace length: 53.0 ± 0.6 mm; body weight: 30.4 ± 1.1 g; **Supplementary Figure 1**) were purchased from a turtle farm in Jiaxing, Zhejiang province (120.89°E, 30.77°N). Turtles were individually raised in plastic boxes (length* width*height = 35 cm*25 cm*10 cm) with approximately 5-cm-depth water and housed in an incubator (KB400, Binder GmbH, Tuttlingen, Germany) under a fluctuating temperature of 28 ± 4℃ for 1 week (set at a ten-step programmed ramp temperatures, see details in **Supplementary Figure 2** and **Supplementary Table 1**). The acclimation temperature simulated the average fluctuation range of daily air temperatures in Jiaxing in July and August (1981–2010; data were obtained from "China Meteorological Data

Service Center")[1]. Turtles were provided a 13 L:11D cycle by eight fluorescent lamps in the incubator (light on between 6:00 and 19:00) and fed a commercial diet at 10:00 every day. The water was changed every day when the light was turned off.

After 1-week acclimation, ten randomly selected turtles were left in the incubator and defined as the "control group" (*n* = 10), while the other 20 turtles were assigned to another incubator around 7:00 and were raised as described above, except for exposure to a simulated heat wave (35 ± 4℃, the average temperatures in Jiaxing when heat waves occurred, from July to August, 2011–2017, data were obtained from "https://lishi.tianqi.com"; set at a ten-step programmed ramp temperatures, see details in **Supplementary Figure 2** and **Supplementary Table 1**). One week later, we sampled all the ten turtles from the control group, and randomly selected ten turtles which had been exposed to heat waves (heat wave group, *n* = 10) between 7:00 and 9:00 (**Supplementary Figure 2**). The remaining ten turtles were transferred back to 28 ± 4°C and also sampled between 7:00 and 9:00 after 1 week recovery (recovery group, *n* = 10; **Supplementary Figure 2**). Thus, the ten turtles from the recovery group experienced 1 week heat wave exposure and 1 week recovery. Overall, the thermal regime of the control group covered the set-point temperature range of *M. mutica* (25–29°C, unpublished data), and the thermal peak during the heat wave was approximately 2°C below the critical thermal maximum of *M. mutica* (approximately 41°C, according to Lu et al., 2020).

When sampled, turtles were immediately sacrificed with decapitation followed by pithing using heavy shears according to AVMA Guideline for the Euthanasia of Animals (2013 Edition), to separate the spleen, liver, and skeletal muscle on ice. Fresh liver and spleen were blotted free of blood, weighed (ML204, Mettler Toledo, Switzerland, ± 0.0001 g), and frozen in liquid nitrogen. Blood was collected from the carotid artery immediately and centrifuged at 3,000 rpm for 15 min (Fresco 21, Thermo Fisher Scientific, Waltham, MA, United States) to separate serum and plasma. All samples were stored in a –80°C refrigerator (CryoCube F570, Eppendorf AG, Barkhausenweg, Hamburg, Germany) before analysis.

Antioxidant Indices

Commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used to detect MDA content, T-AOC, and antioxidant enzyme activity (CAT, GSH-Px, and SOD) in the liver and muscle following the manufacturer's instructions. MDA content was evaluated by measuring the absorbance of a red compound produced by thiobarbituric acid and malondialdehyde at 532 nm. T-AOC was measured based on the absorbance of the Fe^{2+}-o-phenanthroline complex produced by o-phenanthroline reacted with Fe^{2+}, from which Fe^{3+} was reduced by the reducing agents at 520 nm. CAT activity was estimated by measuring H_2O_2 consumption at 405 nm. GSH-Px activity was examined based on the GSH consumption rate at 412 nm during the enzymatic reaction of GSH-Px, which catalyzes dithiodinitrobenzoic acid. SOD activity was determined by monitoring the rate of cytochrome c reduction by superoxide ions at 550 nm in the xanthine-xanthine oxidase system.

Immunity Indices

The organo-somatic index (OSI) for the liver and spleen were calculated using the following formula:

OSI = organ weight (g)/body weight (g) × 100

Enzyme-linked immunosorbent assay (ELISA) kits (Shanghai MLBIO Biotechnology Co., LTD, Shanghai, China) were used to measure serum C3 and C4 levels, and plasma lysozyme activity according to the manufacturer's instructions. Samples were combined with anti-fish horseradish peroxidase-linked antibody, and the color was developed using 3,3′,5,5′-tetramethylbenzidine after complete washing. The reaction was terminated by adding a sulfuric acid solution, and absorbance was measured at 450 nm.

Relative Expression of HSPs

Total RNA was extracted from the livers of four randomly selected individuals from each group using the *TransZol* Up Plus RNA kit (TransGen Biotech Co., Ltd., Beijing, China). RNA quality was evaluated by measuring the absorbance ratio at 260/280 nm using an ultraviolet spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, United States). The acceptable range was 1.8–2.1. cDNA was generated from the extracted RNA using a 2 × *EasyTaq*® PCR SuperMix kit (TransGen Biotech Co., Ltd., Beijing, China), according to the manufacturer's instructions.

Real-time quantitative PCR was performed in duplicate for each sample to determine the relative expression of HSP60, HSP70, and HSP90 on a LightCycler 480 II (Roche Diagnostics Ltd., Forrentrasse, Switzerland), using the β-actin gene as an endogenous reference. All primers for RT-PCR (**Table 1**) were designed using Primer Premier 5.0 (PREMIER Biosoft International, United States). The reactions were carried out in a 20-μL reaction mixture containing 10 μL of *TransStart* Top Green qPCR SuperMix (TransGen Biotech Co., Ltd., Beijing, China), reverse-transcribed cDNA templates, and 0.4 μL of each primer. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Integrated Biomarker Response Indices

To combine all the measured biomarkers and evaluate the general responses, integrated biomarker response (IBR) index was calculated following the method of Beliaeff and Burgeot (2002) and modified by Marigómez et al. (2013): (1) calculation of mean (x) and standard deviation (s) for each biomarker from all the groups; (2) standardization of data for each biomarker: $x_i' = (x_i\text{-}x)/s$, where x_i' is the standardized value of the biomarker,

TABLE 1 | The forward and reverse primer sequences for each gene.

Gene	Forward primer	Reverse primer
*hsp*60	AACATCTAGTGCTGGGATGC	ACTGATGCACTGAATGCTACT
*hsp*70	TGCTGCTATTGCTTATGG	CGGTTATCAAAGTCCTCC
*hsp*90	CAGAGGTTCCCCATTATCA	TCAGTTTGGTGTTGGCTTC
β-actin	CCCCAAAGCCAACAGAGA	ACGCCATCACCAGAGTCCA

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[1] http://data.cma.cn

x_i is the mean value of a biomarker from each group; (3) $Z = x_i'$ or $-x_i'$ in the case of biomarker responding to heat wave by activation or inhibition, then the minimum standardized value (x_{min}') for all groups for each biomarker was obtained, and the absolute value of x_{min}' was added to Z, $y_i = Z + | x_{min}'|$; (4) y_i was plotted on a star plot to display biomarker results, indicating the degree of biological effects for each biomarker in response to different thermal conditions; (5) star plot area connecting the ith and the $(i + 1)$th radius coordinates (A_i) was computed as $A_i = [(y_i \times y_{i+1} \times \sin(2\pi/n))]/2$, where y_i and y_{i+1} represent individual standardized values of each biomarker and its next biomarker in the star plot, respectively, and n is the number of biomarkers; (6) IBR = ΣAi as IBR was the total area for each group, and finally IBR/n was presented in the results considering IBR was depend on the number of biomarkers.

Statistical Analysis

Data were checked for normality and homogeneity of variance using the Shapiro-Wilk and Levene's tests, respectively. One-way ANOVA was used to test the differences between groups, followed by the *post hoc* Tukey's HSD test. The Kruskal-Wallis test followed by Mann-Whitney U *post hoc* test was used when the assumptions of one-way ANOVA were violated. Data are expressed as the mean ± SE. The significance level was set to $\alpha = 0.05$. All analyses were performed with Statistica 6.0, and all graphs were drawn using SigmaPlot 12.5, except that calculation of IBR and star plot were performed by Excel (Microsoft 365).

RESULTS

Effects of Heat Wave on Oxidative Stress Indices

MDA content in the liver and muscles was not significantly affected by heat waves, although MDA content tended to increase by more than 50% in the livers of turtles exposed to heat waves (**Figure 1** and **Table 2**).

TABLE 2 | Statistical results of the effects of heat wave on oxidative stress indices in liver and muscle of *Mauremys mutica*.

Variables	Liver	Muscle
MDA content	$H = 3.022$, $P = 0.221$	$F_{(2,\ 27)} = 0.092$, $P = 0.913$
TAC	$F_{(2,27)} = 0.237$, $P = 0.790$	$F_{(2,27)} = 2.956$, $P = 0.069$
CAT	$F_{(2,27)} = 0.044$, $P = 0.957$	$F_{(2,27)} = 0.065$, $P = 0.937$
GSH	$H = 1.912$, $P = 0.384$	$F_{(2,27)} = 3.459$, $\mathbf{P = 0.046}$
SOD	$F_{(2,27)} = 0.979$, $P = 0.389$	$F_{(2,27)} = 0.076$, $P = 0.927$

P-value in bold indicates significant difference.

For all antioxidant components, heat waves only significantly influenced the activity of GSH-Px in the muscle (**Table 2**), which increased after exposure for 1 week to the heat wave and decreased after 1 week of recovery (**Figures 2A–D**).

Effects of Heat Wave on Immune Indices

Heat wave treatment significantly reduced the hepatosomatic index [$F_{(2,\ 27)} = 3.217$, $P = 0.056$] and spleen somatic index [$F_{(2,27)} = 3.825$, $\mathbf{P = 0.034}$] but both organo-somatic indices slightly increased after 1-week recovery (**Figure 3**).

C3 levels did not significantly change during the experiment (C3: $H = 5.544$, $P = 0.063$, **Figure 4B**), while the lysozyme activity and C4 level increased significantly after the heat wave and decreased to the control level after 1 week of recovery [lysozyme: $F_{(2,27)} = 11.593$, $\mathbf{P < 0.001}$, **Figure 4A**; C4: $F_{(2,27)} = 11.137$, $\mathbf{P < 0.001}$, **Figure 4C**].

Effects of Heat Wave on the Relative Expression of HSPs in Liver

Heat waves did not alter the relative expression levels of HSP60, HSP70, or HSP90 mRNA in the liver of *M. mutica* [HSP60: $F_{(2,\ 9)} = 0.04$, $P = 0.959$; HSP70:

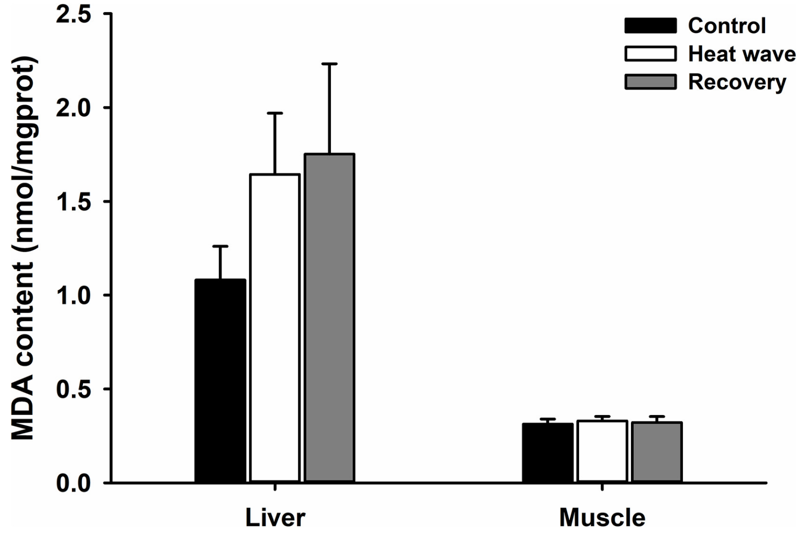

FIGURE 1 | Effects of heat wave on MDA content in the liver and muscle of *Mauremys mutica.* Data were represented as mean ± standard error (SE).

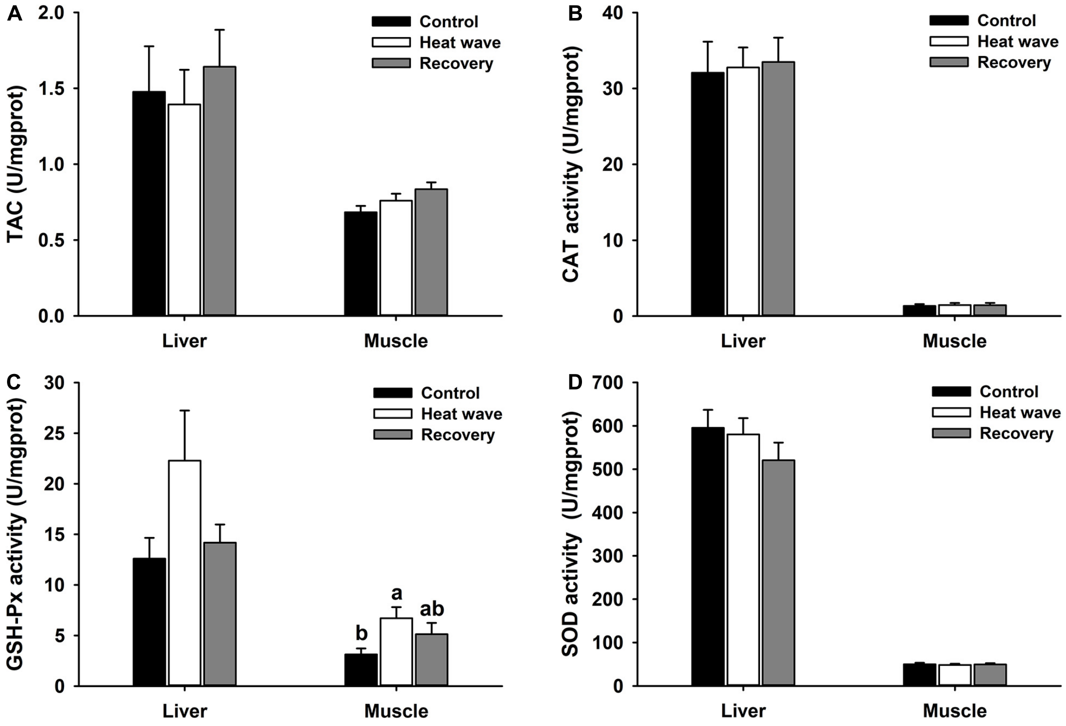

FIGURE 2 | Effects of heat wave on TAC **(A)**, CAT activity **(B)**, GSH-Px activity **(C)**, and SOD activity **(D)** in the liver and muscle of *Mauremys mutica*. Data were represented as mean ± standard error (SE).

$F_{(2,\ 9)} = 0.115$, $P = 0.989$; HSP90: $F_{(2,\ 9)} = 0.108$, $P = 0.898$; **Figure 5**].

Integrated Biomarker Response Indices

All the 18 measured biomarkers were represented in star plot (**Figure 6A**). Most sensitive biomarkers to heat wave were GSH-Px in liver (GSH-Pxl), GSH-Px in muscle (GSH-Pxm), spleen somatic index (SSI), lysozyme activity (Lys), C4 level, HSP60 and HSP90, concerning the magnitude of differences between thermal treatments. The IBR/n index was marginally higher in heat wave group, comparing with control and recovery groups (**Figure 6B**).

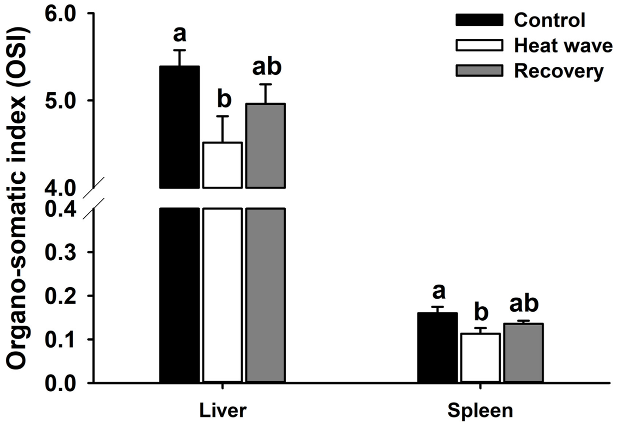

FIGURE 3 | Effects of heat wave on organo-somatic index of *Mauremys mutica*. Data were represented as mean ± standard error (SE).

DISCUSSION

The current study demonstrated that the effects of a simulated heat wave on the biochemistry and physiology of the aquatic turtle *M. mutica* were complex and transient. After exposure to a 1-week heat wave, no significant oxidative damage was found, and only GSH-Px activity of the antioxidant indices was enhanced in *M. mutica*. Heat wave exposure deleteriously affected the liver and spleen, but increased the blood lysozyme activity and C4 level of the turtle. Moreover, heat waves did not affect the relative expression of HSPs in the turtle liver. A slight increasing in IBR/n value was induced by heat wave. Following a 1-week recovery period, the effects of heat waves disappeared, indicating that the protection mechanism was effective in *M. mutica*.

Effects of Heat Wave on Oxidative Stress in *Mauremys mutica*

As the end product of lipid peroxidation induced by reactive oxygen species (ROS), MDA content indirectly reflects the degree of oxidative damage to cell membrane lipids—especially phospholipids, polyunsaturated fatty acids (PUFA), glycolipids and cholesterol (McCay et al., 1976; Parrish, 2013; Ayala et al., 2014). In the present study, MDA content did not significantly

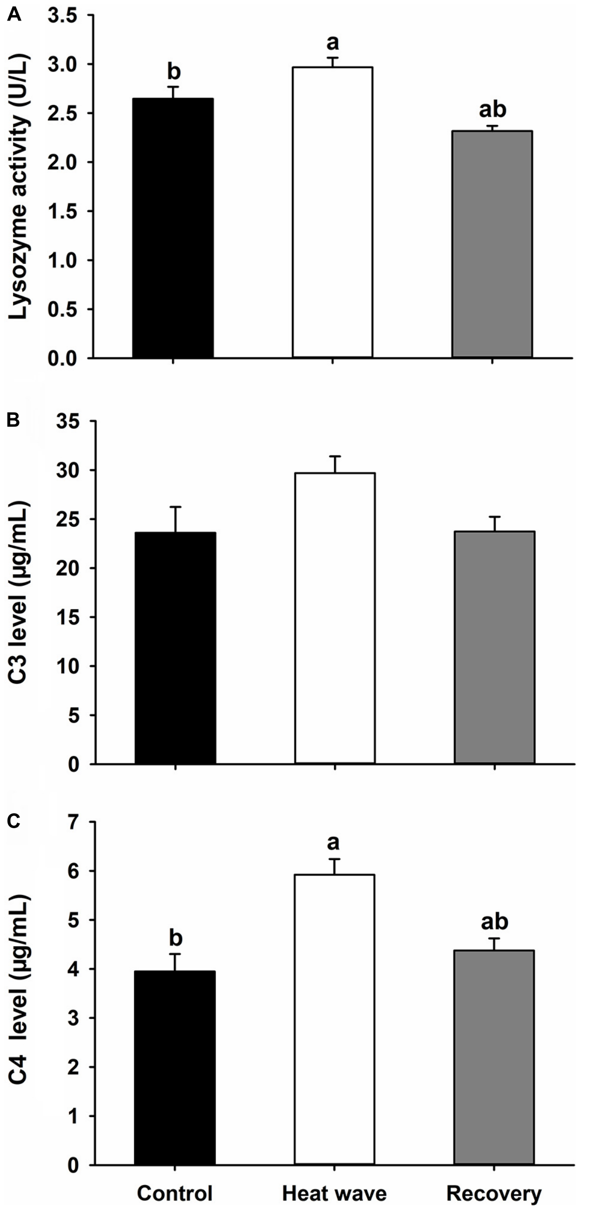

FIGURE 4 | Effects of heat wave on the lysozyme activity **(A)**, C3 level **(B)**, and C4 level **(C)** of *Mauremys mutica.* Data were represented as mean ± standard error (SE).

increase in the liver or muscle of turtles after the heat wave and recovery, indicating that heat waves did not induce visible adverse effects on the lipids in the tissues of *M. mutica*. A similar pattern was reported in the Chinese soft-shell turtle (*Pelodiscus sinensis*) after exposure to acute heat stress and recovery (Zhang et al., 2019) and in two marine gastropods (*Nerita atramentosa* and *Austrocochlea concamerata*) during a 1-week heat wave exposure experiment (Leung et al., 2019). However, increased MDA content induced by heat waves has been observed in other ectotherms, such as a desert lizard (*Eremias multiocellata*) (Han et al., 2020) and marine gastropod (*Austrocochlea constricta*) (Leung et al., 2019). These conflicting results indicate that heat waves have complex effects on the oxidative physiology in ectotherms. Otherwise, *M. mutica* may be somewhat resistant to lipid peroxidation, like some long-living marine mollusks which are usually able to acclimate to new temperatures and/or maintain homeostasis upon exposure to changing temperatures (Munro and Blier, 2012).

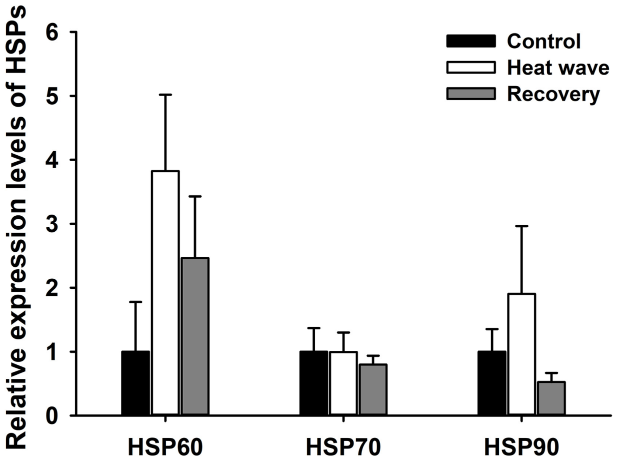

FIGURE 5 | Effect of heat wave on the relative expression levels of HSP60, HSP70 and HSP90 mRNA in liver of *Mauremys mutica.* Data were represented as mean ± standard error (SE).

In the present study, heat waves did not affect T-SOD activity, CAT activity, or T-AOC in *M. mutica* but did elevate GSH-Px activity in the muscle of *M. mutica*. One possible explanation for this is that GSH-Px activity is more sensitive to thermal fluctuations than SOD and CAT. Similarly, a simulated heat wave did not influence SOD activity in the desert lizard *P. przewalskii* (Zhang et al., 2018). Acute heat stress had similar effects on the antioxidant enzymes in ectotherms. For example, in the Chinese soft-shell turtle, acute heat shock only altered GSH-Px activity and had no impact on the enzymatic activity of CAT and SOD (Zhang et al., 2019), whereas the activity of all three enzymes in a marine fish (*Cyprinodon variegatus*) did not change after acute heat exposure (Baker et al., 2020). Furthermore, the response of antioxidant enzymes to temperature change varied among tissues; it has previously been observed in the Chinese soft-shell turtle that GSH-Px activity increased in the kidney but decreased in the liver (Zhang et al., 2019). In the marine fish *Sparus aurata*, SOD activity increased in the liver but decreased in the brain after heat stress (Madeira et al., 2016). Previous studies showed that GSH-Px could prevent lipid peroxidation in cell membranes (McCay et al., 1976; Marinho et al., 1997). Hence, the enhanced GSH-Px activity may be one possible reason for the unchanged MDA content after heat wave exposure in *M. mutica*.

Effects of Heat Wave on Immunity of *Mauremys mutica*

In the current study, the hepatosomatic and spleen somatic indices decreased after a 1-week heat wave, implying potential

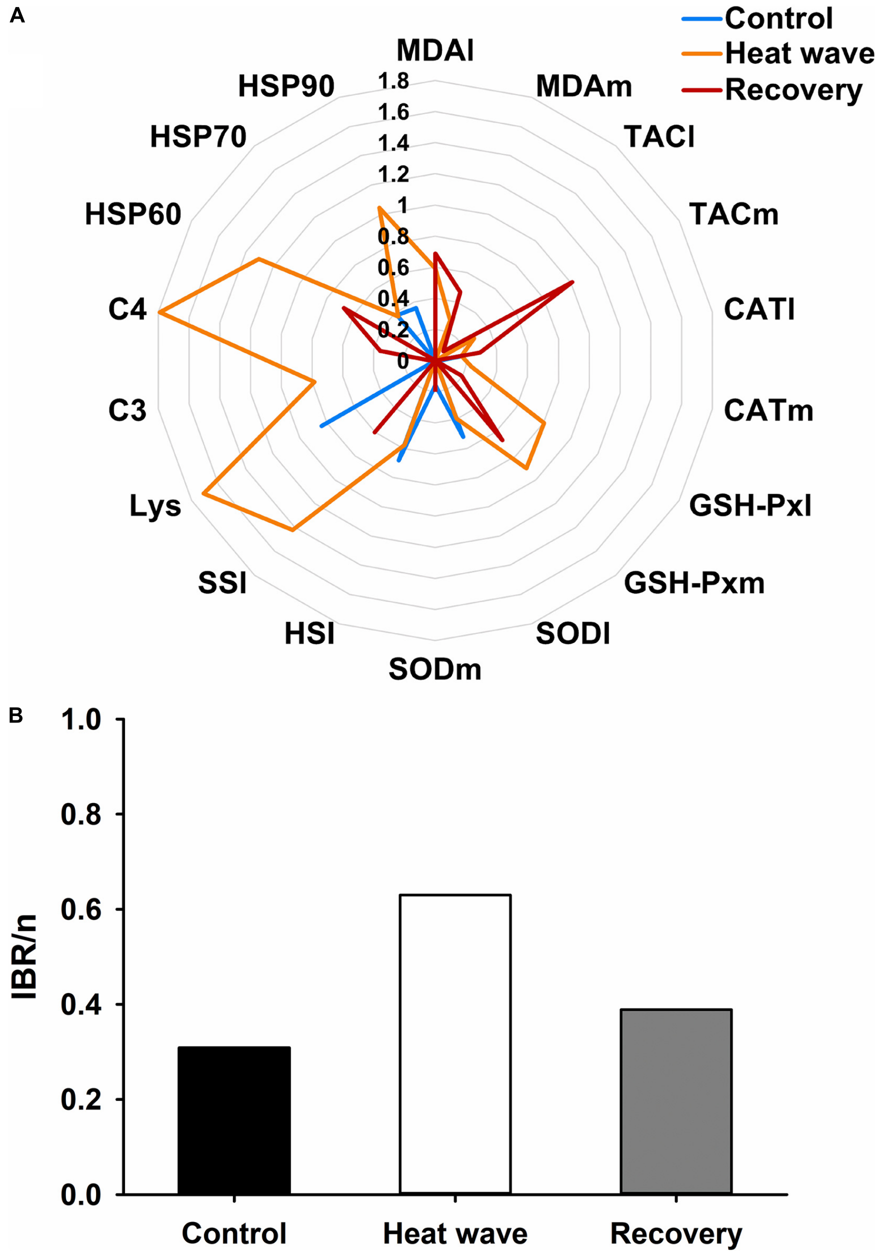

FIGURE 6 | Integrated biomarker response (IBR) star plot **(A)**, and IBR/n values **(B)** for Mauremys mutica after thermal treatments. MDAl, MDA in liver; MDAm, MDA in muscle; TACl:TAC in liver; TACm:TAC in muscle; CATl, CAT in liver; CATm, CAT in muscle; SH-Pxl, GSH-Px in liver; GSH-Pxm, GSH-Px in muscle; SODl, SOD in liver; SODm, SOD in muscle; HIS, hepatosomatic somatic indices; SSI, spleen somatic indices; Lys, lysozyme.

damage in turtle immune organs to heat waves (**Figure 3**). The hepatosomatic index has also been widely used as a rough indicator of the overall health of aquatic animals (Ben Ameur et al., 2012; Blazer et al., 2018). Reduction of the hepatosomatic index may indicate poor health triggered by the stress of heat waves. This result contradicts a previous finding that repeated daily acute heat shock did not change the hepatosomatic index in cold-water fish (*Salvelinus namaycush*) (Guzzo et al., 2019). Considering the key role of the liver in metabolism, another possible explanation for the reduced hepatosomatic index is that heat waves may increase energy expenditure or reduce feeding rate in *M. mutica*; this has been recognized in two fiddler crab species raised under warmer conditions (Vianna et al., 2020). Also, the decline of hepatosomatic index may suggest heat wave caused an energetic trade off in the turtles, as most ectothermic animals switch from a physiological compensation strategy (i.e., allocation of energy to cellular stress responses, repair mechanisms and molecular adjustments) to a metabolic conservation strategy (i.e., blocking stress responses) (Marshall et al., 2011; Yao and Somero, 2013; Vianna et al., 2020). Thus, the lack of responses in antioxidant enzymes as described above may reflect an exhaustion of the cellular machinery has occurred and animals went into metabolic depression. Further research is needed to elucidate this phenomenon by detecting the response of energy metabolism and feeding behavior to heat waves, and determining the level of tissue damage that actually occurred through histopathological analyses in turtles, which was a possible limitation to our study. As the dominant secondary lymphoid organ, the spleen plays an important role in defending against infection by parasites, bacteria, and viruses in reptiles (Zimmerman et al., 2010). In the present study, heat waves decreased the relative size of the spleen and that may impair immunity in *M. mutica*, which could be a potential cause of severe disease infection in *M. mutica* facing frequent heat stress during late spring and early summer (Gao et al., 2021). Cooling strategies for turtles in the farms should be taken into account during heat wave events, such as providing more shelters or increasing water flow in the ponds for turtles. However, the relationship between disease outbreaks and the spleen somatic index remains unclear. The potential damage to the turtle liver and spleen was not permanent, as the two indices increased to control levels after 1-week recovery. But it was also unclear whether turtles' recovery ability would be permanently suppressed when facing recurrent heat wave stress or not.

Contrary to the immune organs, plasma lysozyme activity and serum complement levels in *M. mutica* increased after exposure to the simulated heat wave and decreased to control levels after 1 week of recovery, whereas C3 levels did not show significant changes (**Figure 4**). This discrepancy reflected the varied response of the turtle immune system to temperature changes between organ and biochemical levels. Lysozymes and the complement system are key components in the reptile innate immune system, which mainly functions to kill bacteria or digest pathogens (Zimmerman et al., 2010; Baker et al., 2019). Accordingly, the elevated lysozyme activity and complement levels may indicate enhanced bacteria killing ability of *M. mutica* when exposed to heat waves, which was not directly measured in the current study. This result challenged our hypothesis and was in disagreement with previous studies that reported that heat waves inhibited immune functions of ectothermic animals, including snakes (Stahlschmidt et al., 2017), lizard (Han et al., 2020), fish (Dittmar et al., 2014), and freshwater snail (Leicht et al., 2013). Nonetheless, the current findings are not rare. For example, a previous study showed that a simulated heat wave increased the activity of immune-related (phenoloxidase and lysozyme-like) enzymes and resistance to bacterial infection in a cricket (*Gryllus texensis*) (Adamo and Lovett, 2011). Additionally, high temperature (37°C) did not weaken the immune function of the red-eared slider turtle (*Trachemys scripta*), whose critical thermal maximum was 40–42°C (Zimmerman et al., 2017). Overall, the diverse immune response of ectotherms to temperature change may be due to the variation of treatments from different research, or the varying thermal sensitivity of immune indices among taxa (Stahlschmidt et al., 2017; Zimmerman et al., 2017).

Effects of Heat Wave on the Expression of HSPs in Liver of *Mauremys mutica*

Expression of heat shock proteins was found to be upregulated after acute or gradual heat exposure in many ectotherms, including turtles, protecting organisms from physiological damage caused by high temperatures (Tedeschi et al., 2015; Dang et al., 2018; Harada and Burton, 2019). Unexpectedly, in the present study, heat waves did not induce significant upregulation in the expression of *hsp* gene in the liver of *M. mutica*, even though the turtles experienced 37–39°C for nearly 5 h daily during the heat wave (**Supplementary Figure 2**). A similar phenomenon was also observed in a montane lizard (*Phrynosoma hernandesi*) exposed to acute heat stress at 36°C for 4 h (Refsnider et al., 2021) and in a skink after 24 h exposure to 35°C (*Plestiodon chinensis*) (Dang et al., 2018). One possible explanation was exhaustion of the cellular stress responses indicated by the decreased hepatosomatic index as described above. Another possible reason may be that the heat wave climax or the duration of heat stress was insufficient to activate high expression of HSPs, and high-temperature resistance of hatchling *M. mutica* would be elevated after warm acclimation (Lu et al., 2020). Future work to explore whether 1-week heat waves would enhance turtle tolerance to high temperatures is warranted. Contrary interpretation also makes sense, since basal HSPs expression levels may maintain constantly high in the turtle to protect them against heat stress during summer. This has been previously observed in intertidal ectotherms exposed to steep temperature changes (Madeira et al., 2012). Alternatively, the response of HSPs to heat stress varies among tissues. For example, the expression of HSP60 was upregulated in the embryonic brain of loggerhead sea turtles (*Caretta caretta*) but was not altered in the heart after exposure to 36°C for 3 h (Tedeschi et al., 2015). In addition, the sampling time may be another cause. In the current study, turtles were sampled around 7:00–9:00 on the eighth day, and thus had adapted to moderate temperatures overnight. Previous research found that HSP70 in the liver decayed faster during 1-h post-stress adaptation than in other tissues, such as the lung and muscle in the Chinese soft-shell turtle (Li et al., 2012). Consequently, the expression of HSPs in the liver may not be a suitable biomarker for evaluating the stress of heat waves in turtles. To obtain a more comprehensive understanding of molecular responses to heat waves in turtles, further studies could introduce transcriptome analysis, which has been increasingly used to uncover transcriptional responses to heat stress in reptiles (Bentley et al., 2017; Gao et al., 2021), and assess the temporal and spatial variation in gene expression under heat waves.

Effects of Heat Wave on the IBR Indices of *Mauremys mutica*

Combining the responses of multiple biomarkers, IBR provided a valid tool to assess ecological risk and animal health status in relation to environmental stressors, including chemical contaminants, evaluated CO_2 level and extreme temperatures (Beliaeff and Burgeot, 2002; Campos et al., 2019). In current work, seven biomarkers (GSH-Pxl, GSH-Pxm, SSI, Lys, C4, HSP60, and HSP90) seemed to be more sensitive to heat wave according to the star plot, which was consistent with the response patterns of these biomarkers to temperature treatments, even some did not alter significantly. Heat wave slightly increased the IBR/n index, indicating a mild stress on the turtles by heat wave. The IBR/n values of recovery group and control group were similar, suggesting the stress induced by heat wave diminished after 1 week recovery and the physiological and biochemical protection mechanism was effective in *M. mutica*.

In summary, the current study provides an initial insight into the complex physiological and biochemical effects of heat waves in an aquatic turtle. In *M. mutica*, a simulated heat wave did not induce significant oxidative stress, but may nevertheless cause damage to the immune organs. Meanwhile, the enhanced activity of antioxidant enzymes (GSH-Px) and immune functions of lysozyme activity and C4 levels might protect the turtles from the threat of heat waves. The influence of heat waves on the turtle might be temporary, as all the significant changes had returned to baseline after a 1-week recovery period. Taken together, these results suggest that *M. mutica* may tolerate heat waves under current climate scenarios. It should be noted that turtles used in current study are from a captive population in turtle farm, and further studies are needed to investigate whether wild populations would display similar responses to heat wave with the captive populations. Furthermore, future research should examine the physiological and biochemical responses to more frequent, extended, or intense heat waves, and investigate the long-lasting effects of heat waves on fitness-related traits (e.g., reproductive output and survival) in more ectothermic animals (Adamo and Lovett, 2011; Bauerfeind and Fischer, 2014; Stahlschmidt et al., 2017).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethical and Welfare Committee of Wenzhou University (Approval No. WZU-049).

AUTHOR CONTRIBUTIONS

SL and YZ designed the experiment, analyzed the data, and wrote the manuscript with comments from LX. SL, JL, WC, and ZX carried out the experiment. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.704105/full#supplementary-material

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REVIEW
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Climate Change Impacts on Tropical Reptiles: Likely Effects and Future Research Needs Based on Sri Lankan Perspectives

Buddhi Dayananda[1], Simeon Bezeng Bezeng[2,3]*, Suranjan Karunarathna[4] and Ross A. Jeffree[5]

[1] School of Agriculture and Food Sciences, The University of Queensland, Brisbane, QLD, Australia, [2] Department of Geography, Environmental Management and Energy Studies, University of Johannesburg, Johannesburg, South Africa, [3] Department of Life and Consumer Sciences, University of South Africa, Johannesburg, South Africa, [4] Nature Explorations and Education Team, Moratuwa, Sri Lanka, [5] Jeffree Conservation & Research, Sydney, NSW, Australia

Edited by:
Luke P. Miller,
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Reviewed by:
Nicolas Urbina-Cardona,
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Colombia
Sumudu Rubasinghe,
University of Peradeniya, Sri Lanka

***Correspondence:**
Simeon Bezeng Bezeng
bezengsimmy@gmail.com

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The tropical island nation of Sri Lanka has a rich terrestrial and aquatic reptilian fauna. However, like most other tropical countries, the threat of climate change to its reptile diversity has not been adequately addressed, in order to manage and mitigate the extinction threats that climate change poses. To address this shortfall, a review of the international literature regarding climate change impacts on reptiles was undertaken with specific reference to national requirements, focusing on predicted changes in air temperature, rainfall, water temperature, and sea level. This global information base was then used to specify a national program of research and environmental management for tropical countries, which is urgently needed to address the shortcomings in policy-relevant data, its availability and access so that the risks of extinction to reptiles can be clarified and mitigated. Specifically, after highlighting how climate change affects the various eco-physiological features of reptiles, we propose research gaps and various recommendations to address them. It is envisaged that these assessments will also be relevant to the conservation of reptilian biodiversity in other countries with tropical and subtropical climatic regimes

Keywords: extinction, point endemic, range restricted, temperature, climate warming, reptiles

INTRODUCTION

Climate change now looms as one of the greatest threats to global biodiversity as well as a formidable force of global environmental change (Evans, 2019; Kellogg, 2019). It is already exerting substantial adverse impacts on different hierarchies of the biosphere. As the atmospheric concentrations of carbon dioxide and other greenhouse gases continue to increase, it is predicted that climate change will cause distributional shifts, phenological modifications, and altered interactions in biological communities. In addition, climate change is expected to accentuate species extinction rates and lead to impairment of ecosystem functions in the coming decades (Ackerman, 1980; Pounds et al., 1999; Walther et al., 2002; Thomas et al., 2004; Brondizio et al., 2019).

The scientific community is constantly evaluating the vulnerability of organisms and ecosystems to climate change and formulating mitigation and response strategies that maybe implemented to minimize the compounding effects of climate change (Lee and Jetz, 2008). This is particularly true for tropical island nations like Sri Lanka; ranked as one of the world's biodiversity hotspots due to its rich reptile diversity–with 235 described species, of which 63.4% are endemic (de Silva and Ukuwela, 2017; Karunarathna et al., 2019). Moreover, reptiles as a phylogenetic group have been relatively neglected in biodiversity conservation studies in general, relative to mammals, birds and amphibians, and are clearly in need of greater priority (Gumbs et al., 2018) in relation to climate change impacts.

Key approaches which have been identified as holding promise include the identification of resilient landscapes and climate refugia for native wildlife, carbon-neutral and alternative-energy initiatives, green infrastructure and low-impact development, landscape-scale conservation planning, and sustainable use of natural resources and energy sources (Bickford et al., 2010). However, financial and human resources available for such initiatives are severely limited within a global economy based on immediate monetary gains coupled with conservative socio-political views in both developing and developed nations. Together with many other issues, these factors give rise to low national and international priorities for biodiversity conservation. Given these current economic and political constraints, the most significant challenges for conservation authorities and natural resource managers in the near future are; (i) the identification of species and ecosystems which are most vulnerable to climate change, (ii) assessing species responses to climate change, and (iii) the prioritization of planned conservation actions and responses.

In the 2008 Review of The IUCN Red List of Threatened Species, Foden et al. (2008) introduced the major characteristics that increase an organism's susceptibility to future climate change (but see also review by Bellard et al., 2012). These characteristics of vulnerable species are broadly classified as follows: (1) a highly specialized set of niche dimensions such as unique habitat/resource needs or micro-environmental conditions; (2) narrow environmental tolerances or thresholds; (3) life histories that depend on environmental cues/triggers which are disrupted by climate change; (4) obligatory mutualistic and other forms of symbiotic interspecific interactions which are likely to be disrupted by climate change; (5) poor ability to disperse to or colonize suitable new habitats; and (6) species with a high site fidelity (adapted from Foden et al., 2008).

Several tropical species share one or more of the above features pointing to their enhanced vulnerability to climate change-induced threats. Moreover, numerous studies based on both long-term field surveys and bioclimatic modeling have suggested that tropical ecosystems are likely to be severely impacted by climate change (Hughes, 2000). However, the impacts of climate change on tropical biota remain relatively understudied, and most existing studies focus on south and central American tropical biomes. In contrast, South-Asian tropical biomes have only attracted minimal attention in the climate change literature, and this is particularly the case for the rich biodiverse Indian Oceanic tropical island nation of Sri Lanka. With respect to reptilian diversity in Sri Lanka, nine chelonian species in six families (five marine turtles, three freshwater terrapins, and one land tortoise) are recognized, of which one species (red eared terrapin, *Trachemys scripta*) has been introduced through the pet trade (Karunarathna et al., 2017). Two species of native crocodile and 118 lizard species are found in the country, and of these, 96 species are endemic to Sri Lanka. Among the 118 lizard species, the species-rich families are the Gekkonidae (59 species), Scincidae (32 species), and the Agamidae (21 species) with six endemic genera (*Ceratophora*, *Chalcidoseps*, *Cophotis*, *Lankascincus*, *Lyriocephalus*, and *Nessia*). Furthermore, 106 snake species occur in the island of which 51 species are endemic, including the genus *Aspidura* (de Silva and Ukuwela, 2017; see **Figure 1** and **Table 1**).

In this review, we outline the likely main effects of climate change on reptiles in tropical Sri Lanka with specific reference to changes in air temperatures, rainfall patterns, water temperatures, and sea levels. We also identify the current research gaps and the requirements for future research which are needed to better characterize the most vulnerable reptilian species. As this study provides a body of information to better identify the most vulnerable species of reptiles, it is envisaged as being of benefit to wildlife managers in their choice of the most appropriate management strategies to mitigate climate change impacts for the conservation of reptiles in tropical and sub tropical countries.

Furthermore, such a review for the island of Sri Lanka with its geographic location, small land area, and rich biodiversity is likely to be relevant to other tropical and subtropical regions such as Costa Rica, Caribbean Islands, Southern Florida and Florida Keys, south and central Pacific islands, and the Indo-Malayan region, which have also been identified as areas that need critical conservation planning in the face of climate change. For example, Day (2009) highlighted a range of challenges linked to the scarcity of biodiversity data for the understanding of the impacts of climate change on biodiversity in the Caribbean Islands, which are needed for the development of more effective species conservation action strategies. Thus, a review of the potential impact of climate change on reptiles in tropical countries may initiate some national-level actions while drawing attention from the international scientific community. In the absence of peer-reviewed eco-physiological studies on Sri Lankan herpetofauna, we synthesized all the scientific literature used in Angilletta (2009); Dayananda et al. (2016), and Noble et al. (2017) in order to articulate the comprehensive and critical views of the reptilian eco-physiological knowledge gaps and future research needs in relation to current and future climate change scenarios.

CLIMATE CHANGE IMPACTS ON THE ECO-PHYSIOLOGY OF REPTILES

Effects of Changes in Rainfall and Monsoonal Patterns

In Sri Lanka, the monsoon system has already been observed to have changed appreciably and seasonal rainfall anomalies are already evident (Zubair et al., 2008; Burt and Weerasinghe, 2014). A decrease in the frequency of rainfall, drier periods than previously and increases in the intensity of extreme rainfall

FIGURE 1 | Climatically restricted and threatened reptile species in Sri Lanka. **(A)** *Ceratophora erdeleni*–a critically endangered and endemic lizard species restricted to the 80 km^2 land area in Rakwana hill zone. **(B)** *Geochelone elegans*–a near threatened land tortoise that is found in Dry and Intermediate zone. **(C)** *Aspidura brachyorrhos*–a vulnerable and represent an endemic genus of non-venomous snake species that is found in Central Heighland zone. **(D)** *Calodactylodes illingworthorum*–a vulnerable and endemic gecko found in Uva Savannah zone (please refer to the reptile zone map in **Figure 2A**).

events are now evident, including an increased frequency of flooding; these trends are expected to continue and intensify. Moisture regimes, including factors of soil hydrology, humidity, and the hydrologic characteristics of wetlands and aquatic habitats, are crucial environmental factors that govern the embryonic development of reptiles. Water exchange by eggs with the surrounding environment is particularly important during the incubation process (Warner et al., 2011). For example, freshwater turtles select well-drained, moist soil for their egg-laying in close proximity to wetlands. Similarly, crocodiles and alligators nest adjacent to large, permanent water bodies. With the observed trends in climate change, many areas of the world that are rich in reptile fauna, including Sri Lanka, are getting drier and moisture-stressed (Bickford et al., 2010). As a result, oviparous reptiles can be expected to suffer from egg dehydration which can in turn lead to decreased hatching success and small hatchling size; small-sized juveniles can subsequently suffer increased predation and can be weak in their abilities for dispersal and general motility (Angilletta, 2009). Dehydration-induced physiological stressors during embryonic development may well have adverse, long-term fitness consequences, such as slow growth and development (Ackerman, 1991; Miller and Packard, 1992; Belinsky et al., 2004). Thus, among the Sri Lankan herpetofauna, the following species are identified as likely to suffer from similar climate-driven adversities; the two species of terrapins (native *Melanochelys trijuga* and endemic *Lissemys ceylonensis*), the two species of crocodiles (*Crocodylus palustris* and *Crocodylus porosus*) and 13 species of snakes (*Amphiesma stolatum*, *Aspidura brachyorrhos*, *Aspidura copei*, *Aspidura ceylonensis*, *Aspidura deraniyagalae*, *Aspidura desilvai*, *Aspidura drummondhayi*, *Aspidura guentheri*, *Aspidura ravanai*, *Aspidura trachyprocta*, *Atretium schistosum*, *Fowlea asperrimus*, and *Fowlea piscator*) (Karunarathna et al., 2019).

Effects of Air Temperature Increments

One of the most direct and predictable effects of climate change is an increase in mean air temperature which has also been clearly identified as a major threat to reptiles in coming years (Stocker et al., 2013). Being poikilotherms, reptiles are sensitive to changes in their thermal landscape, thus rising atmospheric temperature may lead to changes in reptilian metabolism (Araújo et al., 2006; Tewksbury et al., 2008; Sinervo et al., 2010). Reptiles depend on external environmental temperatures to optimize their body temperature (T_b), and then their physiological performance and behavioral activities (Huey et al., 2012) such as locomotion, digestion, growth, and reproduction are strongly influenced by environmental temperatures (Huey and Bennett, 1987; Huey and Kingsolver, 1989; Hoffmann et al., 2013). Continued climate warming is thus expected to further push environmental thermal regimes beyond the optimal range to which many reptiles have become adapted. Moreover, recent studies have verified that climate change poses a serious threat to reptile populations (Araújo et al., 2006; Tewksbury et al., 2008; Sinervo et al., 2010; Huey et al., 2012). Changes in environmental temperature can alter the body temperature (T_b) of reptiles and thus their physiological performance, which is closely linked to their vulnerability to extinction (Pounds et al., 1999; Root et al., 2003; Thomas et al., 2004; Araújo et al., 2006; Wake, 2007; Bickford et al., 2010; Huey et al., 2010, 2012; Vitt and Caldwell, 2013).

Many tropical reptile species are known to exist close to their physiological thermal optima. Moreover, tropical environments confer a greater degree of temporal thermal stability. Therefore,

TABLE 1 | Reptile families in Sri Lanka with their geographical distributional zones (please refer to the reptile zones map in **Figure 2A**), number of species and current conservation status (CR = Critically Endangered, EN = Endangered and VU = Vulnerable; After Ministry of Mahaweli Development and Environment, 2019).

Total			**IUCN Status**			**Reptiles Zones**								
Families	**Species**	**Endemics**	**CR**	**EN**	**VU**	**Dry and Intermediate**	**Central Highland**	**Knuckles Hill**	**Lowland Wet**	**Marine**	**Northern Arid**	**Rakwana Hill**	**Southern Coastal**	**Uva Savannah**
Crocodylidae	2	0	0	1	0	2	0	0	0	0	2	0	2	1
Geoemydidae	1	0	0	0	0	1	1	1	1	1	1	0	1	1
Cheloniidae	4	0	0	4	0	0	0	0	0	4	0	0	0	0
Dermochelidae	1	0	1	0	0	0	0	0	0	1	0	0	0	0
Testudinidae	1	0	0	0	0	1	0	0	0	0	1	0	1	1
Trionychidae	1	1	0	0	0	1	0	1	1	0	1	0	1	1
Emydidae	1	0	0	0	0	0	0	0	1	0	0	0	1	0
Agamidae	22	19	5	8	2	8	10	9	7	3	3	7	5	6
Chameleonidae	1	0	0	1	0	1	0	0	0	0	1	0	0	0
Gekkonidae	62	52	24	14	9	20	15	10	16	8	7	10	9	12
Lacertidae	2	0	0	0	0	2	0	0	0	0	2	0	0	2
Scincidae	34	29	2	7	4	16	7	10	14	0	10	4	6	8
Varanidae	2	0	0	0	0	2	1	2	2	0	2	1	2	2
Acrochordidae	1	0	0	0	1	1	0	0	1	0	1	0	1	0
Erycidae	1	0	0	0	0	1	0	0	0	0	1	0	1	1
Pythonidae	1	0	0	0	0	1	1	1	1	0	1	1	1	1
Cylindrophiidae	1	1	0	0	1	1	1	1	1	0	0	1	0	1
Colubridae	47	26	3	7	8	29	25	26	31	0	15	18	19	24
Homalopsidae	3	0	0	1	0	3	0	0	1	0	2	0	1	0
Elapidae	20	2	0	1	0	4	3	3	2	15	3	2	3	3
Gerrhopilidae	2	2	1	1	0	0	2	0	2	0	0	0	0	1
Typhlopidae	8	6	2	2	0	7	2	2	2	0	5	0	1	2
Uropeltidae	18	18	3	8	1	7	5	3	5	0	3	1	0	6
Viperidae	6	3	0	1	1	4	3	3	4	0	3	4	3	3
Total species	242	159	41	56	27	112	76	72	92	32	64	49	58	76

tropical species are likely to be more vulnerable to climate-change-induced temperature changes than more temperate species. A confirmatory example is provided by Sinervo et al. (2010), which showed in their recent surveys of 48 Mexican lizard species at 200 sites that 12% of local populations have gone extinct since 1975, and by 2080 39% of local lizard populations and 20% of worldwide lizard species are predicted to go extinct due to climate change.

The capacity of reptiles to perform physiological and behavioral functions at different body temperatures is described by their thermal performance curve (Huey et al., 2012). It falls within critical limits (Araújo et al., 2006; Huey et al., 2009, 2012; Sinervo et al., 2010) and performance reaches a maximum within an optimal body temperature region. If their body temperature increases above the optimal body temperature then their performance drops rapidly. Body temperatures higher than the optima are known to create physiological stress, reduced performance and increased disease susceptibility, ultimately leading to population declines and extinctions (Root et al., 2003; Huey et al., 2010; Sinervo et al., 2010). Due to projected temperature increases of 1.1–6.4°C by the year 2100, the metabolic rates of ectotherms could increase by 10–75% (Bickford et al., 2010). Such increases in metabolic rates, coupled with reduced foraging time, could be expected to negatively affect population growth rates (Bickford et al., 2010).

However, it is true that some reptiles do inhabit environments that are cooler than their optimum average temperatures (Deutsch et al., 2008), and they may actually benefit from rising temperatures (Araújo et al., 2006). For example, in common lizards, *Lacerta vivipara* positive correlations have been found between rising summer temperatures and body size, clutch size and total reproductive effort (Chamaillè-Jammes et al., 2006). In side-blotched lizards (*Uta stansburiana*) higher night-time temperatures increased reproductive success, hatchling size and hatchling survival (Clarke and Zani, 2012). Some species maybe able to buffer small temperature changes, but large temperature increases may force many species to shift their distributions to cooler climates. For example, open-habitat species may be forced

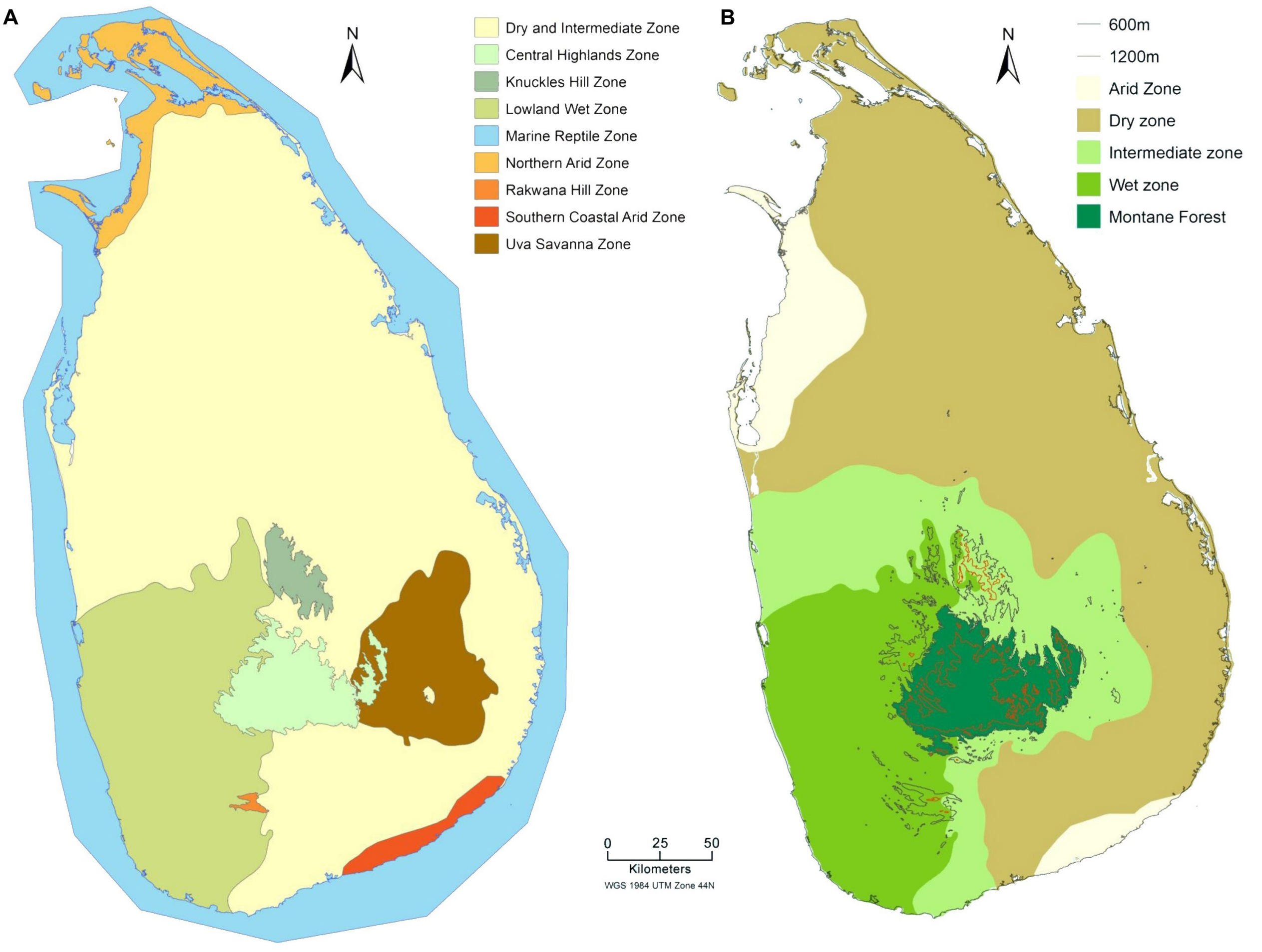

FIGURE 2 | Sri Lanka climatic zone maps. **(A)** Nine distinctive reptile zones (Ministry of Mahaweli Development and Environment, 2019) and **(B)** Five distinctive bioclimatic zones.

to invade closed forest environments (Huey et al., 2009). In Spain, changes in the distributions of reptiles were associated with increases in temperature, and 22 reptile species shifted pole-wards by an average of 15.2 km from 1940–1975 to 1991–2005, which is equivalent to approximately 0.5 km/year (Moreno-Rueda et al., 2012).

Nevertheless, many reptile species are unlikely to cope with changing climates because of habitat fragmentation and natural barriers to species movements. For example, in Europe 98% of reptiles may experience range contractions by 2050, if they have no dispersal capacity (Araújo et al., 2006). Furthermore, based on mean annual dispersal distance to favorable habitats under current conditions, future distribution (under climate change scenarios), it is predicted that up to 41% of the Australian arid zone gecko's (*Gehyra variegata*) current population would fail to colonize in their favorable microclimates by 2070 (Duckett et al., 2013).

With specific regard to Sri Lanka (also in tropical and subtropical regions) the degree of habitat fragmentation is much more severe in its southwestern region where much of the wilderness remains as forest fragments which are smaller than 100 km^2, mainly due to infrastructure development, particularly road construction and industrial agriculture. In fact southwest Sri Lanka together with the Western Ghats of India is said to support the highest human population density among the world's biodiversity hotspots (Cincotta et al., 2000). If warming trends continue in tropical countries unabated, then the inhabitants of the lowland habitats will be forced to migrate to higher elevations in search of the optimal conditions. The lack of landscape-scale connectivity in southwestern Sri Lanka is expected to appreciably impede such species' movements.

The magnitude of the effects of warming on reptiles will depend on physiological and/or behavioral plasticity and their evolutionary adaptive capacities (Williams et al., 2008; Chevin et al., 2010; Hoffmann, 2010; Hoffmann et al., 2013; Monasterio et al., 2013). Most reptiles are able to control their body temperature precisely via behavioral and postural adjustments (Huey et al., 2003; Leal and Gunderson, 2012). Phenotypic plasticity is a significant factor in organisms in response to fluctuating environmental conditions (Walther et al., 2002;

Parmesan and Yohe, 2003; Root et al., 2003; Berteaux et al., 2004). However, in organisms where changes in traits are simply a result of phenotypic plasticity, such changes occur much faster than evolutionary genetic changes, and are thus likely to have a more immediate and direct influence on responses to climate change (Williams et al., 2008; Chevin et al., 2010). Although behavioral thermoregulation is widespread in reptiles, unfortunately, so far we have a very limited understanding of how reptiles respond to temperature changes via evolutionary genetic changes.

Increased air temperature regimes are also expected to alter "reptiles" interactions with predators, with such changes being already evident. For example, they can be exposed to new predators when birds change their hunting grounds as a consequence of rising air temperatures (Low, 2007). Furthermore, brief exposure to temperatures above the critical thermal maximum (CT_{max}) can cause the death of an individual which is unable to prevent its overheating; hatchlings will need to move between hotter and cooler surfaces more often, potentially exposing them to predators (Webb and Whiting, 2005) and lowering their chance of survival (Dayananda and Webb, 2017). Furthermore, high environmental temperatures may restrict an individual's ability to perform important activities such as foraging, defense or mating (Sinervo et al., 2010).

Effects of Climate Warming During Embryonic Development

In oviparous reptiles, temperature plays an important role during embryonic development and incubation temperature can affect the offspring in a variety of ways (Angilletta, 2009). Increases in air temperatures cause higher temperatures inside lizard nests (Dayananda et al., 2016) and recent research has predicted significant declines in lizard populations over the next century (Sinervo et al., 2010). Thus, one of the most significant impacts of climate change on reptiles is expected to be derived from the effects of warming during their embryonic development.

The effect of the thermal environment during embryonic development has been examined in a wide variety of reptilian taxa. For example, laboratory studies indicate that in oviparous reptiles, incubation temperature directly affects a range of factors, including the duration of incubation, embryo survival and the size, shape, behavior, sex, and performance of hatchlings (Janzen, 1994; Downes and Shine, 1999; Du and Ji, 2003; Deeming, 2004; Parker and Andrews, 2007). These experimental information can be integrated into forecasts of species survival and geographical distribution under projected climate warming regimes.

However, these effects appear to vary between species. For example, in wall lizards (*Podarcis muralis*) higher incubation temperatures produced smaller and lighter hatchlings which ran more slowly than hatchlings from eggs that were incubated at cooler temperatures (Brana and Ji, 2000). In general, hotter incubation temperatures increase locomotor performance in some turtles and lizards (but not in others–see Booth et al., 2000) and increases in locomotor performance would generally be expected to increase the ability of hatchlings to escape from predators (Janzen, 1993). For example, in chelonians, increases in incubation temperature increased the swimming performance and body size of *Apalone mutica* (Janzen, 1993); similarly, in *Pelodiscus sinensis* (Du and Ji, 2003) and *Chelonia mydas* (Booth et al., 2004), *Saproscincus mustelina*, *Lampropholis delicata* (Downes and Shine, 1999) but also actual decreases in *P. muralis* (Van Damme et al., 1992), *Nannoscincus maccoyi* (Downes and Shine, 1999), *Takydromus wolteri* (Chen et al., 2003), and *Takydromus septentrionalis* (Du and Ji, 2006).

Incubation temperature can also influence the growth rate of hatchling reptiles. There is a general trend for post-hatching growth rates to increase at higher incubation temperatures (Hutton, 1987; Webb and Cooper-Preston, 1989; Spotila et al., 1994; Roosenburg and Kelley, 1996; Demuth, 2001; Ji et al., 2003; Booth et al., 2004; Nelson et al., 2004; Andrews, 2008).

The gender of oviparous reptiles is determined by the environmental temperature during incubation and cannot be predicted by zygotic genotype [temperature-dependent sex determination (TSD)]. The following three patterns of TSD can be observed in reptiles: (a) species with TSD Ia [male-female (MF)] produce males at low temperatures and females at high temperatures; (b) species with TSD Ib [female-male (FM)] produce females at low temperatures and males at high temperatures, and (c) species with TSD II [female-male-female (FMF)] produce females at low and high temperatures and males at intermediate temperatures. TSD Ia has been reported for turtles, TSD Ib for tuatara, lizards and crocodilians and TSD II for turtles, lizards, and crocodilians (Valenzuela, 2004). Therefore, changes in the climate are expected to result in appreciable differences in the hatchling sex ratio, which can then alter the operational sex ratio. In the longer term, these trends can result in dramatic changes in the population sex ratio and can ultimately drive a population to become either male or female limited. Such changes can consequently have negative impacts on mate selection strategies. Cumulatively, these impacts are expected to lead to reduced recruitments in the population, gradually leading to declining numbers.

However, this picture is complicated as it is not entirely clear that all reptiles possess TSD and additionally, TSD and GSD are not necessarily mutually exclusive (Holleley et al., 2015). For example, incubation temperature does not affect the sex ratio of the Chinese soft-shelled turtle *P. sinensis* (Ji et al., 2003), and the Brisbane river turtle *Emydura signata* also possesses GSD (Booth et al., 2004). In essence, TSD has been found in all crocodilians, tuatara, and it is prevalent in the turtles but less frequent in lizards (Valenzuela, 2001, 2004; Nelson et al., 2004). Furthermore, TSD differs in occurrence between families of reptiles. For example, many turtles exhibit TSD, whereas no example has been reported from the families Trionychilidae and Chelidae. In lizards, TSD is common in the Agamidae and Gekkonidae, but reports are absent for the Teiidae, Phrynosomatidae, and Polychrotidae (Valenzuela, 2004). Theoretically, TSD species with larger clutches deposit their eggs at various depths in the nest and eggs at the top are exposed to the high temperature than those at the bottom of the nest; hence, these more prolific species will have different sex ratios within the same nest (Georges et al., 2004).

Incubation temperature is also known to affect the "lizards" behavior and survival. For example, larger side-blotched lizards (*U. stansburiana*) and tree lizard (*Urosaurus ornatus*) hatchlings had a higher probability of survival compared to their smaller counterparts (Ferguson and Fox, 1984; Miles, 2004) and higher incubation temperatures decrease the survival of the velvet gecko (Dayananda et al., 2016). Furthermore, at higher temperatures Yucatan banded geckos (*Coleonyx elegans*) were less active than individuals from low incubation temperature regimes, in relation to their anti-predator behaviors (Trnik et al., 2011). Learning ability of hatchlings has also been demonstrated to be affected by incubation temperature; in Velvet gecko (*Amalosia lesueurii*) eggs incubated at elevated temperatures hatchlings showed reduced learning abilities and associated lower survival rates in the wild (Dayananda and Webb, 2017; Dayananda et al., 2017b).

Also, a strong link has been established between incubation temperature and the critical thermal tolerance limits in hatchlings of the velvet gecko. Hatchlings from high-temperature incubators had a lower critical thermal maximum (38.7°C) and a higher critical thermal minimum (6.2°C) compared to hatchlings from cold-temperature incubators (Dayananda et al., 2017a). Decreased thermal tolerance may also reduce the time available for hatchlings to forage by forcing them to remain inside shelters for longer periods and also species living in thermally stressful environments at high environmental temperatures can force body temperatures to reach the critical thermal maximum (Sinervo et al., 2010).

Effects of Sea Level and Temperature Increase on Aquatic Reptiles

Water temperature is one of the most important factors which directly affects fitness, reproductive traits and geographical distributions of aquatic reptiles (Elsworth et al., 2003; Pike et al., 2006; Mazaris et al., 2008). For example, water temperature has been correlated with nesting dates in Loggerhead Sea turtle *Caretta caretta*, and the extent of its nesting season decreased by about 43 days due to warmer sea surface temperatures (Pike et al., 2006). Furthermore, over the last 17 years, leatherback turtle (*Dermochelys coriacea*) populations in the North Atlantic have moved their northern boundary distribution by 330 km (McMahon and Hays, 2006).

The geographical distributions of aquatic reptile also clearly depend on the availability of their prey and changes to their foraging areas and availabilities of food resources can also be dependent on rising water temperatures/mean annual sea surface temperature (Witt et al., 2007). For example, redistribution or/and decreasing prey populations has affected (a) the growth and reproductive output and also the duration of the interval between breeding seasons in Green turtles (*C. mydas*) (Broderick et al., 2003), and (b) the nesting abundance of Loggerhead turtle (*C. caretta*) (Chaloupka et al., 2008).

Similar observations have also been made in freshwater reptiles. For instance, semi-aquatic snakes such as Garter snakes and water snakes tend to shift their foraging grounds as the seasons progresses (Southwood and Avens, 2010). With the predicted climate change, the seasonal shifts can become more abrupt and the length of the growing season can also change. These climatic modification can be expected to also impact the life histories of reptilian prey, and thereby, will induce changes in both spatial and temporal distribution of suitable foraging grounds.

Sea levels are projected to rise by a further 0.5–2.0 m by 2100 (Nicholls et al., 2011) with a current mean sea level rise of 3.6 mm year^{-1} (IPCC, 2019) which directly links with the global temperature increment of increase (Vermeer and Rahmstorf, 2009). Rising sea levels have already led to the intrusion of saltwater upstream, increasing the salinity of freshwater wetlands in Northern Australia (Mulrennan and Woodroffe, 1998), and this effect will continue to negatively impact the freshwater reptiles in many regions as sea levels continue to rise. For example, the Roti Island snake-necked turtle (*Chelodina mccordi*) has been predicted to disappear due to rising water levels on the low-lying islands this species inhabits (Bickford et al., 2010). Increasing salinity in freshwater habitats has already influenced the distribution of the American alligator (*Alligator mississippiensis*) and has led to reduced growth rates and survival in the American crocodile (*Crocodylus acutus*) (Mazzotti and Brandt, 1994; Mazaris et al., 2009). Furthermore, increasing salinity in freshwater habitats in Southern Sri Lanka has already influenced the Estuarine crocodile (*C. porosus*) in its distribution, population reductions and increased level of human-crocodile conflict (Amarasinghe et al., 2015). Rising sea levels also threaten other coastal and brackish-water habitats such as sand dunes, coastal grasslands and scrublands, and tidal marshes. Reptiles specialized for these unique ecosystems (e.g., Diamond-backed terrapins) may also lose their critical habitats due to rising sea levels (Bickford et al., 2010).

With specific reference to Sri Lanka there are five species of marine turtles that employ its coastal zone for nesting. Among them, Leatherback Turtle (*D. coriacea*), Loggerhead Turtle (*C. caretta*), and Olive Ridley Turtle (*Lepidochelys olivacea*) are already classified as Vulnerable and the Green Turtle (*C. mydas*) is classified as Endangered. The Hawksbill Turtle (*Eretmochelys imbricata*) is classified as Critically Endangered but how rising sea levels may affect them has not been ascertained to date (**Supplementary Table 1** highlighting all reptile families and species in Sri Lanka with their geographical distributional zones and IUCN Red List status). However, in the Caribbean islands up to 32% of the total current beach area which is important nesting ground for both Hawksbill (*E. imbricata*) and Loggerhead (*C. caretta*) turtles could be lost if sea levels rise by 0.5 m (Fish et al., 2005). It is also predicted that in the northwestern Hawaiian islands up to 40% of the Green turtle's (*C. mydas*) nesting beaches could be lost following a 0.9 m sea level rise (Baker et al., 2006). In the northern Great Barrier Reef in Australia up to 38% of available nesting area will be lost by the largest green turtle population in the World due to the projected sea-level rises (Fuentes et al., 2010). Generally, rising sea level predominantly affects the availability of nesting sites for sea turtles, with females needing to find alternative nesting sites along neighboring coastlines. Coastal erosion may limit the number of alternative nesting sites with the outcome of several females choosing the same location which is similar to a communal nesting behavior. This higher nest density can increase the risk of nest destruction by other nesting females which also leads to the increasing embryo mortality and reduced overall reproductive success

(Mazaris et al., 2009; Fuentes et al., 2010). Thus it is clear from these international studies that all marine turtles in Sri Lanka are potentially vulnerable to rising sea levels and that increased coastal erosion may significantly reduce their nest sites, increase embryonic mortality and thereby reduce their reproductive success, ultimately causing their populations to decline.

Moreover, reptilian pathogen densities, distributions and population dynamics can be indirectly influenced by the environmental factors mediated by climate disruption. For example, due to enhanced rainfall, two tick species *Aponomma hydrosauri* and *Amblyomma limbatum* have changed their boundary positions and the density of ticks on lizards in regions flanking the boundary zone has increased for *A. hydrosauri* and decreased for *A. limbatum* (Bull and Burzacott, 2001).

REPTILIAN BIODIVERSITY RESEARCH GAPS AND FUTURE REQUIREMENTS IN SRI LANKA UNDER CLIMATE CHANGE

Human-induced pressures on national biodiversity continue unabated, and it is increasingly important that conservation efforts are prioritized to maximize conservation returns in a world of limited human and financial resources. As such, conservation scientists continue to evaluate the vulnerability of organisms to climate change to support the implementation of evidence-based management strategies to mitigate the impacts of climate change in order to conserve threatened species (Lee and Jetz, 2008).

By assessing the biodiversity impacts of both state and non-state actors, we can identify their contributions to biodiversity conservation efforts (Mair et al., 2021). However, the assessment of a species' response to ongoing climate warming, and the conservation actions required to mitigate the impacts, are among the most significant and controversial challenges for ecologists in the future. Specifically, efficient conservation planning and the desired actions needed to abate threats to species loss is hampered by key biodiversity knowledge gaps (Hoveka et al., 2020). For reptiles in particular, to better predict which species are at risk, accurate data is are required on species' exposure to thermal stressors in the environment, their sensitivity to such stressors, and their ability to adapt to such stressors (Williams et al., 2008).

This is especially true for the Indian Oceanic tropical island nation of Sri Lanka, where gaps in our knowledge of species distribution under current and future climate change scenarios are still limited. To avert the impacts of climate change on this rich reptilian fauna requires a collaborative effort of government working closely with civil society and local communities to ensure that biodiversity is properly conserved and sustainably utilized. To achieve this, we propose that targeted conservation actions for Sri Lankan reptiles under the climate change scenarios should be geared toward identifying Environmentally Sensitive Areas (ESA), in order to conserve significant Sri Lankan ecosystems that are outside protected areas and using reptiles as an indicator taxon. This can be achieved by conducting research studies on climate change impacts on ecosystems and reptiles, e.g., (a) identification of indicator species for long term population monitoring and research priorities, (b) modeling impacts of climate change on reptiles, their changing distribution patterns, and boundary shifts of climatic zones, (c) lifecycle studies on their sex ratios, and (d) *ex situ* conservation) (**Figure 2**). Additionally, an enhanced reptile protection can be achieved by developing mechanisms to establish and improve the skills of conservationist throughout the country and by partnering with local communities in sustainable management strategies in the agriculture and tourism sectors of the economy. Addressing the insufficient level of protection for reptiles will represent a significant contribution toward tackling the Leopoldean shortfall in Sri Lanka (see Hoveka et al., 2020 for further details). Key research gaps that need to be prioritized include those described below.

Tracking Shifts in Species Distribution Under Climate Change Scenarios

Environmental niche models (ENMs) have become popular tools for forecasting the potential impacts of future climate change on the geographical distributions of species (Sillero, 2011). These models are used first to analyze the geographic distribution of a species' niche resulting from existing climatic conditions and then to generate projections of future range shifts under climate change. This is achieved by correlating the records of a "species" occurrences with a set of environmental variables under a range of climate change scenarios (Araújo and Pearson, 2005; Bezeng et al., 2017).

The main advantages of ENMs are their relatively low data requirements and capacity for rapid analysis, which can provide the baseline information on the status of species that may require immediate e conservation actions. Thus ENMs have been used to assess species vulnerability to climate change across numerous taxonomic groups, and in many regions of the world (Araújo and Luoto, 2007).

Current and future climate data, including 19 Bioclimatic variables layers, is available for such ENM modeling in various databases such as WorldClim (Fick and Hijmans, 2017). Unfortunately, in Sri Lanka the availability of "species" occurrences (i.e., presence/absence) data is not publicly available for research due to personnel authorships and access restrictions by the relevant governmental organizations which are responsible for the preparation of the IUCN Red List. Due to these constraints on data access, ENMs are still new to tropical countries and are yet to be employed in order to investigate the impact of climate change on organisms, especially ectotherms (but see Bezeng et al., 2017). Additionally, ENM approaches are still limited in handling other forms of species data (e.g., life history traits), which are fundamental to enhancing the predictive accuracy (but see Huang et al., 2020). Amidst these challenges, ENMs hold huge potential as a conservation tool for wildlife management in tropical regions (see review by Cayuela et al., 2009 and references therein). Specifically, many megadiverse countries in Latin American have successfully used ENM approaches to model the range shifts of various species across different biogeographic realms in order to identify pre-emptive conservation management strategies to avoid extinction from climate related causes (Urbina-Cardona et al., 2019). Particularly, the adoption of ENM approaches that incorporate both climate and eco-physiological data for reptiles (as shown in section "Climate Change Impacts on the Eco-physiology of Reptiles") will help improve prediction of a species' potential for range shifts under climate change scenarios. The obvious first step that Sri Lankan researchers could take is to establish an open access biodiversity and species occurrence data repository. One regional example for consideration in tropical and subtropical countries is the Atlas of Living Australia[1], which provides biodiversity data from multiple sources and makes it freely available online for all researchers to use in their investigations.

Physiological Sensitivity of Adults to Elevated Temperatures

The increases in environmental temperature are the most direct outcome of climate change. It is well established that such increases alter body temperature of reptiles and consequently their physiological performance and vulnerability (Huey et al., 2012). The complete thermal performance or fitness curve of an organism provides robust understanding of their sensitivity and response to temperature. Thus measurement of the temperature at which performance is maximized (Topt), the critical thermal maximum (CT_{max}) and the critical thermal minimum (CT_{min}) values provide critical information on how tropical and subtropical reptiles will respond to increased environmental temperature and allows assessment of their physiological consequences. The thermal tolerance limits CT_{max} and CT_{min} constitute the endpoints of thermal performance curves which are generally associated with loss of the righting response (LRR) (Lutterschmidt and Hutchison, 1997a). It provides critically important information on how an organism's physiology, distribution and ecology are influenced by climate (Lutterschmidt and Hutchison, 1997b).

Performance curves illustrate the ability of reptiles to perform a specific activity at different body temperatures (Huey et al., 2012). Common traits for performance curves include locomotor speed, endurance, prey capture ability, development time, reproductive rate, growth rate, and net energy gain (Huey and Stevenson, 1979; Hertz et al., 1988; Huey et al., 1990, 2012; Angilletta et al., 2002). Among these traits, locomotion is the most frequently measured indicator of performance (Waldschmidt and Tracy, 1983; Huey and Kingsolver, 1989; Rall and Woledge, 1990; Adolph and Porter, 1993; Swoap et al., 1993; Hawkins, 1995; Kubisch et al., 2011; Woolrich-Piña et al., 2012; Aidam et al., 2013). Studies on lizards suggest that locomotor performance, which is tightly linked to an individual's body temperature, influences fitness (Irschick and Garland, 2001). Thus, analysis of reptile performance is a useful proxy for studying their relationship between environmental temperature, physiological adaptations, their ecological relevance and relates to their evolutionary traits (Huey and Stevenson, 1979; Huey and Bennett, 1987; Huey and Kingsolver, 1989). Lizards living in lowland forests and open habitats are at high risk from climate warming and they are already experiencing body temperature at or above their optimal body temperature (Huey et al., 2009). Hence, performance experiments on tropical lizards, particularly those living in the dry zone (see **Figure 2**) and also along the latitudinal gradient is highly desirable to understand their current physiological adaptations for the following reasons:

(i) There are no studies to date on reptile performance so far conducted with Sri Lankan species which would inform on their thermal requirements and behavioral thermoregulation capacities;
(ii) Although Sri Lanka publishes consecutive national Red Lists, the impacts of climate change on species is not well-considered or evaluated (see Foden et al., 2008). For example, the ability of IUCN criteria to detect risks imposed by potentially slow-acting threats such as climate change is very limited as a criterion to detect rates of population decline over periods of 5 years.
(iii) As it is well documented that tropical lizards are highly vulnerable to climate change (Huey et al., 2009; Sinervo et al., 2010), it is imperative that the most threatened reptile taxa in Sri Lanka are identified in order to establish science-based conservation initiatives and management actions before populations go locally extinct.

Physiological Sensitivity of Embryos to Elevated Temperatures

Climate warming is also predicted to cause higher temperatures inside reptilian nests (Dayananda et al., 2016). Predicting the vulnerability of oviparous reptiles to climate warming therefore, also requires knowledge of how increases in incubation temperature affect sensitive life stages, particularly embryos (Booth et al., 2000; Valenzuela, 2001; Huey et al., 2012; Urban et al., 2014). Thermal environments during embryonic development have been examined in a wide variety of reptilian taxa, and incubation experiments suggest that incubation temperatures are able to affect incubation length as well as embryo survival, size, shape, behavior and sex, and the performance of hatchlings (Deeming, 2004). Additionally, thermal sensitivity of oviparous embryos depends on their upper thermal limits, and increased thermal stress beyond these upper limits will lead to cardiac arrest of embryos (Angilletta et al., 2013). Therefore, we propose the following two steps to better predict the physiological sensitivity of reptilian embryos under climate change scenarios;

(a) An understanding is required of the correlation between nest temperature and environmental temperature, particularly for species which lay their eggs in open nests, inside crevices or underneath rocks and which lay their eggs communally (e.g., *Calodactylodes illingworthorum* and *Cnemaspis* species). These eggs are particularly vulnerable to heatwaves that are predicted to increase in frequency and duration in future. Strong positive linear correlation between air temperatures and nest temperatures demonstrate that those hotter air temperatures will produce hotter nests in the future (Dayananda et al., 2016).

(b) Programmable incubators to be employed that can be set at natural nest temperatures and the predicted future nest

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[1] www.ala.org.au/

temperatures, to incubate lizard eggs in order to measure and compare morphology, physiology, performance and sex of resulting hatchlings.

(c) Investigations of the physiological and biochemical adaptations of embryos leading to their heat tolerance by analysis of heat shock proteins (HSPs), particularly HSP70. It is well known that HSPs are upregulated when organisms are exposed to extreme temperatures (Feder and Hofmann, 1999; Sørensen, 2010). However, production of HSPs reduce the rates of synthesis of other proteins and can lead to decreasing fecundity, developmental and survival rates of hatchlings (Feder and Hofmann, 1999). For example, high incubation temperatures associated with overexpression of HSP70 during embryonic development of hatchling turtles led to their decreased heat tolerance (Gao et al., 2014).

Maternal Sensitivity and Responses to Climate Change

Gravid female reptiles can protect their embryos against the higher nest temperatures by laying eggs earlier, digging deeper nests, or choosing cooler/shadier nest sites (Doody et al., 2006). Information is required on how gravid females actually do respond to higher environmental temperatures during their nesting period and also their plasticity in nesting behaviors. In particular, these studies are important for marine turtles and dry zone lizards because they are already identified as the most vulnerable groups in the future (**Figure 2**).

CONCLUDING REMARKS

Based on the eco-physiological studies which have already been conducted in other parts of the world, it is reasonable to assume that tropical reptiles will face a range of detrimental effects of climate change with declining population sizes, and altered community structures and ultimately extinctions. Thus, particularly for Sri Lanka, we need to establish and conduct a set of comprehensive studies to explore how future climate change is most likely to affect the persistence of reptiles, given the current deficit of information required to predict species-specific vulnerabilities to climate change. Furthermore, longer-term studies are also necessary to determine whether warming will contribute to local extinctions, as has occurred elsewhere (Sinervo et al., 2010). This recommended research program will accordingly increase our understanding of the potential impacts of climate change on tropical reptiles and benefit Conservation Biologists by the provision of data that is necessary to both model and mitigate the impacts of climate change on reptiles. Documentation of the contemporary status of reptilian taxa based on IUCN Red List standard has continued for more than 20 years in Sri Lanka. However, the applied research and experimentation on the probable effects of climate change have not been achieved to date. The evidence to predict threatened reptile species and their responses to environmental change is still not available. Thus it is crucial that such research is conducted to identify the taxa most vulnerable to climate change, in order to reduce the likelihood that they may go extinct, and so that society continues to benefit from the many ecosystem services provided by reptiles.

AUTHOR CONTRIBUTIONS

BD conceived and designed the project. BD, SB, SK, and RJ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.688723/full#supplementary-material

Supplementary Table 1 | Reptile families and species in Sri Lanka with their geographical distributional zones (please refer to the reptile zones map in **Figure 2A**), and current conservation status (After Ministry of Mahaweli Development and Environment, 2019; Gibson et al., 2020).

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Effects of Soil Temperature and Moisture on the Development and Survival of Grasshopper Eggs in Inner Mongolian Grasslands

Tingjuan Wu[1,2], Shuguang Hao[1] and Le Kang[1]*

[1] State Key Laboratory of Integrated Management of Insect Pests and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, China, [2] College of Pharmacy, Henan University of Chinese Medicine, Zhengzhou, China

Grasshopper eggs overwinter in soil for almost half a year. Changes in soil temperature and moisture have a substantial effect on grasshopper eggs, especially temperature and moisture extremes. However, the combinatorial effect of temperature and moisture on the development and survival of grasshopper eggs has not been well studied. Here, we examined the effects of different soil moistures (2, 5, 8, 11, 14% water content) at 26°C and combinations of extreme soil moisture and soil temperature on the egg development and survival of three dominant species of grasshopper (*Dasyhippus barbipes*, *Oedaleus asiaticus*, and *Chorthippus fallax*) in Inner Mongolian grasslands. Our data indicated that the egg water content of the three grasshopper species was positively correlated with soil moisture but negatively correlated with hatching time. The relationship between hatching rate and soil moisture was unimodal. Averaged across 2 and 11% soil moisture, a soil temperature of 35ºCsignificantly advanced the egg hatching time of *D. barbipes*, *O. asiaticus*, and *C. fallax* by 5.63, 4.75, and 2.63 days and reduced the egg hatching rate of *D. barbipes* by 18%. Averaged across 26 and 35°C, 2% soil moisture significantly delayed the egg hatching time of *D. barbipes*, *O. asiaticus*, and *C. fallax* by 0.69, 11.01, and 0.31 days, respectively, and decreased the egg hatching rate of *D. barbipes* by 10%. The hatching time was prolonged as drought exposure duration increased, and the egg hatching rate was negatively correlated with drought exposure duration, except for *O. asiaticus*. Overall, the combination of high soil temperature and low soil moisture had a significantly negative effect on egg development, survival, and egg hatching. Generally, the response of grasshopper eggs to soil temperature and moisture provides important information on the population dynamics of grasshoppers and their ability to respond to future climate change.

Keywords: climate change, embryonic development, grasshopper, soil moisture, soil temperature

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Lin Zhang,
Hubei University of Chinese Medicine,
China

Reviewed by:
Zhu Wanlong,
Yunnan Normal University, China
Shigui Wang,
Hangzhou Normal University, China

****Correspondence:***
Le Kang
lkang@ioz.ac.cn

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INTRODUCTION

Experiments and models have indicated that climate change has significant effects on the population dynamics, phenology and distribution of insects (Walther et al., 2002; Parmesan and Yohe, 2003; Root et al., 2003; Parmesan, 2006; Poniatowski et al., 2020). For example, experimental warming and precipitation interactively modulate the mortality rate and timing of spring emergence of a gallmaking tephritid fly (Xi et al., 2016). Warmer and drier weather in the preceding year increase the winter mortality of honey bees (Switanek et al., 2016). In recent decades, the frequency of extreme weather events (e.g., drought and high temperature) has increased (Deutsch et al., 2008), and these events have affected the development and survival of insects (Katz and Brown, 1992; Bale et al., 2002; Jentsch et al., 2009; Allen et al., 2010). Although many studies have focused on the effects of average variation in climatic factors on the development and survival of insects (Masters et al., 1998; Frampton et al., 2000; Srygley, 2014; Gherlenda et al., 2016), the responses of insects to extreme environments remain unclear. An improved understanding of the effects of extreme environments on insects may greatly enhance our knowledge of how climate change affects the distribution, phenology and population dynamics of insects.

Grasshoppers are important primary consumers in temperate grassland ecosystems. Female grasshoppers lay their eggs in the soil and endure a severe season (hot and dry summer or cold and wet winter). Thus, the egg stage is the most crucial developmental period for grasshoppers, as it determines their abundance in the following year. The embryonic development, survival, and hatching time of grasshopper eggs largely depend on soil temperature and moisture (Mukerji and Gage, 1978; Wagner et al., 1984; Honek and Kocourek, 1990; Briere et al., 1999; Hao and Kang, 2004a,b; Walter et al., 2018). Furthermore, grasshopper life cycles vary with elevation (Dingle and Mousseau, 1994; Telfer and Hassall, 1999), latitude (Groeters and Shaw, 1992), topography (Coxwell and Bock, 1995), and soil type (Poniatowski et al., 2020), all of which are associated with variation in soil temperature and moisture. Grasshopper population outbreaks are closely associated with environmental conditions, such as flooding, drought, or warm winters experienced during the egg stage (Powell et al., 2007; Yu et al., 2009; Zhang et al., 2009). Grasshopper species vary in the strategies they employ to adapt to unpredictable changes in environmental factors. For example, grasshoppers have evolved different embryonic diapause traits (facultative, obligate, or no diapause) (Hao and Kang, 2004a; Guo et al., 2009) to alter egg hatching time based on environmental conditions. Thus, studying the responses of grasshopper egg development and survival to soil temperature and moisture may provide valuable insights into the ecological mechanisms underlying the adaptation of grasshoppers to environmental changes.

The Inner Mongolian grasslands are located in arid and semiarid regions, where organisms often face year-round drought stress. However, severe drought and high temperature tend to be more common in late spring and summer. Precipitation is typically concentrated in the fall. In Inner Mongolian grasslands, the dominant grasshopper species *Dasyhippus barbipes*, *Oedaleus asiaticus*, and *Chorthippus fallax* can generally be divided into early-, mid- and late-season species, respectively, based on their phenology. These grasshopper species have divergent life history traits and occupy distinct spatial and temporal ecological niches (Kang and Chen, 1994a). Our previous studies have shown that these grasshopper species respond differently to temperature and moisture treatments in laboratory experiments (Hao and Kang, 2004a,b; Zhao et al., 2005; Kang et al., 2007). The early-season species *D. barbipes* has obligate diapause, and warming does not significantly advance its egg hatching time. In contrast, warming advances the egg hatching time of the late-season species *C. fallax*, which does not have diapause (Guo et al., 2009). These studies suggest that diapause traits of grasshopper species can buffer them from the effects of environmental change (Guo et al., 2009; Wu et al., 2012; Kearney et al., 2018) or increase the sensitivity of embryonic development to environmental change. In addition, insect hatching in spring may be more strongly affected by environmental forces compared with the later hatching of other species (Walter et al., 2018). Therefore, study of the responses of different grasshopper species to soil temperature and moisture can improve our ability to predict how their distribution will change in response to climate change.

In this study, we examined three dominant species of grasshoppers that vary in their phenology and habitat use. *D. barbipes* and *O. asiaticus* are early- and mid-season species, respectively, that prefer xeriphilic environments. *C. fallax* is a late-season species that prefers humid environments. We carried out a series of individual and cross over experiments of soil temperatures and moisture to investigate their combinatorial effect on the embryonic development and survival of grasshopper eggs under laboratory conditions. The aim was to test the hypotheses that the effects of soil temperature and moisture on grasshopper egg development and survival are species-specific and that late-season grasshopper species are more sensitive to lower soil temperature and moisture than early- and mid-season grasshopper species.

MATERIALS AND METHODS

Egg Collection

We obtained eggs of three grasshopper species, *D. barbipes*, *O. asiaticus* and *C. fallax,* by collecting adult grasshoppers in the grasslands of Duo Lun County (42°02′N, 116°17′E, 1324 m a.s.l.) of Inner Mongolia, China. In this region, the long-term (1953–2007) mean annual air temperature is 2.1°C, and the monthly mean temperature ranges from 18.9°C in July to −17.5°C in January. The mean annual precipitation is 383 mm, with 90% falling between May and October.

Field-collected adult grasshoppers were reared in cages in the laboratory to produce egg pods under a 14:10 (light:dark) photoperiod. 60-W tungsten filament bulbs were used to provide light from 8:00 a.m. to 10:00 p.m. and 26 °C air temperatures in daytime for adult rearing cages. We fed the grasshoppers fresh gramineous plants collected from the field daily. To collect eggs, we placed a plastic pot filled with moistened sand on the bottom of the cage so that adult females could lay eggs. The egg pods were then sieved out from the pot every 2 days. We transferred these egg pods to a small plastic cup filled with sterile sand containing 5% water content by mass. We kept these cups in a 5°C refrigerator before using them in the manipulative experiments.

Experimental Substrate and Preparation

The experimental substrate was sand, which was sifted through a 2-mm^2 sieve, washed with water to clean out the clay, oven-dried at 180°C for 12 h for sterilization, and then moistened to 2, 5, 8, 11, and 14% of the water content by gross mass. To ensure that egg pods had the same water content at the start of the experiment, we placed all egg pods in soil with 5% moisture and at 18°C for 3 days prior to starting the experiment. Twenty egg pods of each grasshopper species were randomly selected and placed in a plastic cup (inner diameter 7.5 cm and height 8.5 cm, holds about 250 g dry sand) filled with sand with different moisture levels. Each cup was a replicate of each treatment, which consisted of two temperatures and 6 levels of moisture. We sealed the cups with parafilm and replaced sand weekly to maintain constant soil moisture.

Effect of Soil Moisture on the Egg Water Content

To measure the effects of soil moisture on the egg water content of each grasshopper species, we placed the cups with egg pods and different soil moistures (described above) into an incubator at 18°C (to slow the embryonic growth rate to minimize the effect of embryonic development on the egg water content) for 5 days. We weighed the wet mass of egg pods, oven-dried the egg pods at 65°C for 72 h, and reweighed the egg pods for dry mass to calculate the egg water content with a precision of +/− 0.01 mg. All treatments had 6 replicates.

Main Effects of Soil Moisture on Egg Hatching Time and Survival

To measure the effect of soil moisture on hatching time and survival, we incubated the cups with egg pods at moistened with 2, 5, 8, 11 and 14% and at 26°C (the favorable temperature for grasshopper egg development). The egg pods were examined daily to record the number of hatchlings and the number of dead grasshopper eggs in each treatment. After 60 days, we considered any remaining eggs to have entered diapause. We incubated these eggs in diapause at 4°C for 60 days to break diapause and then moved them to 26°C. We kept all treatments at 26°C for another 60 days in post-diapause development and recorded the number of hatchlings daily. At the end of the experiment, all egg pods were dissected to examine the number of living eggs. All treatments had 6 replicates.

Interactive Effects of Soil Temperature and Moisture on Egg Hatching Time and Survival

We used 2 and 11% as the lowest and highest soil moisture based effect of soil moisture on egg water content, and 26°C and 35°C as normal and high soil temperatures based on local field meteorological data (Wu et al., 2012) to form four cross treatments (11% and 35°C, 11% and 26°C, 2% and 35°C, 2% and 26°C), respectively. First, we incubated plastic cups with twenty egg pods of each grasshopper species and silver sand of 2 and 11% moisture at 26°C for 60 days to confirm that all eggs had entered diapause. Second, we incubated all cups at 4°C for 60 days to terminate egg diapause. Finally, we incubated cups at either 26°C or 35°C, resulting in four cross treatments of temperature and moisture. We recorded the number of hatched eggs and deaths from all treatments daily for 60 days. Because *C. fallax* is a non-diapause species, we skipped the incubation at 26°C for prediapause development and 4°C for diapause termination and directly placed *C. fallax* eggs into the four cross treatments of temperature and soil moisture. At the end of the experiment, we dissected all the egg pods and recorded the number of unhatched eggs alive or dead. All treatments had 6 replicates.

Effect of Embryonic Stage and Drought Duration on Egg Hatching Time and Survival

To determine the embryonic stage during which eggs are most sensitive to drought and how drought exposure duration affects the hatching time and hatching rate of the three grasshopper species, we designed experiments in which the embryonic stage (eggs with prediapause development for 5 and 15 days before we initiated drought treatment) and drought exposure duration varied. We took grasshopper eggs that had developed for 5 and 15 days at 26°C and 8% sand moisture. Eggs were then transferred to cups filled with anhydrous sand (0% moisture) for 0, 5, 10, 15, or 20 days at 26°C. Afterward, we returned the eggs to 8% soil moisture conditions for 60 days and continually incubated unhatched eggs at 4°C for 60 days to terminate diapause. We finally incubated postdiapause eggs at 26°C and 8% moisture for another 60 days. We recorded the number of hatchlings and dead eggs during pre- and postdiapause development daily. We dissected egg pods and recorded the number of live and dead eggs at the end of the experiment. All treatments had 6 replicates.

Generally, egg development at 5 and 15 days under normal conditions is associated with the embryonic stages of prediapause (stages 10–11 and stages 16–17, respectively). In this experiment, we defined a standard variation index (VI) to compare the sensitivity of the response of egg hatching time and hatching rate to drought exposure duration among the different grasshopper species. VI = (egg hatching time (or hatching rate) of drought treatment—egg hatching time (or hatching rate) of control treatment)/egg hatching time of control treatment. Higher values indicate greater sensitivity of the hatching time or hatching rate of grasshopper eggs to drought exposure duration.

Data Analysis

We used three- and two-way ANOVAs to examine the main and interactive effects of species, soil moisture, soil temperature, embryonic development stage, and drought duration on egg hatching time and hatching rate of the three grasshopper species. We performed one-way ANOVAs to assess variation in egg water content, egg hatching time and hatching rate among different soil moisture levels for each grasshopper species. We used linear regressions and polynomial analyses to measure the relationships between soil moisture and egg water content as well as between egg hatching time and hatching rate for each grasshopper species. The slopes of the regression models were used to compare the sensitivity of the three grasshopper species to changes in soil moisture. We followed ANOVAs with *post hoc* analyses (Duncan test; alpha = 0.05) to assess differences among soil moisture levels. In this study, we referred to hatching time as the duration required for 50% of eggs to hatch in each treatment and the hatching rate as the number of eggs hatched at the end of each trial/the number of eggs at the beginning of each trial. We conducted all statistical analyses using SPSS 16.0 software (SPSS Inc., Chicago, Illinois, United States).

RESULTS

Effect of Soil Moisture on Grasshopper Egg Water Content, Hatching Time and Hatching Rate

Before treatments, the egg water content of the mid-season species, *O. asiaticus* (39.65%), and late-season species, *C. fallax* (38.43%), was much higher compared with the early-season species, *D. barbipes* (30.86%). When incubated at 18^{o}C for 5 days, the egg water content differed among grasshopper species prior to any manipulative experiments [$F_{(2,\ 60)} = 145.26$, $P < 0.01$]. The egg water content of the three grasshopper species was positively correlated with soil moisture (**Figures 1A–C**), but the response was stronger in the xeriphilic species *D. barbipes* and *O. asiaticus* than in the mesic species *C. fallax*. ANOVA indicated that soil moisture levels significantly affected the egg water content of *D. barbipes* [$F_{(4,20)} = 46.91$, $P < 0.01$; **Figure 1A**] and *O. asiaticus* [$F_{(4,20)} = 9.96$, $P < 0.01$; **Figure 1B**]. Soil moisture only had a marginally significant effect on the egg water content of *C. fallax*. The egg water content was only markedly decreased under 2% soil moisture compared with eggs at other soil moisture levels [$F_{(4,20)} = 2.83$, $P = 0.05$; **Figure 1C**].

At the five levels of soil moisture at 26°C, there were significant differences in egg hatching time [$F_{(2,75)} = 713.57$, $P < 0.01$; **Figures 1D–F**] among the three grasshopper species. The eggs of the mid-season species *O. asiaticus* required a longer period to reach 50% hatching (24.38 days) compared with the eggs of the late-season species *C. fallax* (14.94 days) and early-season species *D. barbipe*s (12.50 days). The egg hatching time of the three grasshopper species was significantly negatively correlated with soil moisture (**Figures 1D–F**). The egg hatching times of *D. barbipes* and *O. asiaticus* were more sensitive to soil moisture than those of *C. fallax*. The egg hatching times of *D. barbipes* [$F_{(4,25)} = 64.83$, $P < 0.1$; **Figure 1D**] and *O. asiaticus* [$F_{(4,25)} = 7.01$, $P < 0.1$; **Figure 1E**] in the 2% soil moisture treatment were significantly longer than those in the other soil moisture treatments. In contrast, there was no difference in egg hatching time among moisture levels for *C. fallax* [$F_{(4,25)} = 1.39$, $P = 0.27$; **Figure 1F**].

There were significant differences in egg hatching rate [$F_{(2,75)} = 567.69$, $P < 0.01$; **Figures 1G–I**] among the three grasshopper species. The egg hatching rate of *C. fallax* (79.47%) was significantly higher than that of *D. barbipe*s (35.80%) and *O. asiaticus* (12.50%) Regardless of grasshopper species, the relationships between egg hatching rate and soil moisture were unimodal, and the highest egg hatching rates were observed at 8 and 11% soil moisture (**Figures 1G–I**). The egg hatching rates of *D. barbipes* at 5, 8, and 11% soil moisture were significantly higher than those at the two extreme soil moisture levels (2 and 14%) [$F_{(4,25)} = 10.24$, $P < 0.01$; **Figure 1G**]. The egg hatching rate of *O. asiaticus* was higher at 11% soil moisture than at other soil moisture levels [$F_{(4,25)} = 3.17$, $P = 0.03$; **Figure 1H**]. The egg hatching rate of *C. fallax* was not significantly affected by soil moisture [$F_{(4,25)} = 0.51$, $P = 0.73$; **Figure 1I**]. In general, 8 and 11% soil water content at 26°C resulted in the shortest hatching time and the highest hatching rates regardless of species.

Interactive Effects of Soil Temperature and Moisture on the Egg Hatching Time and Rate

Averaged across the two soil moisture treatments, the higher temperature (35°C) significantly shortened the egg hatching time and reduced the hatching rate of the three grasshopper species compared with the treatments at 26°C (all $P < 0.01$; **Table 1** and **Figures 2A–C**). Averaged across the two soil temperatures and three grasshopper species, 2% soil moisture prolonged the egg hatching time and decreased the hatching rate compared with 11% soil moisture (all $P < 0.01$; **Table 1** and **Figures 2A–C**). There were no interactive effects of soil temperature and moisture on the egg hatching time and hatching rate of the three grasshopper species. Furthermore, there were interactive effects between soil temperature and grasshopper species and between soil moisture and grasshopper species on the hatching time and hatching rate of the three grasshopper species (all $P < 0.01$; **Table 1**). Thus, grasshopper species significantly differed in their responses to soil temperature and moisture combinations.

Soil temperature and moisture have significant effects on the egg hatching time and hatching rate of each grasshopper species. Averaged across the two soil moistures, 35°C soil temperature significantly shortened the hatching time of *D. barbipes*, *O. asiaticus* and *C. fallax* by 5.63, 4.75, and 2.73 days, respectively (all $P < 0.05$). However, the soil temperature at 35°C significantly reduced the egg hatching rate of *D. barbipes* by 18%comparedwiththat at 26°C. Averaged across the two soil temperatures, 2%soil moisture increased the hatching time of *D. barbipes*, *O. asiaticus* and *C. fallax* by 0.69, 11.01, and 0.31 days, respectively (all $P < 0.05$). The soil moisture at 2% reduced the egg hatching rate of *D. barbipes* by 10% compared with that under 11% soil moisture ($P < 0.01$; **Figure 2D**). No interactive effects of soil temperature and moisture on the egg hatching time and hatching rate of the three grasshopper species were detected (**Table 1** and **Figures 2E,F**).

Effects of Embryonic Developmental Stage and Drought Duration on Egg Hatching Time and Hatching Rate

A standard VI was used to compare the response sensitivity of egg hatching time and hatching rate to drought exposure duration between different grasshopper species. Averaged over the three grasshopper species, embryonic stage had no effect on the response of VI of hatching time to drought exposure duration. The hatching time was prolonged with increasing

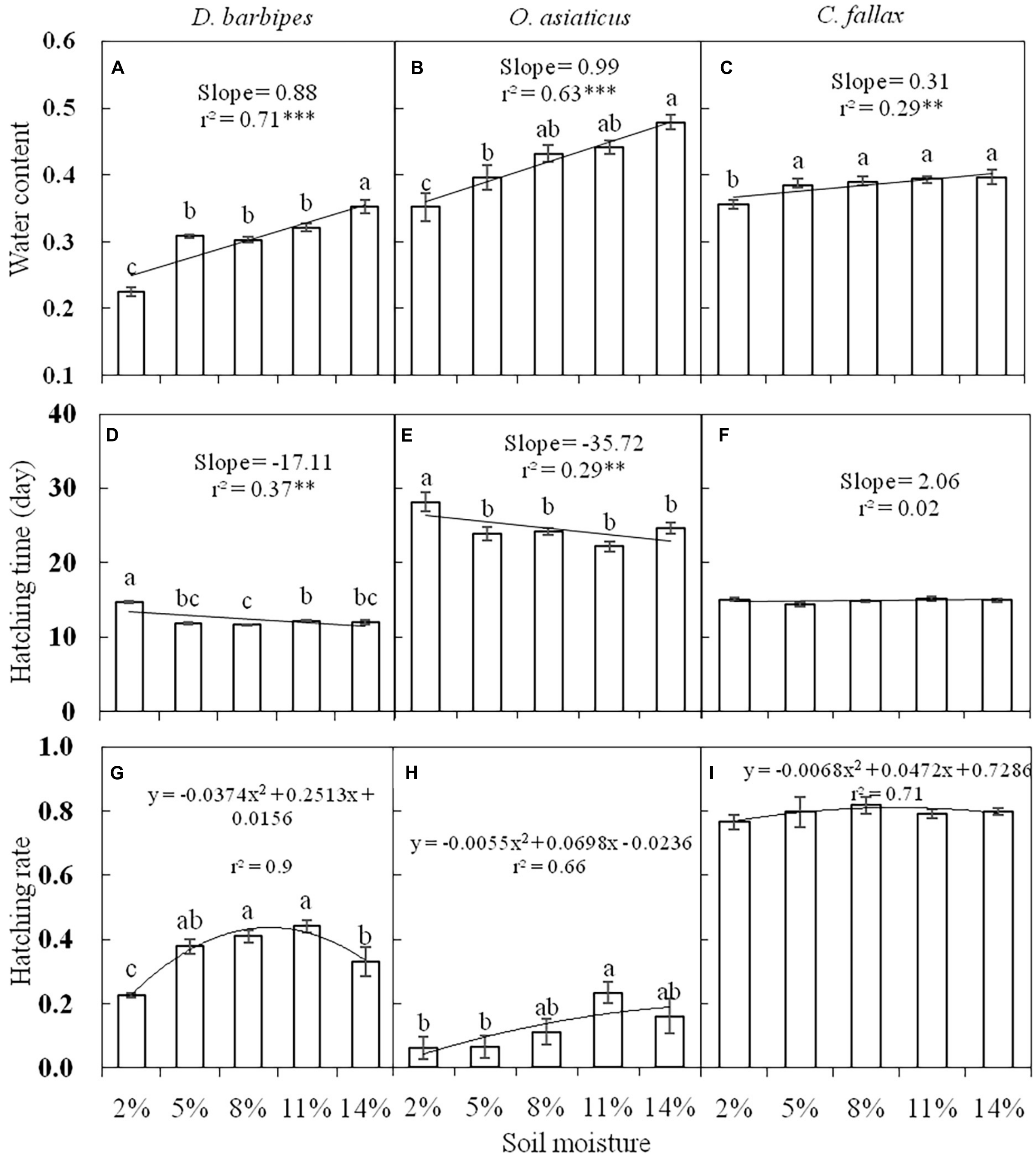

FIGURE 1 | The water content **(A,B,C)**, hatching time **(D,E,F)**, and hatching rate **(G,H,I)** of eggs of *D. barbipes, O. asiaticus* and *C. fallax* in different soil moisture treatments. Regression parameters were estimated using linear models with soil moisture as a continuous predictor. Significant regressions are indicated by *($P < 0.05$), **($P < 0.01$), and ***($P < 0.001$). Lowercase letters above bars indicate within-species differences based on Tukey HSD *post hoc* analyses following ANOVA. Data are mean ± SE ($n = 6$).

drought exposure duration ($P < 0.01$). There was no interactive effect of embryonic stage with drought exposure duration on the VI of hatching time of grasshopper eggs (**Table 1**).

There were significant interactive effects between drought exposure duration and grasshopper species on the hatching time of grasshopper eggs [$F_{(6,120)} = 44.36$, $P < 0.01$]. Drought exposure duration significantly increased the VI of hatching time of *D. barbipes* [$F_{(3,\ 40)} = 7.58$, $P < 0.01$], *O. asiaticus* [$F_{(3,40)} = 4.25$, $P < 0.05$] and *C. fallax* [$F_{(3,40)} = 52.76$, $P < 0.01$]. The eggs of the three grasshopper species were sensitive to varied drought exposure durations. The VI of egg hatching time for *C. fallax* (0.87) was much larger than that of *D. barbipes* (0.03) and

TABLE 1 | Results (*F* values) of two-way ANOVAs of the effect of soil temperature (ST) and soil moisture (SM), embryonic development stage, and drought exposure duration on the variation index (VI) of hatching time and hatching rate of eggs of three grasshopper species.

Source	***Df***	***D. barbipes***		***O. asiaticus***		***C. fallax***	
		Hatching time	**Hatching rate**	**Hatching time**	**Hatching rate**	**Hatching time**	**Hatching rate**
ST	1	1378.53^{c}	36.94^{c}	5.68^{a}	0.60	344.93^{c}	0.00
SM	1	20.80^{b}	10.48^{b}	30.58^{c}	1.68	4.47^{a}	0.51
ST × SM	1	2.53	0.32	0.69	0.02	1.46	0.94
Error	20						
Stage	1	0.03	3.55	4.83^{a}	0.86	0.04	10.33^{c}
Drought	3	7.58^{b}	2.27	4.25^{b}	1.73	52.76^{c}	8.70^{c}
Stage × Drought	3	1.64	1.58	0.57	0.45	1.70	1.16
Error	40						

$n = 6$ (Each treatment was repeated 6 times). Superscript letters denote significant differences between treatments. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

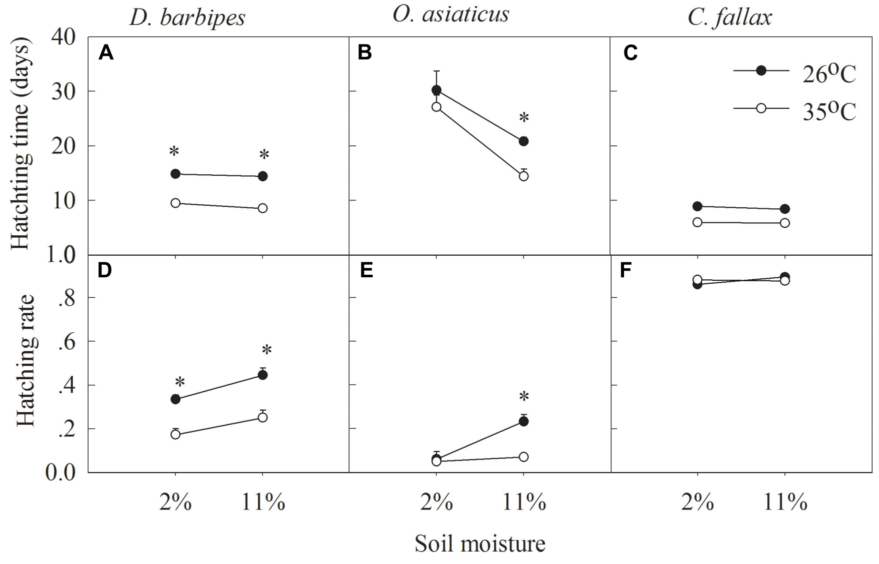

FIGURE 2 | Hatching time **(A,B,C)** and hatching rate **(D,E,F)** of eggs of *D. barbipes, O. asiaticus* and *C. fallax* in different combined treatments of soil temperature and moisture. Data are mean ± SE ($n = 6$). *Indicates a significant difference between the two temperature treatments at the same soil moisture level at $P < 0.05$.

O. asiaticus (0.07) [$F_{(2,120)} = 519.86$, $P < 0.01$; **Figures 3A–C**]. Thus, the egg hatching time of *C. fallax* was the most sensitive to prolonged drought duration in the three grasshopper species. Regression analysis also showed that the VI of egg hatching time of *D. barbipes* and *C. fallax* was positively correlated with drought exposure duration at the two embryonic stages.

Averaged over the three grasshopper species, the embryonic stage had no effect on the VI of the hatching rate of grasshopper eggs. Drought exposure duration had a significant effect on the VI of the hatching rate of grasshopper eggs [$F_{(3,40)} = 4.30$, $P < 0.01$]. Drought exposure duration significantly reduced the VI of the hatching rate of *C. fallax* but had no effect on that of *O. asiaticus* and *D. barbipes* (**Table 1** and **Figures 3D–F**). There were no interactive effects of embryonic stage and drought exposure duration on the VI of the hatching rate of grasshopper eggs (**Table 1**).

There were interactive effects of embryonic stage and grasshopper species on the VI of the hatching rate of grasshopper eggs [$F_{(2,120)} = 3.59$; $P < 0.05$]. The VI of the hatching rate of *D. barbipes* eggs with prediapause development for 15 days was marginally significantly [$F_{(1,40)} = 3.55$; $P = 0.07$] higher than that of eggs with prediapause development for 5 days. No differences existed in the VI of the hatching rate of *O. asiaticus* at 5 and 15 days of prediapause development. However, the VI of the hatching rate of *C. fallax* at 15 days of prediapause development was significantly lower than that at 5 days of prediapause development (**Figures 3D–F**).

The responses of egg hatching rate to drought exposure duration in the three grasshopper species varied. The VI of the egg hatching rate of *D. barbipes* was negatively correlated with drought exposure duration at the embryonic stage of prediapause development for 5 days. The VI of the egg hatching rate of *O. asiaticus* varied with drought exposure duration in the form of a binomial curve. The VI of the egg hatching rate of *C. fallax* was negatively correlated with drought exposure at the two embryonic stages.

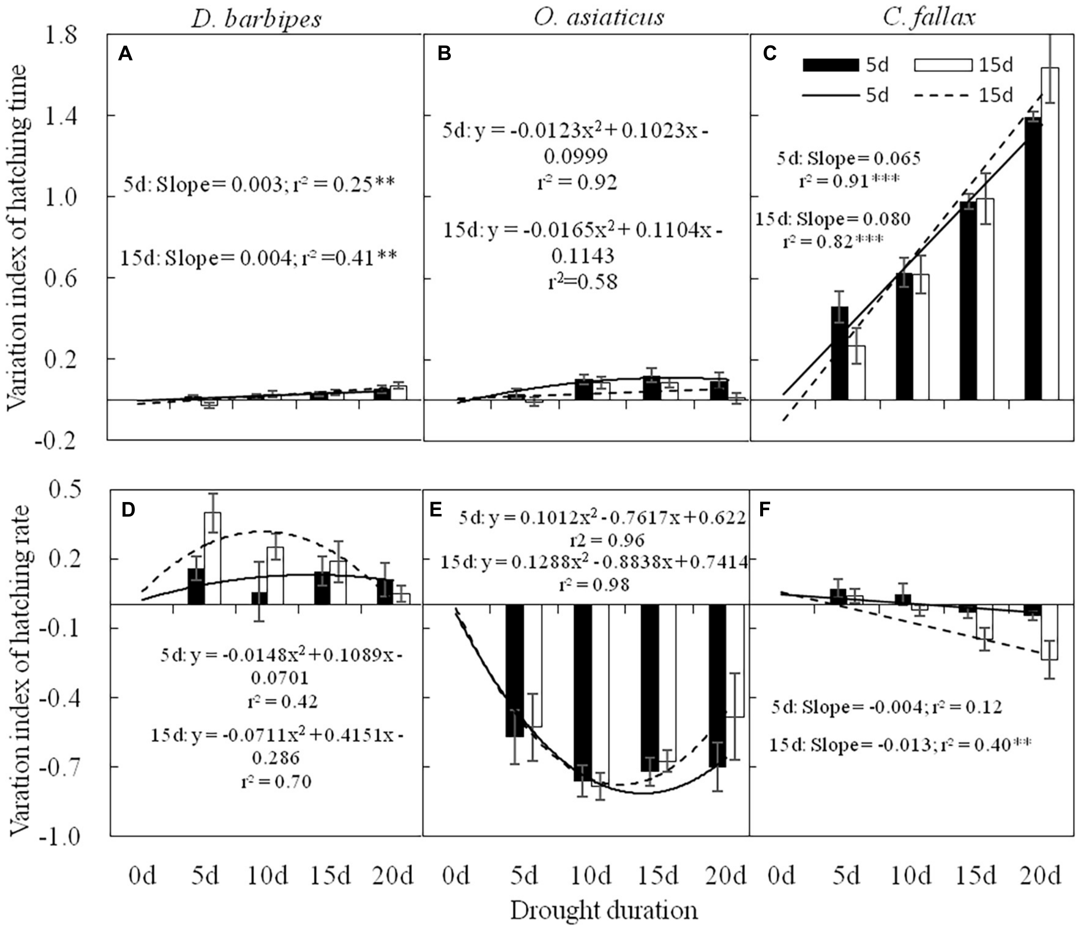

FIGURE 3 | Variation index (VI) of hatching time **(A,B,C)** and hatching rate **(D,E,F)** of eggs of *D. barbipes, O. asiaticus* and *C. fallax* predeveloping for 5 and 15 days under different drought durations. VI = (treatment-control)/control. VI > 0 indicate an increase in the treatment compared with the control; VI = 0, means no change in the treatment compared with the control; and < 0, indicates a decrease in the treatment compared with the control. Regression parameters were estimated using linear models with drought duration as a continuous predictor. Significant linear regressions are indicated by *($P < 0.05$), **($P < 0.01$), and ***($P < 0.001$). Data are mean ± SE ($n = 6$).

DISCUSSION

In this study, the water content, hatching time and hatching rate of eggs of three grasshopper species exhibited species-specific responses to variation in soil moisture and soil temperature. The embryonic development stages were highly sensitive to drought exposure duration. The response of the egg hatching rate to soil moisture clearly differed from that of egg hatching time and egg water content in the three grasshopper species. The distinct responses of grasshopper eggs reflect species-specific ecological traits associated with their phenology and habitat use (Stauffer et al., 2011).

Grasshopper Species Show Distinct Responses to Soil Moisture Variation

The egg water content of the three grasshopper species was positively correlated with changes in soil moisture, although the changes in soil moisture did not greatly affect the water content of *C. fallax*. The different responses of the eggs of the three grasshopper species to soil moisture may contribute to the different structures of egg pods. Egg pods of *D. barbipes* and *C. fallax* were generally wrapped by a compound of froth and sand, which can permit eggs to with stand dehydration or saturation under extreme soil conditions; the crust of the egg pod of *C. fallax* was harder and tighter than that of *O. asiaticus* and *D. barbipes*. Therefore, the egg water content of the egg pods of *C. fallax* was more stable in extremely dry or moist environments egg pods. The weak response of the egg water content of *C. fallax* to soil moisture also supports this observation. The linear responses of the egg water content of *O. asiaticus* to increasing soil moisture were similar to those of the migratory locust (*Locusta migratoria*) (Qi et al., 2007) because their egg pods both have weakened and spongy crust structures. Therefore, the higher sensitivity of *O. asiaticus* to soil moisture compared with *D. barbipes* and *C. fallax* suggested that the crust of the egg pods had a greater buffer capacity to changes in soil moisture.

In addition, soil type, texture and pH, which also affect the water uptake of grasshopper eggs from soil, are important for the embryonic development and survival of grasshopper eggs (Mukerji and Gage, 1978; Monk, 1985; Johnson and Worobec, 1988; Skinner and Child, 2000). Overall, based on the relationships between egg water content and soil moisture in our experiments, nearly 8% soil moisture is optimal for the egg development and survival of the three grasshopper species.

The different responses of egg hatching time and hatching rate of the three grasshopper species to soil moisture may be attributed to their distinct life history strategies. The egg water content, hatching time and hatching rate of *D. barbipes* and *O. asiaticus* were more sensitive to changes in soil moisture compared with *C. fallax*. In fact, *D. barbipes* and *O. asiaticus* occur in relatively dry habitats, whereas *C. fallax* occupies mesophilous environment with tall grass with higher soil moisture (Kang and Chen, 1994a,b). This indicates that grasshopper species occupying wet habitats are better adapted to higher soil moisture, whereas grasshopper species occupying dry habitats are better adapted to lower soil moisture. Although the egg hatching time and hatching rate of *C. fallax* were not affected by 5–14% soil moisture, they were delayed and reduced, respectively, under increased drought exposure duration. These results indicate that *C. fallax* was more sensitive to drought than to increases in soil moisture. Habitat, including vegetation structure, orientation of the sun, and the extent of shelter from wind, can greatly affect the adaptation of insects to environmental moisture (Wingerden et al., 1991; Stauffer et al., 2011). Thus, difference in the crust structure of the egg pods, habitat use and diapause traits can account for the distinct responses of the three grasshopper species to variation in soil temperature and soil moisture.

Soil Temperature and Soil Moisture Were Important Factors Affecting the Development and Survival of Grasshopper Eggs

Higher soil temperature shortened the hatching time of the three grasshopper species. Soil temperature in early spring and late autumn is close to the low threshold for grasshopper development, and warming in spring or autumn significantly facilitates the embryonic development of grasshopper eggs (Wu et al., 2012). Another study indicated that soil moisture and temperature could account for 99% of the variance in egg hatching time (Mukerji and Gage, 1978; Powell et al., 2007). Moreover, higher soil temperature significantly reduced the egg hatching rate of early-season *D. barbipes*, indicating that *D. barbipes* is better adapted to lower soil temperatures. Soil temperature is thus an important environmental factor affecting the hatching and survival of grasshopper eggs, especially for early-season grasshopper species. Our data suggested that climate warming may affect the fitness and distribution of grasshoppers in temperate grasslands by altering their phenology and survival.

The hatching time of the three grasshopper species was prolonged at 2% soil moisture compared with 11% soil moisture. In addition, the reduced hatching rate of *D. barbipes* at 2% soil moisture indicated that soil moisture was another crucial environmental factor regulating the hatching time and survival of grasshopper eggs. Our results are consistent with the fact that soil moisture could explain 83% of the variance in egg mortality (Mukerji and Gage, 1978).

Although increased drought duration delayed the egg hatching time and reduced the egg hatching rate of the three grasshopper species, 2% soil moisture and 20 days drought exposure could not prevent egg hatching. Eggs of a tropical grasshopper species can hatch in soil moisture as low as 1% (Gehrken and Doumbia, 1996). Therefore, within a specific temperature and moisture spectrum, soil temperature may have greater effects than soil moisture on egg hatching time and hatching rate, especially for early-season grasshopper species.

There were no marked interactive effects of soil temperature and moisture on the egg hatching time and hatching rate of the three grasshopper species. This result is inconsistent with previous studies, in which warm and dry weather in spring have been shown to potentially favor the completion of embryonic development and hatching (Pickford, 1966). Warm and dry conditions in autumn or cool and wet conditions in spring are not favorable for grasshopper populations because these conditions reduce embryonic development and the survival of eggs (Powell et al., 2007). Indeed, some studies have shown that the mortality of locust eggs increases under long-term exposure to low temperature and high moisture (Qi et al., 2007). These studies indicate that the combined effects of temperature and moisture are seasonal or species-specific.

Species-Specific Responses of Hatching Time and Hatching Rate at Different Embryonic Stages to Drought Exposure Duration

The embryonic stages sensitive to environmental drought exposure varied among the different grasshopper species. Previous studies have reported that the inception of diapause is dependent on the temperature and moisture conditions at the time when eggs are laid (Wardhaugh, 1980). The diapause stage of grasshopper eggs is more tolerant of extreme soil drought conditions than the no-diapause stage (Gehrken and Doumbia, 1996; Bale et al., 2002). Therefore, diapause can buffer eggs from the negative effects of drought on embryonic development in *D. barbipes* and *O. asiaticus,* which enter diapauses early (stages 16–17) after developing for 15 days at normal temperature. Therefore, the egg hatching rates of *D. barbipes* and *O. asiaticus* after development for 15 days at normal temperature were not significantly affected by drought exposure duration. Our results indicated that the egg diapause of grasshoppers plays an important role in resisting environmental drought stress. Similarly, summer egg diapause in a matchstick grasshopper synchronizes the life cycle and buffers thermal extremes (Kearney et al., 2018).

The unchanged responses of eggs developing for 5 days at normal temperature of the three grasshopper species may be attributed to the early embryonic stage, as the physiological processes of grasshopper eggs may not yet be sensitive to soil moisture change. Grasshopper eggs require little water to develop before the anatrepsis stage (Pickford, 1966; Gehrken and Doumbia, 1996). The physiological sensitivity and biotic properties of grasshopper eggs in postdiapause development are important for estimating their biological response to climate change (Deutsch et al., 2008; Laws and Belovsky, 2010).

CONCLUSION

Although the development and survival of grasshopper eggs are regulated by complex environmental factors, our results suggested that soil temperature was the most important factor affecting the hatching time and hatching rate of grasshopper eggs in temperate grassland. The difference in habitat use, the crust structure of the egg pods, and diapause traits can account for the distinct responses of the three grasshopper species to variation in soil temperature and soil moisture. These findings enhance our ability to predict future changes in grasshopper populations and aid the sustainable control of temperate grasslands.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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No Evidence for Effects of Ecological and Behavioral Factors on Eye Size Evolution in Anurans

Chuan Chen[1,2,3†], Ying Jiang[1,2,3†], Long Jin[1,2,3†] and Wen Bo Liao[1,2,3*]

[1] Key Laboratory of Southwest China Wildlife Resources Conservation (Ministry of Education), China West Normal University, Nanchong, China, [2] Key Laboratory of Artificial Propagation and Utilization in Anurans of Nanchong City, China West Normal University, Nanchong, China, [3] Institute of Eco-adaptation in Amphibians and Reptiles, China West Normal University, Nanchong, China

Eye size varies markedly among taxonomic levels, and this variation is often related to the patterns shaped by phylogeny and ecological and behavioral factors. The selective pressures underlying eye size evolution are especially studied in fishes, anurans, birds, and mammals. However, selective pressures underlying the eye size evolution in anurans have inconsistent scaling rules. Here, we investigated the links between eye size and both ecological (e.g., light availability and habitat type) and behavioral factors (e.g., activity time, foraging mobility, defensive strategy, and mating system) among 252 species of anurans by using phylogenetically controlled generalized least-squared (PGLS) regression. Results show that anuran eye size scales hypo-allometrically with body size. However, eye size was not significantly influenced by ecological and behavioral factors, including habitat type, activity time, light availability, foraging mobility, defensive strategy, and mating system. Therefore, neither ecology nor behavior plays a key role in promoting eye size evolution in frogs.

Keywords: anurans, behavioral factors, body size, eye size, hypo-allometrical relationship

Edited by:
Bao-jun Sun,
Institute of Zoology, Chinese Academy of Sciences (CAS), China

Reviewed by:
Yang Wang,
Hebei Normal University, China
Shaobin Li,
Yangtze University, China
Guangzhan Fang,
Chinese Academy of Sciences (CAS), China

****Correspondence:***
Wen Bo Liao
Liaobo_0_0@126.com

†These authors have contributed equally to this work

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INTRODUCTION

The eyes can extract and exploit the information transmitted by light in animals at taxonomic groups (Land and Nilsson, 2012). Eye size varies extensively from tiny to giant among different animals (Martin, 1993; Land, 2009; Land and Nilsson, 2012). The size and dimension of an eye heavily affect the visual system and the abundance and quality of visual information, where animals extract from different environments (Walls, 1942). Although eye acuity is directly associated with eye size and lens diameter, it is also associated with other factors such as quality of the optic component, the angular spacing of the receptors, and the diameter of the photoreceptor (Veilleux and Kirk, 2014). Larger eyes possess larger abundance of photoreceptors and size of images, resulting in longer focal length for determining the retinal area size, where the images of the objects spread (Martin, 2007). Hence, selection for the increased resolving power leads to the enlarged eye size through the longer focal length (Martin, 1983).

While larger eyes can obtain information more effectively from changing environments compared with normal ones, eye size is physically and developmentally constrained (Huber et al., 1997; Moran et al., 2015). Considering the cost associated with benefiting from better vision by providing increased image resolution or enhance sensitivity to light, the eyes may reduce or even lose during over evolutionary periods in species that move into dark or murky ecosystems (Caves et al., 2017; Porter and Sumner-Rooney, 2018). In elasmobranchs, relative and absolute eye size varies considerably and species with smaller eyes tend to be costal, while species with larger eyes are oceanic species living in deep and dark environments (Lisney and Collin, 2007). Moreover, eye size is affected by the activity time, where nocturnal species have larger eye size associated with higher acuity than diurnal species (e.g., mammals: Veilleux and Kirk, 2014; birds: Garamszegi et al., 2002; Hall and Ross, 2007; geckos: Werner and Seifan, 2006).

Eye size is associated with behavioral factors, such as predator avoidance and prey tracking (Garamszegi et al., 2002; Møller and Erritzøe, 2010; Cronin et al., 2014; Thomas et al., 2020). For instance, predator pressure promotes eye size evolution in preys, because the better processing of visual information in species with large eyes enhance the survivability of birds and fishes (Douglas and Hawryshyn, 1990; Land and Nilsson, 2012; Starunov et al., 2017). Moreover, the cutaneous secretion of toxin is one of the main defensive mechanisms against predator risk, and species with granular glands may experience lower predator risk in anurans (Toledo and Jared, 1995; Prates et al., 2012; Huang et al., 2019). As a result, species with granular glands are associated with smaller eye size compared with species with non-granular glands.

For anurans, investigating the relationships of eye size and both ecological and behavioral factors among 44 species have revealed that eye size is not associated with activity time, foraging mobility, habitat type, defensive strategy, mating system, and water turbidity (Huang et al., 2019). However, a recent study has evaluated the eye size and six traits of natural history hypothesized to be related to eye size evolution among 220 species of anurans, and the results indicate that eye size is correlated with adult mating habitat and activity patterns (Thomas et al., 2020). Although anuran eye size was studied outside of a few families, potential relationships between eye size and ecological and behavioral factors are unclear.

In the present study, we investigated the relationships between eye size and both ecological (e.g., light availability and habitat type) and behavioral factors (e.g., defensive strategy, activity time, foraging mobility, and mating system) in 252 anuran species within seven families in China. First, we predicted that species living in aquatic habitats would display increased eye size, because they inhabit light environments. Second, we predicted that nocturnal species would have large eyes to maximize sensitivity in low-light conditions. Third, we predicted that species approaching slowly and capturing prey would have smaller eyes than species approaching quickly and often chasing prey. Finally, we predicted that species with granular glands would possess small eyes, because they experience low predator risk.

MATERIALS AND METHODS

Data Collection

The data of eye diameter and snout-vent length (SVL) were collected from "Fauna Sinica," which includes 3,171 males and 1,820 females in 252 anuran species (Fei et al., 2009). We calculated the average eye size and SVL based on males and females for all analyses (**Supplementary Table 1**). All species in this study have available phylogenetic information.

Categorical Variables

Following the method of Thomas et al. (2020) and our filed observation, adult habitat type for each species on a six-point scale were classified into scansorial [primarily associated with plants; up off the ground (arboreal/shrubs/reeds)], ground-dwelling (primarily active on the ground), subfossorial (primarily active under leaf litter; shallow burrowers), fossorial [primarily active in deeper burrows (not burrowing simply for aestivation/long periods of inactivity)], aquatic (primarily found in water; rarely/never leaves water), and semi-aquatic (strongly associated with/commonly found in water but also frequently uses land habitats). Activity time was classified on a two-point scale, namely, nocturnal (primarily active at night), and both diurnal and nocturnal (active during both day and at night) (Huang et al., 2019). Foraging mobility was categorized based on behavioral phenotype, including species approaching slowly and capturing prey and species approaching quickly and often actively chasing prey (Huang et al., 2019). Light availability of species was classified into strong light (i.e., forest is an open environment) and weak light (i.e., light does not penetrate much) (Huang et al., 2019). Defensive strategy was also classified on a two-point scale, namely, species with granular glands in dorsal and ventral skin and species with non-granular glands in skin (Huang et al., 2019). Mating system for each species was classified as polyandry and monandry (Zeng et al., 2016).

Phylogeny

The molecular phylogeny was constructed in the 252 species based on three mitochondrial genes and three nuclear genes (**Figure 1**). The mitochondrial genes included the large and small subunits of the mitochondrial ribosome genes (12S/16S) and cytochrome b (CYTB). The nuclear genes included the rhodopsin (RHOD), the tyrosinase (TYR), and the recombination-activating gene 1 (RAG1). All sequences were aligned using the MUSCLE function in MEGA v.6.0.6 (Tamura et al., 2013), and we determined the best nucleotide substitution model of each gene by using the Akaike Information Criterion in jModelTest v.2.1.2 (Darriba et al., 2012). The best substitution model was TYR, HKY + G for RAG1 and RHOD, GTR + G for 12S and GTR + I + G for CYTB and 16S. Considering the lack of fossil dates, we used these models to construct the phylogenies based on BEAUTi and BEAST v.1.8.3 (Drummond et al., 2012; Mai et al., 2019; Mai et al., 2020), with a relaxed uncorrelated lognormal clock, unlinked substitution models, a Yule speciation process, and no calibration points. The effective sample size values for each tree statistics showed the satisfying convergence of the Bayesian chain and adequate model mixing in Tracer v.1.6.0 (Rambaut and Drummond, 2014). Then, we generated a maximum clade credibility tree with a 20% burn-in and mean node heights before ending the analysis by using TreeAnnotator v.1.8.3 (Drummond et al., 2012; Chen et al., 2021).

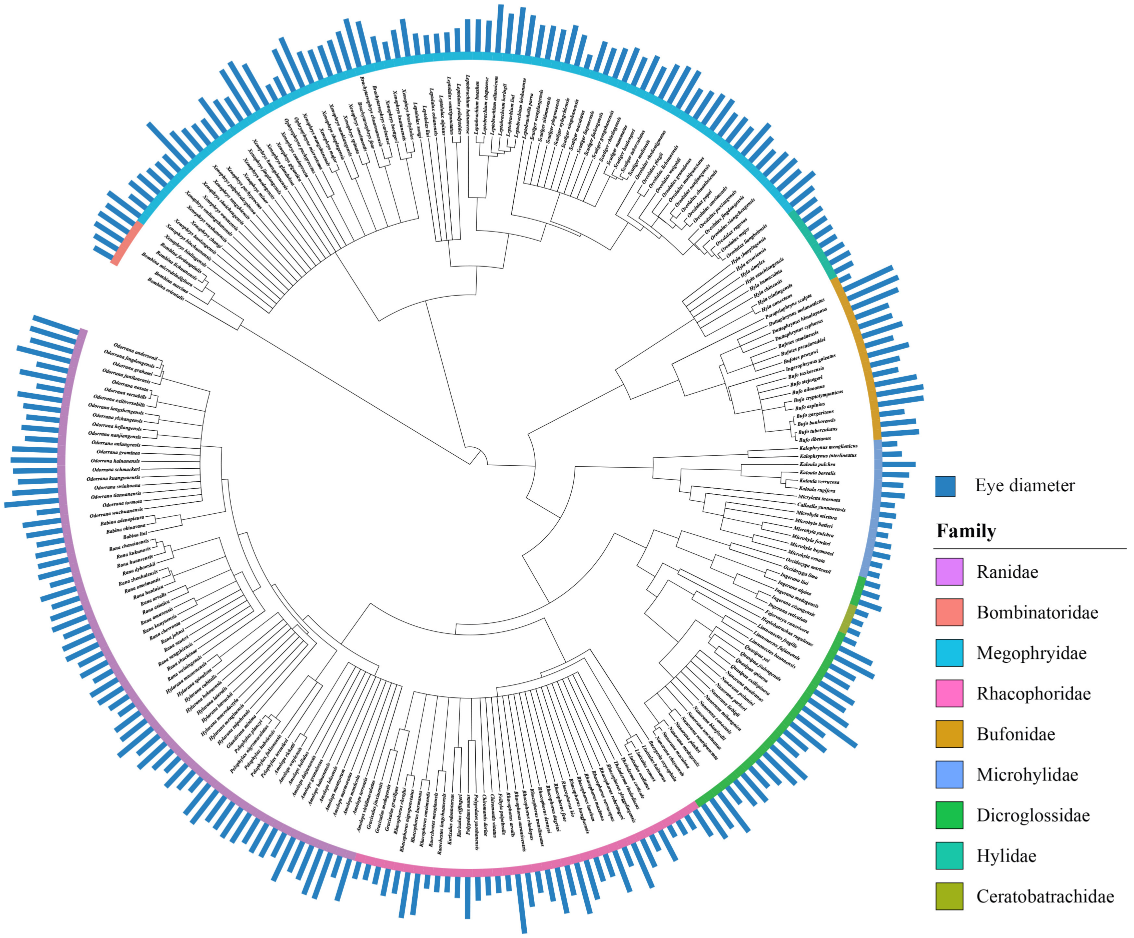

FIGURE 1 | Phylogenetic tree of the 252 Chinese anuran species on the basis of the three nuclear genes (RAG1, RHOD, TYR) and the three mitochondrial genes (CYTB, 12S, 16S) using TreeAnnotator v.1.8.3 in the comparative analysis. Phylogeny shows means of eye diameter in anurans of species. The color indicates family in frogs.

Data Analysis

All data analysis were performed in the statistical software R v. 4.1.0 (R Development Core Team, 2021). We analyzed the evolutionary link between eye and body size among species by using phylogenetically controlled generalized least-squared (PGLS) regression with $\log_{10}$-transformed data in the APE-package in R software (v. 5.4-1) (Paradis, 2012; Cai et al., 2020; Huang et al., 2020). In PGLS analysis, we estimated the phylogenetic scaling parameter (λ) by using the maximum-likelihood method. In the model residuals, λ represented the phylogenetic signal. The parameter λ estimated the effect of phylogenetic signal on the relationship between eye and body size ($\lambda = 1$ indicates strongly phylogenetic signal, and $\lambda = 0$ indicates no phylogenetic signal). For comparison with allometric studies, where the phylogenetical relationships were corrected, we used the ordinary least-squares (OLS) regressions in stats v. 4.1.0 (R Development Core Team, 2021) and the standardized major axis (SMA) regressions in smatr v. 3.4.8 (Warton et al., 2012) with the same $\log_{10}$-transformed data.

For testing the effect of ecological and behavioral factors on eye size, we used multiparameter fixed analysis in PGLS regression to test whether variables were associated with eye size. To test the effect of each of ecological and behavioral factors on eye size evolution, we first use phylogenetic ANCOVAs conducted through caper package of PGLS models with SVL as a covariate (e.g., ED∼SVL* habitat type) and adult habitat, activity time, foraging mobility, light availability, defensive strategy or mating system as a fixed effect (Thomas et al., 2020). Besides, we ran three models (including OU, BM, and Pagel) in PGLS for each variable to avoid overparameterizing model data redundancy (Thomas et al., 2020).

RESULTS

Eye Size Scales Hypo-Allometrically With Snout-Vent Length

A hypo-allometric (slope < 1) interspecific scaling was observed between eye size and SVL in 252 species of anurans (PGLS:

slope = 0.818, R^2 = 0.821, $P < 0.0001$, λ = 0.415; **Figure 2**). The OLS and SMA models produced similar fits to PGLS models, though the slopes of SMA models were the highest (slope = 0.889, $P < 0.001$), whereas those of PGLS were the lowest. SMA tests also indicated that the scaling of eye size with SVL was hypo-allometric.

Eye Size Correlates With Ecological and Behavioral Traits

Analysis with phylogenetic ANCOVA in separate models showed that the relative eye size was not affected by habitat type (**Supplementary Table 2**; $F_1 < 0.001$, $p = 0.988$), activity time ($F_1 = 0.128$, $p = 0.721$), foraging mobility ($F_1 = 0.017$, $p = 0.897$), light availability ($F_1 = 0.996$, $p = 0.319$), defensive strategies ($F_1 = 0.632$, $p = 0.427$), and mating system ($F_1 < 0.001$, 0.992).

PGLS in a single model revealed that the relative eye size was not affected significantly by habitat type, activity time, light availability, foraging mobility, defensive strategy, and mating system (**Table 1**). Nocturnal species did not possess larger eyes than diurnal and nocturnal species (**Figure 3A**). Species approaching slowly and capturing prey did not have smaller eyes than species approaching quickly and often actively chasing prey (**Figure 3B**). Species living under strong light did not have smaller eyes than species living in weak light (**Figure 3C**). Species with granular glands in dorsal and ventral skin did not have smaller

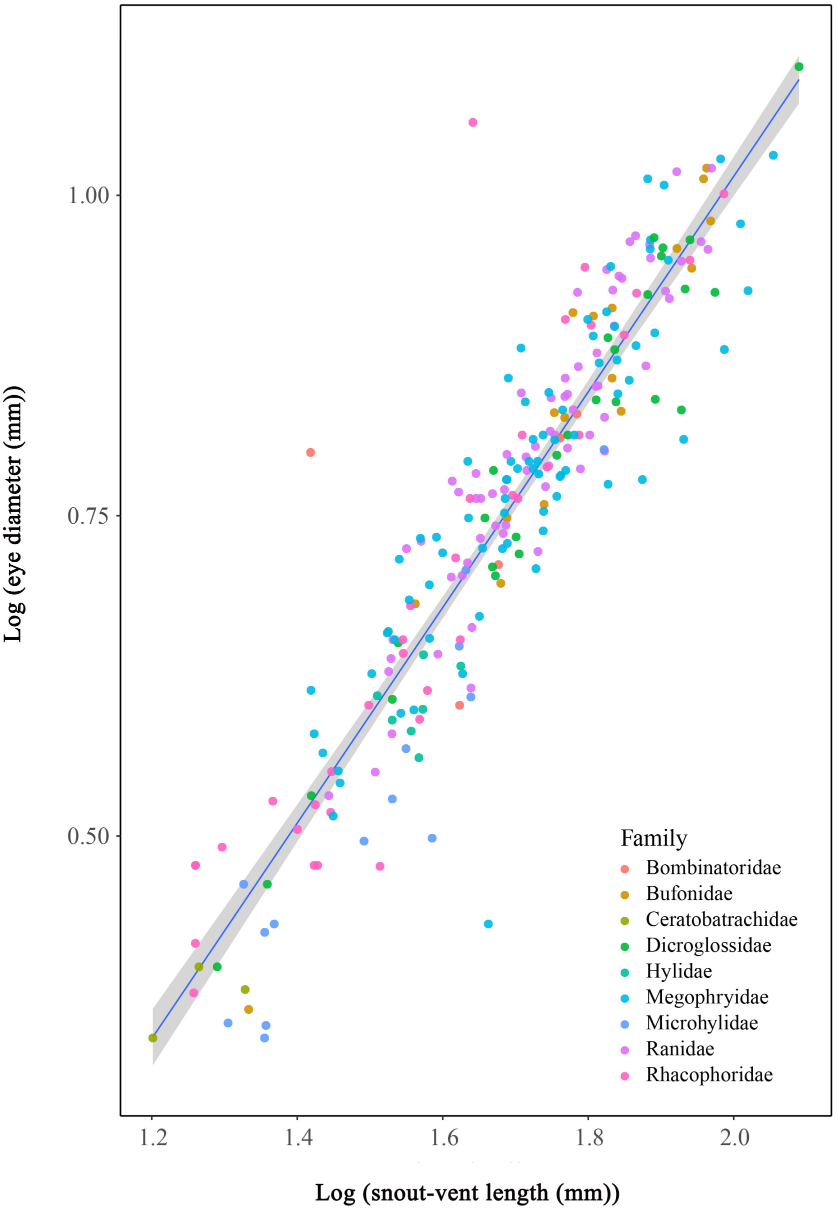

FIGURE 2 | Hypo-allometrical relationships between eye diameter and snout-vent length across 252 anurans species.

TABLE 1 | Evolution of eye size in relation to various predictor variables across 252 anurans species using phylogenetic generalized least squares model.

Predictors	Eye size				
	λ	β	*t*	R^2	*P*
Habitat type	$0.3800^{1,<0}.^{001}$	−0.0004	−0.1461	0.0001	0.8840
Activity time		−0.0018	−0.2161	0.0002	0.8291
Light availability		0.0078	0.7680	0.0024	0.4432
Foraging mobility		0.0045	0.3501	0.0005	0.7266
Defensive strategy		−0.0086	−0.7629	0.0024	0.4463
Mating system		0.0019	0.0753	0.00002	0.9400
SVL		0.8157	31.4329	0.8019	< 0.001

eyes than species with non-granular glands in skin (**Figure 3D**). The effects of habitat type and mating system on eye size is shown in **Supplementary Figures 1A,B**.

DISCUSSION

Eye size scales with body size among 252 species of anurans in this study, in which species with larger bodies have larger eye size, indicating a hypo-allometric relationship between body and eye size. However, eye size in anurans is not affected by ecological and behavioral factors (e.g., adult mating habitats, activity time, foraging mobility, light availability, defensive strategy, and mating system). Scansorial species do not have larger eyes than ground-dwelling, fossorial, and aquatic species. Nocturnal species living under weak light do not possess larger eyes than both nocturnal and diurnal species living under strong light. Moreover, species approaching quickly and often actively chasing preys do not display larger eyes than species approaching slowly preys. Moreover, species with granular glands did not have larger eyes than those without granular glands. Hence, these traits are not correlated with eye size when correcting for body size and phylogeny, suggesting that light availability and foraging behavior cannot play a key role in shaping eye size evolution in anurans.

Body size is the strongest predictor of eye size, which is expected as larger species usually possess larger eyes than smaller species in sharks, reptile, birds, and mammals (Brooke et al., 1999; Kiltie, 2000; Garamszegi et al., 2002; Ross et al., 2006; Werner and Seifan, 2006; Lisney and Collin, 2007; Liu et al., 2012). For anurans, eye size scales hypo-allometrically with SVL (Huang et al., 2019; Thomas et al., 2020) and we found the same patterns as Huang' and Thomas' that larger species had larger eyes, displaying hypo-allometrical relationship between eye size and body size.

Eye size varied among adult habitats in animal groups. For instance, evidence supports that larger eye in anurans are beneficial to scansorial species by accommodating fast temporal resolutions during jumping process and high acuity in visually complex arboreal habitats (Thomas et al., 2020). Likewise, fossorial species reduced eye size as adaptations to dark and/or abrasive habitats in mammals (Borghi et al., 2002), fishes (Eagderi and Adriaens, 2010), caecilian amphibians (Mohun et al., 2010), and reptiles (Yovanovich et al., 2019). However, the variation in the eye size of the 44 species of anurans investigated seems largely independent of adult habitats, possibly because all specimens were obtained from one, albeit large, geographical region (Huang et al., 2019). In the present study, eye size did not increase from fully fossorial to subfossorial to non-fossorial (e.g., ground-dwelling, semiaquatic, scansorial) species, indicating that the fossoriality degree cannot determine eye size evolution across 252 species of anurans.

Many nocturnal vertebrates display enlarged eye size to maximize visual sensitivity (e.g., primates: Kirk, 2006; birds: Hall and Ross, 2007; reef fish: Schmitz and Wainwright, 2011). For instance, nocturnal birds evolve larger eyes to provide a wide pupil and improve light sensitivity during night compared with diurnal birds (Martin, 1985; Brooke et al., 1999). Significant influences were observed in the activity pattern of relative eye size among the 220 species of anurans, in which nocturnal species evolved larger eyes compared with both diurnal species and nocturnal and diurnal species (Thomas et al., 2020). However, previous studies have indicated that activity pattern cannot affect variation in relative eye size among species because most of the 44 species of anurans can be seen foraging and mating also at night (Huang et al., 2019; Mai et al., 2020). Similar to the findings of Huang et al. (2019), we found that activity pattern did not affect relative eye size among the 252 species of anurans, possibly because the common anuran ancestor is likely to be nocturnal, and the majority of the species of extant anurans retain this active pattern (Anderson and Wiens, 2017). Large eyes are associated with larger abundance of photoreceptors and produce larger image sizes, thereby collecting more light per solid angle of image than small eyes (Martin, 2007). For fishes, the larger eyes increase the chance of photon capture when detecting small bioluminescent flashes at low levels of sunlight (Warrant, 2000; Warrant and Locket, 2004). Moreover, to improve light sensitivity at night, it may be beneficial to enlarge not only retinal area, but also pupil diameter in dim light conditions (Martin, 1985). However, light availability did not affect the eye size evolution in anurans, where species living under weak light did not have larger eyes than those living under strong light. Hence, nocturnal species with higher light availability did not evolve larger eyes than diurnal species, suggesting that light availability was not correlated with detection of predator risks.

Vision plays an important role in searching for foods for birds, as indicated by marked differences in eye size across the types of foraging mobility, where species approaching quickly and actively capturing preys displayed an increase in eye size, whereas those approaching slowly preys displayed a decrease in eye size (Garamszegi et al., 2002). In a previous study on 44 species of anurans, foraging mobility did not affect eye size evolution, suggesting that anurans approaching quickly preys may live in low-light conditions, which cannot lead to increased eye size compared with anurans approaching quickly preys (Huang et al., 2019). All the 44 used species are possibly at least partially active at night. However, after sampling a larger number of species in our study, the results did not confirm the positive effect of capture behavior on eye size evolution.

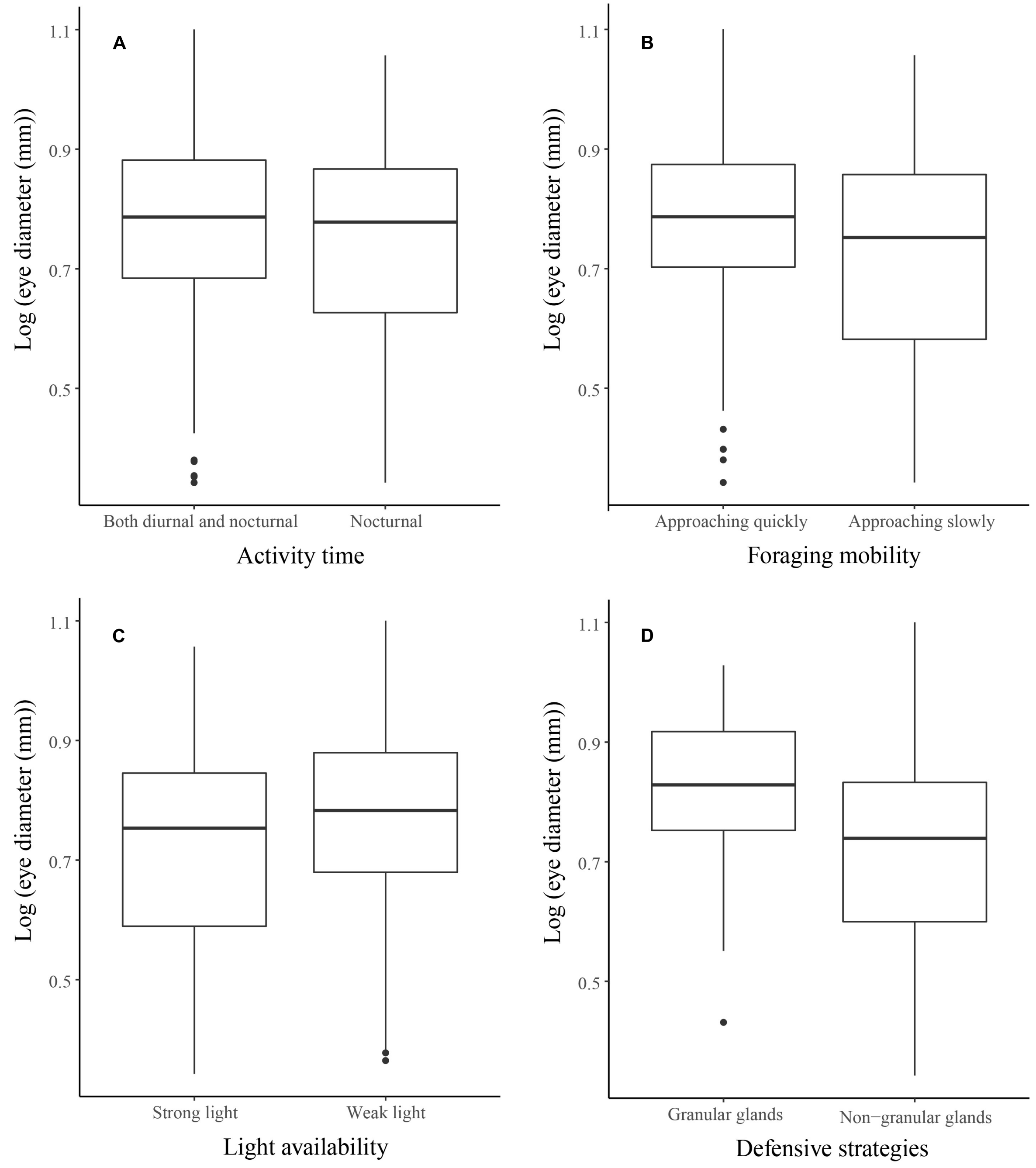

FIGURE 3 | Relationships between eye diameter and **(A)** activity time, **(B)** foraging mobility, **(C)** light availability, and **(D)** defensive strategies across 252 anuran species. Black points indicate the outliers.

Predator risk is the strongest selective pressure in shaping eye size evolution (Garamszegi et al., 2002). Considering species with larger eyes can easily spot predators early (Striedter, 2005; Kotrschal et al., 2015), stronger predator risk coincides with the larger-eyed birds (Møller and Erritzøe, 2010). The relationship between eye size and predator risk may be as prominent as it is for instance in birds with eye size that is positively correlated with fight distance (Blumstein et al., 2004). Consistent with a previous study (Huang et al., 2019), we found that species with granular glands in skin did not exhibit smaller eyes than species with non-granular glands in skin, suggesting that species depending on chemical defense cannot shape eye size evolution in anurans. Moreover, relative eye size is neither correlated with sexual size dimorphism nor mating system among the 44 species of anurans (Huang et al., 2019). In the present study, we found that eye size variation in 252 species of anuran cannot be explained by mating system contrary to the prominent role of sexual selection in eye size evolution.

In conclusion, eye size variation cannot be explained by ecological and behavioral factors including activity time, light availability, foraging mobility, and defensive strategy in our sample of 252 Chinese frogs. Eye size is not correlated with activity pattern and light availability, showing that a potential nocturnal bottleneck is not observed in anuran eyes evolution. Moreover, foraging mobility is not associated with the eye size in anurans, highlighting that foraging behavior cannot shape eye size evolution. Considering that defensive strategy (i.e., poison glands) does not affect eye size, the anti-predator ability in anurans cannot promote the enlarged eye size.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by China West Normal University.

AUTHOR CONTRIBUTIONS

YJ and CC participated in laboratory work, data analysis, and manuscript drafting. LJ and WBL conducted data analysis and visual representation of the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.755818/full#supplementary-material

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The Thermal Biology of *Takydromus kuehnei* Indicates Tropical Lizards From High Elevation Have Not Been Severely Threatened by Climate Change

Shiang Tao[1,2], Kunming Cheng[2], Xinghan Li[2], Xingzhi Han[3], Jichao Wang[4]*†, Rongquan Zheng[1]*† and Baojun Sun[2]

[1] College of Chemistry and Life Sciences, Zhejiang Normal University, Jinhua, China, [2] Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China, [3] College of Wildlife and Protected Area, Northeast Forestry University, Harbin, China, [4] Key Laboratory for Ecology of Tropical Islands, College of Life Sciences, Ministry of Education, Hainan Normal University, Haikou, China

Climate change poses different threats to animals across latitudes. Tropical species have been proposed to be more vulnerable to climate change. However, the responses of animals from tropical mountains to thermal variation and climate change have been scarcely studied. Here, we investigated the thermal biology traits of a tropical lizard (*Takydromus kuehnei*) distributed at high elevations (>950 m) and evaluated the vulnerabilities of *T. kuehnei* by thermal biology traits, thermal safety margin, and thermoregulatory effectiveness. The average active body temperatures of *T. kuehnei* in the field were 26.28°C and 30.65°C in April and June, respectively. The selected body temperature was 33.23°C, and the optimal temperature for locomotion was 30.60°C. The critical thermal minimum and critical thermal maximum temperatures were 4.79°C and 43.37°C, respectively. Accordingly, the thermal safety margin (1.23°C) and thermoregulatory effectiveness (1.23°C) predicted that *T. kuehnei* distributed in tropical mountains were not significantly depressed by environmental temperatures. This study implies that high-elevation species in tropical regions may not be severely threatened by ongoing climate change and highlights the importance of thermal biology traits in evaluating the vulnerability of species to climate change.

Keywords: climate change, tropical mountains, lizards, thermal biology, thermoregulatory effectiveness, thermal safety margin, *Takydromus kuehnei*, high elevation

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Giovanna Battipaglia,
University of Campania Luigi Vanvitelli,
Italy

Reviewed by:
Liyun Yin,
Hebei Normal University, China
Lei Shi,
Xinjiang Agricultural University, China

***Correspondence:**
Jichao Wang
wjc@hainnu.edu.cn
Rongquan Zheng
zhengrq@zjnu.cn

†These authors have contributed equally to this work

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INTRODUCTION

Climate change has negatively affected animal distribution and abundance (Root et al., 2003; Thomas et al., 2004; Medina et al., 2016). Although the latitudinal pattern of the vulnerabilities of animals to climate change is still controversial, increasing investigations claim that tropical animals are profoundly vulnerable to climate change, considering higher metabolic rates (e.g., Dillon et al., 2010), narrow thermal-safety margins (TSM) (e.g., Sunday et al., 2014), and depressed life-history cycles (e.g., Blouin-Demers and Weatherhead, 2001; Deutsch et al., 2008). For instance, the environmental temperature in tropical regions is higher than that in other latitudes, making it possible to exceed the physiological thermal-tolerance limits of animals (Huey et al., 2009; Sunday et al., 2014).

However, animals can migrate toward high latitudes and high elevations to avoid the risk of being threatened by climate change (e.g., Forero-Medina et al., 2011; Freeman et al., 2018). High latitudes and elevations can provide lower average temperatures and more fluctuating temperatures, which provide retreat (i.e., cool places) for thermoregulation. Therefore, tropical mountains may provide refuge for tropical animals to escape from exposure to warming temperatures (Bonebrake and Deutsch, 2012). However, it is largely unknown whether the high-elevation species in tropical regions are depressed by ongoing climate change (Freeman et al., 2018). Understanding the vulnerabilities of animals from tropical mountains is important in not only revealing the thermal adaptation of tropical species at high elevations but also evaluating the availabilities of migrating toward high elevations to escape from climate change (Ghalambor et al., 2006).

As ectotherms, reptiles have been threatened by climate change in the past decades and are projected to encounter more severe threats in the future (Huey et al., 2010; Sinervo et al., 2010; Barnosky et al., 2011; Diele-Viegas et al., 2020; Taylor et al., 2020). Lizards regulate body temperatures by an external heat source, and thus, their biological functions are subject to the effect of thermal environments on body temperatures (Huey, 1982; Hertz et al., 1993). Therefore, compared to other taxa, lizards are particularly sensitive to thermal variations and constitute an appropriate study system for evaluating the vulnerability of animals to climate change (Huey et al., 2012; Taylor et al., 2020). The thermal biology traits, thermal safety margin, and thermoregulation effectiveness are important for thermal adaptation and are also indispensable proxies for determining the vulnerability to climate change in lizards (Huey et al., 2012; Clusella-Trullas et al., 2021). The effects of climate change on lizards depend on the interaction of thermal environments and the integration of thermal biology traits, thermoregulation effectiveness in maintaining body temperature, and thermal safety margins (Sunday et al., 2014; ObregÓn et al., 2020). For example, lizards would maintain their active body temperature in an appropriate range by thermoregulation, which is approximate to the range of optimal body temperatures for various physiological processes (Huey and Kingsolver, 1989; Angilletta et al., 2002). The critical thermal minimum (CT_{min}) and critical thermal maximum (CT_{max}) temperatures are thermal limits of permitting performance (Angilletta et al., 2002; Taylor et al., 2020), which generally depend on the thermal environment of species (Ji et al., 1996; Chen et al., 2003; Zhang and Ji, 2004). In addition, the thermal sensitivity of locomotion and resting metabolic rates (RMR) is essential for evaluating the thermal adaptation in ectotherms (e.g., Dillon et al., 2010; Shu et al., 2010; Sun et al., 2014, 2018b). Therefore, thermal biology traits, including body temperature, selected body temperature range, and thermal tolerance, can be integrated to determine the thermoregulatory effectiveness and TSM, which are critical for evaluating the vulnerability of reptiles to climate change (Hertz et al., 1993; Sunday et al., 2014).

The *Takydromus* lizards have a wide distribution across latitudes in China, ranging from tropical to cold-temperature regions (Zhao, 1993; Lin et al., 2002; Portniagina et al., 2019). *Takydromus kuehnei* is a small lacertid lizard (snout-vent length, SVL < 60 mm), mainly distributed in tropical regions across southern China. The population on Hainan Island is a typically tropical population in China. According to previous predictions of species vulnerability across latitudes, *T. kuehnei* of the Hainan population may be the most vulnerable in *Takydromus* lizards to climate change in China. In the Diaoluoshan Mountains, *T. kuehnei* is distributed across low to high elevations ranging from 400 to 1000 m (Wang, 2014). Therefore, the high-elevation population of *T. kuehnei* is an excellent study system for analyzing the response of high-elevation tropical species to thermal variation, considering the interaction between warming climates and thermal biology traits.

With high-elevation population of *T. kuehnei* from the Diaoluoshan Mountains, this study monitored thermal environments, determined thermal biology traits, and calculated thermoregulatory effectiveness and TSM. By comparing the thermal biology traits, thermoregulatory effectiveness, and TSM with published data across latitudes (e.g., Zhang and Ji, 2004; Sunday et al., 2014; Hao et al., 2020), we aimed to evaluate the vulnerabilities of tropical species (i.e., *T. kuehnei* in this study) from high elevation to climate change and thus test whether mountains are potential refuges for tropical species under ongoing climate change. Base on the assumption that the ambient temperatures at high elevation in tropical areas are low on average with high variations, which are similar to that from medium and high latitudes (e.g., Ghalambor et al., 2006; Freeman et al., 2018), and the effects of thermal environments on thermal biology traits in *Takydromus* lizards (Hao et al., 2020), we predicted that the thermal biology traits of *T. kuehnei* from high elevation are similar to congeners from medium or high latitudes. Accordingly, we also predicted that *T. kuehnei* are not vulnerable to climate change by high thermoregulation effectiveness and big thermal safety margin. Further, we predicted that the high elevations (i.e., tropical mountains) can be potential refuges for tropical species to escape from exposure to climate change.

MATERIALS AND METHODS

Study Species Collection and Active Body Temperatures in the Field

Fieldwork was performed at the Mt. Diaoluo National Reserve in Hainan, China (18°43′N, 109°52′E). We collected 20 adult *T. kuehnei* (12 males and 8 females) from a high-elevation population (height > 900 m) using either a noose or by hand.

During collection, we measured the active body temperature of *T. kuehnei* once the lizard was captured. We measured cloacal temperatures using an electronic thermocouple within 30 s of capture (UNT T-325, Shanghai, China). The active body temperatures of the lizards were also measured repeatedly in June when they were released.

Operative Temperatures

During lizard collection, we also monitored the operative temperatures (T_e) using copper tube models. The copper tube models mimicked the size of the lizards (diameter × length: 15 mm × 70 mm). Each model was inserted with an iButton (DS1921, MAXIM Integrated Products Ltd., United States). We randomly set the copper models exposed to full sun and shaded sites. The iButtons recorded the hourly temperature. We used two iButtons for each site, and the average of two iButtons for each site was calculated as T_e. The T_e collection lasted from April to June when the lizards were released.

Lizard Husbandry

After collection in April, we transported the lizards to the laboratory built in the Mt. Diaoluo National Reserve. We weighed (body mass, ± 0.01 g) and measured (SVL, ± 0.1 mm) the lizards and arranged every five lizards with different sexes in each plastic terrarium (150 cm × 40 cm × 50 cm, length × width × height) with moist soil. The terraria were set in a room where the temperature was maintained at 18 ± 1°C. A supplementary heating lamp was suspended above one end of each terrarium to create temperatures ranging from 18 to 40°C from 06:00 to 20:00. Food (crickets and larval *Tenebrio molitor*) and water were provided *ad libitum*. After three days of rearing, we started to determine the thermal biology traits. During the test, if the females were pregnant, we supplemented the test of the traits after they laying the eggs.

Selected Body Temperature

The measurements of T_{sel} were also conducted in a temperature-controlled room at 18 ± 1°C. To create a thermal gradient, we placed a large tank (1000 × 500 × 300 mm, length × width × height) and set a 275 W incandescent lamp above one end of the tank. The heating period was 06:00 to 20:00, which mimicked the natural light period. We followed two established protocols to measure the selected body temperature (T_{sel}). To facilitate comparison of the T_{sel} of *T. kuehnei* to published data from other *Takydromus* lizards, we first followed an old protocol (Ji et al., 1996; Zhang and Ji, 2004). Briefly, four to five lizards were introduced into the cold end of the terrarium at 16:00 on the first day. The next day, we measured body temperature (cloacae, within 30 s) for each individual at 09:00 and 15:00. The average of two measurements for each lizard was calculated as the selected body temperature (T_{sel}). To facilitate thermoregulatory accuracy and effectiveness comparison, we also determined the selected temperature range using a new protocol (Li et al., 2017). In brief, we measured body temperatures (cloacae, within 30 s) at each hour from 08:00 to 18:00. The selected temperature range was calculated as the central 50% of all recordings for each lizard.

Thermal Tolerance

We determined the critical thermal minimum (CT_{min}) and critical thermal maximum (CT_{max}) in a programmed incubator (KB 240, Binder, Germany). Before determination, the candidate was acclimated to 28°C for 2 h. Thereafter, the lizards were cooled/heated from 28°C at a rate of 1°C per 10 min. During the cooling/heating period, we constantly monitored the righting responses of the lizards. Once lizards lost the capacity to right themselves after being turned over, the body temperature (cloacae) was recorded as CT_{min}/CT_{max}. Thereafter, the lizards were immediately moved to 28°C for recovery. If lizards could not recover from the test, their records were eliminated from further analysis. In this study, all lizards recovered to normal after cooling or heating.

Locomotor Performance

The locomotor performance of lizards was estimated by sprint speed at six test temperatures ranging from 18 to 38°C (18, 22, 26, 30, 34, and 38°C, in a randomized sequence). Before the measurement, the lizards were acclimated in a temperature-controlled incubator at the test temperature for 2 h. Sprint speed was measured in a customer-made wood racetrack (1200 mm × 100 mm × 150 mm, length × width × height, with intervals marked every 200 mm) in a temperature-controlled room. The lizard was introduced into the racetrack from one end and stimulated by a paintbrush to run through the racetrack. The running processes were recorded using an HD video camera (DCR-SR220E, Sony, Japan). Each lizard was tested twice at the test temperature with an interval of 1 h for rest. We analyzed the video of locomotion with established methods (Shu et al., 2010; Sun et al., 2014). For each lizard, we recorded the fastest speed at 200 mm intervals for each test by raw numbers, and the average of the fastest records from each test was calculated as the sprint speed.

Resting Metabolic Rates

The resting metabolic rate of *T. kuehnei* was determined at six test temperatures (18, 22, 26, 30, 34, and 38°C) in a random order using a respirometry system (Sable Systems International, Henderson, NV, United States). We estimated the RMR by measuring the CO_2 production rate as a proxy for published protocols (Sun et al., 2018b, 2020). The lizards were fasted for at least 12 h before the test and were acclimated for 2 h at the test temperature. The RMR of the lizards was determined using a closed-circuit system (volume = 281.4 mL). The lizard was housed at the test temperature in the chamber, which was placed in a temperature-controlled incubator (MIR554-PC, Sanyo, Japan). First, we set the system open to the air that had sucked up moisture through a tube with 300 ml/min flow rate to stabilize the baseline. After 3 min of opening to the air, the circuit system was transferred to be closed. We continuously recorded the rate of carbon dioxide production (VCO_2) for at least 7 min in a closed-circuit system. The RMR was calculated as the carbon dioxide production per gram of body mass per hour (mL g^{-1} h^{-1}), using the equation $RMR = VCO_2 \times volume/body\ mass$, where VCO_2 is the CO_2 production rate in percentage (%/h) in the closed-circuit system.

Thermoregulatory Accuracy and Effectiveness, and Thermal Safety Margin

For the calculation of the thermoregulation accuracy (d_b), we followed published protocols (Li et al., 2017). If T_b was below (or above) the selected temperature range, d_b was calculated as the difference between the T_b and the lower (or upper) bound of the selected temperature range. Alternatively, if T_b was within the selected temperature range, d_b was zero. The T_e values at day time from 07:00 to 17:00 were used to calculate *de*. If T_e was below (or above) the selected temperature range, d_e was calculated as the difference between T_e and the lower (or upper) bound of the selected temperature range. When T_e was within the selected temperature range, d_e was zero. Finally, we used two indices, *E* and $d_e - d_b$, to estimate thermoregulatory effectiveness. *E* was calculated using the equation $E = 1 - \text{mean } d_{b/}\text{mean } d_e$, following published methods (Hertz et al., 1993; Li et al., 2017). The thermal safety margins (TSM) were calculated as the difference between $T_{e,max}$ (the mean maximum hourly T_e) and CT_{max}, following the published protocol (Sunday et al., 2014).

Statistical Analysis

The normality and homogeneity were checked using the *Shapiro–Wilk* test and *Bartlett*'s test, respectively. First, we analyzed sex differences in $T_{sel,}$ CT_{min}, and CT_{max} by one-way ANOVA. Thereafter, we analyzed the T_a and T_e with *Kruskal*–test between April and June, because they are independent in test time. We analyzed the difference in T_e between the open and shaded locations using the *Wilcoxon* test. We tested between-month differences in T_a, T_e, d_b, and d_e using the *Wilcoxon* test. We also analyzed the difference between T_{sel} and $T_{a,}$ and T_{sel} and T_e using the *Wilcoxon* test. For the analysis of locomotion, we used the modified Gaussian regression to fit the dependence of sprint speed on test temperatures using a published method (Hao et al., 2020). Subsequently, the optimal temperature for locomotion was calculated according to the regression. The thermal dependence of the resting metabolic rates was analyzed using the allometric growth curve to body temperature. If there was a significant difference between sexes in thermal biology traits, we supplementarily show the data with males and females separately.

RESULTS

Operative Temperature and Field Body Temperature

T_e differed between open and shaded sites ($W = 482732$, $p < 0.001$), and full open sites had significantly higher T_e than sites under full shade (full shade, 24.07 ± 0.13°C; full sun, 27.26 ± 0.16°C) (**Figure 1A**). T_e varied over time (*Kruskal–Wallis* Chi-squared = 1601.4, $df = 23$, $p < 0.001$) (**Figure 1B**). In addition, daily time T_e in April (30.19 ± 0.28°C) was significantly higher than that in June (27.78 ± 0.21°C) ($W = 227946$, $p < 0.001$).

The T_a of *T. kuehnei* depended on the time of day in both April (*Kruskal–Wallis* Chi-squared = 115.62, $df = 30$, $p < 0.001$) (**Figure 2A**) and June (*Kruskal–Wallis* Chi-squared = 59.814, $df = 31$, $p = 0.001$) (**Figure 2B**). In contrast to the variation in T_e between April and June, the T_a in April (26.28 ± 0.21°C) was significantly lower than that in June (30.65 ± 0.22°C) ($W = 2051.5$, $p < 0.001$) (**Figure 2**).

Thermal Biology Traits

The selected body temperature (T_{sel}) for *T. kuehnei* was 33.23 ± 0.28°C by the old protocol. The range of T_{sel} for *T. kuehnei* was 28.2°C to 36.4°C, with the average value of T_{sel} being 32.12 ± 0.21°C by the new protocol. Sex did not affect T_{sel} by the old ($F_{1,17} = 0.705$, $p = 0.413$) or new protocol ($F_{1,68} = 0.495$, $p = 0.484$) (**Table 1**). The CT_{min} and CT_{max} for *T. kuehnei* were 4.79 ± 0.18°C and 42.37 ± 0.11°C, respectively. We found significant differences in CT_{min} ($F_{1,17} = 8.828$,

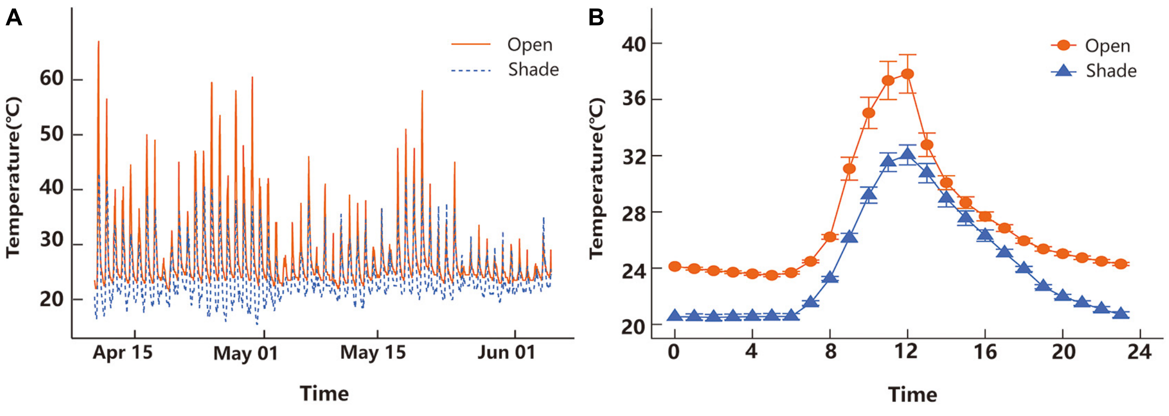

FIGURE 1 | Hourly **(A)** and daily average **(B)** operative temperatures (T_e) for high-elevation *T. kuehnei* in the field. **(A)** The Orange solid line and blue dash line indicate the T_e of open and shade sites, respectively. **(B)** Orange spots and blue triangles indicate the T_e of open and shade sites across the daytime, respectively. Data are shown as mean ± SE.

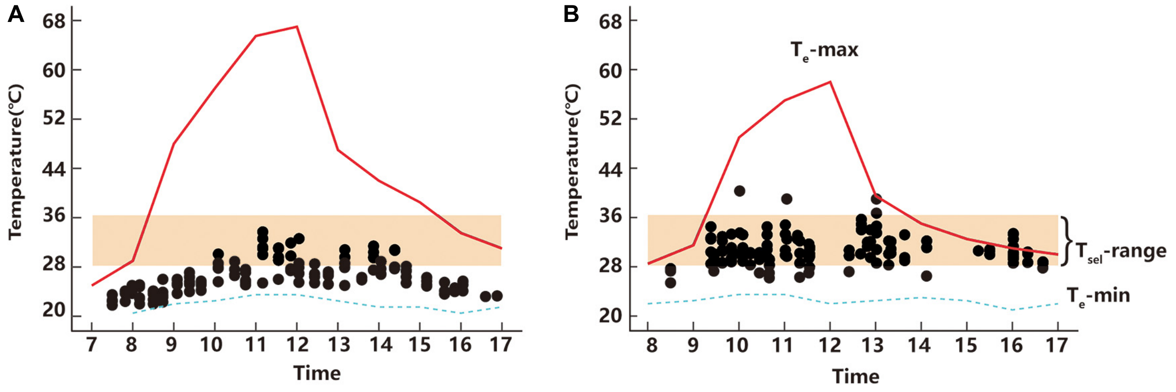

FIGURE 2 | Active body temperatures of *T. kuehnei* of April **(A)** and June **(B)**, respectively. The black spot represents the active body temperatures of each individual. The red solid and blue dash lines indicate the maximum and minimum operative temperatures, respectively. The orange shaded area represents the selected temperature range.

$p = 0.009$) and $\text{CT}_{\max}$ ($F_{1,17} = 6.241$, $p = 0.023$) between males and females (**Table 1**).

Locomotor Performance and Metabolic Rates

The sprint speed for *T. kuehnei* was significantly dependent on body temperature; it increased with body temperature within the range of 18 to 30°C and then decreased at higher body temperatures from 30 to 38°C. According to the modified Gaussian regression, the optimal body temperature for maximum sprint speed was 30.60°C (**Figure 3A**). Metabolic rates were dependent on body temperature, with increasing allometrically against test temperatures ($R^2 = 0.82$, $p < 0.0001$) (**Figure 3B**).

TABLE 1 | Selected body temperatures (T_{sel}), the critical thermal minimum (CT_{rmmin}), critical thermal maximum ($\text{CT}_{\max}$), thermoregulatory accuracy (d_b, d_e), thermoregulatory effectiveness (*E*), and thermal-safety margins (TSM) of *T. kuehnei*.

	Mean	SE
$\text{T}_{\text{sel(newprotocol)}}$	32.12°C	0.21
$\text{T}_{\text{sel(oldprotocol)}}$	33.23	0.28
$\text{CT}_{\min}$	4.79°C	0.19
$\text{CT}_{\text{min(male)}}$	4.40°C	0.24
$\text{CT}_{\text{min(female)}}$	5.34°C	0.15
$\text{CT}_{\max}$	42.37°C	0.11
$\text{CT}_{\text{max(male)}}$	42.57°C	0.13
$\text{CT}_{\text{max(female)}}$	42.09°C	0.15
d_b	1.33°C	0.11
d_b (April)	2.34	0.16
d_b (June)	0.21	0.06
d_e	2.56°C	0.09
$d_{e(\text{April})}$	2.78	0.15
$d_{e(\text{June})}$	2.35	0.11
E	0.48	
$E_{(\text{April})}$	0.16	
$E_{(\text{June})}$	0.92	
$d_e - d_b$	1.23°C	
$d_e - d_{b(\text{April})}$	0.44°C	
$d_e - d_{b(\text{June})}$	2.14°C	
TSM	1.23°C	

Data are shown as mean and SE.

Thermoregulatory Accuracy, Effectiveness, and Thermal Safety Margin

Operative temperatures was significantly lower than the average T_{sel} ($W = 20659$, $p < 0.001$). Most T_e values ($N = 706$) were below the lower threshold of the selected temperature range, 156 values were higher than the upper threshold of the selected temperature range, and 380 values were within the range of selected body temperatures. Accordingly, d_e did not vary between seasons ($W = 189527$, $p = 0.8171$). T_a was lower than T_{sel} in both April ($W = 797$, $p < 0.001$) and June ($W = 2541$, $p < 0.001$). In April, most T_a values ($N = 115$) were below the selected temperature range, and the remaining values ($N = 28$) were within the selected temperature range. In June, most T_a values ($N = 109$) were in the selected temperature range, and four values were higher than those above the selected temperature range. Accordingly, the d_b in June was lower than in April ($W = 15748$, $p < 0.001$, **Table 1**). In addition, the d_e of *T. kuehnei* was higher than that of the d_b in April and June. Therefore, the d_e - d_b and *E* values were positive (**Table 1**). Similarly, all TSM values were positive, and the average TSM for *T. kuehnei* was 1.23°C (**Figure 4** and **Table 1**).

DISCUSSION

Tropical (i.e., low latitude) species are proposed to be more vulnerable to climate change than those from subtropical and temperate regions (i.e., medium and high latitudes) (e.g., Deutsch et al., 2008; Dillon et al., 2010; Sunday et al., 2019). However, high-elevation regions (i.e., tropical mountains) are

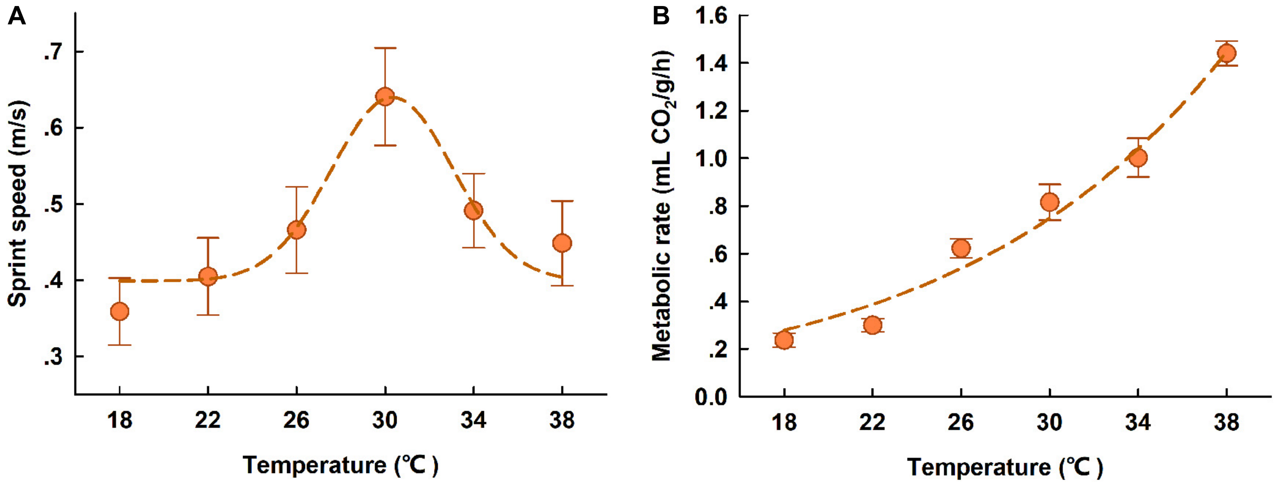

FIGURE 3 | Locomotor performance **(A)** and resting metabolic rates **(B)** of high-elevation *T. kuehnei* at different body temperatures. The spots indicate the sprint speed **(A)** and RMR **(B)** at each test temperature, and the dash lines indicate the regression. The data are shown as mean ± SE.

potential refuges for species under climate change (Forero-Medina et al., 2011; Freeman et al., 2018; Meng et al., 2019). Therefore, it is critical to determine the thermal biology and thus the vulnerabilities to climate change of species from tropical mountains (i.e., high elevations), providing a reference for species migration toward high elevations (Huey et al., 2009). According to the thermal biology traits, thermoregulatory effectiveness, and thermal safety margin, we found that the high-elevation population of *T. kuehnei* is not a severe threat to climate change, implying that tropical mountains are available for tropical species in escaping the exposure to climate change (e.g., Freeman et al., 2018).

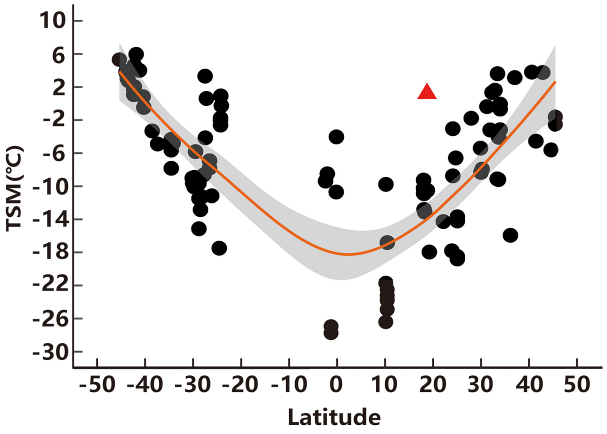

FIGURE 4 | Thermal-safety margins (TSM) of lizards across latitudes. Each spot indicates a TSM of each species and the red triangle represents the TSM of high-elevation *T. kuehnei*. The orange line represents the fitting curve of TSM along the global latitude and the gray area represents the 95% confidence interval. The database was collected from published analysis (Sunday et al., 2014).

Thermal Biology Traits of *Takydromus kuehnei* Did Not Follow the Existing Latitudinal Pattern

As ectotherms, most aspects of behavior and physiology in reptiles are profoundly affected by temperature, making reptiles an important model for studying the vulnerabilities of species to climate change (Huey et al., 2010, 2012). Accordingly, numerous studies have focused on the response of reptiles to thermal variation and climate change (see details in Huey and Berrigan, 2001; Taylor et al., 2020). Comparing biological traits among populations and species is an essential way to evaluate the responses of species to climate change across geographical clues (Bacigalupe et al., 2018; Taylor et al., 2020). *Takydromus* lizards are distributed from the northernmost Heilongjiang province to the southernmost Hainan province in China (Du et al., 2010; Cai et al., 2012; Ma et al., 2019; Portniagina et al., 2019). Previous studies have summarized the thermal biology traits of the genus *Takydromus* distributed in China across latitudes (Hao et al., 2020). The T_{sel} of the genus *Takydromus* lizards tends to decrease toward low latitudes. However, in contrast to the current pattern, we found that T_{sel} of *T. kuehnei* from the high-elevation population is 33.23 ± 0.28°C, which is the highest among the five *Takydromus* lizards with published data collected by the same protocols (i.e., old protocol in this study) (Ji et al., 1996; Chen et al., 2003; Zhang and Ji, 2004; Hao et al., 2020). Similarly, thermal tolerance does not fit the current latitudinal pattern. Our previous study indicated that the thermal tolerance range increased toward high latitudes by increasing CT_{max} and decreasing CT_{min} (Hao et al., 2020). However, in the high-elevation population of *T. kuehnei*, CT_{max} and CT_{min} are similar to those of medium-altitude species (i.e., *T. septentrionalis*) (Ji et al., 1996; Hao et al., 2020).

Thermoregulatory Effectiveness and Thermal Safety Margin Indicate High-Elevation *Takydromus kuehnei* Are Not Seriously Threatened by Climate Change

In this study, the T_a for *T. kuehnei* from the high-elevation population was lower than congeners (i.e., *T. septentrionalis*, Sun et al., 2018a) in both April (26.28°C) and June (30.65°C), although it is a tropical lizard under high T_e (**Figures 2A,B**). In addition, although T_a exhibited significant seasonal variation, only 1.6% (4/246) records were higher than the selected body temperature range, which is opposite to the current situation in tropical species where most body temperatures are near or higher than selected body temperatures (Huey et al., 2009). Furthermore, d_b in June was lower than in April, indicating that the lizards in June expressed higher thermoregulatory accuracy in maintaining their body temperatures within the range of thermal preference. The seasonal variation in higher T_a under lower T_e (**Table 1** and **Figures 1**, **2**) appears opposite to the general knowledge that the body temperatures of lizards are subject to ambient temperatures (Huey, 1982). Lizards have different thermoregulatory strategies to adapt to seasonal temperatures (Vicente Liz et al., 2019). Lizards may select a low body temperature in the hot season to avoid exposure to ambient heat temperatures (Firth and Belan, 1998). The higher selected body temperatures in the mild season are probably because mild-season temperatures can relieve lizard exposure to the critical thermal limits (Huey, 1982; Vicente Liz et al., 2019). Accordingly, lizards may enhance activity and facilitate the maintenance of the optimal temperature at a low cost in a mild season (Huey and Slatkin, 1976). In this study, the thermoregulatory effectiveness (i.e., E) of high-elevation *T. kuehnei* in June was higher than that in April (**Table 1**), indicating that *T. kuehnei* in the mild season (i.e., June) allows higher effectiveness of thermoregulation. In comparison, high-elevation *T. kuehnei* buffers the impacts of warming temperatures by thermoregulation, indicating a high E value ($E = 0.48$) relative to other tropical lizards. For example, an endemic arboreal lizard from the tropical islands of Mexico, *Anolis allisoni* ($E = -0.3$), and *Ctenosaura oaxacana* ($E = 0.13$) have low thermoregulatory effectiveness (Valenzuela-Ceballos et al., 2015; Medina et al., 2016).

Thermal-safety margins has been considered as a primary index for evaluating the vulnerability of species to extreme heat (e.g., Deutsch et al., 2008; Sunday et al., 2014). Previous studies revealed that TSM decreased toward low latitudes (i.e., tropical areas), where the TSM of most species was below zero (see details in Sunday et al., 2014). However, this study found that the TSM of high-elevation *T. kuehnei* is higher than zero, as high as that of lizards from medium and high latitudes around 40° (**Figure 4**). The TSM was determined using CT_{max} and T_e max. However, the CT_{max} in lizards is conservative across latitudes because lizards cannot effectively increase the temperature tolerance in the face of rapidly rising temperatures due to climate change (e.g., Sunday et al., 2011; Logan et al., 2014). Therefore, the mild thermal environment, thus T_e, can result in a high TSM for high-elevation *T. kuehnei*.

Generally, high thermoregulatory accuracy and large TSM predict high-elevation *T. kuehnei* would not be threatened by ongoing climate change in the future. Higher E allows *T. kuehnei* to regulate body temperatures with high effectiveness under thermal fluctuance (e.g., Li et al., 2017), and large TSM indicates that *T. kuehnei* is not under the stress of extreme heat (e.g., Sunday et al., 2014).

Tropical Mountains Are Plausible Refuges for Tropical Species

The primary reason for the lower risk of threat of *T. kuehnei* in this study may be that the population is collected from high elevations (>900m). In contrast, other *Takydromus* lizards were collected from low elevations (i.e., ∼200 m to ∼ 290 m) (Hao et al., 2020). High elevation in tropical regions can provide more fluctuant but lower average temperatures than low elevations (e.g., Leuschner, 2000; de Carvalho et al., 2019), which facilitates behavioral thermoregulation and increases the TSM. A large TSM at high elevation is possibly induced by decreased T_e max, although normal CT_{max} (Sunday et al., 2019). The climate variability hypothesis (CVH) posits that tropical organisms in warm and stable thermal environments should possess lower plasticity in their behavioral and physiological responses to thermal variation (Ghalambor et al., 2006; Gaston et al., 2009). In contrast, species from high-latitude or high-elevation habitats in more fluctuating thermal environments, and thus can tolerate or thrive over a broader range of temperatures, indicating a larger thermal safety margin due to being more resilient to climate change (Addobediako et al., 2000; Sunday et al., 2019). For example, the population of *Podarcis hispanica* from high elevation selected significantly higher body temperatures in the thermal gradient than the population with low elevation (Gabirot et al., 2013).

In summary, *T. kuehnei* from high elevation expresses similar thermal biology traits to medium-latitude congeners and allows high thermoregulatory accuracy and effectiveness, as well as a large thermal safety margin. Therefore, *T. kuehnei* could maintain a safe state in tropical regions under climate change. However, this study has some limitations. First, we only focused on the alpine population (>900m), lacking a comparison to the low-elevation population, or some other species with a narrow distribution in elevation/latitude. In the future, further studies with more tropical populations across elevations are needed to understand the vulnerability of tropical species to climate change (Ghalambor et al., 2006). With more intra- and inter-population comparisons, we can also understand the diversity of adaptive strategies to climate change of tropical species (Huey et al., 2009).

DATA AVAILABILITY STATEMENT

The data supporting the conclusions of this article can be found in the **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committees at the Institute of Zoology, CAS (IOZ14001).

AUTHOR CONTRIBUTIONS

BS, JW, and RZ designed the studies and wrote the draft. ST, KC, XL, and XH collected the data. ST and BS analyzed the data. All authors contributed to revisions of the manuscript.

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SUPPLEMENTARY MATERIAL

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Influence of High Temperatures and Heat Wave on Thermal Biology, Locomotor Performance, and Antioxidant System of High-Altitude Frog *Nanorana pleskei* Endemic to Qinghai-Tibet Plateau

***Jie He*[1]†*, Xiaolong Tang*[1]*†*, Peng Pu*[1]*, Tao Zhang*[1]*, Zhiyi Niu*[1]*, Fei Meng*[1]*, Lu Xi*[1]*,
Ming Ma*[1]*, Juan Wu*[1]*, Miaojun Ma*[2] *and Qiang Chen*[1]**

[1] Department of Animal and Biomedical Sciences, School of Life Sciences, Lanzhou University, Lanzhou, China, [2] State Key Laboratory of Grassland and Agro-Ecosystems, School of Life Sciences, Lanzhou University, Lanzhou, China

Edited by:
Jigang Xia,
Chongqing Normal University, China

Reviewed by:
Yongpu Zhang,
Wenzhou University, China
Fernando Diaz,
Center for Scientific Research and Higher Education in Ensenada (CICESE), Mexico

Correspondence:
Xiaolong Tang
tangxl@lzu.edu.cn
Qiang Chen
chenq@lzu.edu.cn

†These authors have contributed equally to this work

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Investigating how highland amphibians respond to changes in ambient temperature may be of great significance for their fate prediction and effective conservation in the background of global warming. Here, using field individuals as the control group, we investigated the influence of high temperatures (20.5 and 25.5°C) and heat wave (15–26.6°C) on the thermal preference, critical thermal limits, locomotor performance, oxidative stress, and antioxidant enzyme activities in high-altitude frog *Nanorana pleskei* (3,490 m) endemic to the Qinghai-Tibet Plateau (QTP). After 2 weeks of acclimation to high temperatures and heat wave, the thermal preference (T_{pref}), critical thermal maximum (CT_{max}), and range of tolerable temperature significantly increased, while the critical thermal minimum (CT_{min}) was significantly decreased. The total time of jump to exhaustion significantly decreased, and burst swimming speed significantly increased in frogs acclimated in the high temperature and heat wave groups compared with the field group. In the high temperature group, the level of H_2O_2 and lipid peroxide (malondialdehyde, MDA), as well as the activities of glutathione peroxidase (GPX), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD), and total antioxidant capacity (T-AOC) significantly increased in the liver or muscle. However, in the heat wave group, the MDA content significantly decreased in the liver, and antioxidants activities decreased in the liver and muscle except for CAT activities that were significantly increased in the liver. These results indicated that *N. pleskei* could respond to the oxidative stress caused by high temperatures by enhancing the activity of antioxidant enzymes. The heat wave did not appear to cause oxidative damage in *N. pleskei*, which may be attributed to the fact that they have successfully adapted to the dramatic temperature fluctuations on the QTP.

Keywords: thermal biology, locomotor performance, oxidative stress, antioxidant defense, high-altitude amphibian

INTRODUCTION

In the background of global warming, the annual average air temperature in Qinghai Tibet Plateau (QTP) is increasing, with increase in frequency and intensity of extreme temperature events (Stocker et al., 2014; Zhao et al., 2014; Yi et al., 2018). Poikilothermic vertebrates, such as amphibians and non-avian reptiles, although have behavioral and weak physiological thermoregulatory mechanisms, their growth, reproduction, foraging, immunity, and competitiveness are easily affected by environmental temperatures (Panov and Mcqueen, 1998; Cherkasov et al., 2006; Pörtner and Farrell, 2008; Peng et al., 2020). Since 1980, 122 species have gone extinct, and more than 32% of amphibian species are vulnerable, endangered, or critically endangered because of climate change, habitat loss and so on (Collins and Storfer, 2003; Stuart et al., 2004; Mendelson et al., 2006; Wake and Vredenburg, 2008). Han et al. (2016) have demonstrated that temperature significantly affects the daily food intake, sprint speed, and length of continuous locomotion in Qinghai Plateau toad-headed lizard *Phrynocephalus vlangalii*. Peng et al. (2020) found that high temperature accelerates the development of tadpoles and reduces their body size during metamorphosis in Tibetan brown frog *Rana kukunoris*. The body mass of the corn snake *Pantherophis guttatus* is decreased significantly in a simulated heat wave (Stahlschmidt et al., 2017). Therefore, understanding how highland amphibians respond to climate warming and heat wave events may have great significance for their effective conservation.

The thermal biology of ectotherms comprise preferred body temperature (T_{pref}) and thermal tolerance (critical thermal maximum CT_{max}, and critical thermal minimum, CT_{min}) (Angilletta et al., 2010b). The T_{pref}, CT_{max}, and CT_{min} of ectotherms are affected by many factors, such as thermal acclimation trials (Avalos et al., 2020), light cycle (Lapwong et al., 2020), and ontogeny (Wollmuth et al., 1987). For example, T_{pref} is higher in the warm/wet season than in the cold/dry season in the terrestrial toad *Rhinella icterica* (César et al., 2018). Furthermore, the common coqui frog *Eleutherodactylus coqui*, acclimated to a cold temperature (16°C, 30 days), has CT_{min} lower than that of those acclimated to a high temperature (25°C) (Haggerty, 2016). The plasticity of thermal biology of amphibians can help them to adapt to the warming ecological environment and meet the needs of different physiology (Hernandez-Sandoval et al., 2018). The locomotion performance of individuals largely determine their adaptability, such as foraging, reproduction, and avoiding predators (Clusella-Trullas et al., 2010), while the locomotion performance of ectotherms is closely related to ambient temperature (Chown and Nicolson, 2004; Stillwell and Fox, 2005). The individual locomotion ability gradually decreases once the ambient temperature goes beyond optimal temperature range (Hertz et al., 1983; Bennett, 1990). However, some locomotion properties, i.e., stamina and burst performance, show plasticity after acclimation to different temperatures (Clusella-Trullas et al., 2010). For instance, the tropical clawed frog *Xenopus tropicalis,* acclimated to lower temperature (24°C, 2 months), has stronger stamina than individuals acclimated to high temperature (29°C, 2 months) (Padilla et al., 2019). The soft-shelled turtle *Pelodiscus sinensis,* acclimated to higher temperature (30°C, 4 weeks), has faster swimming speed than those acclimated to a low temperature (10 and 20°C, 4 weeks) (Wu et al., 2013). Nevertheless, there are limited studies that reported whether high-altitude amphibians also exhibit plasticity in thermal biology and locomotion performance after acclimation to high temperatures and heat wave.

The oxidative stress and antioxidant defense ability of organism are closely related to the ambient temperature (Zhang et al., 2021). A high-temperature environment can increase metabolism and accelerate oxygen consumption, which will increase the production of reactive oxygen species. For example, hydrogen peroxide (H_2O_2) content significantly increases in the liver of the giant spiny frog (*Quasipaa spinosa*) under heat stresses (Liu et al., 2018). The antioxidant defense system includes enzymatic antioxidants, such as catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), superoxide dismutase (SOD), and some non-enzymatic antioxidants, such as glutathione (GSH; Cheng et al., 2015; Paital et al., 2016). Liu et al. (2018) found that the antioxidant enzyme system in the liver of giant spiny frogs (*Q. spinosa*) was enhanced to cope with oxidative stress when they were exposed at 30°C for 48 h. Furthermore, the antioxidant system does not change in corn snake (*P. guttatus*) when acclimated to a simulated heat wave, although its oxidative damage level significantly decreases (Stahlschmidt et al., 2017). The telomere length of desert lizard (*P. przewalskii*) shortens while SOD activity did not change in a stimulated heat wave (Zhang et al., 2018).These research studies suggest that high temperature and heat wave may have a different impact on individuals.

Nanorana pleskei is endemic to the Qinghai Tibet Plateau and belongs to the Dicroglossinae family. It is distributed at 2,800 to 5,100 m above sea level and is considered to be one of the amphibians with highest altitude distribution in the world (Zhang et al., 2012). Niu et al. (2018) investigated oxidative stress and antioxidant defense in hibernating *N. parkeri*, and the results showed that the level of oxidative stress and oxidative damage was significantly increased, but that the total antioxidant capacity was significantly decreased compared with the summer frogs. Zhang et al. (2021) found that long-term cold acclimation would inhibit the antioxidant defense system of *N. pleskei*. However, there is little know about how *N. pleskei* responds to the warming environment at the organism's performance and physiological and molecular levels.

Herein, to investigate the influence of high temperatures and heat wave on the high-altitude *N. pleskei* in the background of global and plateau warming, we measured the thermal biology, locomotor performance, oxidative stress, and antioxidant defense system in liver and muscle of frogs acclimated to high temperatures and heat waves, and compared the results with that of the field individuals. Our results provide a reference for studies on the physiological and biochemical mechanisms of high-altitude amphibians adapted to high temperatures, and for survival prediction and protection of high-altitude amphibians.

MATERIALS AND METHODS

Ethics Statement

This study was carried out under the approval of the Ethics Committee of Animal Experiments at Lanzhou University and on the basis of principles from the China Council on Animal Care. Every effort was made to minimize the numbers used and any suffering experienced by the animals in the experiment.

Animal Acclimation

Adult *N. pleskei* frogs (n = 84) were captured in Maqu County (33.77°N, 101.74°E, 3,490 m asl), Gansu province, China, at the end of July, 2020. They were transported to the research station of alpine meadow and wetland ecosystems of Lanzhou University in Maqu, Azi Branch Station (33.67°N, 101.87°E, 3,512 m asl). The frogs used in the experiment were captured on post-breeding period, and the sex ratio was 1:1 in the measurement of biochemical indexes. Acclimation to high temperatures and heat wave, and the measurement of thermal biology and locomotor performance were all performed in the research station 15 km from the sampling site. The frogs were randomly separated into four groups (n = 21 for each group). The thermal trials included three treatments consisting of two high-temperature treatments and one heat wave treatment. The frogs captured directly from the field were assigned to the control group. We set two high temperature groups in this study, ETem20 (20.5 ± 0.5°C) and ETem25 (25 ± 0.5°C), according to the preferred body temperature of field frogs (21 ± 0.5°C) and the monthly maximum (22.5 ± 0.5°C) in August in Maqu County from 2005 to 2017, respectively (**Figure 1A**). To further simulate the temperature fluctuation experienced by frogs during the active period, the heat wave group (HWG) was set based on the monthly maximum (12.1 ± 0.5°C) and minimum (1.2 ± 0.5°C) average temperatures in August in Maqu County from 2005 to 2017 (**Figure 1A**). The temperatures of the ETem20, ETem25, and heat wave groups were controlled 2 2 weeks by an automatic temperature control system, and three temperature data loggers (iButton DS1922G; CA, United States) were fixed in the bottom of each aquarium to record temperature changes in real-time (**Figure 1B**). According to our records of temperature data loggers during the acclimations, the average temperature of the heat wave group was 19.3°C, and the range of diurnal temperature fluctuation was 15–26.6°C (**Figure 1B**).

The frogs (n = 21 for each group) were placed in glass aquaria (48.7 cm × 35.5 cm × 28.5 cm) containing water and grass from the wild. The animals were fed with insects that were captured near the habitat of frogs, such as mosquitoes, screwworms, and locust larvae. The water and grass were replaced every 3 days. The photoperiod was 12 L: 12 D. Before the experiment, the cloacal temperature, snout-to-vent length (SVL), and body mass of all the frogs were measured. Body mass index (BMI, body mass × SVL^{-2} × 100%) was calculated before and after the acclimation treatments. Then, 13 frogs from each group were used to measure thermal biology and locomotor performance parameters, and 8 frogs from each group were sacrificed to collect the whole liver and hind leg muscle tissues. The hepatosomatic index (HSI, liver mass × body mass $^{-1}$ × 100%) and muscle index (MI, muscle mass × body mass $^{-1}$ × 100%) were also calculated. All tissues were quick-frozen in liquid nitrogen immediately and stored at -80°C until the measurement of biochemical parameters.

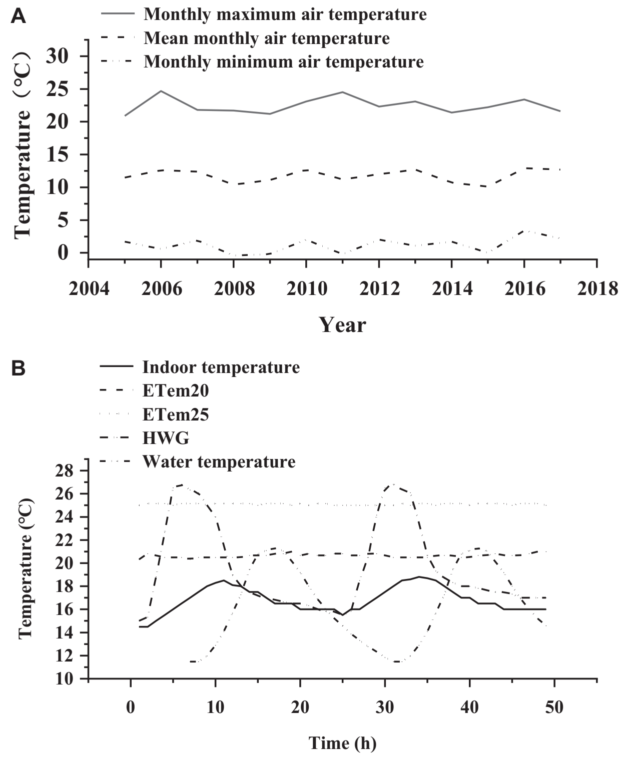

FIGURE 1 | (A) Monthly maximum air temperature, mean monthly air temperature, and monthly minimum air temperature in August in Maqu County from 2005 to 2017. **(B)** Changes in temperature in ETem20, ETem25, and HWG, and changes in indoor temperature and water temperature of *Nanorana pleskei* for 2 consecutive days. ETem20, ETem25, HWG, and water temperature represent the group that elevated temperature to 20°C, the group that elevated temperature to 25°C, water temperature at sampling site heat, and wave group, respectively.

Thermal Biology

Preferred body temperature (T_{pref}) and ambient temperature (T_{amb}) were measured in a homemade cuboid-shaped glass tank (120 cm × 15 cm × 30 cm), which was covered up with a shading cloth in all the trials to create a completely dark environment (César et al., 2018). A layer of moist gauze was spread on the bottom of the tank to ensure an adequately humid environment in the trials. One side of the tank was warmed using a ceramic lamp placed inside, and the other side was cooled using ice packs placed outside. Three temperature data loggers were placed on both ends and the middle to record temperature changes in real time. Our results showed that the range of temperature gradient was 14.6 ± 0.5 to 39.1 ± 0.5°C, and that the temperature in the middle was 20.7 ± 0.5°C. Frogs (n = 13 for each group) were placed in the temperature gradient for about 1 h to adapt before each trial. After 1 h, the cloacal temperatures of the frogs were measured every 30 min using a handheld electronic thermometer (TM6801b, China). Each frog was tested three times, and the average was defined as T_{pref}. The ambient temperature (T_{amb}) in the location of each frog was also measured three times using the same thermometer, and the average was defined as T_{amb}.

The frogs used for measuring T_{pref} and T_{amb} were used further to measure thermal tolerances after resting for 24 h. The thermal tolerances of frogs comprise the non-lethal critical thermal maximum (CT_{max}) and minimum (CT_{min}), which were the cloacal temperatures at which frogs lose their ability of righting reflex at high and low temperatures (Lowe and Vance, 1955). The time interval between the measure of CT_{max} and CT_{min} was 48 h. Low-temperature thermostat bath (HMDC-2006, China) was used to control temperatures in the measurement of CT_{max} and CT_{min}. In the process of measuring CT_{max} and CT_{min}, water temperature was gradually increased or decreased at a rate of.33°C min^{-1}. In the measurement of thermal tolerances, the frogs were checked every 5 min at the beginning, and then checked every 1 min when their movement slows down until individuals lose their ability of righting reflex, and then the cloacal temperature was recorded as the CT_{max} and CT_{min}. The measurement time of each group was not more than 30 min to avoid short-term adaptation.

Stamina and Burst Swimming Speed

After 48 h of measuring CT_{max} and CT_{min}, the stamina of frogs was measured in a 2.7-m homemade runway with a water temperature of about 16°C. An individual was chased by a glass stick until it was exhausted and lost the ability of righting reflex, and then the total distance and time of jumping to exhaustion were recorded to characterize the stamina of frogs. The burst swimming speed of frogs was further measured after 48 h of rest. The frogs were placed in a custom-made cuboid-shaped glass tank (120 cm × 15 cm × 30 cm) containing 10-cm deep water collected from the field, and a ruler with an accuracy of.1 cm was fixed at the bottom of the tank to record the distance of swimming. A digital camera (Canon A610; Canon, Japan) was fixed above the tank to videotape the swimming movements of the frogs when they were being stimulated with a glass stick on the tail. Then, the videos were analyzed to measure the swimming distance during the 1 s using the Adobe Premiere Pro 2020 frame-by-frame. Each video was analyzed 10 times, and the maximum distance during the 1 s was regarded as burst swimming speed (cm/s).

Measurement of Oxidative Damage and Antioxidant Activity Indices

The liver and muscle tissues from the eight frogs in each group were accurately weighed, and then we immediately added a quantitative pre-cooled.8% saline solution in a 1:4 ratio in 1.5-ml centrifugal tubes to thoroughly grind, and then the supernatant fluid was collected by centrifuging the tissue fluid at 3,000 rpm and 4°C for 10 min. Afterward, the supernatant fluid was diluted in proportion to 10, 1, 0.3, and.1% tissue solution with the pre-cooled.8% saline solution. Chemical assay kits were used to determine the total protein content, MDA, H_2O_2, GPX, SOD, CAT, GR, T-AOC, GSH, and GSSG (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions. The GSH-eq was calculated as the sum of GSH and 2 × GSSG.

Statistical Analysis

All the data were represented as means ± standard error and analyzed on the SPSS 20.0 (IBM SPSS statistics 20.0). The data were checked by Kolmogorov–Smirnov test for normality. The statistical differences ($P < 0.05$) of each measured parameter among the high temperatures, heat wave, and field groups were analyzed by one-way ANOVA. The results of homogeneity variance were showed in the one-way ANOVA analysis, and LSD *post hoc* test for data in homogeneity and Tamhane's T2 test for data in heterogeneity were used in the *post hoc* multiple comparisons to detect the differences between every two groups ($P < 0.05$). Furthermore, a paired *t*-test was performed to compare the significant difference in measured parameters before and after acclimation of each group. All graphs were generated with Origin 2018 64Bit (OriginLab, American).

RESULTS

Morphological Characteristics

There was no significant difference in body mass (BM) and body size (SVL) of the frogs among the field groups, ETem20, ETem25, and HWG (BM: $F_{3,82} = 0.271$, $P < 0.05$; SVL: $F_{3,77} = 0.914$, <0.05), at the beginning of the acclimation trial (**Table 1**). After 2 weeks of acclimation to high temperatures and heat wave, the body mass in ETem25 ($t_{21} = 1.926$, $P < 0.05$) and the BMI in ETem20 ($t_{21} = 2.31$, $P < 0.05$) and ETem25 ($t_{21} = 3.342$, $P < 0.01$) was significantly decreased compared with before acclimation, but there was no significant difference in HWG (**Table 1**). In addition, the hepatosomatic index was significantly decreased in ETem25 ($F_{3,28} = 2.441$, $P < 0.05$) compared with the field groups, ETem20 and HWG. The muscle index was remarkably increased in ETem20 compared with field group, ETem25, and HWG ($F_{3,28} = 5.035$, $P < 0.01$).

Thermal Biology

Thermal preference was significantly increased in ETem20 (23.2 ± 0.61°C, $F_{3,41} = 19.007$, $P < 0.01$) and ETem25 (23.57 ± 0.21°C, $F_{3,41} = 19.007$, $P < 0.01$) compared to the that in field group (21.73 ± 0.69°C) (**Figure 2**). Moreover, T_{pref} was decreased in HWG compared with ETem25 ($F_{3,41} = 19.007$, $P < 0.001$). T_{amb} was also significantly increased in ETem20 (24.84 ± 1.14°C, $F_{3,44} = 3.412$, $P < 0.01$), ETem25 (23.39 ± 0.53°C, $F_{3,44} = 3.412$, $P < 0.01$), and HWG (23.85 ± 0.59°C, $F_{3,44} = 3.412$, $P < 0.01$) compared to the field group (21.23 ± 0.78°C). T_{amb} did not have a significant difference among the high temperature groups and heat wave group (**Figure 2**).

The CT_{max}, CT_{min}, and thermal tolerance range of *N. pleskei* are illustrated in **Figure 3**. Compared with the field group (CT_{max} = 31.62 ± 0.33°C, CT_{min} = 3.17 ± 0.34°C), CT_{max} in

TABLE 1 | Morphological comparison of *Nanorana pleskei* in different temperature acclimation groups and field group.

		N	FG	ETem20	ETem25	HWG
Body mass, BM(g)	BA	23	3.61 ± 0.13	3.62 ± 0.18	3.75 ± 0.13	3.70 ± 0.17
	AA	23		3.68 ± 0.21	3.47 ± 0.11*	3.83 ± 0.18
SVL (cm)	BA	23	3.75 ± 0.092	3.65 ± 0.065	3.75 ± 0.025	3.65 ± 0.075
	AA	23		3.60 ± 0.058	3.87 ± 0.074	3.52 ± 0.056
Body mass index, BMI(%)	BA	23	25.52 ± 0.89	26.80 ± 0.68	25.68 ± 0.57	27.20 ± 0.70
	AA	23		24.79 ± 0.60*	23.77 ± 0.45*	26.94 ± 0.72
Hepatosomatic index, HSI(%)		8	6.52 ± 0.81^A	6.24 ± 0.64^A	4.68 ± 0.21^B	6.76 ± 0.67^A
Muscle index, MI(%)		8	6.80 ± 0.74^A	8.68 ± 0.42^B	6.45 ± 0.58^A	5.65 ± 0.32^A

*BA, before acclimation; AA, after acclimation. FG, ETem20, ETem25, and HWG represent field group, the group that elevated temperature to 20°C group, the group that elevated temperature to 25°C and heat wave group, respectively. Body mass, SVL, and body mass index were compared between BA and AA in the same treatment group. Hepatosomatic index and muscle index were compared for all the groups. The significant difference was shown with asterisks by comparing all the groups, and different characters mean P < 0.05. The statistical significance, by comparing each group before and after acclimation, was shown with different characters, *P < 0.05.*

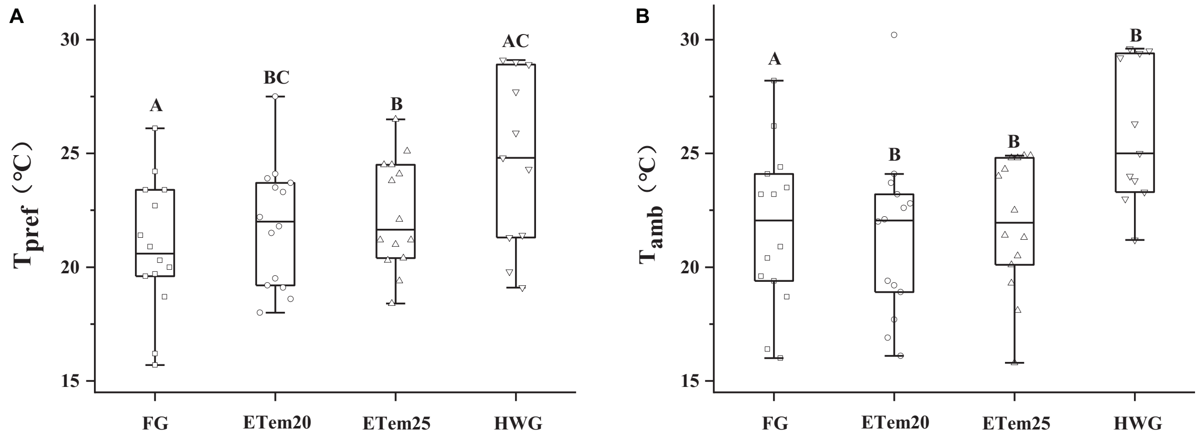

FIGURE 2 | Comparison of **(A)** thermal preference (T_{pref}) and **(B)** ambient temperature (T_{amb}) of *N. pleskei* (n = 13) in different temperature acclimation groups and field group. The line inside the box plot denotes median, the borders of the box depict 25th and 75th quartiles, and the whiskers represent the 5th and 95th percentiles. The dots indicate the outliers and data points. The significant difference was shown by comparing all the groups. Different characters mean $P < 0.05$.

ETem20 (33.39 ± 0.3°C, $F_{3,44}$ = 8.707, $P < 0.001$), ETem25 (32.63 ± 0.14°C, $F_{3,44}$ = 8.707, $P < 0.001$), and HWG (32.56 ± 0.16°C, $F_{3,44}$ = 8.707, $P < 0.01$) were markedly elevated, and CT_{min} was significantly reduced in ETem20 (2.16 ± 0.25°C, $F_{3,38}$ = 11.652, $P < 0.01$), ETem25 (1.74 ± 0.06°C, $F_{3,38}$ = 11.652, $P < 0.001$), and HWG (1.47 ± 0.11°C, $F_{3,38}$ = 11.652, $P < 0.001$) (**Figures 3A,B**). Besides, compared with ETem25 and HWG, CT_{max} significantly increased in ETem20 ($F_{3,44}$ = 8.707, $P < 0.05$). The thermal tolerance range (ΔCT, the value of CT_{max} minus CT_{min}) was significantly increased in ETem20 (31.74 ± 0.42°C, $F_{3,42}$ = 18.499, $P < 0.001$), ETem25 (30.93 ± 0.23°C, $F_{3,42}$ = 18.499, $P < 0.001$), and HWG (31.13 ± 0.22°C, $F_{3,42}$ = 18.499, $P < 0.001$) compared with the field group (28.25 ± 0.57°C) (**Figure 3C**). There was no significant difference in CT_{min} and thermal tolerances range among the high temperatures group and heat wave group (**Figures 3B,C**).

Stamina and Burst Swimming Speed

The total time of jumping to exhaustion was significantly influenced by acclimation temperatures, but there was no significant difference in the total distance to exhaustion among the ETem20, ETem25, and heat wave groups ($F_{3,38}$ = 0.66, $P > 0.05$) (**Table 2**). Compared with the field group, the total time to exhaustion was markedly shortened ($F_{3,38}$ = 3.337, $P < 0.001$; $F_{3,38}$ = 3.337, $P < 0.001$; $F_{3,38}$ = 3.337, $P < 0.001$), and the burst swimming speed ($F_{3,38}$ = 12.32, $P < 0.01$; $F_{3,38}$ = 12.32, $P < 0.001$; $F_{3,38}$ = 12.32, $P < 0.001$) was significantly increased in ETem20, ETem25, and HWG, respectively (**Table 2**). The total time to exhaustion was significantly decreased in ETem20 compared with HWG ($F_{3,38}$ = 3.337, $P < 0.01$), and the burst swimming speed was significantly decreased in ETem20 compared with ETem25 ($F_{3,38}$ = 12.32, $P < 0.05$).

Oxidative Stress and Oxidative Damage in Liver and Muscle

The results of *post hoc* test showed that the level of MDA in the liver was significantly increased in ETem20 ($F_{3,22}$ = 21.361, $P < 0.01$) but significantly decreased in ETem25 ($F_{3,22}$ = 21.361, $P < 0.001$) and HWG ($F_{3,22}$ = 21.361, $P < 0.05$) compared with the field group, and that it was remarkably enhanced in ETem20 compared with ETem25 and HWG ($F_{3,22}$ = 21.361,

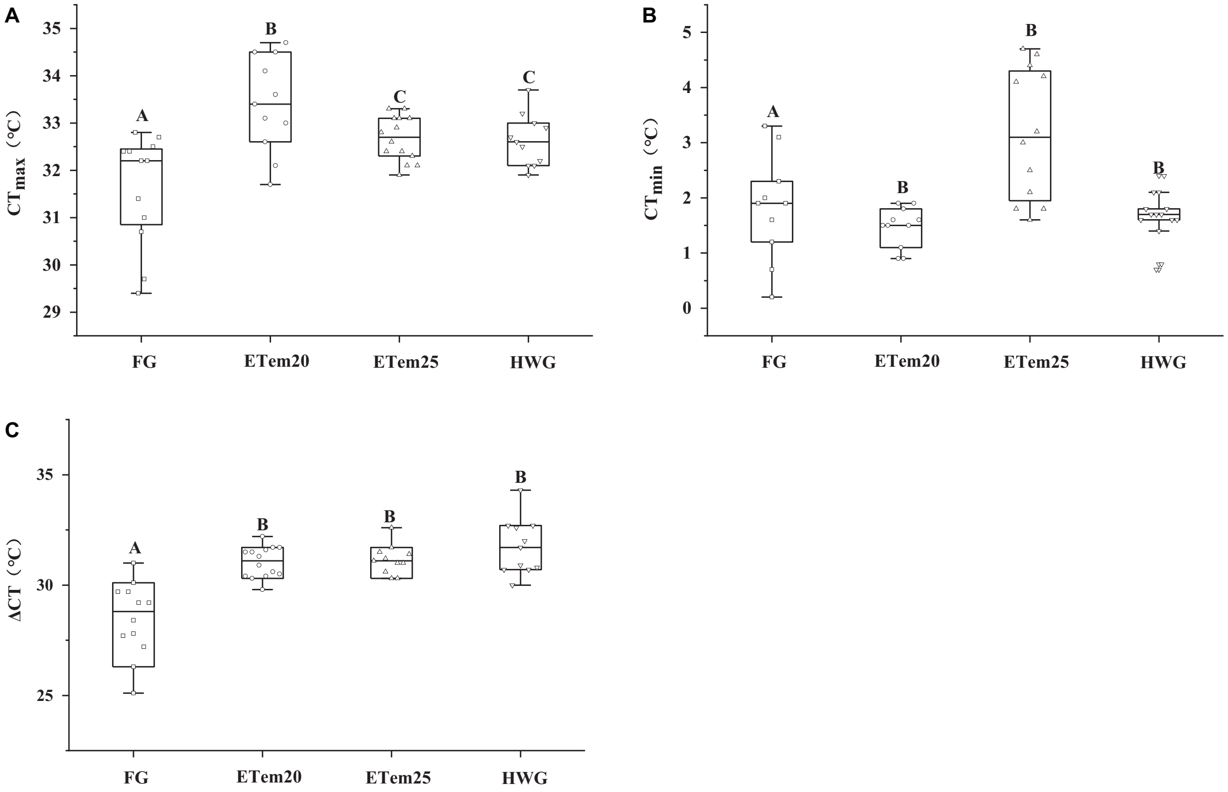

FIGURE 3 | Comparison of **(A)** critical thermal maximum (CT_{max}), **(B)** critical thermal minimum (CT_{min}), and **(C)** thermal tolerance range (ΔCT) of *N. pleskei* (*n* = 13) in different temperature acclimation groups and field group. The significant difference was shown by comparing all the groups. Different characters mean $P < 0.05$.

TABLE 2 | Stamina and burst swimming performance comparison of *N. pleskei* in different temperature acclimation groups and field group.

	N	The total distance to exhaustion(cm)	The total time to exhaustion(s)	Burst swimming speed(cm/s)
FG	13	1422.7 ± 97.2	635.90 ± 76.6^{A}	19.18 ± 0.87^{A}
ETem20	10	1435.4 ± 37.4	260.63 ± 21.1^{B}	23.76 ± 1.08^{B}
ETem25	13	1165.2 ± 137.1	303.38 ± 33.2^{BC}	28.03 ± 1.44^{C}
HWG	11	1473.8 ± 151.7	368.75 ± 24.9^{C}	27.18 ± 0.47^{BC}

The significant difference was shown by comparing all the groups. Different characters mean P < 0.05.

$P < 0.001$). Furthermore, the level of MDA in the muscle was significantly decreased in ETem20 ($F_{3,23} = 363.323$, $P < 0.001$) and increased in ETem25 ($F_{3,23} = 363.323$, $P < 0.001$) compared with the field group, and significantly increased in the ETem25 ($F_{3,23} = 363.323$, $P < 0.001$) compared with ETem20 and HWG (**Figure 4A**). The level of H_2O_2 in the liver was significantly elevated in ETem20 ($F_{3,23} = 13.785$, $P < 0.001$) and ETem25 ($F_{3,23} = 13.785$, $P < 0.001$) compared with the field group and HWG. It was also significantly increased in the ETem25 ($F_{3,23} = 75.799$, $P < 0.001$) compared with the field group, ETem20, and HWG in the muscle (**Figure 4B**).

The level of GSH, an effective antioxidant, in the liver ($F_{3,26} = 23.92$, $P < 0.001$; $F_{3,26} = 23.92$, $P < 0.001$; $F_{3,26} = 23.92$, $P < 0.001$) and muscle ($F_{3,20} = 153.889$, $P < 0.001$; $F_{3,20} = 153.889$, $P < 0.001$; $F_{3,20} = 153.889$, $P < 0.001$) was remarkably decreased in ETem20, ETem25, and HWG compared with the field group, respectively. In addition, it was significantly increased in HWG ($F_{3,26} = 23.92$, $P < 0.05$) compared with ETem20 and ETem25 in the liver, but significantly decreased in ETem20 ($F_{3,20} = 153.889$, $P < 0.001$) compared with ETem25 and HWG in the muscle (**Table 3**). The ratio of GSH/GSSG, an oxidative stress index, was significantly decreased in the liver ($F_{3,25} = 24.089$, $P < 0.001$; $F_{3,25} = 24.089$, $P < 0.001$; $F_{3,25} = 24.089$, $P < 0.001$) and muscle ($F_{3,21} = 60.417$, $P < 0.001$; $F_{3,21} = 60.417$, $P < 0.001$; $F_{3,21} = 60.417$, $P < 0.001$) of ETem20, ETem25, and HWG compared with the field group. Furthermore, it was significantly increased in HWG ($F_{3,25} = 24.089$, $P < 0.05$) compared with ETem20 and ETem25 in the liver, and was significantly decreased in ETem20 ($F_{3,21} = 60.417$, $P < 0.001$) compared with ETem25 and HWG in the muscle (**Table 3**).

Antioxidant Activity in Liver and Muscle

The acclimation to high temperatures had a significant effect on antioxidant enzymes activities and total antioxidant capacity

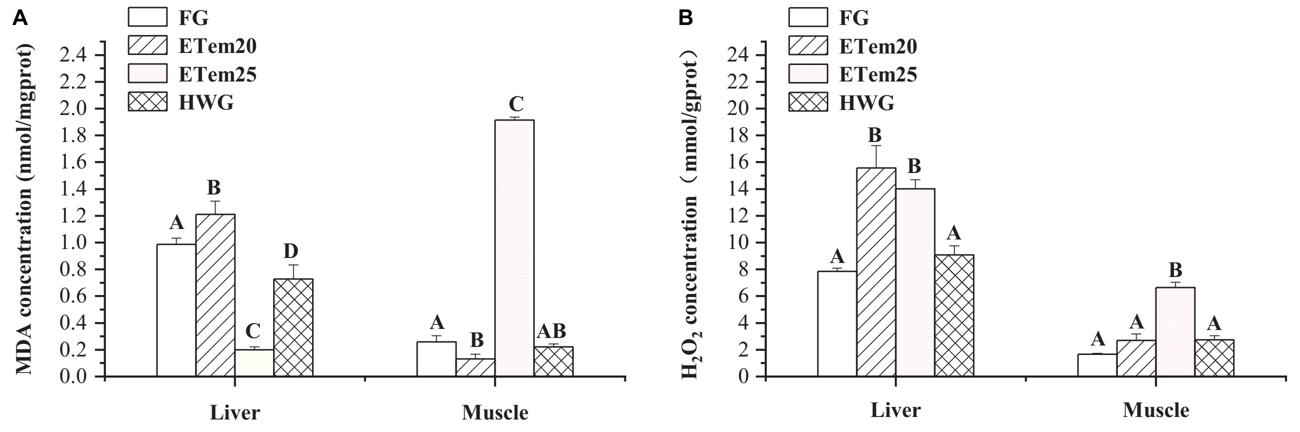

FIGURE 4 | Changes in panel **(A)** malondialdehyde (MDA) and **(B)** H_2O_2 level in the liver and muscle of *N. pleskei* ($n = 8$) in different temperature acclimation groups and field group. The sample size of each group was eight. The significant difference was shown by comparing all the groups. Different characters mean $P < 0.05$.

TABLE 3 | Glutathione status in the liver and muscle of *N. pleskei* in different temperature acclimation groups and field group.

	N		**FG**	**ETem20**	**ETem25**	**HWG**
Liver	8	GSH-eq(μmol/gprot)	206.9 ± 6.85^{A}	387.4 ± 39.6^{B}	248.0 ± 12.9^{C}	201.9 ± 10.1^{A}
	8	GSH(μmol/gprot)	78.6 ± 5.46^{A}	32.1 ± 1.21^{B}	32.7 ± 3.19^{B}	57.7 ± 11.0^{C}
	8	GSSG(μmol/gprot)	57.9 ± 5.9^{A}	171.5 ± 22.3^{B}	118.8 ± 14.3^{C}	75.1 ± 7.8^{AC}
	8	GSH/GSSG	1.40 ± 0.18^{A}	0.199 ± 0.02^{B}	0.33 ± 0.03^{B}	0.77 ± 0.11^{C}
Muscle	8	GSH-eq(μmol/gprot)	113.8 ± 5.31^{A}	177.4 ± 18.6^{B}	103.2 ± 6.42^{A}	85.4 ± 1.71^{A}
	8	GSH(μmol/gprot)	39.9 ± 0.47^{A}	6.18 ± 1.47^{B}	18.6 ± 0.44^{C}	15.5 ± 1.82^{C}
	8	GSSG(μmol/gprot)	39.6 ± 2.22^{A}	88.5 ± 11.3^{B}	35.1 ± 6.86^{A}	39.3 ± 5.02^{A}
	8	GSH/GSSG	1.10 ± 0.15^{A}	0.05 ± 0.00^{B}	0.44 ± 0.01^{C}	0.43 ± 0.04^{C}

Significance was determined by comparing the livers and muscles of N. pleskei among all the groups. Different characters mean P < 0.05.

(T-AOC; **Figure 5**). The activity of CAT increased by 2-fold in ETem20 ($F_{3,27} = 16.714$, $P < 0.001$) and HWG ($F_{3,27} = 16.714$, $P < 0.001$) in the liver compared with the field group and ETem25, but was decreased by 1.9-fold in ETem20 ($F_{3,26} = 8.121$, $P < 0.001$), 2.3-fold in ETem25 ($F_{3,26} = 8.121$, $P < 0.001$), and 2.6-fold in HWG ($F_{3,26} = 8.121$, $P < 0.001$) in the muscle compared with the field group (**Figure 5A**). The activity of GR in the liver increased by 1.7-fold in ETem20 ($F_{3,25} = 386.082$, $P < 0.001$), but decreased by 3.2-fold in ETem25 ($F_{3,25} = 386.082$, $P < 0.001$) and 1.6-fold in HWG ($F_{3,25} = 386.082$, $P < 0.001$), whereas in the muscle, it decreased by 1.8-fold in ETem20 ($F_{3,26} = 133.269$, $P < 0.001$), 2-fold in HWG ($F_{3,26} = 133.269$, $P < 0.001$), and increased by 2.7-fold in ETem25 ($F_{(1,13)} = 20.049$, $P < 0.001$) compared with the field group (**Figure 5B**). In addition, it significantly increased in ETem20 ($F_{3,25} = 386.082$, $P < 0.001$) compared with ETem25 and HWG in the liver, and increased in ETem25 ($F_{3,26} = 133.269$, $P < 0.001$) compared with ETem20 and HWG in the muscle. Compared with the field group, the activity of GPX increased by 2.7-fold in ETem20 ($F_{3,25} = 33.278$, $P < 0.001$), 2.3-fold in ETem25 ($F_{3,25} = 33.278$, $P < 0.01$) and decreased by 1.9-fold in HWG ($F_{3,25} = 33.278$, $P < 0.05$) in the liver, and it decreased by 2.6-fold in ETem20 ($F_{3,25} = 67.267$, $P < 0.01$), 4.1-fold in HWG ($F_{3,25} = 67.267$, $P < 0.001$) and increased by 1.3-fold in ETem25 ($F_{3,25} = 67.267$, $P < 0.001$) in the muscle (**Figure 5C**). Moreover, it increased in the ETem20 and ETem25 compared with the HWG ($F_{3,25} = 33.278$, $P < 0.001$) in the liver, and in the ETem25 ($F_{3,25} = 67.267$, $P < 0.001$) compared with ETem20 and HWG in the muscle (**Figure 5C**). The activity of SOD increased by 2.2-fold in ETem20 ($F_{3,27} = 24.413$, $P < 0.001$), 1.2-fold in ETem25 ($F_{3,27} = 24.413$, $P < 0.01$) and decreased by 1.3-fold in HWG ($F_{3,27} = 24.413$, $P < 0.05$) in the liver compared with the field group, but it decreased by 2-fold in ETem20 ($F_{3,26} = 18.291$, $P < 0.01$) and 1.8-fold in HWG ($F_{3,26} = 18.291$, $P < 0.01$) in the muscle compared with the field group and ETem25 (**Figure 5D**). In addition, it increased in the ETem20 ($F_{3,27} = 24.413$, $P < 0.001$) in the liver compared with the ETem25 and HWG (**Figure 5D**). The T-AOC in the liver increased by 2.7-fold in ETem20 ($F_{3,20} = 47.238$, $P < 0.001$), 1.6-fold in ETem25 ($F_{3,20} = 47.238$, $P < 0.01$), and decreased by 1.1-fold in HWG ($F_{3,20} = 47.238$, $P < 0.05$) compared with the field group. Furthermore, it increased in ETem20 ($F_{3,20} = 47.238$, $P < 0.01$) in the liver compared with ETem25 and HWG (**Figure 5E**). There was no significant difference in the muscle among all the groups (**Figure 5E**).

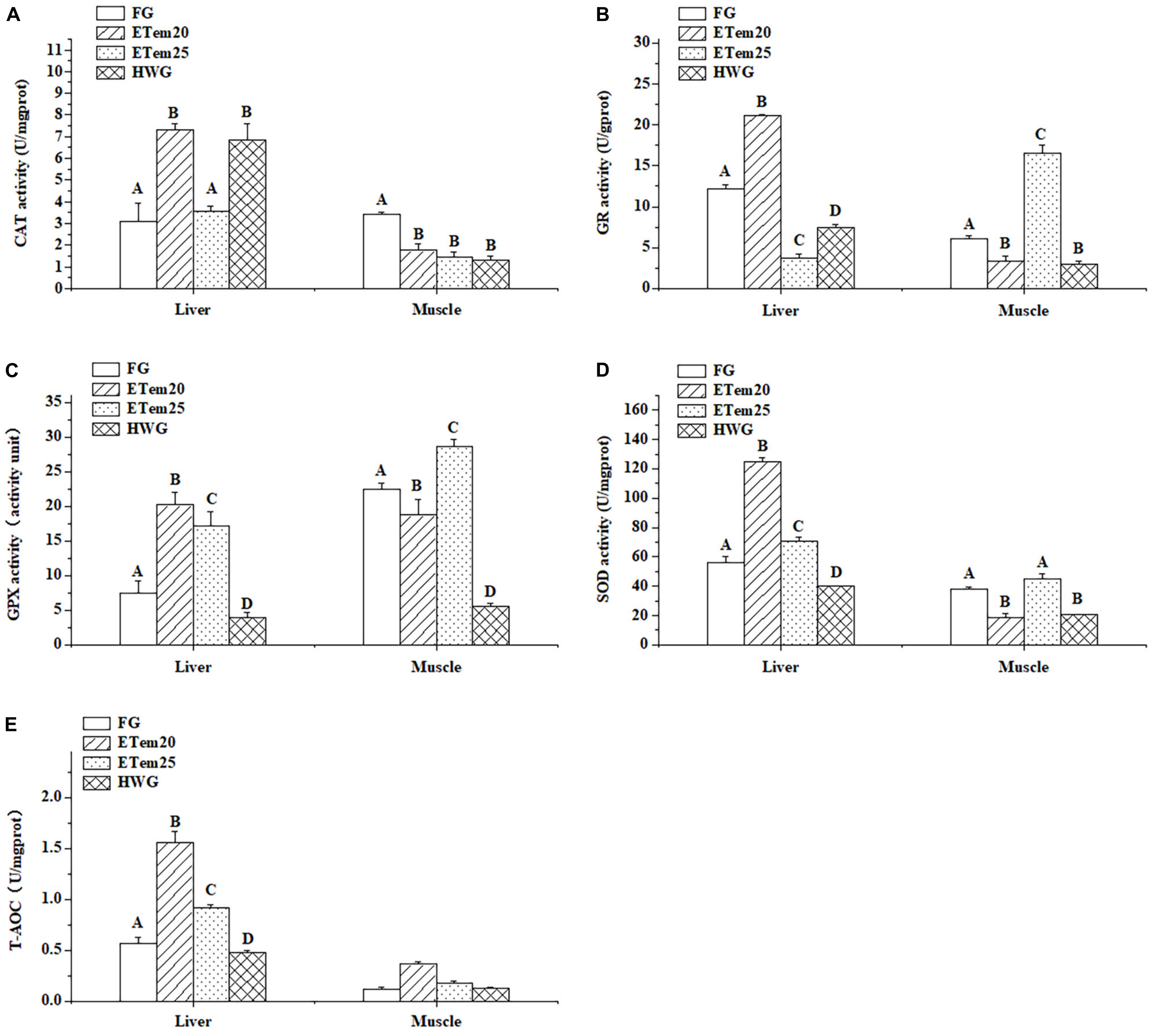

FIGURE 5 | Changes in panel **(A)** catalase (CAT), **(B)** glutathione reductase (GR), **(C)** glutathione peroxidase (GPX), and **(D)** superoxide dismutase (SOD) activity, and **(E)** total antioxidant capacity (T-AOC) in the liver and muscle of *N. pleskei* ($n = 8$) in different temperature acclimation groups and field group. The sample size of each group was 8. The significant difference was shown by comparing all the groups. Different characters mean $P < 0.05$.

DISCUSSION

This study investigated the effect of high temperatures and heat wave on thermal biology, locomotion performance, oxidative stress, and antioxidant defense systems in the high-altitude frog *N. pleskei*. The results indicated that thermal biology and locomotion performance showed plasticity for *N. pleskei* acclimated for 2 weeks at high temperatures and heat wave. The level of oxidative stress, oxidative damage, and antioxidant activity were remarkably enhanced in frogs acclimated at high temperatures. However, heat wave nearly has no significant influence on the oxidative stress of *N. pleskei* and even decreased the oxidative damage and activities of mostly antioxidant enzymes.

Preferred Body Temperature and Thermal Tolerances

The increasing ambient temperature may reduce the activity time of poikilotherms, and further affect their fitness-related activities, such as foraging and mating, which will directly lead to the decline in population size and even lead to species extinction (Gvozdík, 2012). Although behavioral thermoregulation enables them to maintain their body temperature within an optimal range, it also comes at the cost of increased energy consumption and predation risk (Sartorius et al., 2002). T_{pref} is closely related to the optimal temperature range of biochemical and physiological activities of organisms (Angilletta et al., 2010a). In this study, the T_{pref} of *N. pleskei* was significantly increased after acclimation to high temperatures and heat wave, compared with field frogs. The significant effect of thermal acclimation on T_{pref} has been confirmed in other ectotherms, such as terrestrial toad (*R. icterica*) (César et al., 2018), fresh-water prawn (*Macrobrachium tenellum*) (Hernandez-Sandoval et al., 2018), river shrimp (*Cryphiops caementarius*) (Avalos et al., 2020), and soft-shelled turtle (*P. sinensis*) (Wu et al., 2013). The great ability of thermal acclimation makes them remain active at high temperature, and expand the time window for activity, so as to partially or completely compensate for the impact of climate change (Gvozdík, 2012).

CT_{max} and CT_{min} are usually defined as the upper limit and lower limit of individual survival, and animals will experience movement disorder, spasm, and loss of righting reflex or balance when the ambient temperature reaches or exceeds the high-temperature and low-temperature limits (Lowe and Vance, 1955). We found that both the CT_{max} (31.62°C) and CT_{min}(3.17°C) of unacclimated frog *N. pleskei* were higher than the annual maximum (24.7°C) and minimum(−24.4°C) temperatures in their distribution range. Ectothermic animals have thermal tolerance after a short period of sublethal temperature, which is an acute response, enabling them to cope with short-term fluctuations in ambient temperature (Hong et al., 2009). That was aligned with the results, 2 weeks of acclimation to high temperatures significantly increased the temperature tolerance range of *N. pleskei* (CT_{max} was significantly increased, and CT_{min} was significantly decreased). The plasticity of thermal tolerance has also been demonstrated in other exothermic animals, such as *Galaxias zebratus* (Olsen et al., 2021), *Argyrosomus regius* (Kır et al., 2017), and *Eleutherodactylus coqui* (Haggerty, 2016).

The T_{pref}, CT_{max}, and CT_{min} of frogs acclimated in heat wave also showed plasticity. The T_{amb} and CT_{max} increased and CT_{min} decreased significantly, although there was no significant difference in the T_{pref} compared with field frogs. Brusch et al. (2016) investigated the effects of microhabitat type, species, and SVL on thermal tolerances of 10 lizards from Costa Rica and revealed that microhabitat type is an important factor to predict the maximum tolerated temperature regardless of size or evolutionary relationship. The climate conditions in Tibetan plateau are complex and changeable, such as windy and dry in spring and winter, rainy in summer and autumn, and great circadian temperature difference (Zhang et al., 2006). In addition, Zhao et al. (2014) found that the summer days and daily maximum and minimum air temperature have all risen, while the freezing and frost days have all declined in the QTP from 1963 to 2012. The warming trend of lowest temperature was more striking than that of highest temperature. Chen et al. (2020) predicted that the number of heat wave days in the Qinghai Tibet Plateau will significantly increase in the future. Therefore, we suggested that the plasticity of thermal biology in frogs acclimated to high temperatures and heat wave may be closely related to the climate of microhabitat of *N. pleskei* on the plateau, and that it would show great adaptability to the variability of habitat climate. The thermal tolerance of high-altitude *N. pleskei* may be due to their adaptation mechanism to the changing thermal environment, enabling them to survive in the short-term exposure at high temperatures. A similar phenomenon has also been found in the anuran species that have transformed from aquatic to terrestrial or semi-terrestrial during ontogenesis, and their increased thermal tolerance is also important for their survival, as they may be exposed to a wide range of diurnal temperature fluctuations (Sherman, 1980; Rome et al., 1992).

Stamina and Burst Swimming Speed

As found in most frogs and toads (Knowles and Weigl, 1990; Wilson and Franklin, 1999; Wilson et al., 2000; Padilla et al., 2019), the locomotor performance of highland *N. pleskei* was also affected by the acclimation temperature. Compared with the field group, the total time of jumping to exhaustion significantly decreased while the burst swimming speed of the frogs was significantly increased after the acclimation to high temperatures and heat wave. The locomotor performance of the animals, especially the burst performance, is important for avoiding predators and catching prey. However, stamina may be more important in finding and copulating with mates. Probably there is a trade-off between these two locomotor properties, as they depend on different underlying physiological mechanisms (Padilla et al., 2019). Our results demonstrated that *N. pleskei* was able to jump faster until exhaustion and swim faster after the acclimation to high temperature and heat wave, and that this may be more likely to contribute to their foraging and avoiding predators successfully to maintain normal physical condition (Preest and Pough, 2003). Accordingly, we speculated that the strengthening of burst ability and the attenuation of endurance may be beneficial to the survival of frogs in a short time, but with the continuous rising of global temperatures, further research is needed to assess the impact of extreme high temperature on the survival of plateau amphibians.

Oxidative Stress and Oxidative Damage

The oxygen consumption and metabolism of animals increase with rise in temperature within a certain range, resulting in increased generation of reactive oxygen species (ROS; Paital et al., 2016). The overproduction of ROS will damage DNA, proteins, lipids, and other biomolecules (Cheng et al., 2015). The thermal acclimation significantly increased the level of H_2O_2 (**Figure 4B**), and significantly decreased GSH content and GSH/GSSG ratio in the liver and muscle in *N. pleskei* (**Table 3**). This suggests that high-temperature-acclimated *N. pleskei* is in a state of oxidative stress compared to the field frogs. The body mass index (BMI) and muscle index (MI) values significantly decreased in the frogs after acclimation in high temperatures compared with those before acclimation, and the hepatosomatic index (HSI) was lowest in ETem25 among all the groups (**Table 1**). These may be the result of the energy budget among antioxidation, growth, and development for *N. pleskei* in response to high temperatures (Ferral et al., 2020). Niu et al. (2018) and Zhang et al. (2021) have demonstrated that the content of GSH-eq and GSSG in *N. parkeri* was markedly decreased in winter and long-term cold acclimation. On the contrary, the content of GSH-eq and GSSG obviously increased in the liver and muscle in *N. pleskei* acclimated at high temperatures and heat wave. This inconsistency may be related to food intake. The frogs were unfed during winter and cold acclimation, but food was plenty during thermal acclimation in our experiment. Therefore, it is possible that the *de novo* synthesis of glutathione increased in the frogs during our thermal acclimation, so the GSH-eq content in the liver and muscle significantly increased (Zhang et al., 2012; Niu et al., 2018). GSH is one of the important non-enzymatic antioxidants in cells to inhibit the production of ROS (Meister and Anderson, 1983). In our study, GSH is greatly oxidized to GSSG by the catalysis of GPX to inhibit the further production of ROS, because the activity of GPX was significantly enhanced in *N. pleskei* acclimated at high temperatures and heat wave (**Figure 5C**). This maintains the redox status in hepatocytes and muscle cells of frogs under thermal acclimation, which may be the main reason for the increase in GSSG content and decrease in GSH/GSSG ratio in the liver and muscle.

The acclimation to heat wave did not significantly influence the level of H_2O_2 in the liver and muscle, but it remarkably reduced the lipid oxidative damage in the liver (**Figure 4**). This may be due to the increase in CAT activity (**Figure 5A**), because it decomposes H_2O_2 into H_2O and oxygen (Lledías et al., 1998). The GSH content in the liver and muscle, and GSH/GSSG ratio in the muscle significantly decreased after the acclimation to heat wave. These results correspond with the study on corn snake (*P. guttatus*), in which the acclimation to heat wave reduced its oxidative stress (Stahlschmidt et al., 2017). However, the telomere length and overwinter survival of desert toad-headed agama (*Phrynocephalus przewalskii*) were shortened after exposure to a stimulated heat wave (Zhang et al., 2018). In Asian yellow pond turtle (*Mauremys mutica*), heat wave does not significantly affect the level of MDA in the liver and muscle (Li et al., 2021). Therefore, heat wave has different physiological effects on different species, positive or negative. Our study indicated that simulated heat wave does not induce oxidative stress, and even significantly decreased oxidative damage in frogs. In addition, the BMI did not significantly change in the frogs acclimated in heat wave compared with that before the acclimation to heat wave. Therefore, we suggested that heat wave may have positive effects on *N. pleskei*. The possible reason for the non-significant or positive effects of heat wave is that the thermal regimes of the heat wave group may be too mild for frogs and they can recover from heat stress in the low-temperature period.

Antioxidant Defense System

Under normal conditions, the production and elimination of ROS maintain a dynamic balance in cell metabolism. Thermal stress could impair the balance between oxidant and antioxidant defense mechanisms, causing oxidative stress and oxidative damage in tissues of organisms (Maud et al., 2006; Tu et al., 2012). As we have mentioned above, higher temperature acclimation induced oxidative stress and oxidative damage in *N. pleskei* (**Figure 4**). To offset the adverse effects of peroxides, the organism usually activates the antioxidant enzymes and some non-enzymatic antioxidants to resist oxidative stress and prevent or repair oxidative damage (Tu et al., 2012; Duan et al., 2016). In this study, compared to the field group, the activities of GPX, GR, CAT, SOD, and T-AOC in the liver were significantly increased in ETem20 (**Figure 5**). In addition, the activities of GPX and SOD and T-AOC in the liver, and GPX and GR activity in the muscle were significantly increased in ETem25 (**Figure 5**). These results indicated that the overall antioxidant capacity of *N. pleskei* is enhanced after high-temperature acclimation. Similar inference was found in giant spiny frog (*Quasipaa spinosa*) (Liu et al., 2018), puffer fish (*Takifugu obscurus*) (Cheng et al., 2015), and ark shell (*Scapharca broughtonii*) (An and Choi, 2010).

In terms of the effect of heat wave on antioxidant defense, our results showed that the activities of GR, GPX, and SOD decreased in the liver and muscle, but that the activity of CAT in the liver increased in high-altitude frog, *N. pleskei* (**Figure 5**). This may be the reason for the significant decrease in lipid peroxide level. Similar results were found in *P. guttatus* in the acclimation to simulated heat wave, in which the antioxidative capacity did not change but the oxidative damage level obviously decreased (Stahlschmidt et al., 2017). On the contrary, although the activity of T-SOD and CAT, and T-AOC are not affected by heat wave, the GPX activity in the muscle in *M. mutica* significantly increased (Li et al., 2021). The activities of antioxidant enzymes were attenuated for *N. pleskei* in the acclimation to simulated heat wave, but its oxidative damage level dramatically decreased. In the process of long-term adaptation to the plateau environment, *N. pleskei* may have evolved a unique mechanism to deal with temperature fluctuations. Therefore, their oxidative damage and antioxidant enzyme activity did not significantly change and even attenuated with simulated heat wave treatment.

CONCLUSION

In conclusion, thermal preference, thermal tolerance, stamina, and burst performance showed plasticity under thermal acclimation and simulated heat wave. In addition, thermal acclimation disturbs the redox state, which is manifested as oxidative stress and oxidative damage in the liver and muscle of the high-altitude frog *N. pleskei*. The antioxidant capacity was enhanced significantly after high-temperatures acclimation. Unexpectedly, the heat wave did not cause oxidative stress and oxidative damage, and even decreased the activity of antioxidant enzymes. In summary, the impact of heat wave on amphibians is not as great as we expected, while the short-term extreme high temperatures have a greater impact on them. These results can provide a reference for studies on the adaptation of other high-altitude amphibians and protection of the QTP ecosystem.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of Animal Experiments at Lanzhou University.

AUTHOR CONTRIBUTIONS

QC and JH: conceptualization. QC, JH, PP, MM, and ZN: methodology. JH: formal analysis and writing – original draft. QC, JH, TZ, FM, MM, and LX: investigation. QC, JH, and XT: data curation. PP, XT, and QC: writing – review and editing. JH and PP: visualization. QC, MM, and XT: supervision. All the authors read and approved the submitted version.

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Assessment of Amphibians Vulnerability to Climate Change in China

Chunlin Zhao[1,2], Jianping Jiang[1], Feng Xie[1], Cheng Li[1] and Tian Zhao[1]*

[1] CAS Key Laboratory of Mountain Ecological Restoration and Bioresource Utilization and Ecological Restoration Biodiversity Conservation Key Laboratory of Sichuan Province, Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, China, [2] Key Laboratory of Bio-Resources and Eco-Environment (Ministry of Education), College of Life Sciences, Sichuan University, Chengdu, China

Global climate change is considered to be one of the main threats to organisms. As poikilothermic animals, amphibians are in particular sensitive because they cannot adapt to the dramatic climate change through active physiological regulation. Using 104 representative species, the present study conducted an assessment of amphibians vulnerability to climate change in China through the combination of two approaches. Specifically, 18 vulnerability criteria belonging to five categories (i.e., thermal tolerance, individual reproductive, population diffusion and diversity, food and habitat, and climate conditions) were first selected and scored based on literatures and experts opinions. Species were then ranked into three levels of climate change vulnerability (i.e., high, moderate, and low) by calculating vulnerability scores and conducting natural breaks analyses, as well as performing a principal coordinate analysis (PCoA) and *k*-means cluster analyses, respectively. To integrate the two results, a matrix with the ranks from each result was developed to produce a final integrated list. Our results indicated that the 104 amphibian species were classified into three types by natural breaks, with 54 low vulnerable species, 41 moderately vulnerable species, and nine highly vulnerable species. Based on the results of PCoA and *k*-means cluster analyses, five species were highly vulnerable, 38 species were moderately vulnerable, and 61 species were low vulnerable. The combination of the two ranks suggested that 36 species such as *Hyla tsinlingensis* and *Liangshantriton taliangensis* were of low vulnerability, 54 species such as *Echinotriton chinhaiensis* and *Hynobius chinensis* were of moderate vulnerability, and 14 species such as *Ichthyophis kohtaoensis* and *Zhangixalus prasinatus* were of high vulnerability. Overall, our results indicated that climate change could have strong potential effects on amphibians in China. And the highly vulnerable species such as *Ichthyophis kohtaoensis*, *Zhangixalus prasinatus*, and *Theloderma corticale* should be the priority in future conservation activities.

Keywords: climate change, amphibians, natural breaks, vulnerability assessment, conservation

Edited by:
Bao-jun Sun,
Institute of Zoology, Chinese Academy of Sciences (CAS), China

Reviewed by:
Wei Chen,
Anhui University, China
Zhiqiang Guo,
Hainan University, China

****Correspondence:***
Tian Zhao
zhaotian@cib.ac.cn

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INTRODUCTION

Biodiversity and ecosystems have been in the Anthropocene phase due to the expansion of human population since the twentieth century (Elmqvist et al., 2013). Accordingly, global changes (e.g., land use change, habitat fragmentation, biological invasions, and climate change) induced by human activities have caused dramatic impacts on the biological attributes of ecosystems (Gaston et al., 2010; Bellard et al., 2012). This is considered to be the main reason leading to the changes of organisms. For instance, previous studies indicated that global changes induced the distribution of terrestrial organisms (e.g., birds, mammals, and plants) to be shifted from low to high elevations/latitudes (Chen I.C. et al., 2011), less diverse of reef communities (Hoegh-Guldberg et al., 2007), and the change of birds species composition in tropical forest ecosystems (Pounds et al., 1999). As poikilothermic animals, amphibians are considered to be more susceptible to global changes (Carey and Alexander, 2003; Li et al., 2013). This is because they have a group of specific life history traits, such as permeable skin, eggs without shell, and aquatic living larvae but terrestrial living adults (Wake and Vredenburg, 2008). Indeed, global changes have caused the decline of one third (32%) of the amphibian populations all over the world (IUCN, 2016). This is especially true in China, as a previous study indicated that around 43% of the amphibians were strongly affected by global changes, including one extinct species, one regional extinct species, and 176 under threatened species in China (Jiang et al., 2016).

Climate change is one of the most important facets of global changes affecting the survival of amphibians (Grimm et al., 2008; Wang and Feng, 2013). Specifically, the increasing of global mean temperature can induce the phenological shifts, and thus affect amphibians life history traits (e.g., breeding time; Parmesan, 2007, hibernation; Reading, 2007; Sheridan and Bickford, 2011; Gao et al., 2015). Moreover, the fluctuation of precipitation can strongly disturb amphibians habitat quality, tadpoles metamorphosis, as well as adults population dynamics (Lowe, 2012). In addition, recent works have demonstrated that amphibians distribution patterns can be also altered by climate change (e.g., *Odorrana hainanensis*; Huang et al., 2017, *Andrias davidianus*; Zhao et al., 2020). However, these studies only focused on single species, integrative work is still needed to evaluate the vulnerability of a group of amphibian species to climate change at both regional and global scales (but see Li et al., 2013).

The vulnerability of a species to climate change is the extent to which is threatened or extinct owing to climate change (Dawson et al., 2011). Therefore, it is typically assessed by selecting criteria related to species exposure, sensitivity, and adaptive capacity to climate change (Williams et al., 2008; Foden et al., 2013). Specifically, exposure is the degree of climate change likely to be experienced by species. It is usually assessed based on scenario projections from general circulation models (GCMs), and is strongly depended on external factors such as temperature change, precipitation fluctuation, severe weather events, and sea level rise (Dawson et al., 2011; Rowland et al., 2011). Sensitivity refers to the degree of change of species survival, physiology, behavior, population dynamics, or life history when facing the stress of climate change. It can be assessed by empirical, model, and observational studies (Dawson et al., 2011; Rowland et al., 2011). Adaptive capacity is the ability of species to adapt to climate change through persistence, migration, dispersal colonization, and evolutionary responses. Similar to sensitivity, it can be also assessed by empirical, model, and observational studies (Nicotra et al., 2015; Beever et al., 2016).

In the present study, we selected representative amphibian species to assess the vulnerability of this taxa to climate change in China. We hope our results can provide useful information to determine the prior protection levels of amphibians, and provide information related to the extinction risks of different species caused by climate change to policy makers and animal protection departments.

MATERIALS AND METHODS

Representative Species

According to Fei et al. (2009), Fei et al. (2012), and the new species published in recent years, there are 585 amphibian species in China by the end of November, 2021.[1] However, not all the species have large populations in the wild. In ecological studies, species with high abundance in the field are usually selected as representative species to assess the effects of human/natural disturbance on animal communities (Park et al., 2006). Based on this criterion, and also considering some narrow distributed endemic amphibians on mountains, a total of 104 representative species were selected. Specifically, at least one species was selected from each genus, and two to three species were selected from genera that contained more than six species in China (e.g., *Scutiger*, *Rana*, and *Zhangixalus*; **Supplementary Table 1**).

Vulnerable Factors

Based on the report of IPCC in 2001, previous studies usually selected factors related to species exposure (e.g., temperature, rainfall changes, and migration barriers), sensitivity (e.g., habitat specificity, interspecific relationship, migration ability, and food diversity), and adaptability (e.g., environmental pollution, human disturbance, future adaptation to habitat change, adaptability to habitat availability, and food availability) to assess the vulnerability of species to climate change (e.g., Bagne et al., 2011; Rowland et al., 2011; Foden et al., 2013; Li et al., 2017). However, recent studies suggested that only considering these criteria cannot completely reflect the vulnerability of organisms to climate change (Fortini and Schubert, 2017). It is also necessary to incorporate factors related to species migration, habitat adaptation, thermal tolerance, and evolution, which reflect the life history response of species to climate change (Foden et al., 2013; Fortini and Schubert, 2017). Following these suggestions, a total of 18 vulnerable factors belonging to five categories (i.e., thermal tolerance, individual reproductive, population diffusion and diversity, food and habitat, and habitat climate conditions) were selected in the present study (**Supplementary Table 2**).

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[1] http://www.amphibiachina.org/

Data Acquisition

All the vulnerable factors were categorical variables. The data of species thermal tolerance, individual reproductive, population diffusion and diversity, food and habitat evaluation were acquired from Fei et al. (2006, 2009, 2012), the published literatures (e.g., Hou et al., 2014; Sung et al., 2016; Wang et al., 2017; Zeng et al., 2017), as well as the experts opinions. Data of habitat climate conditions was extracted from Worldclim database (Version 1.4).[2] Then, we divided the species vulnerability to each factor into three levels (1: Low vulnerability; 2: Moderate vulnerability; 3: High vulnerability), and the definition of each level related to each vulnerable factor was obtained following the criteria of World Wide Fund for Nature (WWF).[3] Details of the vulnerable factors and their definitions are as follows (**Supplementary Table 2**):

Thermal Tolerance

Thermal Tolerance

Amphibians have their own thermal tolerance ranges (Khatiwada et al., 2020), which reflect their resilience to temperature change (Bernardo and Spotila, 2006). We scored this factor as:

1—High thermal tolerance (Low vulnerability).
2—Moderate thermal tolerance (Moderate vulnerability).
3—Low thermal tolerance (High vulnerability).

Published information on the thermal tolerance of amphibians in China is very limited. Therefore, our uncertainty scores were determined according to the experts opinions rather than empirical evidences.

Breeding

Spawning Sites and Clutch Size

Amphibians can spawn in different sites such as trees, streams, and ponds (Duellman, 1992). Amphibians can also have varied clutch size, from a few to thousands (Fei et al., 2012). Moreover, these species with distinct spawning sites and diverse clutch size can exhibit a gradient of sensitivity to climate change (Carr et al., 2013; Foden et al., 2013). For instance, the pool-breeding amphibians are highly vulnerable to climate change (Scheele et al., 2012), and species with larger clutch size are of low vulnerability (Combes et al., 2018). Therefore, we defined breeding associated with spawning sites and clutch size as:

1—Site type of oviposition is land/clutch size is $\geq$ 1,000 (Low vulnerability).
2—Site type of oviposition is lotic water and trees/clutch size is between 100 and 1,000 (Moderate vulnerability).
3—Site type of oviposition is lentic water/clutch size is $\leq$ 100 (High vulnerability).

Metamorphosis Period

Rapidly metamorphic amphibians are less affected by climate change (Semlitsch, 1987; Rowe and Dunson, 1995; Ultsch et al., 1999; Carey and Alexander, 2003; Corn, 2005). This is because species with rapid metamorphosis can avoid death in a prolonged drought. Therefore, we defined breeding associated with metamorphosis period as:

1—Completed the metamorphosis $\leq$ 3 months (Low vulnerability).
2—Completed the metamorphosis between 3 and 12 months (Moderate vulnerability).
3—Completed the metamorphosis $\geq$ 12 months (High vulnerability).

Maturation Age

Species with fast sexual maturity are lowly vulnerable. This is because delayed reproduction may increase vulnerability to compete for limited resources when facing extreme conditions. Moreover, recruitment of long maturated species is more relied on long term of suitable conditions to allow their offspring to reach size-based resistance to extreme conditions (Luhring and Holdo, 2015). Hence, we defined breeding associated with maturation age as:

1—The sexual maturity time is $\leq$ 1 year (Low vulnerability).
2—The sexual maturity time is between 1 and 3 years (Moderate vulnerability).
3—The sexual maturity time is $\geq$ 3 years (High vulnerability).

Food and Habitat

Feeding

Temperature rise will increase amphibians metabolic rates, which may subsequently increase their food demands (Li et al., 2013). Therefore, feeding generalists can quickly obtain sufficient food, which will be less affected. However, feeding specialists may be more affected as they strongly relied on specific food items, which may be not easily acquired in the field. According, we defined feeding as:

1—Feeding generalists (Low vulnerability).
2—Between feeding specialists and generalists (Moderate vulnerability).
3—Feeding specialists (High vulnerability).

Abundance of Potential Food Resources

Climate change could indirectly affect amphibians survival rate and reproductive success *via* changing the abundance of food resources (Gouveia et al., 2013). Therefore, this item was considered as:

1—Wide range of food resources (Low vulnerability).
2—Moderate range of food resources (Moderate vulnerability).
3—Limited food resources (High vulnerability).

Primary Habitat

Arboreal type species can better adapt to drier conditions through the variation of body shape (Castro et al., 2021). However, increasing temperature may easily impact body condition and decrease the survival of terrestrial/water type species, such as *Bufo bufo* (Reading, 2007), *Xenopus laevis* (Ruthsatz et al., 2018),

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[2] http://www.worldclim.org/
[3] https://www.worldwildlife.org/initiatives/adapting-to-climate-change

and *Rana cascadae* (Kissel et al., 2019). Accordingly, we defined primary habitat as:

1—Arboreal type species (Low vulnerability).
2—Terrestrial type species (Moderate vulnerability).
3—Water type species (High vulnerability).

Habitat Type Diversity

Climate change induced habitat loss leading to the decline of amphibian populations (Li et al., 2013). Amphibians with more habitat types may be less effected by climate change. Therefore, we defined habitat type diversity as:

1—High diversity of habitat types (Low vulnerability).
2—Moderate diversity of habitat types (Moderate vulnerability).
3—Low diversity of habitat types (High vulnerability).

Population Status

Population Size

Amphibians with small populations are greatly affected by climate change. This is because populations of these species can be hardly restored once they declined due to climate change. Hence, we defined population size as:

1—Large population size (Low vulnerability).
2—Moderate population size (Moderate vulnerability).
3—Small population size (High vulnerability).

Population Distribution

Species with larger distribution range usually exhibit stronger environmental tolerance (Khatiwada et al., 2020), which can be considered as low vulnerability. Therefore, we defined populations distribution as:

1—Large distribution range (Low vulnerability).
2—Moderate distribution range (Moderate vulnerability).
3—Small distribution range (High vulnerability).

Primary Movement Methods

Movement methods are related to amphibians migration ability, with swimming species exhibiting the lowest migration ability. And they are more easily affected by climate change (Corn, 2005; Lawler et al., 2009; Warren et al., 2013). Hence, we defined primary movement methods as:

1—Jumping species (Low vulnerability).
2—Climbing and walking species (Moderate vulnerability).
3—Swimming species (High vulnerability).

Genetic Diversity

Species with low genetic diversity have fewer new characteristics that can adapt to new climatic conditions (Foden and Young, 2016). Therefore, we defined genetic diversity as:

1—High genetic diversity of species (Low vulnerability).
2—Moderate genetic diversity of species (Moderate vulnerability).
3—Low genetic diversity of species (High vulnerability).

Breeding Migration

Some amphibian species in temperate regions migrate from terrestrial habitats to aquatic habitats for short-term reproduction (Duellman and Trueb, 1985). Since rainfall plays a crucial role in determining amphibians breeding migrations (Greenberg and Tanner, 2004; Arnfield et al., 2012), climate change may cause stronger effects on these species. Thus, we defined breeding migration as:

1—Species without migration behavior (Low vulnerability).
3—Species with migration behavior in the breeding period (High vulnerability).

Habitat Climate Conditions

Extent to Which Species Is Currently Exposed to Climate Variability

Species distributed in areas that have been strongly affected by climate change (e.g., Qinghai-Tibet Plateau) could be more easily affected (Zhou et al., 2014). Therefore, we defined the extent to which species is currently exposed to climate variability as:

1—Low levels of the extent to which species is currently exposed to climate variability (Low vulnerability).
2—Moderate levels of the extent to which species is currently exposed to climate variability (Moderate vulnerability).
3—High levels of the extent to which species is currently exposed to climate variability (High vulnerability).

Extent to Which Air Temperature Is Expected to Change Within the Range of Species Distribution

Species distributed in areas with high variability of air temperature (e.g., tropics and plains) may be more susceptible (Collins and Storfer, 2003; Toranza and Maneyro, 2014). Thus, we defined this item as:

1—Low levels of the extent to which air temperature is expected to change within the range of species distribution (Low vulnerability).
2—Moderate levels of the extent to which air temperature is expected to change within the range of species distribution (Moderate vulnerability).
3—High levels of the extent to which air temperature is expected to change within the range of species distribution (High vulnerability).

Extent to Which Precipitation Is Expected to Change Within the Range of Species Distribution

Species distributed in areas with high levels of precipitation variability (e.g., eastern China) may be more easily affected (Chen S. et al., 2011). Hence, we defined it as:

1—Low levels of the extent to which precipitation is expected to change within the range of species distribution (Low vulnerability).
2—Moderate levels of the extent to which precipitation is expected to change within the range of species distribution (Moderate vulnerability).

3—High levels of the extent to which precipitation is expected to change within the range of species distribution (High vulnerability).

Other Threat Factors

There are many others threats for amphibians, including habitat loss, habitat alteration, and pollution, etc. (Alford and Richards, 1999; Pimm and Raven, 2000; Collins and Storfer, 2003; Stuart et al., 2004). Species with less effects of these factors can be considered as low vulnerability. These were mainly determined according to the experts opinions as follows:

1—Low levels of other threat factors (Low vulnerability).
2—Moderate levels of other threat factors (Moderate vulnerability).
3—High levels of other threat factors (High vulnerability).

Statistical Analyses

Two approaches are incorporated to assess the vulnerability of amphibians to climate change in China. First, the climate change vulnerability index was calculated for each species based on the formula as follows:

$$VI = \prod_{i=1}^{k} \left(\sum_{i=1}^{n} VF_i \right)$$

where VI is the vulnerability index (the larger the value, the higher the vulnerability), VF_i is the criterion of each category, n is the total number of factors within each category, and k is the total number of categories. After that, natural breaks analyses were used to determine the vulnerability of 104 species to climate change following Gardali et al. (2012). Second, a principal coordinate analysis (PCoA; Gower, 1966) based on the Bray-Curtis index of the distance matrix created by Euclidean distance between species was conducted following Villéger et al. (2008). PC axes with their eigenvalue ≥ 1 were selected to construct a multi-dimensional vulnerability space. Then, k-means cluster analyses were carried out according to the distribution of all species in the space. Finally, according to the method provided by Gardali et al. (2012), the two results for each species were integrated by using the comprehensive list matrix to determine the vulnerability for each species (**Table 1**).

RESULTS

According to the International Union for Conservation of Nature (IUCN) red list, the 104 representative amphibian species included one Extinct (ET), three Critically Endangered (CR), eight Endangered (EN), 11 Vulnerable (VU), five Near Threatened (NT), 54 Least Concern (LC), and 22 No Evaluated/Data Deficient (NE/DD).

Results Based on Natural Breaks

The results from species vulnerability index and natural breaks analyses indicated that 54 species belonged to the low vulnerability type (e.g., *F. multistriata, Hoplobatrachus chinensis*, *Rhacophorus bipunctatus*, *Rana omeimontis*, and *Oreolalax major*), accounting for 51.92% of all the studied species. 41 species were moderate vulnerability type (e.g., *A. davidianus*, *Rana kukunoris*, *Hynobius chinensis*, *Amolops granulosus*, and *Scutiger glandulatus*), accounting for 39.42% of all the studied species. And nine species were high vulnerability type (e.g., *Glyphoglossus yunnanensis*, *Cynops wolterstorffi*, *Amolops medogensi*, *Batrachuperus pinchonii*, *Glandirana emeljanovi*, *Hylarana cubitalis*, *Pachyhynobius shangchengensis*, *Odorrana zhaoi*, and *Onychodactylus zhangyapingi*; **Supplementary Table 3**), accounting for 8.65% of all the studied species.

Results Based on PCoA and *k*-Means Cluster Analyses

The first four principal components had their eigenvalue ≥ 1, which explained 55% of the total inertia (PC1 = 21.13%, PC2 = 14.40%, PC3 = 10.96%, PC4 = 8.51%). Specifically, PC1 was mainly positively correlated with spawning sites, but negatively correlated with clutch size, metamorphosis period, maturation age, habitat type diversity, population size, population distribution, and genetic diversity. PC2 was mainly positively correlated with thermal tolerance, metamorphosis period, maturation age, extent to which species is currently exposed to climate variability, extent to which air temperature is expected to change within the range of species distribution, and negatively correlated with extent to which precipitation is expected to change within the range of species distribution. PC3 was mainly positively correlated with primary movement methods, but negatively correlated with metamorphosis period. In addition, the interpretation rate of each factor to PC4 was relatively small ($<$ 50%; **Supplementary Table 4** and **Figure 1**).

The results of k-means cluster analyses indicated that 61 species (e.g., *R. kukunoris*, *R. omeimontis*, *Pelophylax plancyi*, *Tylototriton kweichowensis*, *F. multistriata*, *O. major*, and *Hoplobatrachus chinensis*) were low vulnerable species, accounting for 58.65% of all the studied species. 38 species (e.g., *Hynobius chinensis*, *A. davidianus*, *G. minima*, *A. granulosus*, *S. glandulatus*, and *G. yunnanensis*) were moderately vulnerable species, accounting for 36.54% of the total studied species. Finally, five species (i.e., *Ichthyophis kohtaoensis*, *K. eiffingeri*, *T. corticale*, *R. bipunctatus*, and *Zhangixalus prasinatus*; **Supplementary Table 3**) were highly vulnerable species, accounting for 4.8% of the total species.

The Integrated Results Based on Two Approaches

Comprehensive list matrix was created according to the integrated results from the two approaches. Our results demonstrated that 36 species were finally classified as low vulnerability type (e.g., *F. multistriata, O. major*, *R. omeimontis*, and *Hoplobatrachus chinensis*). 54 species were moderate vulnerability (e.g., *A. davidianus*, *Hynobius chinensis*, *R. kukunoris*, *G. minima*, and *A. granulosus*). And 14 species were high vulnerability (i.e., *I. kohtaoensis*, *P. shangchengensis*, *B. pinchonii, O. zhangyapingi*, *C. wolterstorffi*, *Glandirana emeljanovi*, *Hylarana cubitalis*, *A. medogensi*, *O. zhaoi*,

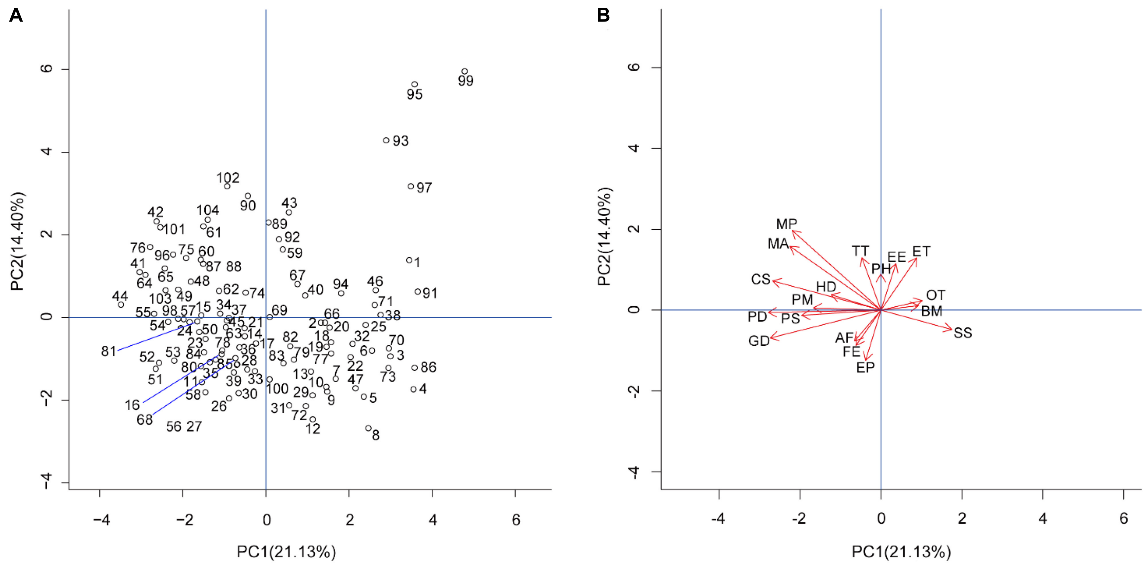

FIGURE 1 | Principal coordinate analysis of 104 species and 18 vulnerable factors. **(A)** Distribution of the 104 species. **(B)** Contribution of the 18 vulnerable factors. Codes of species are in **Supplementary Table 1**. Abbreviation of vulnerable factors are as follows: MP, metamorphosis period; MA, maturation age; CS, clutch size; HD, habitat type diversity; PM, primary movement methods; PD, population distribution; PS, population size; GD, genetic diversity; AF, abundance of potential food resources; FE, feeding; EP, extent to which precipitation is expected to change within the range of species distribution; SS, spawning sites; BM, breeding migration; OT, other threats factors; ET, extent to which air temperature is expected to change within the range of species distribution; EE, extent to which species is currently exposed to climate variability; PH, primary habitat; TT, thermal tolerance.

K. eiffingeri, *T. corticale*, *R. bipunctatus*, *Z. prasinatus*, and *G. yunnanensis*; **Supplementary Table 3**).

DISCUSSION

The selection of representative species was considered to be feasible to study the response of animal groups to the natural/human induced disturbance (Park et al., 2006). In the present study, 104 representative species were selected from amphibians in China (at least one for each genus, and two to three were selected from genera that contained more than six species; e.g., *Scutiger*, *Rana*, and *Zhangixalus*) to investigate the vulnerability of this taxa to climate change by using the combination of two approaches. Our results indicated that 14 species were highly vulnerable to climate change, with most of them distributing in low latitude areas. For instance, *I. kohtaoensis*, *H. cubitalis*, *C. wolterstorffi*, *T. corticale*, and *G. yunnanensis* are mainly distributed in Yunnan, Guangxi, Guangdong, and Hainan, while *Z. prasinatus* is only distributed in Taiwan. And *A. medogensis* and *O. zhaoi* are distributed in Moto, Tibet. Therefore, our results supported the claims that species in low latitude areas were more vulnerable to climate change (Deutsch et al., 2008). This is because species at lower latitudes always live at optimum temperature, they thus are more sensitive to temperature changes (Deutsch et al., 2008). Within the 14 high vulnerability species, five species (36%) were endemic, and most of their IUCN categories were above NT. This result is consistent with Li et al. (2013), suggesting that the endemic and endangered species were more vulnerable to climate change because of their narrow distribution and small populations. In the present study, most of these species are distributed in the eastern Qinghai-Tibet plateau and Hengduan mountains, which exhibited specific habitats. For instance, *C. wolterstorffi* preferred shallow water bodies around Dianchi Lake, in which had abundant aquatic plants and plankton (He, 1998). Furthermore, *O. zhaoi* can survive and reproduce only under the habitat conditions of small montane streams and small waterfalls (Fei et al., 2012). The effects of climate change on these species could be the reduction of body length (Sheridan and Bickford, 2011), migration to suitable habitats (Duan et al., 2016), and faster metamorphosis. At the same time, climate change (e.g., drought, flood, and high temperature) will also lead to strong changes in habitat characteristic (McMenamin et al., 2008) and food sources (Donnelly and Crump, 1998) of other high vulnerability species such as *I. kohtaoensis*, *P. shangchengensis*, *B. pinchonii*, and *O. zhangyapingi*, resulting in a decline in their populations.

TABLE 1 | Matrix that integrates the ranks of amphibians vulnerability to climate change from natural breaks and *k*-means cluster analyses.

	Integrative rank		
	K-means cluster rank		
Natural breaks rank	1	2	3
1	**1**	**2**	**3**
2	**2**	**2**	**3**
3	**3**	**3**	**3**

1, low vulnerability; 2, moderate vulnerability; 3, high vulnerability.

There were 54 moderately vulnerable species, probably because the life history traits of these species may be able to cope with the impact of climate change within a certain range (Naya et al., 2011). This is because some species, such as *Polypedates megacephalus* and *Bufotes pewzowi*, can be in response to climate change through more evolved reproductive patterns (e.g., laying eggs on the land/tree; Li and Jiang, 2016, and larger clutch sizes; Fei et al., 2012). Overall, 68 species (about 65% of the total number of studied species) were assessed as high and moderate vulnerability to climate change, indicating that climate change has a strong impact on amphibians in China, which may be one of the main reasons causing the decline of amphibian populations (Wu and Li, 2004). More importantly, the IUCN categories of 20 out of these 68 species were above VU. Since conservation should be conducted for endemic and/or endangered species with limited individuals in the field (Myers et al., 2000; Kier et al., 2009), we suggested that the following eight species should also be considered in future climate change studies (i.e., *A. davidianus*, *G. minima*, *Echinotriton chinhaiensis*, *Ranodon sibiricus*, *H. chinensis*, *Rana sauteri*, *Liuixalus ocellatus*, and *Parapelophryne scalpta*).

Overall, our study investigated that more than 60% of the amphibians (i.e., high and moderate vulnerability) were strongly affected by climate change in China. This can be attributed to these species specific life history traits, population status, and habitat conditions. According, these species, in particular highly vulnerable species, should be the priority in future conservation activities. In recent decades, a growing number of natural reserves have been established in China to protect amphibians. However, since the climate condition of habitats may be no longer suitable for amphibians due to climate change in the future, more work (e.g., the adjustment of natural reserves in time) is still needed (Ma and Jiang, 2005). More importantly, our results also suggested that the vulnerability of climate change research cannot only focus on large vertebrates (e.g., *Ailuropoda melanoleuca* and *Panthera uncia*), amphibians should also be considered and better protected in response to future climate change.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of Chengdu Institute of Biology.

AUTHOR CONTRIBUTIONS

TZ contributed to the development of ideas. TZ and CZ analyzed the data and wrote the first draft of the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Hypoxia Induces Oxidative Injury and Apoptosis *via* Mediating the Nrf-2/Hippo Pathway in Blood Cells of Largemouth Bass (*Micropterus salmoides*)

Yu Xin[1,2†], Zhixin Yang[2†], Yuke Zhu[2], Yixuan Li[2], Jie Yu[2], Wanqing Zhong[2], Yanhan Chen[2], Xiaohui Lv[3], Junru Hu[3], Jinjiang Lin[4], Yutao Miao[2,5] and Lei Wang[2,5]*

[1] College of Fisheries, Guangdong Ocean University, Zhanjiang, China, [2] Institute of Modern Aquaculture Science and Engineering (IMASE), Guangdong Provincial Key Laboratory for Healthy and Safe Aquaculture, Guangzhou Key Laboratory of Subtropical Biodiversity and Biomonitoring, Key Laboratory of Ecology and Environmental Science in Guangdong Higher Education, School of Life Sciences, South China Normal University, Guangzhou, China, [3] Guangdong Key Laboratory of Animal Breeding and Nutrition, Key Laboratory of Animal Nutrition and Feed Science in South China, Ministry of Agriculture and Rural Affairs, Guangdong Academy of Agricultural Sciences, Institute of Animal Science, Guangzhou, China, [4] Uyu (Guangzhou) Technology Company Ltd., Guangzhou, China, [5] Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai), Zhuhai, China

Edited by:
*Lin Zhang,
Hubei University of Chinese Medicine,
China*

Reviewed by:
*Zhou Meng,
Zhongkai University of Agriculture
and Engineering, China
Yiping Luo,
Southwest University, China*

***Correspondence:**
*Lei Wang
wanglei@scnu.edu.cn*

*† These authors have contributed
equally to this work*

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Investigating how aquatic animals respond to hypoxia brought about by changes in environmental temperature may be of great significance to avoid oxidative injury and maintain the quality of farmed fish in the background of global warming. Here, we investigated the effects of hypoxia on oxidative injury and environment-sensing pathway in blood cells of *Micropterus salmoides*. The total blood cell count (TBCC) and Giemsa staining showed that hypoxia could lead to damage of blood cells. Flow cytometry analysis confirmed that the apoptosis rate, Ca^{2+} level, NO production and ROS of blood cells were significantly increased under hypoxia stress. Environment-sensing pathways, such as Nrf2 pathway showed that hypoxia resulted in significant up-regulation of hiF-1 alpha subunit (Hif-1α), nuclear factor erythroid 2-related factor 2 (Nrf2) and kelch-1ike ECH- associated protein I (Keap1) expression. Meanwhile, the expression of Hippo pathway-related genes such as MOB kinase activator 1 (MOB1), large tumor suppressor homolog 1/2 (Lats1/2), yes-associated protein/transcriptional co-activator with PDZ-binding motif (YAP/TAZ), protein phosphatase 2A (PP2A) were significantly increased in blood cells after hypoxia exposure. In addition, hypoxia stress also increased the expression of catalase (CAT) and glutathione peroxidase (GPx), but decreased the expression of superoxide dismutase (SOD). Consequently, our results suggested that hypoxia could induce oxidative injury and apoptosis *via* mediating environment-sensing pathway such as Nrf2/Hippo pathway in blood cells of *M. salmoides*.

Keywords: climate warming, hypoxia, *Micropterus salmoides*, blood cells, Nrf2/Hippo pathway

INTRODUCTION

The effects of global warming have become increasingly prevalent in marine ecosystems around the world, where consistently increasing seawater temperatures have exposed marine species to environmental stress conditions. Climate warming has already led to many changes in key environmental factors such as water temperature, seawater pH and oxygen concentration in the oceans (Breitburg et al., 2018; Vagner et al., 2019). Numerous factitious and environmental factors such as high temperature, water pollution and high-density fish farming in the water body will inevitably lead to hypoxic conditions in fish (Wang et al., 2012; Levin and Breitburg, 2015). Hypoxia is one of the main reasons that threatens the survival and growth of fish, it causes massive fish deaths and significant economic losses in the aquaculture industry, and seriously hinder the sustainable and healthy development of the aquaculture industry (Zhao et al., 2020).

Hypoxia may induce oxidative stress by breaking the balance between the production of reactive oxygen species (ROS) and the removal of ROS. Excess ROS are generated, which can damage critical cellular components and activate the antioxidant signaling pathway in fish (Azimi et al., 2017). Further, large amounts of ROS could cause apoptosis, lipid oxidation, tissue damage, and DNA and protein degradation (Jls et al., 2020). Nitric oxide (NO), as a cellular signaling molecule, has also been found to play a primary role of vasorelaxation in hypoxic situations and can be synthesized by nitric oxide synthase (NOS; Cao et al., 1981). When activated by pathogens or cytokines, such as ROS (Kumar et al., 2018), NO could be produced by NOS (Dimmeler and Zeiher, 1997). Further, NO reacts with ROS and forms peroxynitrite (ONOO−), which is highly reactive toward a wide variety of biomolecules (Curtin et al., 2002). There is also growing evidence that NO damages mitochondria, leading to the opening of transition pores, followed by cytochrome c release and caspase activation leading to apoptosis (Brüne, 2003). Earlier studies indicated that when compared with the population at sea level, the contents of NO and its products in Tibetan circulation are significantly higher, indicating that NO had effects on vascular tension, blood flow and oxygen delivery (Erzurum et al., 2007). Meanwhile, *Danio rerio* data support this possibility, accompanied by significant NO production, vasodilation and lower blood pressure (Jensen, 2007). Apoptosis is a critical component in maintaining homeostasis and growth in all tissues and plays a significant role in immunity and cytotoxicity, and it can initiate and clear damaged cells to maintain homeostasis when exposed to hypoxia (Luo et al., 2017; D'Arcy, 2019). However, the challenge that hypoxia poses to oxidative stress and the mechanisms that hypoxia induce oxidative injury and apoptosis in fish are not as well understood despite intensive research effort (Borowiec and Scott, 2020).

The antioxidant system can alleviate the negative effects of hypoxia on the body by preventing the production of ROS or by eliminating ROS, and it is mainly mediated by the transcription factor nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf-2), which can induce the expression of genes related to exogenous detoxification and cell protection (Barrera et al., 2021). As an environment-sensing pathway, Nrf-2 and its repressor, Kelch-like ECH-associated protein 1 (Keap1) can help the body defend against oxidative stress, thereby maintaining the body's homeostasis balance (Bellezza et al., 2018). Some research indicated that the interruption of Nrf-2-Keap1 signaling may occur during human aging and cause sarcopenia (Mondal et al., 2018). When oxidative stress occurs, Keap1 is oxidized at the active cysteine residue, causing Keap1 to inactivate, Nrf-2 to stabilize and translocate into the nucleus (Motohashi et al., 2002; Tonelli et al., 2018). The Hippo pathway also is an environment-sensing pathway, which always be a master regulator of tissue homeostasis (Hong et al., 2020). It is an evolutionarily conserved signal cascade that regulates many biological processes, including cell growth and fate determination, organ size control and regeneration (Ma et al., 2019). In previous studies, sterile 20-like kinase 1/2 (Mst1/2) kinases in Hippo pathway are associated with Salvador family WW domain containing protein 1 (SAV1) and Mps one binder kinase activator-like 1A and 1B (MOB1A/B or collectively, MOB1) to phosphorylate large tumor suppressor 1/2 (Lats1/2). Lats1/2 subsequently phosphorylate Yes- associated protein (YAP) and its paralog, WW domain–containing transcription regulator protein 1 (TAZ), causing them to bind *via* 14-3-3 (Zhao et al., 2007; Hao et al., 2008; Lei et al., 2008; Oka et al., 2008). Hereafter, YAP and TAZ are prevented from entering the nucleus, interacting with transcription factors (i.e., TEAD family members and others) in the cytoplasm and regulating downstream gene targets to initiate a series of signal cascades (Zhao et al., 2008; Zhang et al., 2009; Lehmann et al., 2016; Meng et al., 2016). Studies have shown that Nrf-2 could uses the Hippo pathway effector TAZ to induce tumorigenesis (Barrera et al., 2021). Meanwhile, the excessive ROS generated by hypoxia, superoxide dismutase (SOD) can efficiently convert superoxide anion (O_2^{-}) into H_2O_2 and O_2. After this process, H_2O_2 could be transformed into H_2O and O_2 under the combined action of catalase (CAT) and glutathione peroxidase (GPx), so that the body could be protected from hypoxic damage (Cao et al., 2012). However, there have been relatively few detailed examinations about how hypoxia induces oxidative damage and apoptosis through the Nrf-2/Hippo pathway in hypoxia-tolerant fish.

Largemouth bass (*Micropterus salmoides*), which is a world-famous economic fish, has been widely cultivated and become the major freshwater aquaculture species in China (Li et al., 2017; Yu et al., 2018). But the recent increase in global warming, high-density farming, and deterioration of water quality have increased the probability of hypoxia, which will inevitably lead to hypoxia stress (Sun et al., 2020c). Blood is not only an important part of the digestive and respiratory systems, but also plays important role in the immune system (Dichiera et al., 2020). Previous studies have also shown that acute hypoxic stress can lead to apoptosis and inflammatory responses in fish blood cells, and in severe cases can cause a dramatic reduction in the oxygen-carrying capacity of fish blood cells, resulting in mass mortality (Huang et al., 2019; Cai et al., 2020). Understanding the physiological changes of fish blood cells under hypoxic conditions is one of the keys to understanding how environmental stress causes oxidative damage and cell apoptosis in fish. However, in recent years, research on largemouth bass under hypoxic stress has mainly focused on some other tissue such as liver and gill (Sun et al., 2020b). For example, hypoxia could induce an increase in anaerobic glycolysis in the liver and caused histological lesions in the gill tissue of the fish (Gaulke et al., 2014; Crans et al., 2015; Yang et al., 2017). It is little known about the mechanism of how hypoxia induces oxidative damage and apoptosis in blood cells of largemouth bass.

In this study, we simulated an acute hypoxia environment in the laboratory and analyzed the physiological parameters, environment-sensing pathways such as Nrf-2/Hippo pathway and antioxidant ability of largemouth bass exposed to acute hypoxic environment for various durations. The dynamic pattern of physiological parameters and the pattern of Nrf-2/Hippo pathway during hypoxic exposure would provide a theoretical basis for future studies on healthy aquaculture of largemouth bass and facilitate our understanding of the mechanisms in hypoxia stress and global warming adaptation.

MATERIALS AND METHODS

Animals

Largemouth bass were obtained from the Panyu aquaculture base of South China Normal University. Fish with no disease, no injury, and strong vigor were selected. The average weight of experimental fish was 108.4 ± 5.2 g and the average body length was 18.5 ± 3.1 cm. Before the trial, the water quality indicators were detected daily (pH 7.6 ± 0.2, temperature 25 ± 1°C, ammonia nitrogen $\leq$ 0.05 mg/L, and dissolved oxygen(DO) $\geq$ 6.5 mg/L). The fish were temporarily cultured with continuous ventilation for 2 weeks of domestication and were fed by Commercial feed (Guangdong Evergreen Feed industry Co., Ltd., Guangdong, China) during domestication. The fish were fasted for 24 h before the experiment.

Hypoxia Treatment and Sample Collection

After an 2-week acclimation period prior to the experiments, 160 largemouth bass which held in one big tank (1000 cm × 525 cm × 100 cm, water volume 520 L) were seperated into 16 plastic aquaria (50 cm × 35 cm × 30 cm, water volume 52 L). 10 largemouth bass were placed in each aquarium and each group contains four aquaria. The initial dissolved oxygen concentration of the culture water was around 6.50 mg/L. Before hypoxia treatments, three largemouth bass were randomly selected from four aquaria (Control group, DO = 6.50 mg/L) for sample collection. In order to start the hypoxia state, the aquaria were not subjected to oxygenation and circulation, and the mouth of aquaria were sealed with a thin film, and the dissolved oxygen in the tanks was measured once per 5 min. The oxygen in the aquaria gradually decreased with the consumption of largemouth bass. Three samples were collected from each experimental group after around 20 min (H1 group, DO = 4.33 mg/L), around 40 min (H2 group, DO = 3.25 mg/L) of hypoxia exposure. After acute hypoxic stress, the remaining largemouth bass were re-oxygenated for 50 min and the dissolved oxygen reached 7.09 mg/L, which was recorded as the R group and then three fish were sampled. Blood cells were withdrawn from the tail vein and immediately transferred into individual plastic tubes. All samples were kept on ice to prevent blood cells clumping.

Total Blood Cell Count and Giemsa Staining

A glass microscope slide was placed on a horizontal surface, and 10 μL blood was added onto one end of the slide. A coverslip was used, lying across the glass slide and keeping the coverslip in contact with the blood. The coverslip was moved down the glass slide to the opposite end. The blood smear is made after air-dried. Blood smears were stained with Reisser-Giemsa dye (Biosharp Biotechnology Co., Ltd., Anhui, China). Garris-Giemsa A solution was dropped on the smear, and the whole smear was stained with the dye for 0.5 min. Then drop the Reisch-Jimsa B solution onto liquid A, blow the breeze with the mouth or ear ball to make the liquid surface ripple, make the two liquids fully mixed, dyeing for 1 min before being washed in distilled water. Finally, it was observed with polarized light microscope (Leica, Germany) and photographed after drying. At the same time, the blood cells after multiple dilutions were counted on the counting board under an ordinary microscope.

Flow Cytometry

The blood was centrifuged at 500 × *g*, 4°C for 10 min, and the supernatant was discarded after centrifugation. Then red blood cell lysis buffer was added to the cell pellet to lyse mature erythrocytes for 5 min. After lysis, the supernatant lysate was removed by centrifugation, and proper amount of PBS was added to re-precipitate the suspension. Then the blood cells were analyzed with a BD FACS Aria III flow cytometer (BD, NJ, United States).

Detection of Cell Apoptosis

Annexin V-FITC/PI Fluorescence double staining apoptosis assay kit (Elabscience Biotechnology Co., Ltd., Hubei, China) was used to stain the cells. The cells were suspended with 100 μL diluted 1 × Annexin V Binding Buffer, then 2.5 μL Annexin V-FITC and 2.5 μL PI staining solution were added to cell suspension. The mixed buffer were incubated for 15 min at room temperature in the dark. 400 μL of diluted 1 × Annexin V Binding Buffer was added to make the cell concentration 1.0×10^5 cells/mL and mixed the samples. The samples were placed on ice in darkness and tested by flow cytometry within 1 h and data were analyzed by the Flow Jo 10 software (version 10.2).

Intracellular Reactive Oxygen Species Measurement

Intracellular ROS was estimated by using a fluorescent probe, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). The cells were stained by Meilun Reactive Oxygen Species Assay

Kit (Dalian Meilun Biotechnology Co., Ltd., Liaoning, China). After the cells were collected, the cells were suspended and added with an appropriate volume of diluted DCFH-DA working solution. The cells were incubated in a cell culture box at 37°C for 30 min under dark conditions, and mixed upside down every 5 min to make the probe and cells fully contact. After incubation, the cells were washed with PBS 3 times to fully remove DCFH-DA that did not enter the cells. Collected to make single-cell suspensions. Then the fluorescence was determined by flow cytometry.

Nitric Oxide Measurement

DAF-FM DA (NO fluorescent photoprobe) (Dalian Meilun Biotechnology Co., Ltd., Liaoning, China) was used to stain the cells, and the cells were suspended with diluted DAF-FM DA and incubated for 20 min in a cell culture box at 37°C. Mix upside down every 3–5 min so that the probe is in full contact with the cells. After incubation, the cells were washed with PBS 3 times to fully remove the DAF-FM DA that did not enter the cells. Then the cells were re-suspended by PBS to prepare single-cell suspension for flow cytometry.

Intracellular Calcium Levels Measurement

Fluo-3 and AM ester (eBioscience Biotechnology, MA, United States) were used to stain the cells. The calcium ion (Ca^{2+}) fluorescent probe Fluo-3 and AM ester were added to the cells and incubated at 37°C for 20 min in the dark. Cells were washed three times with PBS and collected to make single-cell suspensions. The intracellular fluorescence intensity measured by flow cytometry represented the Ca^{2+} level.

Real-Time Quantitative RT-PCR Analysis

Total RNA was extracted from the blood cells of fish with Trizol (Takara Biotech, Kyoto, Japan) and their quality and quantity were determined by a Nanodrop2000 spectrophotometer (Thermo, MA, United States), and t the integrity was assessed by 1% agarose gel electrophoresis (Wang et al., 2015). The cDNA was synthesized by reverse transcription with PrimeScript reverse transcriptase (Takara Biotech, Kyoto, Japan) in a 20 μL reaction volume containing 1 μg total RNA. The reaction mixture was stored at −20°C for future use. Quantitative real-time RT-PCR (qRT-PCR) experiments were performed in a CFX96 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., CA, United States) using SYBR Mixture (Takara Biotech, Kyoto, Japan). Use β-actin as an internal reference gene. Each sample was paralleled three times. PCRs were performed on a total reaction volume of 20 μL containing 4 μL of cDNA, 0.5 μL of primer, 10 μL of SYBR Mixture, and 5 μL of ddH_2O. The thermal cycling program was as follows: activation at 95°C for 10 min, followed by 35 cycles of 95°C for 10 s, several annealing temperatures for 30 s, and 72°C for 32 s; melt curve detection of 60°C for 5 s to 95°C increments 0.5°C. Quantitative analyses were performed using the $2^{-\Delta\Delta Ct}$ method. The PCR cycling protocol was 95°C for 60 s, 40 cycles of 94°C for 5 s and 60°C for 30 s, followed by 95.0°C for 5 sec, followed by melting curve analysis from 65.0 to 95.0°C (increment 0.5°C, 0:05). Quantitative analyses were performed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are described in **Table 1**.

Statistical Analyses

All analyses were carried out using the SPSS software (version 26.0). The data were analyzed by single-factor analysis of variance (ANOVA) and multiple comparisons by LSD method. The data were expressed as mean ± standard deviation (SD, $n = 3$). $P < 0.05$ indicated significant differences.

RESULTS

Effect of Acute Hypoxia and Reoxygenation on Cell Morphology and Total Blood Cell Count

The results of the Giemsa staining (**Figure 1A**) of the blood cells showed a clearly significant decrease in the H1 and H2 group relative to control group. Interestingly, macrophage-like cells could be found in hypoxia groups, especially in H2 group.

After oxidative stress, compared to the control group ($1.70 \times 10^9 \pm 0.36 \times 10^9$ cell/ml), the TBCC of treatment group decreased at H1 ($1.26 \times 10^9 \pm 0.1 \times 10^9$ cell/ml) ($P < 0.05$) and H2 ($1.01 \times 10^9 \pm 0.21 \times 10^9$ cell/ml) ($P < 0.05$), and remain unchanged in the R group ($0.94 \times 10^9 \pm 0.04 \times 10^9$ cell/ml) ($P > 0.05$), while the TBCC of H1 group decreased more than H2

TABLE 1 | Sequences of primers used in qRT-PCR.

Gene		Primer sequence (5′–3′)
MOB1	F	ACACCCGAAACAGACGAGAC
	R	AATATCGCTGGCAGGACGAG
Lats1/2	F	GAGTCATGTGTCCAGCGGAA
	R	TTATCCGGCTCGGCATCTTC
YAP/TAZ	F	GCCAAAGTTTTGGTGTCGCA
	R	GAGTGTTTCCTCGGCTGTGA
14-3-3	F	AGATGGCAGTTTTGGGGACT
	R	AGTGTGAGCTCATGGAGGTTG
PP2A	F	ACGAAATCAGCGTGGACAGT
	R	GTTCACAGGACGTCACCCAT
TEAD	F	GAGTGTGTGGACATCAGGCA
	R	ATTGGCTGGTGACACCGTAG
Nrf-2	F	CAGACAGTTCCTTTGCAGGC
	R	AGGGACAAAAGCTCCATCCA
Keap1	F	CAGCATTACATGGCCGCATC
	R	CTTCTCTGGGTCGTAAGACTCC
SOD	F	CCACCAGAGGTCTCACAGCA
	R	CCACTGAACCGAAGAAGGACT
CAT	F	GTTCCCGTCCTTCATCCACT
	R	CAGGCTCCAGAAGTCCCACA
GPx	F	CCCTGCAATCAGTTTGGACA
	R	TTGGTTCAAAGCCATTCCCT
Hif-1a	F	CAGAGGACCTGTTGAATCGTT
	R	TTGTAGATGACAGTGGCTTGG

group (**Figure 1B**). And this result showed a similar trend which was consistent with Giemsa staining.

Effect of Acute Hypoxia and Reoxygenation on Apoptosis

After exposure to acute hypoxic conditions, the early (Q3 region) and late apoptosis (Q2 region) were increased in a dose dependent manner (**Figure 2A**). Further quantified these apoptosis cells by software, the number of apoptotic cells In Q2 and Q3 region were 22.72, 42.9 and 35.77% after treated with H1, H2, and R ($P < 0.05$, **Figure 2B**). Compared to the control group (10.52%), the proportion of apoptotic cells was demonstrated to be significantly increased from H1 to H2, peaking at H2, and subsequently declining (R). These results revealed that acute hypoxic conditions could promote apoptosis.

Effect of Acute Hypoxia and Reoxygenation on Concentration of Calcium

The level of Ca^{2+} fluorescence was low when the largemouth bass treated with control dissolved oxygen, while the level of Ca^{2+} fluorescence in H1 (52.83 ± 0.75) was two times higher than control group (20.53 ± 0.93). With the decrease of dissolved oxygen, the level of Ca^{2+} fluorescence was apparently increased at H1 and H2 group ($P < 0.05$, **Figure 3**), the level of Ca^{2+} fluorescence show the greatest peak in the H2 group, and the fluorescence intensity of the H2 group (61.63 ± 1.27) was about three times that of the control group. In addition, the level of Ca^{2+} fluorescence was decelerated after reoxygenation (58.63 ± 0.68).

Effect of Acute Hypoxia and Reoxygenation on Concentration of Nitric Oxide

After largemouth bass treated with different dissolved oxygen, the level of Nitric oxide (NO) fluorescence was significantly increased when compared to the control group (20.87 ± 0.29) ($P < 0.05$, **Figure 4**). With the decrease of dissolved oxygen, the level of NO fluorescence was increased in a dependent manner at H1 and H2 group (26.7 ± 0.10, 35.47 ± 0.21). And after exposure to reoxygenation, the level of NO fluorescence was shown to be significantly decreased at R group (33.43 ± 0.25) ($P < 0.05$).

Effect of Acute Hypoxia and Reoxygenation on Reactive Oxygen Species Production

The level of ROS production of H1, H2 and R group were determined to be 37.80 ± 0.26, 45.83 ± 0.38 and 43.90 ± 0.26. In comparison with the control (31.97 ± 0.50), ROS formation significantly increase in H1 and H2 ($P < 0.05$, **Figure 5**); which the ROS formation was downregulated at R group. Although there was a subsequent upward trend, he level of ROS fluorescence remained significantly higher than those of control ($P < 0.05$).

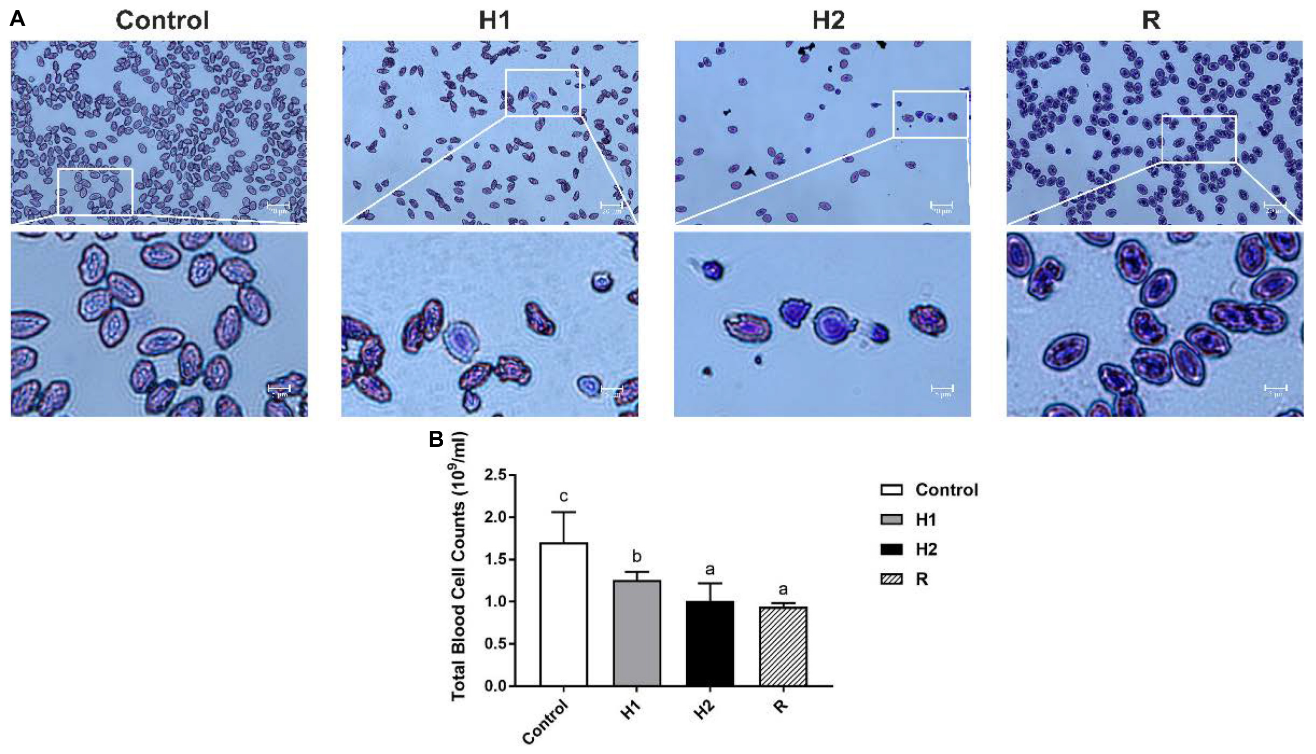

FIGURE 1 | Effects of hypoxia on **(A)** cell morphology and **(B)** total blood cell count in largemouth bass after hypoxia. Scale bar = 20 μm (top), 5 μm (bottom).

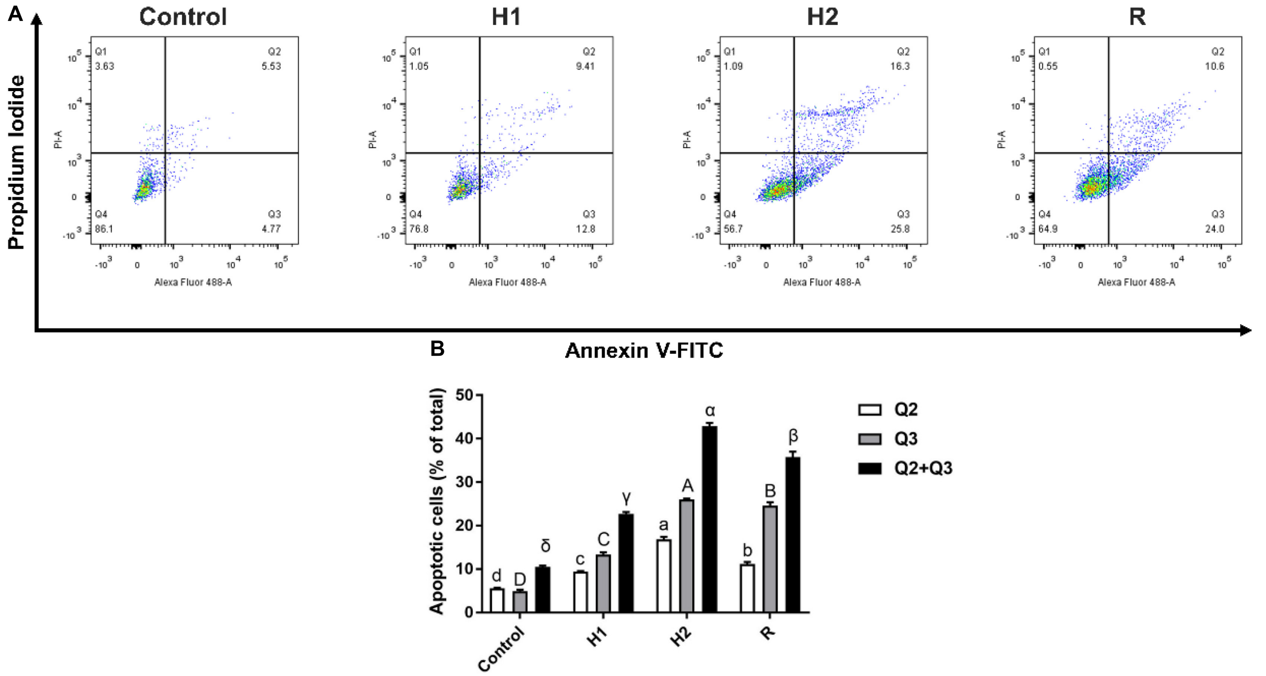

FIGURE 2 | Effect of apoptosis in the blood cell of largemouth bass under acute hypoxia and reoxygenation. **(A)** Cell apoptosis rate was determined by flow cytometry. **(B)** Bar graph of apoptosis due to normoxia and hypoxia. Data are expressed as the mean ± SD (n = 3; one-way analysis of variance followed by the T-test). All experiments were conducted in triplicate. Different letters denote significant differences ($P < 0.05$).

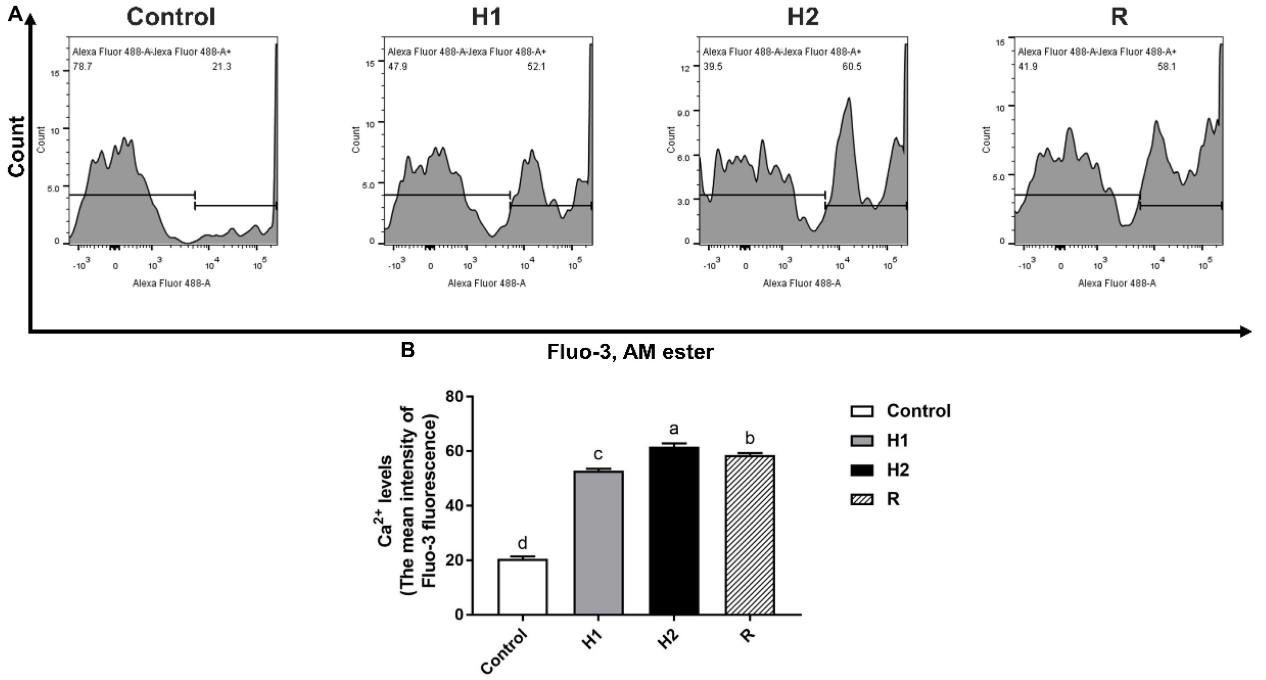

FIGURE 3 | The change of Ca^{2+} levels in the blood cell of largemouth bass under acute hypoxia and reoxygenation. **(A)** The Ca^{2+} levels of blood cell was determined by flow cytometry. **(B)** The Ca^{2+} levels for each cell treatment. Data are expressed as the mean ± SD (n = 3; one-way analysis of variance followed by the T-test). All experiments were conducted in triplicate. Different letters denote significant differences ($P < 0.05$).

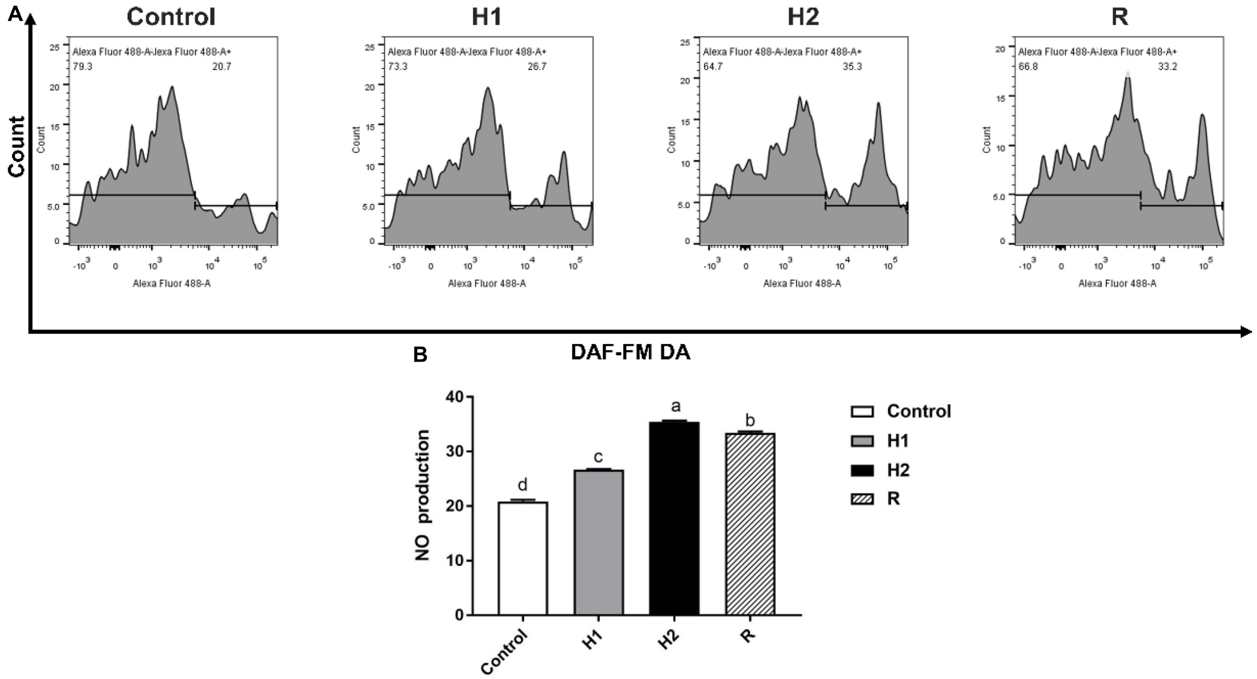

FIGURE 4 | The difference of NO production in the blood cell of largemouth bass under acute hypoxia and reoxygenation. **(A)** The NO production of blood cell was determined by flow cytometry. **(B)** The NO production for each cell treatment. Data are expressed as the mean ± SD ($n = 3$; one-way analysis of variance followed by the T-test). All experiments were conducted in triplicate. Different lowercase letters above each bar represent significant differences ($P < 0.05$).

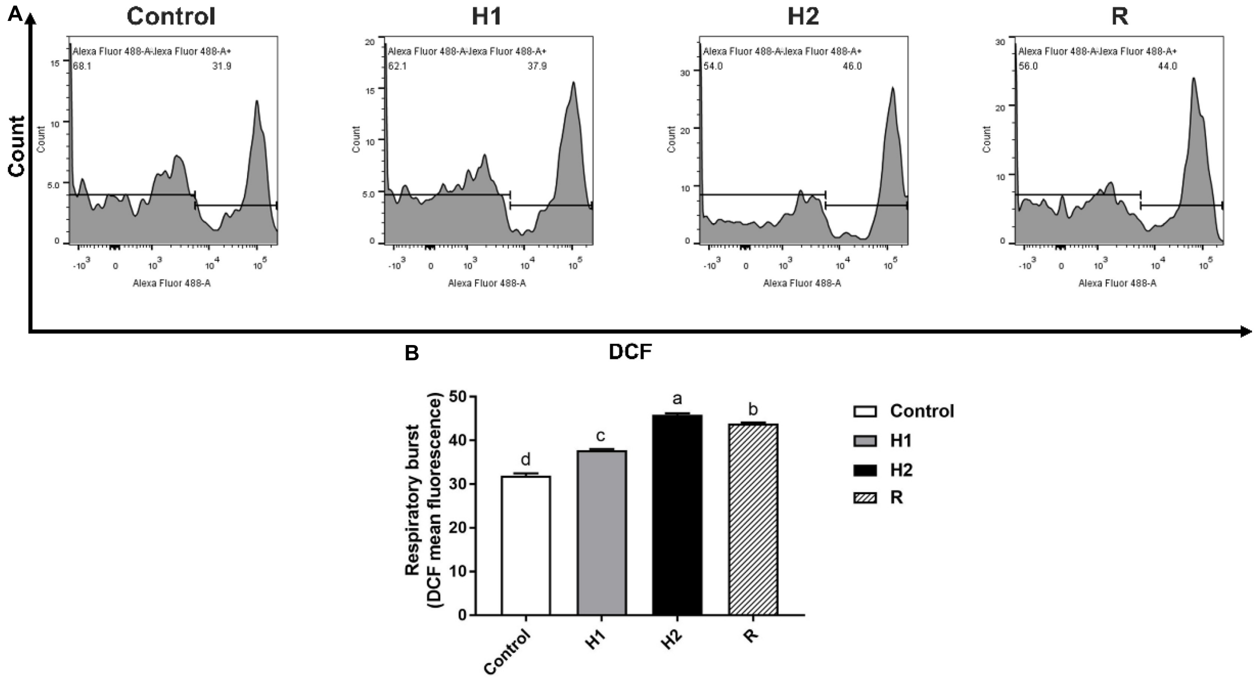

FIGURE 5 | The change of respiratory burst in the blood cell of largemouth bass under acute hypoxia and reoxygenation. **(A)** The respiratory burst of blood cell was determined by flow cytometry. **(B)** The respiratory burst for each cell treatment. Data are expressed as the mean ± SD ($n = 3$; one-way analysis of variance followed by the T-test). All experiments were conducted in triplicate. Different lowercase letters above each bar represent significant differences ($P < 0.05$).

Effect of Acute Hypoxia and Reoxygenation on Nrf-2 Pathway Related Genes

The expression of hypoxic sensor protein Hypoxia-inducible factor-1 alpha (Hif-1α) gene was shown to be continuously increasing in H1 and H2 group during the hypoxic exposure stage, whereas decreasing to its lowest value at R group, which was not significantly different from that in Control ($P < 0.05$). Subsequently, the Hif-1α level was shown to be significantly increased in H1 and H2 group ($P < 0.05$), reaching its highest value in H2, with significant differences observed between Control, H1 and R ($P < 0.05$, **Figure 6A**). The expression level of Nrf-2 gene decreased to the lowest value in H1 first, then significantly increased ($P < 0.05$, **Figure 6B**), peaking at H2 group. During the hypoxic exposure stage, the Nrf-2 gene expression decreased obviously in R, which still higher than that in H1 and control group. Simultaneously, it was significantly different from that in H1 and H2 group ($P < 0.05$, **Figure 6B**). Expression of Keap1 gene was demonstrated to be continuously increased during normoxia to hypoxia stress, with H2 exhibiting the highest value, which was distinctly different from that in Control and H1 group ($P < 0.05$, **Figure 6C**). Nevertheless, after reoxygenation, the expression of Keap1 in Group R was significantly lower than that of H2 ($P < 0.05$, **Figure 6C**), but still obviously higher than that in Control and H1 ($P < 0.05$, **Figure 6C**). Throughout the study, the levels of Hif-1α and Keap1 showed similar trends. More noteworthy phenomenon was that the expression level of Keap1 in H1 was five times higher than that of Nrf-2, and the highest expression level of Keap1 was about three times higher than the highest expression level of Nrf-2. In addition, the expression level of Keap1 and Nrf-2 genes downregulated during the reoxygenation stage, while the expression level of Keap1 was still about five times higher than that of Nrf-2.

Furthermore, compared with the control group, the expression of SOD gene decreased obviously in H1 and H2 group ($P < 0.05$, **Figure 7A**), and there was no significant difference between H1 and H2 group ($P > 0.05$, **Figure 7A**). Moreover, the SOD gene expression in R Group reached the lowest level after reoxygenation ($P < 0.05$, **Figure 7A**). Besides, the CAT expression increased slightly in H1 first in hypoxia environment, with no significant difference with the control group, however, the expression of CAT significantly upregulated in H2 group ($P < 0.05$, **Figure 7**), reaching its peak. In addition, the level of CAT gene subsequently declining in R during reoxygenation, meanwhile, its expression was significantly lower than H2 group ($P < 0.05$, **Figure 7B**), which was shown to be significantly higher than that in the control group and H1 ($P < 0.05$, **Figure 7B**). Ultimately, the expression of GPx gene increased rapidly at H1 and H2 group, which has no significant difference between them ($P > 0.05$, **Figure 7C**), and then downregulated in R group, with distinctly different from that in H1 and H2 ($P < 0.05$, **Figure 7C**). The level of GPx was significantly higher than that of control group throughout the experimental period ($P < 0.05$, **Figure 7C**). Most noteworthy, GPx expression increased significantly in H1 group ($P < 0.05$, **Figure 7**), about three times as much as that of CAT, while the expression of CAT upregulated distinctively in H2 group with its expression two times higher than that of GPx. Additionally, during hypoxia and reoxygenation stage, the expression of CAT and GPx was always higher than that of SOD, with the expression of CAT in H2 group was about 80 times higher than that of SOD as well as the expression level of GPx was about 30 times that of SOD in H2 group.

Effect of Acute Hypoxia and Reoxygenation on Hippo Pathway Related Genes

The expression levels of Hippo pathway-related genes were examined in blood cells from each group (**Figure 8**). Compared with the control group, the H1 group showed almost no significant change, but the H2 group showed a clear upward trend, and then the expression of the MOB1 decreased significantly in the R group. Moreover, MOB1 peaked and remarkably increased 10 times in H2 group ($P < 0.05$, **Figure 8B**), but no significant changes in the H1/R group ($P > 0.05$). Similar trend to MOB1, Lats1/2 expression levels peaked in the H2 group, followed by a decline in the R group, and increased by around 3 and 2.5 times, respectively, when compared to the control group ($P < 0.05$, **Figure 8A**). Moreover, in comparison with the control group, the trend of the YAP/TAZ expression levels was also highly similar to that of the MOB1. However, it is worth noting that expression levels of the H1 group of the YAP/TAZ has increased significantly by nearly 2-fold relative to the control group, and it

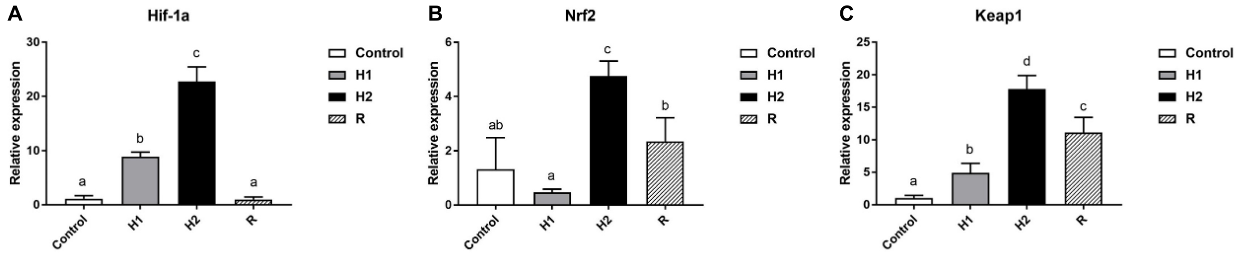

FIGURE 6 | Expression of oxygen sensor in the blood cell of largemouth bass after acute hypoxia and reoxygenation using qRT-PCR analysis. **(A)** HiF-1 alpha subunit (Hif-1α) expression. **(B)** Nuclear factor erythroid 2-related factor 2 (Nrf2) expression level. **(C)** The expression of Kelch-1ike ECH- associated protein l (Keap1). Different lowercase letters above each bar represent significant differences ($P < 0.05$).

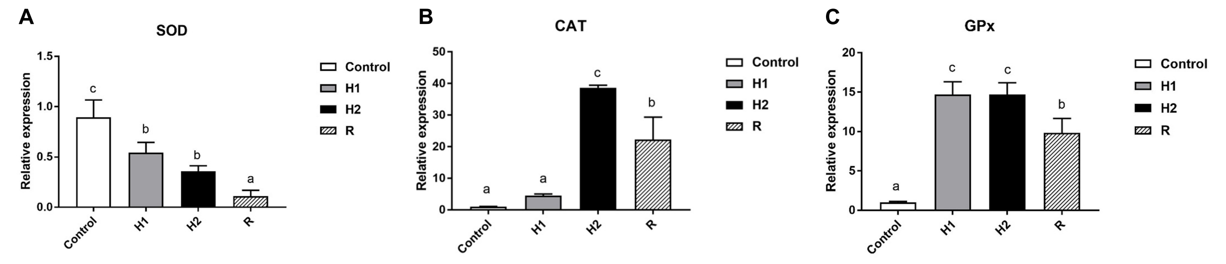

FIGURE 7 | Effects of hypoxia and reoxygenation on antioxidant genes. **(A)** The expression level of Superoxide Dismutase (SOD) gene. **(B)** Catalase (CAT) expression level. **(C)** Glutathione peroxidase (GPx) expression. Different lowercase letters above each bar represent significant differences ($P < 0.05$).

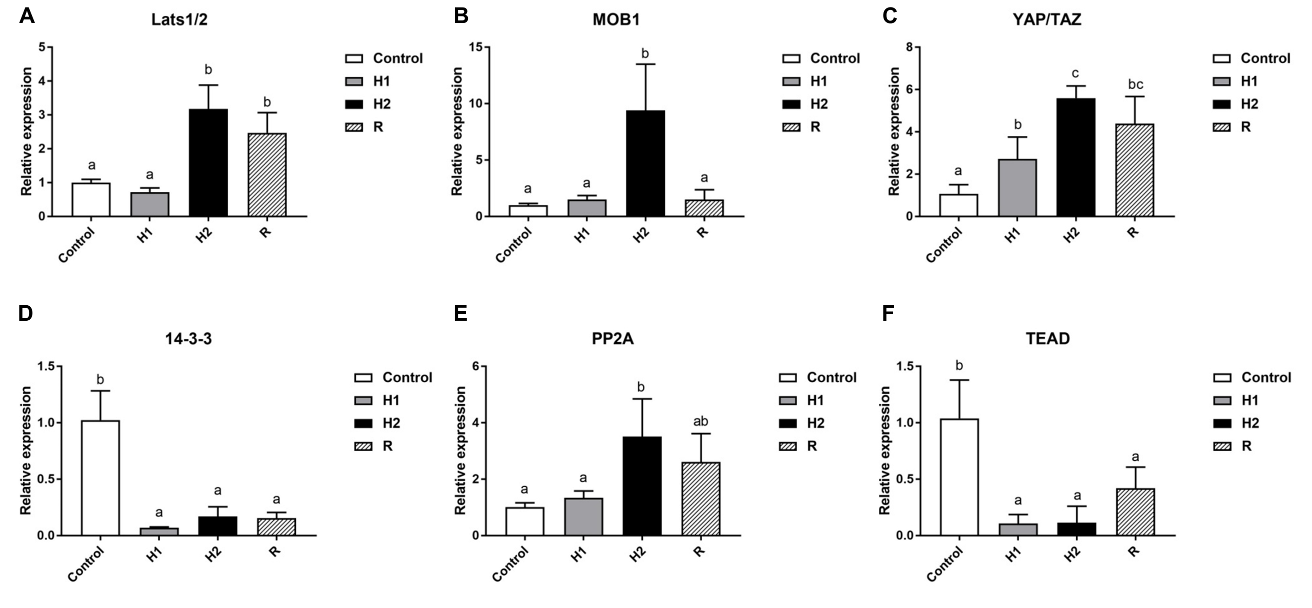

FIGURE 8 | Effects of hypoxia and reoxygenation on Hippo pathway genes. **(A)** The expression level of large tumor suppressor homolog 1/2 (Lats1/2) gene. **(B)** MOB kinase activator 1 (MOB1) expression level. **(C)** Yes-associated protein/transcriptional co-activator with PDZ-binding motif (YAP/TAZ) expression. **(D)** The expression of 14-3-3. **(E)** The expression level of Protein Phosphatase 2A (PP2A). **(F)** The expression level of TEA domain (TEAD) gene. Different lowercase letters above each bar represent significant differences ($P < 0.05$).

reached a maximum in the H2 group, where YAP/TAZ expression rose sixfold, and then dropped a bit in the R group but was still four times as high as in the control group (all $P < 0.05$, **Figure 8C**). The trend of 14-3-3 expression levels was opposite to that of YAP/TAZ, with its expression significantly decreased nearly 2-fold in H1/H2/R group and reached its lowest point in the H1 group, followed by a slight increase in the H2 group and then remained almost the same as in the R group (all $P < 0.05$, **Figure 8D**). As shown in **Figure 8E**, no significant difference was observed between the control group and H1 group regarding the expression of PP2A ($P > 0.05$), while appreciably up-regulated by around four times in H2 group and reached the top, and then had slightly down-regulated in R group (all $P < 0.05$). Furthermore, PP2A expression in the R group was still nearly twice that of the control group. In contrast to the control group, TEAD expression significantly decreased to the lowest point in H1/H2 group and went up in R group, but decreased by around 1 and 0.5 times, respectively, when compared to the control group (all $P < 0.05$, **Figure 8F**). The results indicated hypoxia could remarkably strengthen the expression of YAP/TAZ, a gene central to the Hippo pathway. Taken together, these data demonstrated hypoxia could effectually enhance the antioxidant response in blood cells of *M. salmoides*.

DISCUSSION

A large number of studies have shown that hypoxia can induce apoptosis (Saikumar et al., 1998; Sollid et al., 2003; Sun et al., 2020d). Studies have shown that an apoptotic and cell cycle arrest cascade downstream of HIF-1α involve transcriptional initiation of several genes, such as p53, BNip3 and BAX, which ultimately drive the cells into apoptosis (Harris, 2002). In previous study, the expression of Cyt-C, Caspase-9 and Caspase-3 in the stomach and intestinal tissues of Chinese sea perch (*Lateolabrax maculatus*) was initially increased and then decreased after acute hypoxia, suggesting that hypoxia and reoxygenation induced apoptosis (Sun et al., 2020d). Our work reveals that hypoxic stress leads to an increase in the expression of relevant proapoptotic genes in largemouth bass, which in turn leads to apoptosis and activates inflammatory responses in blood cells, thus forcing largemouth bass to activate a series of resilience mechanisms to cope with and adapt to the adverse effects of hypoxia on its organism.

Studies have proved that hypoxia induced increase of Ca^{2+} in cardiac microvascular endothelial cells, which led to cell apoptosis (Zhang et al., 2016). Recent research found that elevation of intracellular Ca^{2+} level in human glioblastoma cells and mouse Schwann cells triggers ubiquitination of Merlin and activate Lats1,which is an important molecular in Hippo pathway (Wei et al., 2020). Our findings also indicate that hypoxia could induce Ca^{2+} over-produced in hypoxia-damaged blood cells, which show a consonant pattern with previous research. In other research, the mRNA expression levels of NOS did not change significantly in *Carassius carassius* L gills during hypoxia-induced transformation (Sollid et al., 2006). But our study showed that the level of NO fluorescence obviously affected by the amount of dissolved oxygen in the water. This suggests that there are differences in the production of NO induced by hypoxia in different tissues. Studies have shown that YAP/TAZ plays a certain role in ROS production by controlling mitochondrial respiration (White et al., 2019). Many results support the idea that hypoxia can induce ROS. Oxidative stress was caused by hypoxia stress in Przewalski's naked carp *(Gymnocypris przewalskii*), and ROS production in telencephalon cells increased. It is consistent with our findings which indicate that hypoxia induces ROS over-produced in hypoxia-damaged blood cells that may induce cells apoptosis under hypoxia.

Previous studies have demonstrated that hypoxia can cause metabolic disorders, decreased immunity, and respiratory dysfunction in aquatic animals (Han et al., 2017; Peruzza et al., 2018; Sun et al., 2018). Similarly, the negative effects of hypoxia on fishes have long been studied, including metabolic disorders, decreased immunity, and apoptosis (Sun et al., 2020a; Zhao et al., 2020; Wang et al., 2022). In hypoxic responses, the Hif-1α is a master regulator of oxygen sensitivity (Wang J. et al., 2021). Our study demonstrated that the activity of Hif-1α was significantly increased in the blood cell of largemouth bass after exposure to acute hypoxic conditions, consistent with the research on Mandarinfish (*Siniperca chuatsi*) (He et al., 2019; Sun et al., 2020a). Our study found significant differences in the content of Hif-1α with different levels of dissolved oxygen, which suggested that the expression level of Hif-1α may be regulated by the content of dissolved oxygen. Consequently, this study expected to deliberate the relationship between Hif-1α and Nrf-2 pathway, and further revealed the relationship between Hif-1α and antioxidant mechanism. The Nrf-2 is a key component of antioxidant system that its steady state levels are very low under un-stressed conditions, as Keap1 interacts with Nrf-2 primarily *via* its Neh2 domain and targets it for ubiquitin mediated degradation (Yamamoto et al., 2018). However, a variety of stressors such as excessive ROS acting on Keap1's redox-sensitive cysteine residues interfere with its inhibition of Nrf-2, allowing

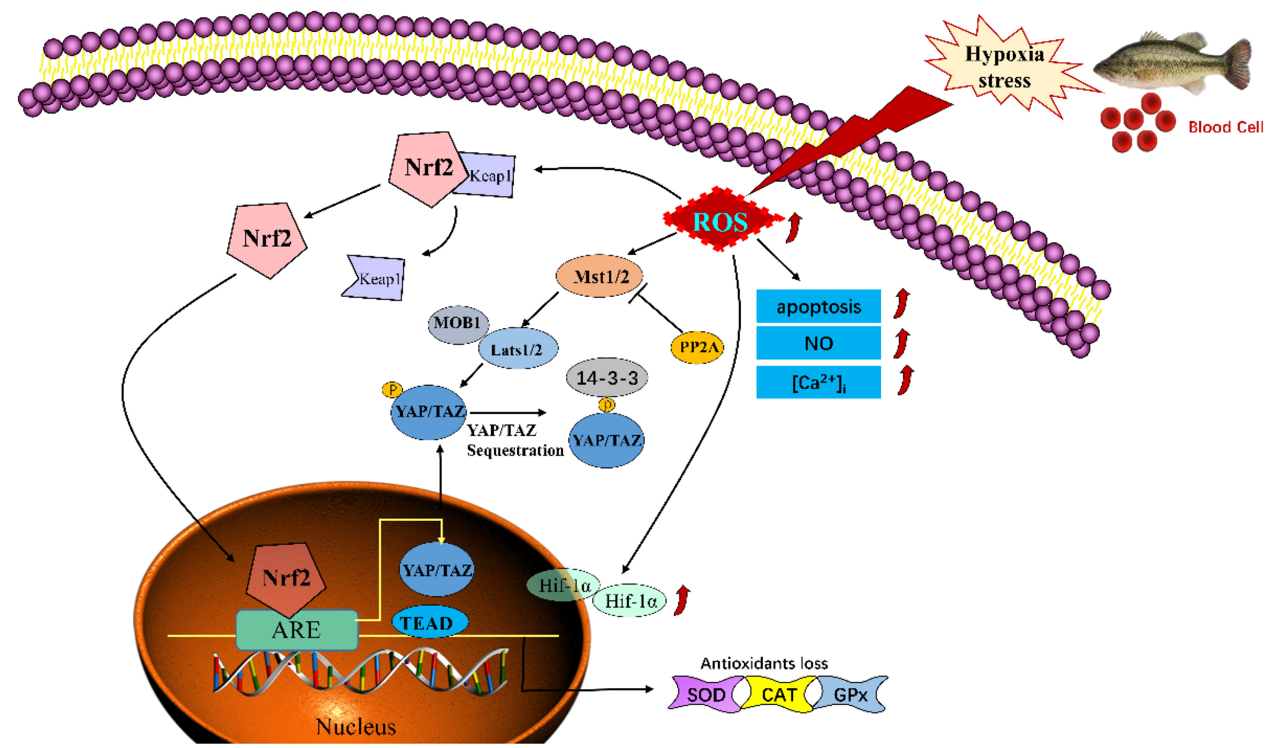

FIGURE 9 | Schematic model of hypoxia -induced injury *via* mediating the Nrf-2/Hippo pathway in blood cells of Largemouth bass (*Micropterus salmoides*).

it to assemble, move to the nucleus, and drive ARE-mediated gene expression, which include antioxidant, detoxification and proteostasis genes (Al-Mubarak et al., 2021). Keap1 and Nrf-2 genes expression was upregulated in blood cell under hypoxia condition, showing that the expression level of antioxidant enzyme genes was apparently consistent with that of Nrf-2. Faced with the excessive production of ROS, and the possible oxidative stress caused by the reoxygenation, the body will increase some antioxidant enzymes under hypoxic conditions to prepare in advance to reduce oxidative damage (Wang M. et al., 2021). In this study, the levels of GPx and CAT increased significantly in hypoxic condition, which was consistent with the previous study in the muscle tissue of largemouth bass (Yang et al., 2017). It is worth noting that the activity of CAT in blood cells did not increase significantly when the dissolved oxygen value was 4.29 $mg \cdot L^{-1}$, while the expression of GPx upregulated obviously in 4.29 $mg \cdot L^{-1}$ of dissolved oxygen, suggesting that the mechanism of response to hypoxia injury in blood cells may be different from that in other tissues. Most noteworthy, the expression of CAT and GPx gene was higher than that of SOD gene during hypoxia stage, which suggested that SOD and CAT, GPx complement each other in hypoxia stress.

As an important pathway prevalent in living organisms, the Hippo pathway is not only a key regulator in controlling organ size and maintaining homeostasis within tissues, but recent studies have also shown that the Hippo pathway controls metabolic processes at the cellular and organismal levels (Ardestani et al., 2018). In the current study, we focused on the role of hypoxia in the regulation of Hippo pathway as well as its negative effect on blood cell of largemouth bass. Previous studies in mammals and Drosophila have established the centrality of YAP/TAZ in the Hippo pathway and found that hypoxia leads to upregulation of YAP/TAZ, which is in good agreement with our findings in blood cells of largemouth bass (Yan et al., 2014). According to the results, hypoxia significantly promoted the expression of YAP/TAZ and remained at a high level after reoxygenation, while MOB1 and Last1/2, upstream regulators of the Hippo pathway, which are highly associated with the regulation of YAP/TAZ expression, also showed a high level of expression in the H2 group. Subsequently, we also found that the expression of 14-3-3, which acts as a repressor of YAP/TAZ entry into the nucleus, was decreased in the hypoxic group, whereas the expression of PP2A, which dephosphorylates YAP/TAZ and thus enters the nucleus to bind to downstream target genes, was a greater increase. These results suggest that hypoxia is able to induce upregulation of YAP/TAZ, the core gene of the Hippo pathway, and further entry into the nucleus (Zhao et al., 2007; Mui et al., 2015). However, the expression of TEAD, which eventually binds to YAP/TAZ after nucleation, showed a dramatic decrease in the H1/H2 group. From our results, we conclude that hypoxia can cause dysregulation of the Hippo pathway and ultimately induce oxidative damage and apoptosis in largemouth bass blood cells. Similarly, results from the study of Dey et al. (2020) also suggested that dysregulated Hippo pathway and YAP/TAZ–TEAD activity is associated with various diseases, most notably cancer in human (Dey et al., 2020).

In conclusion, our findings have demonstrated that hypoxia could induce oxidative injury and apoptosis *via* mediating the Nrf-2/Hippo pathway in blood cells of Largemouth bass (*M. salmoides*) (**Figure 9**). These findings might provide a novel and potential therapeutic approach to understand the mechanisms in hypoxia stress and global warming adaptation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Animal experimental procedures were conducted under protocols approved by the Regulations for Animal Experimentation of South China Normal University.

AUTHOR CONTRIBUTIONS

YX and LW: conceptualization. YX, ZY, YZ, and YL: methodology. YX and ZY: formal analysis and writing – original draft. JY, WZ, and YC: data curation. XL, JH, JL, YM, and LW: writing – review and editing. YZ and WZ: visualization. LW: supervision. All authors read and approved the submitted version.

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Post-exercise Hypothermia Varies Between High- and Low-Altitude Populations in the Asiatic Toad (*Bufo gargarizans*)

***Zhongyi Yao*[1]*, *Kun Huang*[2] *and Yin Qi*[1]**

[1] Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, China, [2] College of Life Science, Sichuan Agricultural University, Ya'an, China

Whether and how poikilothermic animals change their thermal performance to cope with global warming are crucial questions to predict the future of biodiversity. Intraspecific comparison among populations that occur in different climatic zones can provide insight into how poikilotherms may alter their thermal performance under a particular climatic event. We compared populations of the Asiatic toad (*Bufo gargarizans*) from two altitudinal zones (3239 and 926 m above sea level) to explore variations of post-exercise hypothermia, which can lead to lower temperature preference than normal conditions. Common garden experiment was also employed to test plasticity of hypothermic performance in adult toads. As results, exhaustive exercise induced measurable reduction in body temperature for both populations. Furthermore, high-altitude population experienced larger reduction in body temperature than low-altitude conspecifics in both original habitat and common garden conditions. Therefore, low-altitude toads may to enhance their hypothermic reaction if they shift their ranges to higher altitudes to survive warming climate; However, the relatively limited plasticity of hypothermic performance may constraint their adaptative process.

Keywords: hypothermia, exhaustive exercise, high-altitude, common garden, *Bufo gargarizans*

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Bao-jun Sun,
Institute of Zoology (CAS), China

Reviewed by:
Lixia Zhang,
Henan Normal University, China
Wei Chen,
Anhui University, China

****Correspondence:***
Zhongyi Yao
yaozy@cib.ac.cn

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INTRODUCTION

Upward range shift has been recorded in numerous organisms as a response to climatic change in recent decades since cooler environments at higher altitudes may relieve them from overheating (Parmesan and Yohe, 2003; Molina-Martínez et al., 2016; Freeman et al., 2018). During this process, high-altitude environments may impose other severe physiological stressors to species, which may represent new challenges for their survival and reproduction, including hypoxia and intense UV radiation (Bouverot, 1985; Storz et al., 2010; Qiu et al., 2012). Knowledge on how low-altitude poikilothermic organisms cope with high-altitude environments, however, remains limited, which may impede our understanding of the processes and mechanisms of how species react and evolve under climatic change (Pinsky et al., 2019; Sunday et al., 2019; Feldmeier et al., 2020; Jacobsen, 2020).

Body temperature of poikilothermic animals is largely dependent on environmental temperature (Gracey et al., 2004; Harwood, 2007). Meanwhile, body temperature is a key parameter for poikilothermic animals, and has a significant influence on animal physiological processes including energy metabolism, respiration, acid-base balance, and enzyme activity, of which are all closely correlated (Withers, 1978; Vitt and Caldwell, 2014; Abram et al., 2017; Gangloff et al., 2019; Taylor et al., 2021). Consequently, each poikilothermic species has its own optimal temperature range, and they typically reach their ranges by physiological or behavioral thermoregulation (Vitt and Caldwell, 2014; Rozen-Rechels et al., 2019). Because environmental temperature changes along with altitudinal gradient, poikilothermic animals that live in high-altitudes have adapted to the cold environments by regulating their thermal regimes (Bouverot, 1985; Muir et al., 2014; Domínguez-Godoy et al., 2020). Therefore, understanding the variations of preferred temperature and thermoregulation behavior among high- and low-altitude populations is crucial for apprehending the effects of climatic change on range shift of poikilothermic animals (Trochet et al., 2018).

Hypothermia results in a low temperature preference and can be achieved through thermoregulation behavior in poikilothermic vertebrates, including amphibians, reptiles, and fish (Wood and Gonzales, 1996; Moretti et al., 2018; Jones et al., 2019; Duran et al., 2020; Skandalis et al., 2020). Hypothermia can be induced by exhaustive exercise (Tattersall and Boutilier, 1999), hypoxia (Branco et al., 2014; Skandalis et al., 2020), or other stimulations (Moretti et al., 2018; Duran et al., 2020; Skandalis et al., 2020), and is known as lower body temperature than normal state (Romanovsky et al., 2005). Several physiological mediators of low body temperature in poikilotherms have been uncovered, including acidosis and lactate (Wagner et al., 1999; Nedrow et al., 2001). How animals benefit from hypothermia, however, remains controversial. Decreasing body temperature can shift the oxygen dissociation curve leftward, which increases oxygen loading in lungs, reduces energetic cost of ventilatory and cardiac hyperactivity, and restores acid-base balance in blood and tissue (Withers, 1978; Wang et al., 1998; Petersen et al., 2003). Thus, low body temperature in hypoxia likely represents an adaptive response to protect vital organs and reduce energy expenditure (Wood and Gonzales, 1996; Petersen et al., 2003). Furthermore, hypothermia may be a protective mechanism in hypoxic condition, such as at high-altitudes, which can increase survival rate (Bicego et al., 2007; Branco et al., 2014).

Upward range shift in poikilothermic animals has been a key research topic in recent decades (Gangloff et al., 2019; Domínguez-Godoy et al., 2020). Hence, studies on variations of natural thermal performance and tolerance, and the mechanisms behind the formation of variation are highly desirable, especially thermal biology on variations among individuals and populations within species (Taylor et al., 2021). High-altitude environmental stressor, particularly hypoxia and cold, impose significant constraints on animal physiology. Hypoxia typically has an negative impact on life activities, such as reproduction and development (Souchet et al., 2020). How animals living at different altitudes respond to their environments, and how they may change their thermal physiological reaction norms are fascinating questions. Therefore, intraspecific comparison between high- and low-altitudinal populations of poikilotherms represents an ideal paradigm to explore the variations of thermal biology and enhance understanding of how poikilothermic animals cope with climatic change.

Anurans are typical poikilothermic vertebrates, and are sensitive to rapidly elevating environmental temperature, which makes them excellent models for studying impacts of changing environmental temperature (Bodensteiner et al., 2021; Taylor et al., 2021). The Asiatic Toad (*Bufo gargarizans*) is a common anuran species that has a wide distribution in east Asia, and a wide altitudinal range from sea level to over 4,000 m above sea level (a.s.l.; AmphibiaChina, 2020). Therefore, we choose Asiatic toads that live in different altitudes to test intraspecific variations of hypothermic performance.

In this study, we used an exhaustive-exercise induced hypothermia and compared hypothermic performance between high- and low-altitudinal populations of Asiatic toads, both at their original habitat and in a common garden environment. We hypothesized that the high-altitude toads would have a stronger hypothermic reaction (lower body temperature) to exhaustive exercise than the low-altitude toads do, because cold temperature and hypoxia at high-altitudes may interactively affect their thermal physiology and high-altitudes toads may have adapted or acclimated to their environments. Besides, modifications of their thermal physiology could be fixed (evolutionary adaptation) or plastic (phenotypic plasticity).

MATERIALS AND METHODS

Animal Sampling and Experimental Design

Adult Asiatic toads were sampled from a high-altitude site (Kangding, 3239 m a.s.l.) and a low-altitude site (Shimian, 926 m a.s.l.; **Figure 1A**) in Sichuan province of western China during breeding season of 2020. Four males and four females were collected from each site. Because of the delayed phenology at high altitudes, low-altitudinal adults (origin-L) were sampled in January, while high-altitudinal adults (origin-H) were sampled in April (**Table 1** and **Figure 1B**).

We used racetrack trials as the exhaustive exercise, which could lead to post-exercise hypothermia. Racetrack trial was a common method in testing animal locomotor performance (Llewelyn et al., 2010; Hudson et al., 2020; Niu et al., 2021; Wölfer et al., 2021) and was also reliable in testing anurans (Zamora-Camacho, 2018; Rebelo and Measey, 2019; Hudson et al., 2020). Two rounds (round 1 and round 2) of trials were conducted near the sampling sites approximately 48 h after capture. The trials were conducted in January 17 to 18 near Shimian, and in April 22 to 23 near Kangding (**Table 1** and **Figure 1B**). The toads were then moved to the laboratory at the Chengdu Institute of Biology (Chengdu, Sichuan, 523 m a.s.l.) and acclimatized in a common garden environment for at least 2 months. Two rounds of trials (round 3 and round 4) were conducted after the acclimation period in April 1 and 2 with the origin-L individuals, and in July 22 and 23 with the origin-H individuals (**Table 1** and **Figure 1B**).

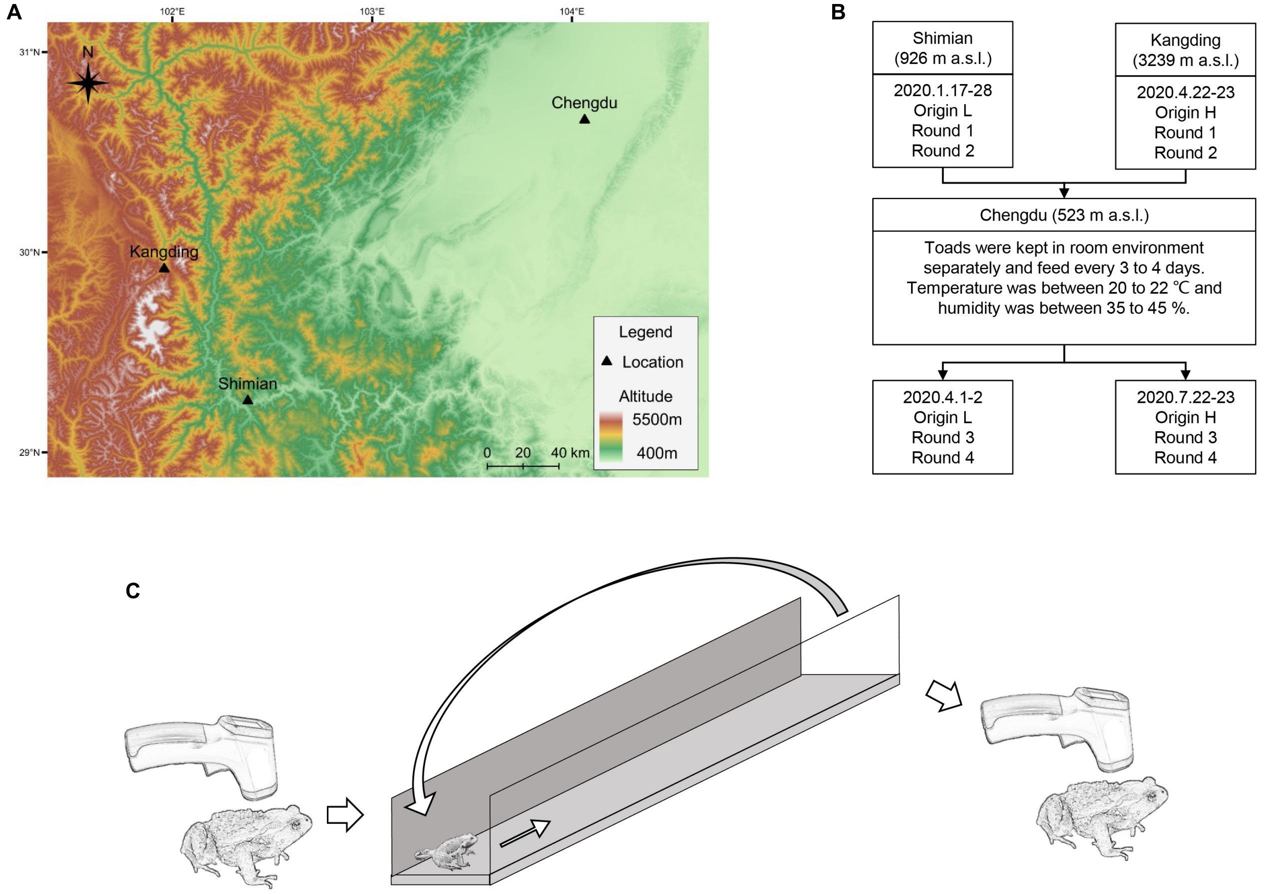

FIGURE 1 | Experimental protocols. **(A)** Sampling sites and common garden site. **(B)** Experimental schedule. **(C)** Setup of racetrack trial.

TABLE 1 | Experimental parameters during each round of racetrack trial.

Round	Date	Location	Sample size	Air temperature/°C	Racetrack temperature/°C	Air humidity/%
1	2020.1.17	Shimian	8 (4 males and 4 females)	21.0	20.3	39
2	2020.1.18	Shimian	8 (4 males and 4 females)	20.9	19.8	38
3	2020.4.1	Chengdu	8 (4 males and 4 females)	21.5	20.2	41
4	2020.4.2	Chengdu	8 (4 males and 4 females)	21.2	19.6	45
1	2020.4.22	Kangding	8 (4 males and 4 females)	21.1	21.5	55
2	2020.4.23	Kangding	8 (4 males and 4 females)	20.8	19.6	47
3	2020.7.22	Chengdu	8 (4 males and 4 females)	21.1	20.4	37
4	2020.7.23	Chengdu	8 (4 males and 4 females)	21.8	20.5	40
5	2020.9.20	Chengdu	10 males	21.7	21.2	35
6	2020.9.21	Chengdu	20 males	21.7	21.1	36

Common Garden Husbandry

The toads were kept individually in boxes with a size of 35 cm × 24 cm ×14.5 cm (length × width × height), and were fed with mealworms (*Tenebrio molitor*). Mealworms were replenished every three to four days, and at the same time the boxes were cleaned. Calcium powder and vitamin powder were added with the mealworms every half month. Refuge and moist sponge mat were also provided to each toad, and the sponge was rehydration every three or four days. Dark and light rhythm was kept at 12:12 h. Meanwhile, the temperature of husbandry room was between 20 to 22°C, and humidity was between 35 % to 45 %.

Exhaustive Exercise and Racetrack Trial

The racetrack was made of a wood board floor with a length of 120 cm and a width of 15 cm, and two acrylic side walls with a height of 30 cm (**Figure 1C**). The racetrack trials were conducted at night between 10 p.m. to 4 a.m. During the trials, toads were released at one end (start point) of the racetrack and allowed to move along racetrack to the other end (end point). A blunt-pointed pen was used to stimulate at the caudal vertebra area of the toads when they stopped (Hudson et al., 2020). Toads were manually moved (with thick gloves to prevent heat conduction) back to the start point to continue the exercise once they arrived at the end point. Exhaustive exercise was terminated after 10 continued pokes without movement, and the toad was considered as exhausted or unwilling to move (Hudson et al., 2020). From round 1 to round 4, a total of 64 trials were completed with 16 individuals. Each animal ran the trial once in each round with randomized orders. Moved distance was recorded for each subject after each trial. Surface body temperature was measured at the center of upper back immediately before and after each trial using an infrared thermometer (FR830, JIACOM) to the nearest 0.1°C. Environmental air temperature and humidity during racetrack trial were controlled at between 20–22°C and 35–55% and recorded (**Table 1**). The temperature of the runway floor was also recorded. The reduction values of surface body temperature (ΔT_S) were estimated as the after-exercise temperature (T_{S2}) minus the before-exercise temperature (T_{S1}) of each trial. To control for personnel effects, all trials was conducted by the same investigator (KH).

To test if surface body temperature could reflect core body temperature well, another two rounds (round 5 and round 6) of trials were conducted in September 20 and 21 (**Table 1**). Ten males of each population were chosen, of which five males had been tested before and the others were novice. In round 5, ten individuals were tested and four of them were poked (between 1 to 2 min) but not allowed to run as negative control. In round 6, 20 individuals were tested and eight of them were negative controls (between 1 to 5 min). A thermometer (UT321, UNI-T) was used to measure cloacal temperature to the nearest 0.1°C (Withers, 1978; Wagner et al., 1999; Duran et al., 2020) before and after each trial, in addition to surface temperature. The reduction of core body temperature (ΔT_C) was calculated in the same way as ΔT_S.

Before each trial, toads were weighted using a digital balance (I-2000, MAXN) to the nearest 0.1 g. A photo with scale was taken for each toad from the back view with a camera (HDR PJ680, Sony) and then the photo was used to measure the snout-vent-length (SVL) to the nearest 0.001 cm using ImageJ 1.53 g (Abramoff et al., 2004).

Statistical Analysis

All continuous variables, including body-mass, SVL, distance, ΔT_S, and ΔT_C, were scaled before statistical analysis. To test how well ΔT_S variation reflect ΔT_C variation, general linear models (GLM) were constructed using package *lme4* (Bates et al., 2014). ΔT_C was the dependent variable and ΔT_S was the independent variable. Model assumptions, including homoscedasticity and normality of residuals, were tested with plots. The outliers that violate model assumptions were removed and models were reconstructed (**Supplementary Tables 1**, **2**).

To test the correlation between moved distance and temperature reduction, a polynomial regression was conducted using package *lme4*, in which ΔT_S was the dependent variable (**Supplementary Table 1**). Distance was the independent variable, which was transformed to binomial expression. There was no violation on model assumptions.

To test effects of all the considered factors on ΔT_S and their interactions, GLMs were constructed. ΔT_S was set as the dependent variable, while the independent variables including distance, origin (population), round, location (places of racetrack trials), sex, body-mass, and SVL (**Supplementary Table 1**). A second model was constructed without body-mass, SVL, and sex, as they were not the target factors and had no significant effect on ΔT_S (**Supplementary Table 1**). Model comparison was conducted with the method of Analysis of Variance (ANOVA), and it indicated that there was no significant difference between the two models (**Supplementary Table 3**). Therefore, model without body-mass, SVL, and sex was selected. Because of the potential interactive effects among the independent variables, interactive effects were introduced into the models (**Supplementary Table 1**). Six combinations of interactive predictors were included:

1) *Location* × *Round*
2) *Origin* × *Location* × *Round*
3) *Distance* × *Origin*
4) *Distance* × *Origin* + *Location* × *Round*
5) *Distance* × *Origin* × *Location*
6) *Distance* × *Origin* × *Location* × *Round*

Model comparison showed that the fourth combination had the lowest AIC score (**Supplementary Table 4**), and therefore, the final model had two interactive effects. Model assumptions were also checked and there was no violation.

We also tested differences of surface body temperature between origin-H and origin-L toads before and after exercise in each trial from round 1 to round 4, as supplementary to model selection. When the scaled temperature data passed tests of normal distribution and homogeneity of variance, an independent-sample *t*-test was conducted. When the data did not pass the tests, an unpaired two-sample Wilcoxon test was conducted.

To test the correlation between moved distance and origin, location, and rounds, GLMs were constructed. The distance was the dependent variable, and origin, location, rounds were set as the independent variables (**Supplementary Table 1**). Model assumptions were also checked and there was no violation.

All analysis was performed using R 4.1.2 (R Core Team, 2020). For visualization, linear models and non-linear models were plotted with package *ggplot2* (Wickham, 2016, 2) and model predictions were extracted using package *ggeffects* (Lüdecke, 2018). The package *interactions* (Long, 2019) was used to plot interactive effects of models.

RESULTS

The average moved distance of the origin-L population in rounds 1 and 2 (total trial $n = 16$) was 44.87 ± 37.73 m (mean ± sd), and in rounds 3 and 4 (total trial $n = 16$) was 61.33 ± 33.40 m. The same measurements of the origin-H population were 14.35 ± 10.51 m and 17.08 ± 10.06 m for rounds

TABLE 2 | Results of polynomial regression between distance and ΔT_S; and linear regression between ΔT_S and ΔT_C.

	Dependent variable:	
	$\mathbf{\Delta T_S}$	$\mathbf{\Delta T_C}$
Distance	–0.807**	
	(0.142)	
Binomial distance	0.254**	
	(0.068)	
ΔT_S		1.363**
		(0.074)
Constant	–0.251*	–0.576**
	(0.112)	(0.059)
Observations	94	27
R^2	0.270	0.931
Adjusted R^2	0.253	0.928
Residual Std. Error	0.864 (df = 91)	0.246 (df = 25)
F Statistic	16.790** (df = 2; 91)	337.379** (df = 1; 25)

*$p < 0.05$, **$p < 0.01$.*

1 and 2 and for rounds 3 and 4, respectively. The ΔT_S of the origin L population in round 1 and round 2 was –1.41 ± 0.76°C, and in round 3 and round 4 was –3.01 ± 1.05. The same measurements of the origin H population were 0.11 ± 0.93°C and –3.05 ± 0.71°C for rounds 1 and 2 and for rounds 3 and 4, respectively.

In rounds 5 and 6, the ΔT_S was –1.61 ± 0.61°C in the experimental group, and was 0.06 ± 0.31°C in the control group. The ΔT_C in the two groups were –1.17 ± 0.58°C and 0.08 ± 0.17°C, respectively. The control group were not allowed to move in trials, so the moved distance was zero, and the distance of the experimental group was 24.65 ± 28.52 m.

Furthermore, the adjusted R^2 from the GLM between ΔT_S and ΔT_C was 0.90. After removing three outliers, the adjusted R^2 increased to 0.93 (**Supplementary Table 2**). Therefore, variations between the two measurements were within an acceptable level (estimate ± se = 1.363 ± 0.074; $p < 0.01$; **Table 2** and **Figure 2A**), and ΔT_S was a valid substitute for ΔT_C.

Model analysis indicated a non-linear correlation between quadratic moved distance and ΔT_S (0.254 ± 0.068, $p < 0.01$; –0.807 ± 0.142, $p < 0.01$; **Table 2** and **Figure 2B**). The results clearly demonstrated that exhaustive exercise caused hypothermia. Body temperature reduction increased with prolonged moved distance, but the slope of changing temperature gradually became flat along with the distance.

The constructed linear model, which contained two interactive effects, supported a significant effect of all independent variables on ΔT_S. The total moved distance was negatively correlated with ΔT_S (–0.731 ± 0.299, $p < 0.01$; **Table 3** and **Figure 3A**), which was concordant with the polynomial regression. Round was positively correlated with ΔT_S (0.377 ± 0.187, $p < 0.05$; **Table 3** and **Figure 3B**), which meant the ΔT_S was significantly increased in round 2 and round 4, compared to round 1 and round 3, except at the Kangding location (**Figure 3B**). When compared to the origin-H population, the origin-L population had a positive relationship with ΔT_S (0.579 ± 0.223, $p < 0.05$; **Table 3** and **Figure 3A**). When compared to the Chengdu location, the Kangding location had a significantly positive correlation with ΔT_S (4.804 ± 0.787, $p < 0.01$; **Table 3** and **Figure 3B**), but the Shimian location was not (0.853 ± 0.784, $p < 0.05$; **Table 3**).

A better way to understand the results was through the interactive effects. Interactive effect of distance and origin was significantly correlated with ΔT_S (*distance* × *Shimian*: 0.632 ± 0.312, $p < 0.05$; **Table 3**), which meant when moving the same distance, a toad from the origin-L group had a larger ΔT_S than a toad from the origin-H group (**Figure 3A**). Another set of significantly interactive variables was round and location (*round* × *Kangding*: –1.344 ± 0.328, $p < 0.01$; **Table 3**), which predicted a negative relationship between round and ΔT_S when the racetrack trials were conducted in Kangding, and the slope was neither similar to slope in Shimian nor similar to slope in Chengdu (**Figure 3B**).

The origin-H toads had lower body temperature before exercise than origin-L toads did in round 1 and round 2 (round 1: $W = 0$, $p < 0.001$; round 2: $W = 11$, $p < 0.05$; **Supplementary Table 5** and **Supplementary Figure 1A**). However, origin-H toads had higher body temperature than origin-L toads did in rounds 3 and 4 (round 3: $T = 2.147$, $p < 0.05$; round 4: $W = 56$, $p < 0.05$; **Supplementary Table 5** and **Supplementary Figure 1A**). As for the post-exercise body temperature, there was no significant difference between the two population (**Supplementary Table 5** and **Supplementary Figure 1B**).

Furthermore, origin had significant correlation with moved distance from the linear model (origin-L: 1.289 ± 0.287, $p < 0.01$; **Supplementary Table 6** and **Supplementary Figure 2**).

DISCUSSION

Our results showed that high-altitude population of Asiatic toads had a stronger hypothermic reaction to exhaustive exercise than low-altitude conspecific toads did. The high-altitude population had lower body temperature at round 2 than round 1 at its original habitat, which was different from toads of low-altitude. When the effect of moved distance was controlled, the high-altitude population had larger body temperature reduction than low-altitude population at both original habitats and common garden location. In other words, high-altitude population did not change the extent of hypothermic reaction to exhaustive exercise after acclimation.

Our results confirm the exhaustive-exercise induced hypothermia in the Asiatic Toad, a phenomenon that has previously been reported for a few other poikilothermic animals (Petersen et al., 2003). Behavioral hypothermia has been proposed as a protective strategy to reduce metabolic demands during hypoxia or after exercise (Wagner et al., 1999; Hicks and Wang, 2004; Branco et al., 2006). Poikilothermic animals in hypothermic state may reduce oxygen consumption, increase affinity of hemoglobin for oxygen, and reduce energetically costly responses (Steiner and Branco, 2002). In turtles (*Chrysemys picta*), for instance, the oxygen-hemoglobin dissociation curve shifts rightward at high temperatures, meaning that oxygen

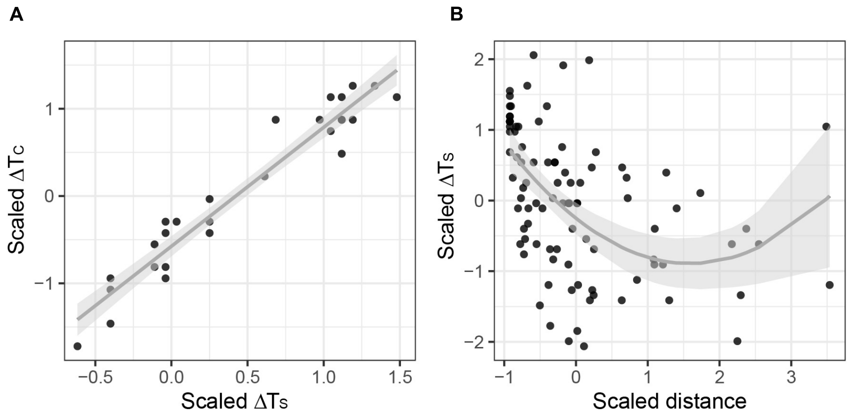

FIGURE 2 | (A) Linear relationship between ΔT_S and ΔT_C. **(B)** Binomial regressions between moved distance and ΔT_S.

TABLE 3 | Results from linear models with interactive effects on ΔT_S.

	Dependent variable:
	ΔT_S
Distance	–0.731*
	(0.299)
Origin (L)	0.579*
	(0.223)
Location (Kangding)	4.804**
	(0.787)
Location (Shimian)	0.853
	(0.784)
Round	0.377*
	(0.187)
Distance: origin (L)	0.632*
	(0.312)
Location (Kangding): round	–1.344**
	(0.328)
Location (Shimian): round	0.454
	(0.324)
Constant	–2.622**
	(0.674)
Observations	64
R^2	0.784
Adjusted R^2	0.752
Residual Std. Error	0.525 (df = 55)
F Statistic	24.896** (df = 8; 55)

*$p < 0.05$, **$p < 0.01$.*

saturation changes to a lower level at the same PO_2 at high temperature than at low temperature (da Silva et al., 2013). Besides, glucose metabolism is positively correlated with oxygen availability (Gullino et al., 1968; Storz and McClelland, 2017), and experiment on vitro muscle of the American Bullfrog (*Lithobates catesbeianus*) suggests that net rate of glucose metabolism at low temperature is reduced compared to that at high temperature (Petersen and Gleeson, 2009). Therefore, hypothermia may increase survival rate of poikilothermic animals under various metabolism interruptions such as hypoxia or exhaustion (Wood and Gonzales, 1996; Petersen et al., 2003; Morris, 2004).

An interesting difference between high- and low-altitude toads is the predicted slope between moved distance and body temperature reduction, which indicates a lower body temperature of high-altitude toads than that of low-altitude conspecifics when moving the same distance. We postulate that this variation may represent an adaptative shift of hypothermic performance in high-altitude environments. For poikilothermic animals living at high altitudes, the cost of allocating energy to thermoregulation to achieve a high body temperature as a response to stress (e.g., infection) may offset the benefits of the high body temperature in cold and hypoxic environments (Duran et al., 2020). Thus, these poikilothermic animals tend to lower their body temperature to save energy for other more important life activities (Duran et al., 2020). On the other hand, oxygen uptake at resting state changes along with body temperature in most animals (Deluen et al., 2022), and animals in a hypoxic environment may suffer from hyperthermia (Wood and Gonzales, 1996). In turn, hyperthermia favors higher metabolism and promotes body recovery but also demands higher oxygen uptake. Amphibians like *Bufo marinus* have a low temperature preference during hypoxic exposure, and this behavioral hypothermia increases arterial saturation and reduces oxygen uptake (Wood and Malvin, 1991). For poikilothermic vertebrates, low temperature also eases ventilatory responses in hypoxic conditions (da Silva et al., 2013). Another possible explanation is the metabolic cold adaptation (MCA) hypothesis, which suggests poikilotherms in cold environments would have a high standard metabolic rate to ensure life activity at low temperatures (Gaston et al., 2009; Deluen et al., 2022). High-altitude populations of the Pyrenean Brook Newts have a higher baseline of the relationship between standard metabolic rate and body temperature compared to low-altitude populations, which supports the MCA (Deluen et al., 2022). Thus, the high-altitude toads may have a metabolic level at a low body temperature similar to that of low-altitude toads at a high body temperature. Hence, more exploration on the physiological significance of hypothermic performance in high-altitudinal poikilotherms is needed.

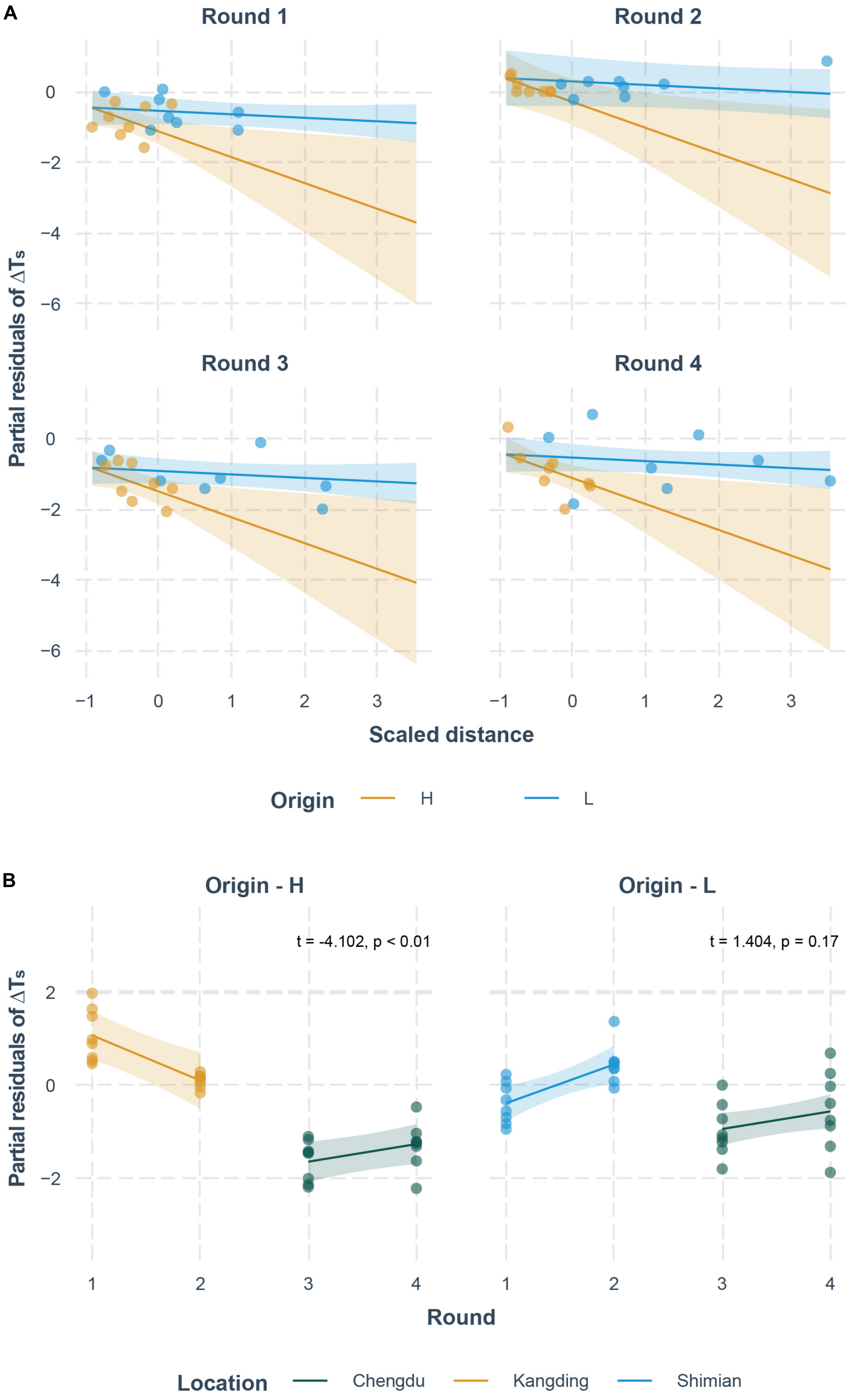

FIGURE 3 | Correlation between interactive effects and ΔT_S. **(A)** Interactive effect of distance and origin on ΔT_S. **(B)** Interactive effect of round and location on ΔT_S. The partial residuals of ΔT_S are plotted after controlling for effects of all controlled variables.

The observed variations of hypothermic reaction may predict a potential changing direction for low-altitude populations when facing warming climate. Warming temperature affects important physiological processes such as metabolism, thus increased temperature may push poikilothermic organisms to higher altitudes or latitudes for cooler environments (da Silva et al., 2013; Gangloff et al., 2019; Feldmeier et al., 2020; Jacobsen, 2020). Low-altitude toads may change their hypothermic performance to match their high-altitude conspecifics when they shift to high-altitude environments.

Another key challenge to poikilotherms is whether thermal trait modifications can keep pace with the warming climate (Bodensteiner et al., 2021). Poikilotherms have a relatively low plasticity in thermal tolerance, which means physiological adjustments of thermoregulation may not be sufficient for poikilotherms when facing rapid climate warming (Gunderson and Stillman, 2015; Gangloff et al., 2019). According to the common garden comparison of toad populations, both high- and low-altitude toads retain their own extents of hypothermic reaction. Thus, coping with climate change relying on plasticity of hypothermic performance may not be optimistic for the Asiatic Toad.

CONCLUSION

Exhausting-exercise induces a hypothermic response in both high- and low-altitude toads, and this reaction leads to measurable reduction in body temperature. Besides, high-altitude toads have lower body temperature than low-altitude conspecifics do when moving the same distance. However, the common garden comparison suggests minimum capacity on plasticity of hypothermic performance for both high- and low-altitude toad populations.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee at the Chengdu Institute of Biology, Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

KH: conceptualization and data curation. ZY and KH: methodology. ZY: software, formal analysis, writing – original draft, and visualization. YQ: investigation, supervision, and project administration. ZY and YQ: writing – review and editing. All authors read, edited, and approved the final submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

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A Multilevel Assessment of Plasticity in Response to High-Altitude Environment for Agama Lizards

Yin Qi[1]†, Tao Zhang[2]†, Yayong Wu[3], Zhongyi Yao[1], Xia Qiu[1], Peng Pu[2], Xiaolong Tang[2], Jinzhong Fu[1,4] and Weizhao Yang[1]*

[1] Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, China, [2] Department of Animal and Biomedical Sciences, School of Life Science, Lanzhou University, Lanzhou, China, [3] College of Life Sciences and Food Engineering, Yibin University, Yibin, China, [4] Department of Integrative Biology, University of Guelph, Guelph, ON, Canada

Upslope range shifting has been documented in diverse species in response to global warming. Plasticity, which refers to the ability of organisms to alter their phenotypes in changing environments, is crucial for the survival of those that newly migrated to a high-altitude environment. The scope and mechanisms of plasticity across biological levels, however, have rarely been examined. We used two agama lizards (genus *Phrynocephalus*) as model systems and a transplant experiment to comprehensively assess their plasticity on multiple organization levels. Two low-altitude (934 m) agama species, *Phrynocephalus axillaris* (oviparous) and *P. forsythii* (viviparous), were transplanted to a high-altitude site (3,400 m). After acclimation for 6 weeks in seminatural enclosures, plasticity was measured from bite force, tail display behavior, gene expression, and metabolome. Both lizards were capable of acclimating to the high-altitude environment without sacrificing their performance in bite force, but they also showed high plasticity in tail display behavior by either decreasing the intensity of a specific display component (*P. forsythii*) or by the trade-off between display components (*P. axillaris*). Genes and metabolites associated with lipids, especially fatty acid metabolism, exhibited significant differentiation in expression, compared to individuals from their native habitats. Improved fatty acid storage and metabolism appeared to be a common response among animals at high altitudes. Despite distinct reproductive modes that may differ in response to physiological pressure, the two lizards demonstrated high concordance in plasticity when they faced a novel environment at high altitudes. Taken together, lizards likely acclimate to high-altitude environments by reducing behavioral activity and increasing energy efficiency after range shifting. Our results provide new insights into our understanding of phenotypic plasticity and its importance in today's changing climate.

Keywords: high altitude, lizards, behavior, performance, gene expression, metabolome, plasticity

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Nicholas Wu,
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Hong-Liang Lu,
Hangzhou Normal University, China

****Correspondence:***
Weizhao Yang
yangwz@cib.ac.cn

† These authors have contributed equally to this work

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INTRODUCTION

Upslope range shifting has been documented in diverse species as a response to global warming because cooler regions likely relieve species from overheating and facilitate their survival (Thomas and Lennon, 1999; Parmesan and Yohe, 2003; Root et al., 2003; Thomas et al., 2006). However, moving up along an elevational gradient comes with several other inevitable environmental stressors, including hypobaric hypoxia and intense UV radiation, which may reduce the successful reproduction and individual survival (Scheinfeldt and Tishkoff, 2010; Kouyoumdjian et al., 2019). Endemic high-altitude species have evolved several specific mechanisms that enable them to tolerate these stressors, such as improved O_2 uptake and modified metabolism (Qu et al., 2013; Zhang et al., 2014; Li et al., 2018). How newly range shifted species survive in the high-altitude environments remains to be explored.

Phenotypic plasticity plays a crucial role in facilitating organisms to live at high altitudes (Wilson and Franklin, 2002; Seebacher, 2005; Scoville and Pfrender, 2010; Corl et al., 2018). Plasticity in metabolism is particularly important (Seebacher, 2005; Horscroft et al., 2017). Abundant evidence has demonstrated that the oxygen transport system is highly plastic in dealing with severe hypoxia (Storz, 2007; He et al., 2013; Lui et al., 2015), while plasticity in metabolic activities also assists animals in coping with cold and hypoxia (Hammond et al., 2001; Seebacher, 2005). The Australian freshwater turtle *Chelodina longicollis*, for example, responds to cold stress by increasing the activity of regulatory enzymes (Seebacher et al., 2004). Correspondingly, gene expression, which connects genotype to metabolism, is highly plastic and also shows large differences between individuals living at different altitudes. For example, deer mice from high altitudes show a large-scale upregulation of genes associated with oxygen and metabolic fuel utilization, compared to low elevation individuals (Cheviron et al., 2014; Scott et al., 2015). Plasticity in animal behavior and performance is well known for buffering environmental changes along altitude gradients, especially for ectothermic species (Kearney et al., 2009; Enriquez-Urzelai et al., 2019). In a new environment, individuals often seek proper retreat sites to avoid the overheating risk (Enriquez-Urzelai et al., 2019) or immediately adjust their behavior patterns to match the local environments (Refsnider et al., 2018).

Despite these advancements, a holistic look at how a newly arrived animal may survive in a natural high-altitude environment is lacking. Previous studies were often carried out in laboratory settings with only one stressor considered, typically temperature or oxygen (Seebacher et al., 2004; Seebacher, 2005), but natural high-altitude environments are complex with multiple stressors. Therefore, animal transplant experiments from low- to high-altitude environments with a natural setting are highly desirable. Furthermore, plastic changes in response to high altitudes likely take place at multiple organization levels, including gene expression, metabolism, and performance. Plasticity in metabolic functions is likely essential for organisms to survive at high altitudes. Different pathways, such as oxidative phosphorylation and tricarboxylic acid cycle, have different capacities in heat production and oxygen utility (Voet and Voet, 1995; Cheviron et al., 2012). Several studies have also shown that the fatty acid metabolic pathway is crucial in dealing with high-elevation environments (Cheviron et al., 2012; Qu et al., 2013; Tang et al., 2013; Lui et al., 2015). Other levels of phenotypic variations, such as morphology and behavior, may also contribute to or depend on metabolic functions.

Toad-headed agama lizards (genus *Phrynocephalus*) provide an excellent system to investigate the plasticity in response to high-altitude environments. As ectothermic species, agama lizards are sensitive to environmental changes. This genus is widely distributed in the Eurasian Arid Belt. Recent studies have shown that they have evolved a series of physiological and behavioral traits that are related to high-altitude habitats (Tang et al., 2013; Yang et al., 2014). In addition, these lizards use tail displays in their social communications, which are regulated by anaerobic metabolism (Zhu et al., 2021; Hu et al., 2022). Also, both oviparity (laying eggs) and viviparity (giving live birth) exist in this group of lizards, and viviparity has often been associated with high-altitude or high-latitude environments (Guo and Wang, 2007). The different reproductive modes may restrict or expand their plasticity in a new high-altitude environment. In this group of lizards, one oviparous species (*Phrynocephalus axillaris*) and one viviparous species (*P. forsythii*) are sympatric at low-altitude habitat, and *P. forsythii* has been assessed to face high extinction risk under global warming (Sinervo et al., 2018). Therefore, a comparison between *P. axillaris* and *P. forsythii* would provide an opportunity to reveal both common and specific patterns in response to high-altitude environments for agama lizards.

In this study, we aimed to make a multilevel assessment of the plasticity in response to high-altitude environments for agama lizards. Specifically, we examined (1) whether agama lizards were capable of acclimation from low- to high altitudes and (2) the patterns of plasticity on performance, behavior, gene expression, and metabolome. To achieve these objectives, we transplanted two sympatric agama species *P. axillaris* (oviparous) and *P. forsythii* (viviparous), from their original low-altitude habitat to high-altitude environments in the Qinghai-Tibetan Plateau. Their bite force was measured to assess their performance, and their gene expression profiles from heart, liver, and muscle, as well as blood metabolomes, were obtained to examine their patterns of plasticity.

MATERIALS AND METHODS

Experimental Design

We used a transplanting design to simulate lizards' upslope range shifting. Two *Phrynocephalus* species, *P. axillaris* and *P. forsythii* from Kuerle, Xinjiang, China (41.50386°N, 86.2290°E, elevation = 934 m a.s.l.), were sampled on June 18–July 2, 2017, and transplanted to a high-altitude environment on July 4, 2017, in Zoige, the eastern part of Qinghai-Tibetan Plateau in Sichuan Province (33.71389°N, 102.48543°E, elevation = 3,400 m a.s.l.). Although a control treatment in the original site would provide consistent conditions for comparison after transplantation, it was difficult to conduct experiments without a well-equipped field workstation in the original site. To minimize bias caused by microhabitat differences, we built six outdoor seminatural enclosures (length × width × height = 5 m × 5 m × 1.5 m, **Figure 1A**), and the settings resembled the original habitat as much as possible. We used sand from a field site of *P. vlangalii*, a sister species of *P. forsythii*, as substrate, and had a fishing net suspended above each enclosure to reduce the risk of bird predation. The lizards were kept in the enclosures for 6 weeks

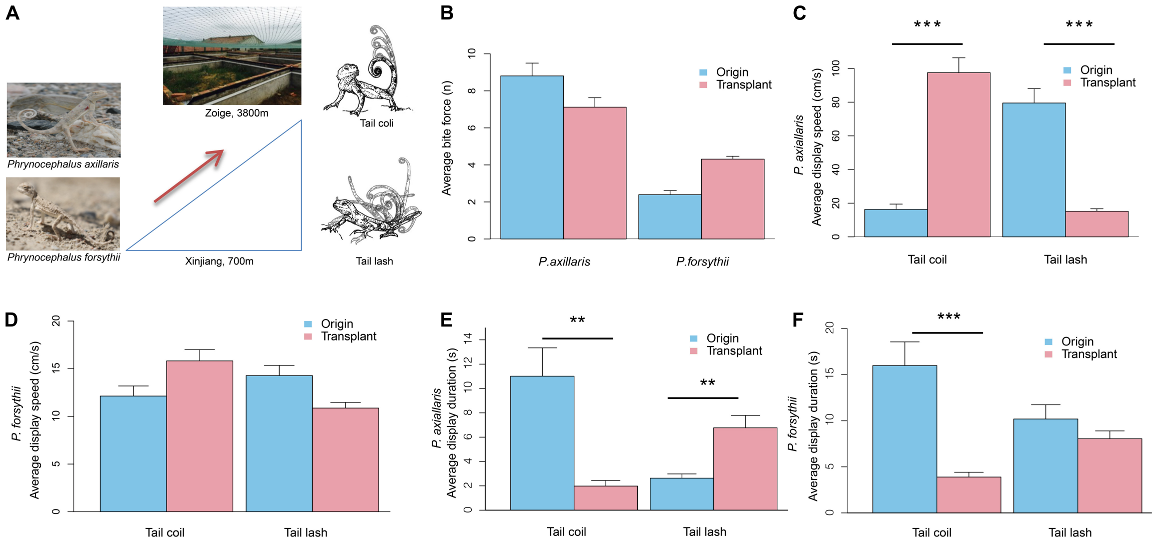

FIGURE 1 | Transplant experimental design and plasticity in performance and behavior for *Phrynocephalus axillaris* and *P. forsythii*. **(A)** Image of *P. axillaris* and *P. forsythii*, the outdoor enclosures used for the common garden in Zoige, the eastern part of the Qinghai-Tibetan Plateau in Sichuan Province, and the schematic drawing of tail coil and tail lash for tail displays. **(B)** Comparison of bite force before and after transplant. **(C)** Comparison of tail coil speed and tail lash speed before and after transplant for *P. axillaris*. **(D)** Comparison of tail coil speed and tail lash speed before and after transplant for *P. forsythii*. **(E)** Comparison of tail coil duration and tail lash duration before and after transplant for *P. axillaris*. **(F)** Comparison of tail coil duration and tail lash duration before and after transplant for *P. forsythii*. In **(B–F)**, bar plots represent the mean values, and error bars represent their standard error. Asterisks indicate statistical significance levels: $^{**}p < 0.01$; $^{***}p < 0.001$.

to acclimate to the new environments, and mealworms were provided every 3 days to offset food shortage.

We examined multiple levels of phenotypic plasticity by comparing lizard states before and after transplanting. The bite force was used to assess lizard body condition and performance in the new environments, while the tail display behavior, gene expression, and metabolism were used to assess the plasticity patterns.

Behavioral Data Collection

We collected data of tail display behavior from 25 males of *P. axillaris* and 26 males of *P. forsythii* from the original site (**Figure 1A**). The detailed protocols on display collection are described by Wu et al. (2018). First, we captured intruder males using noose from a different site 3 km away and measured their snout-vent length (SVL) to the nearest 0.01 mm using caliper. Then, the resident male was approached by a size-matched intruder using a 4-m-long fishing rod. Meanwhile, a video camera (HDR PJ670, Sony, Japan) was set up in front of the resident male and recorded the display response. The trial was terminated after the display stopped, or at a maximum of 5 min if no display was observed. At the conclusion of each trial, a ping-pong ball was placed at the location of the resident and recorded as a scale for subsequent analysis. The resident male was captured using a noose after filming. Immediately after capture, we measured the body temperature from the center of its dorsum with an IR thermometer (HT-866, HCJYET, China). The lizards were kept individually during the experiment. We also measured the SVL and bite force of each lizard. The SVL was measured using a caliper to the nearest 0.01 cm. The bite force was recorded using a piezoelectric force transducer (Type 9203, Kistler Inc., Switzerland) that connected to a charge amplifier (Type 5995A, Kistler Inc., Switzerland) and fitted with two metal bite plates (Vanhooydonck et al., 2005). We encouraged the lizards to bite the plates by gently tapping the mouth using a blunt metal probe. To minimize bias due to motivation and physical condition, we repeated the measurement three times and used the maximum value as the final measurement (Vanhooydonck et al., 2005).

A total of 22 male *P. axillaris* and 23 male *P. forsythii* were transplanted from the original site to the high-altitude site. Individuals of each species were randomly divided into three groups (7–8 for each) and kept in seminatural enclosures. After acclimation for 6 weeks, we recorded the social display behavior and measured SVL and bite force in the same way. All raw data of the behavioral experiment are shown in **Supplementary Table 1**.

Bite Force Plasticity Analysis

We analyzed the correlation between bite force and transplant treatments using linear models in the *nlme* package (Pinheiro et al., 2018) in R version 4.0.5 (R Core Team, 2015). The bite force was log-transformed before modeling to meet the assumption of Gaussian error distribution. We established a linear model with the transplant treatment as the main effect while considering individual body temperature as co-variable.

Display Digitization and Plasticity Analysis

To quantify the tail display, we tracked the motion of tail tips following the methods outlined by Hedrick (2008) in MATLAB

2015b (MathWorks Inc., Natick, MA, United States). The x-y coordinates of tail tips were determined using the DLT dv5 software (Hedrick, 2008) with successive frames. We extracted two tail display variables, the average display speed and display duration. The average display speed was defined as the average distance moved by tail tip within a specific time. The display duration was defined as the time of each display bout. We quantified the two variables for both tail coil display and tail lash display to examine whether tail display plasticity differed between components. As the orientation of the lizard relative to the camera likely affected tail display quantification (Bian et al., 2016), we categorized each display as either facing toward or away from the camera or at the right angle to the camera. Finally, we transformed the grid-based display variables to Euclidean distance in MATLAB using the ping-pong ball as scale.

We analyzed the correlation between tail display and transplant treatments using linear mixed models in the *nlme* package (Pinheiro et al., 2018) in R version 4.0.5 (R Core Team, 2015). All independent variables were log-transformed to meet the assumptions of the linear mixed model. For average tail display speed, we considered transplant treatment and display orientation as fixed effects, with lizard identity as a random effect and assuming a Gaussian error distribution. For display duration, we considered treatment as fixed effects, with lizard identity as a random effect and assuming a Gaussian error distribution. We included the SVL of residents as covariates in both tail display speed and duration models to account for the potential effect of body size.

Transcriptome Sequencing Analysis

For each species, 6 individuals from the original habitat and 6 transplanted individuals were subjected to part of the examination. Tissues of the heart, liver, and muscle were collected immediately after euthanization on the days of collection or immediately after the completion of the transplant experiments. The sample information is provided in **Supplementary Table 2**. Total RNA was extracted from each tissue sample according to the TRIzol protocols (Invitrogen, Carlsbad, California). Paired-end sequencing with a read length of 150 base pairs (bp) was carried out on the Illumina HiSeq2500 platform by Novogene (Beijing, China).

Raw sequencing reads were first cleaned by excluding the adapter sequences and low-quality base calls using a Novogene pipeline. Trimmomatic version 0.35 (Bolger et al., 2014) was used to trim the clean reads with LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15, and MINLEN:70. We checked the read quality before and after trimming using FastQC version 0.11.8 (Andrews, 2010). Subsequently, quality-filtered reads of three different tissue samples from one representative individual of each species (A2, G1, and M2 for *P. axillaris*; C1, I1, and O1 for *P. forsythii*) were used in *de novo* assembly *via* Trinity version 2.8.4 after *in silico* read normalization (Grabherr et al., 2011;

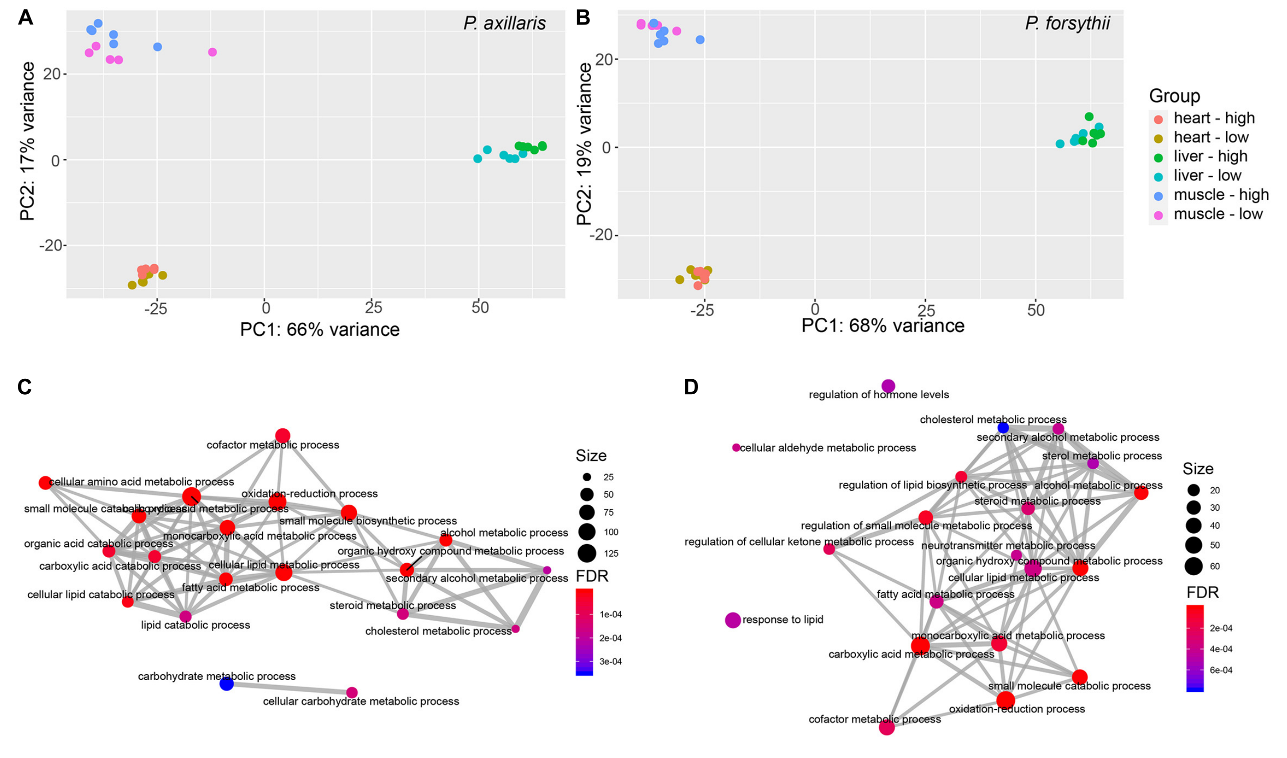

FIGURE 2 | Plasticity in gene expression profiles. **(A)** Principal component analysis (PCA) plot for expression profile of *P. axillaris*. The colors of dots represent the experimental groups. **(B)** PCA plot for expression profile of *P. forsythii*. **(C)** Network for overrepresented Gene Ontology (GO) categories for differentially expressed genes (DEGs) in liver of *P. axillaris*. **(D)** Network for overrepresented GO categories in the liver for *P. forsythii*. In the networks, the size of dots represents the number of genes in each category, and the FDR represents the *p*-values after multiple correction by false discovery rate.

Haas et al., 2013). Since Trinity usually generates a large number of assembled transcripts, among which many may have questionable biological significance, we used kallisto version 0.44.0 (Bray et al., 2016) to quantify the abundance of the assembled transcripts and build expression matrices. Transcripts with "transcripts per million transcripts" (TPM) less than three were removed to generate the final assembly for each species. To obtain orthologous sequences among the two species, a best reciprocal hit (BRH) method was applied for the final assemblies *via* BLAST + version 2.7.1 (Camacho et al., 2009).

The clean reads for each tissue sample were mapped to the transcriptome assemblies for corresponding species by STAR version 2.6 (Dobin et al., 2013). The quantity of reads that matched to the same transcripts was counted using the HTSeq-count tool with the "union" resolution mode (Anders et al., 2015). The expression similarity among samples was calculated by the Euclidean distance and visualized by clustering heatmap after regularized log-transformation (rlog) of normalized counts *via* DESeq2 version 1.20 (Love et al., 2014). Principal component analysis (PCA) was used to assess the relationship between samples. We compared the samples that belonged to the same species separately to examine the expression divergence among different tissue types within one species. Differentially expressed genes (DEGs) were estimated using generalized linear models in edgeR package version 3.22.5 (Robinson et al., 2010; McCarthy et al., 2012). We used a strict thresold to characterize DEGs, with fold-change $\geq$ 2 and adjusted *p*-value < 0.05 [false discovery rate (FDR)].

Functional annotation was applied by aligning the transcripts to the UniProtKB/Swiss-Prot database (release "2018_08") with BLAST hits *E*-value cutoff greater than 10^{-5}. For transcripts with annotation information, overrepresentation test of DEGs was calculated using the clusterProfiler package (Yu et al., 2012) in R with annotation to the Gene Ontology (GO) category and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway databases. The minimum number of transcripts required for each test of a given category was 10.

Metabolomic Assay Analysis

For each species, 10 individuals from the original habitat and 10 transplanted individuals were subjected to this part of the examination. The sampling of blood plasma was conducted immediately after euthanization on the days of collection or the completion of the transplant experiment. In detail, each lizard

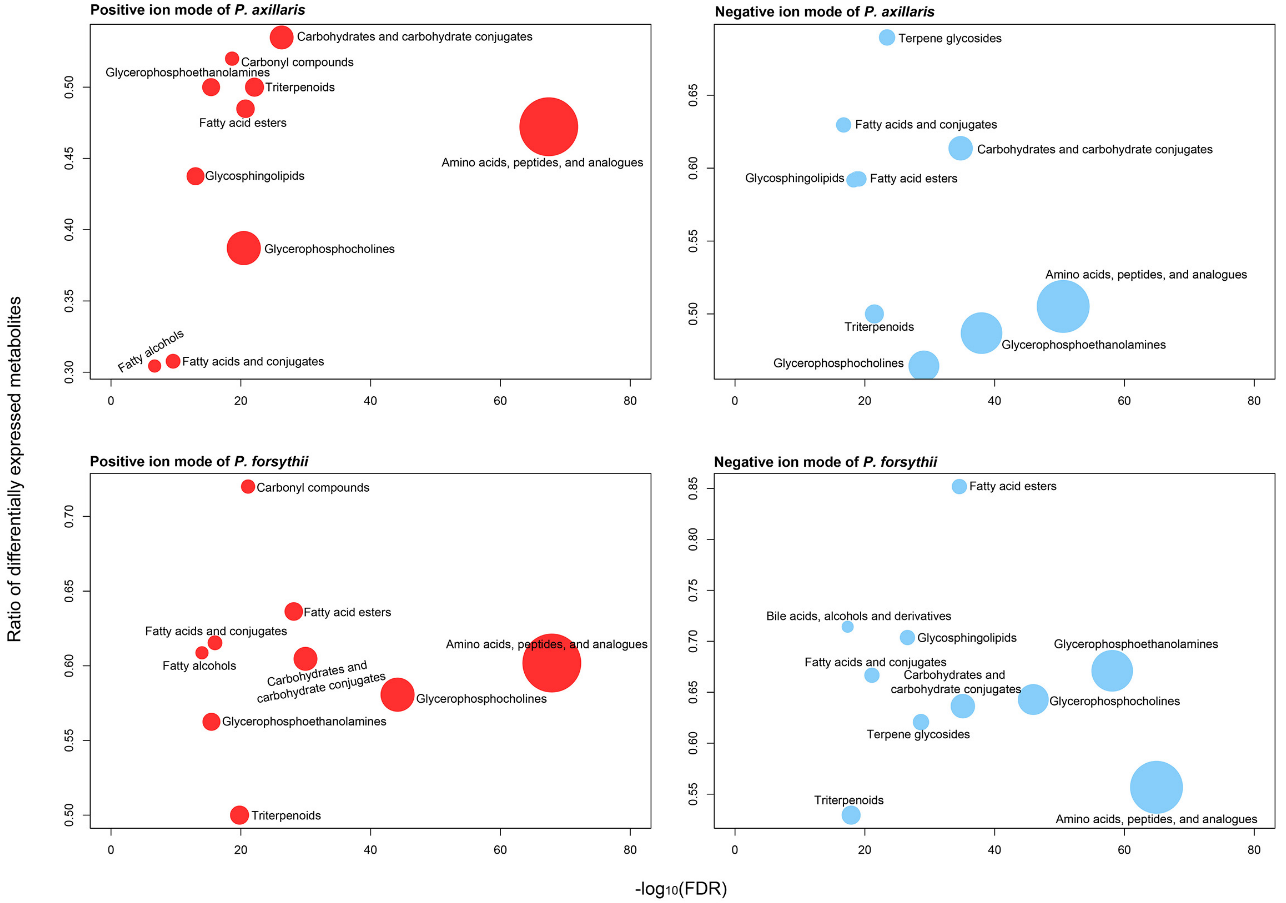

FIGURE 3 | Plasticity in main biochemical clusters of metabolites identified in metabolome assay. Red color denotes metabolites from positive ion mode, and blue color denotes metabolites from negative ion mode. The top panels represent the metabolome in *P. axillaris*, and the bottom panels represent that in *P. forsythii*.

was fixed on a polyvinyl board. Then, we used ophthalmic scissors to remove the skin of the neck and make a cut on the Jugular vein. Notably, 40 μl blood plasma was extracted by a capillary (length: 100 mm; inner diameter: 1.55 mm) from the vein. Finally, the plasma was pushed into a 200-μl tube and then immediately stored in liquid nitrogen. The plasma samples were inputted for metabolome assay using the liquid chromatography-mass spectrometry (LC-MS) method (Brown et al., 2011; Dunn et al., 2011) and an ultra-performance liquid chromatography (UPLC)-TripleTOF 5600 system (AB SCIEX, Majorbio, Shanghai, China). Due to the different properties of metabolites, two modes (i.e., negative and positive ion modes) were used to detect metabolites. The raw data from LC-MS passed through a series of quality control phases, including baseline filtering, peak identification, integration, retention time correction, and peak alignment, and finally were normalized using the software XCMS (Tautenhahn et al., 2012). Final data matrices contained retention time, mass-to-charge ratio, and peak intensity. The identification of metabolites was carried out using substance-matching in the METLIN and HMDB databases and an in-house standard library according to the retention time and m/z. The SIMCA-14.1 (Umetrics, Kinnelon, United States) was used to perform orthogonal partial least squares discriminant analysis (OPLS-DA) for obtaining variable influence on projection (VIP) values. Differentially expressed metabolites (DEMs) were defined using the following cutoff criteria: (1) Benjamini-Hochberg-adjusted *p*-values being less than 0.05; (2) fold-change (expressed as the ratio of average metabolite abundance between groups) being less than 0.5 for downregulation or being larger than 2 for upregulation; (3) VIP being larger than 1.

The Chemical Similarity Enrichment Analysis (ChemRICH; Barupal and Fiehn, 2017) was conducted to classify the metabolites into biochemical clusters from metabolomic database using Chemical Translation Service (CTS) and PubChem Identifier Exchange Service (Barupal et al., 2018). ClassyFire (Feunang et al., 2016) was used to automatedly annotate metabolite classification with default parameters. In addition, to further identify key metabolic processes for various metabolites,

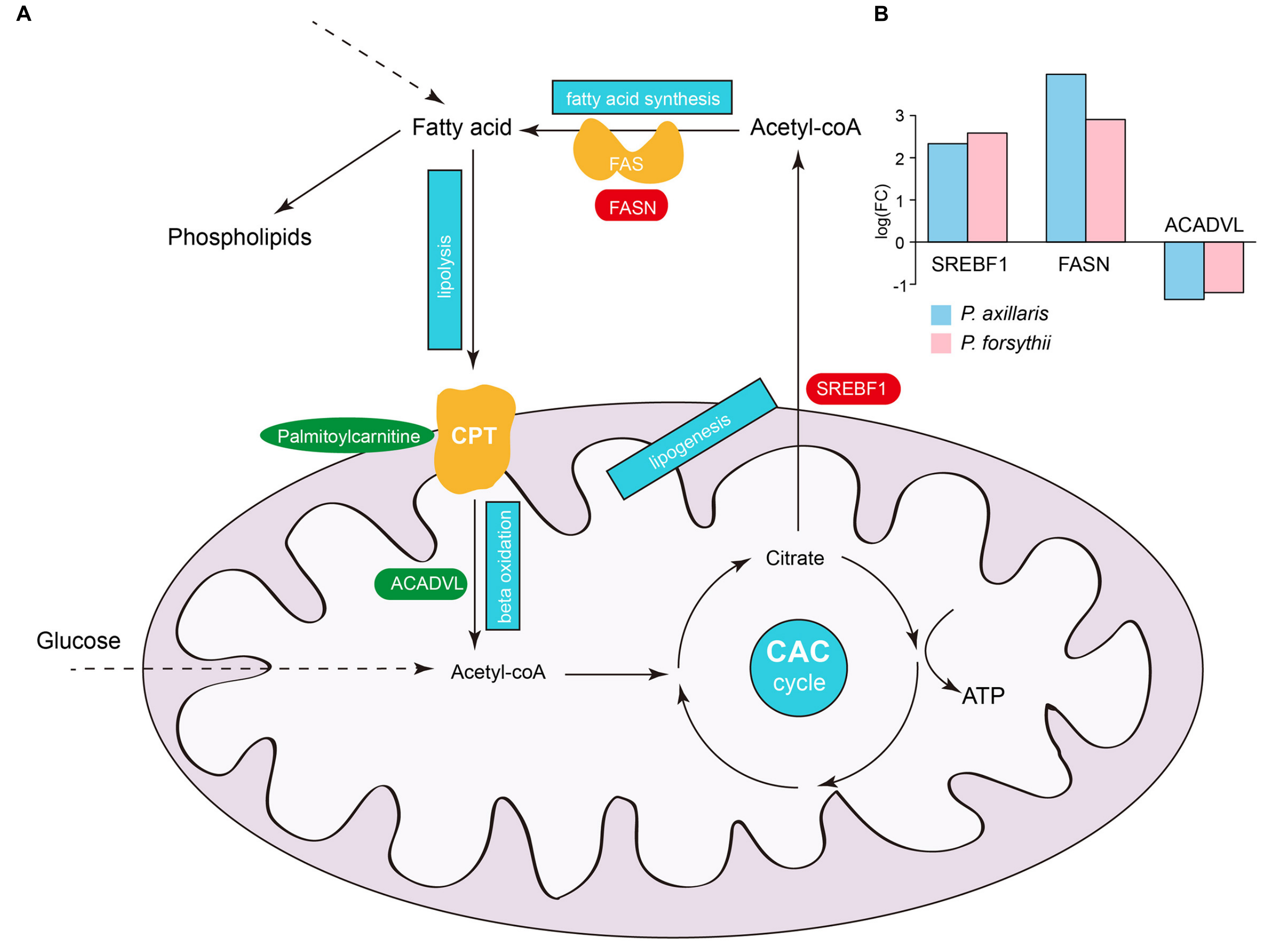

FIGURE 4 | Plasticity in fatty acid metabolism. **(A)** A schematic illustration of fatty acid metabolism in organisms. The blue frames represent the important biological processes in the pathway: lipolysis, beta-oxidation, lipogenesis, citric acid cycle (CAC), and fatty acid synthesis. Crucial genes and metabolites in the pathway are indicated by red or blue colors, which represent upregulation and downregulation of genes or metabolites, respectively. **(B)** The expression fold-change of SREBF1, FASN, and ACADVL in *P. axillaris* and *P. forsythii*.

null diffusion-based enrichment was conducted with the KEGG pathway database by *runDiffusion* function using the FELLA package (Picart-Armada et al., 2018).

RESULTS

Plasticity in Bite Force

We found no significant change in bite force in *P. axillaris* (from 8.81 ± 0.70 n, *n* = 11 to 7.12 ± 0.51 n, *n* = 17; *t* = −0.95, *p* = 0.35; **Figure 1B**) and in *P. forsythii* (from 2.40 ± 0.22 n, *n* = 14 to 4.31 ± 0.16 n, *n* = 18; *t* = 0.74, *p* = 0.47; **Figure 1B**) after being acclimated to high-altitude environments.

Plasticity in Tail Display Behavior

We detected clear plasticity in tail display behavior in both species. In *P. axillaris*, average tail coil speed was significantly increased (from 16.28 ± 3.19 cm/s, *n* = 23 to 97.55 ± 8.82 cm/s, *n* = 20; *t* = 5.06, *p* = 0.0004; **Figure 1C**), while average tail lash speed was significantly decreased (from 79.50 ± 8.52 cm/s, *n* = 22 to 15.19 ± 1.49 cm/s, *n* = 22; *t* = −5.28, *p* = 0.0005; **Figure 1C**) after being acclimated to high elevation area. Meanwhile, the average tail coil duration decreased significantly from 11.01 ± 2.33 s (*n* = 23) to 1.98 ± 0.46 s (*n* = 20; *t* = −3.31, *p* = 0.007), while average tail lash duration increased significantly from 2.63 ± 0.35 s (*n* = 22) to 6.77 ± 1.02 s (*n* = 22; *t* = 4.00, *p* = 0.003; **Figure 1E**). In *P. forsythii*, we found no significant change in average tail coil speed (from 12.13 ± 1.06 cm/s, *n* = 13 to 15.82 ± 1.18 cm/s, *n* = 22; *t* = 1.63, *p* = 0.13; **Figure 1D**), in average tail lash speed (from 14.28 ± 1.07 cm/s, *n* = 20 to 10.87 ± 0.60 cm/s, *n* = 20; *t* = −0.97, *p* = 0.35; **Figure 1D**), and in average tail lash duration (from 10.19 ± 1.55 s, *n* = 20 to 8.05 ± 0.85 s, *n* = 20; *t* = −0.85, *p* = 0.41; **Figure 1F**) after being acclimated to high elevation area, but the average tail coil duration decreased significantly from 15.99 ± 2.57 s (*n* = 13) to 3.89 ± 0.52 s (*n* = 22; *t* = −4.73, *p* = 0.0004; **Figure 1F**).

Plasticity in Gene Expression

A total of 44,000,068–123,811,250 and 42,179,214–91,710,426 raw reads were generated for *P. axillaris* and *P. forsythia* by Illumina sequencing, respectively. After filtering, 41,832,664–118,600,900 and 39,967,100–90,040,782 reads were retained, respectively (**Supplementary Table 3**). For *P. axillaris*, 27,894 transcripts were obtained with an N50 size of 2,372 bp and a mean length of 1,224 bp. For *P. forsythii*, 31,519 transcripts were obtained with an N50 size of 2,185 bp and a mean length of 1,205 bp. By the BRH method, 8,892 orthologous transcripts were identified among the two species.

The PCA plot showed that the tissue-specific expression was very consistent for all types of tissues within the two species, and the expression divergence for any type of tissues between low- and high altitudes was lower than any cross-tissue comparisons (**Figures 2A,B**). Generally, transcriptomes from heart and muscle were less diverged than from liver for both species. As for DEGs of *P. axillaris*, 1,612 were identified in heart between low- and high-altitude groups, with 690 upregulated and 922 downregulated, given low-altitude sample as a reference; 2,792 DEGs were identified in liver, with 1,485 upregulated and 1,307 downregulated; 2,993 DEGs were identified in muscle, with 1,365 upregulated and 1,628 downregulated. Similarly, for *P. forsythii*, 913 DEGs were identified in heart, with 405 upregulated and 408 downregulated; 1,301 DEGs were identified in liver, with 722 upregulated and 579 downregulated; 1,994 DEGs were identified in muscle, with 868 upregulated and 1,126 downregulated.

After functional annotation for DEGs of *P. axillaris*, a total of 32 GO categories and 1 KEGG pathways were identified as overrepresented in heart, most of which were associated with muscle function (e.g., GO: 0006936 muscle contraction, GO: 0003012 muscle system process) and small molecule metabolism (e.g., GO: 0019320 hexose catabolic process, GO:1901135 carbohydrate derivative metabolic process). In muscle, 158 GO categories and 7 KEGG pathways were overrepresented by DEGs, concentrating mostly on muscle function, including GO: 0003012 muscle system process, GO: 0006936 muscle contraction, and GO: 0006941 striated muscle contraction, and also associating with acid metabolism, such as GO: 0043436 oxoacid metabolic process. In liver, different from the other two tissues, 77 GO categories and 13 KEGG pathways were identified as overrepresented by DEGs, which were mostly associated with lipid metabolism, including GO: 0044255 cellular lipid metabolic process, GO: 0006631 fatty acid metabolic process, and GO: 0008202 steroid metabolic process. Similar patterns were also found in overrepresented KEGG pathways, such as map00071 fatty acid degradation and map01212 fatty acid metabolism (**Figure 2C** and **Supplementary Figure 1**).

For the DEGs of *P. forsythii*, four GO categories and one KEGG pathway were overrepresented in heart, which were all related to circadian regulation, including GO: 0032922 circadian regulation of gene expression, GO: 0009649 entrainment of the circadian clock, and pathway hsa04710: circadian rhythm. In muscle, 60 GO categories but no KEGG pathways were overrepresented by DEGs. Those categories were mostly related to cell migration and blood vessel development, including GO: 0030334 regulation of cell migration, GO: 0048514 blood vessel morphogenesis, and GO: 0010594 regulation of endothelial cell migration. In liver, 115 GO categories and 13 KEGG pathways were overrepresented. Similar to *P. axillaris*, most of those functional categories were also associated with lipid metabolism, including GO: 0008202 steroid metabolic process and GO: 0006631 fatty acid metabolic process, and pathways, such as map00071 fatty acid degradation (**Figure 2D** and **Supplementary Figure 1**).

Plasticity in Metabolism

A total of 4,523 features of metabolites were detected in the metabolome assay, among which 4,309 features were retained after filtering. After ChemRICH classification, 1,109 and 985 metabolites were identified with biochemical annotation for positive and negative ion mode of LC-MS, respectively. For *P. axillaris*, 421 DEMs were identified in positive ion mode with 83 upregulated and 338 downregulated; 482 DEMs were identified in negative ion mode with 80 upregulated and 412 downregulated. Similarly, for *P. forsythii*, 565 DEMs were identified in positive ion mode with 106 upregulated and 459 downregulated; 554 DEMs were identified in negative ion mode with 117 upregulated and 437 downregulated. Both positive and negative ion modes suggested that the patterns of metabolome for the two species were quite similar. For all biochemical clusters, amino acids, peptides, and analogs contained the most metabolites for both species, among which approximately 55% of the metabolites were differentially expressed. However, clusters associated with lipids were identified, showing a high ratio of DEMs, such as fatty acid esters, fatty acids and conjugates, and glycosphingolipids (**Figure 3**).

A total of 29 DEMs were mapped to KEGG pathways, among which 16 and 26 were from *P. axillaris* and *P. forsythii*, respectively. A high proportion (50.0% for *P. axillaris* and 53.8% for *P. forsythii*) of DEMs were associated with lipids, including those concordantly regulated in both species, such as the upregulation of 17α-Hydroxypregnenolone (cortisol synthesis and secretion) and sphingosine phosphocholine (sphingolipid metabolism) and the downregulation of cholesterol sulfate (steroid hormones biosynthesis) and L-palmitoylcarnitine (fatty acid degradation). In addition, lipid metabolism-related pathways were overrepresented by DEMs in both species, including fatty acid degradation, primary bile acid biosynthesis, and taurine and hypotaurine metabolism (**Supplementary Table 4**).

Fatty Acid Metabolism

Fatty acid metabolism appeared to be a major source of plasticity for both DEGs and DEMs, and therefore, we further examined detailed expression patterns for genes and metabolites associated with the fatty acid metabolic pathway (**Figure 4A**). We identified a total of 13 genes and 4 symbolic metabolites within the pathway that was differentially expressed in both species. Intriguingly, the patterns of up- or downregulation for those genes were identical in the two lizards, suggesting a common acclimation process in response to high-altitude environments for *Phrynocephalus* species. Among those genes, three play crucial roles in the fatty acid metabolic pathway: sterol regulatory element-binding transcription factor 1 (SREBF1), fatty acid synthase (FASN), and very long-chain-specific acyl-CoA dehydrogenase (ACADVL) (**Figure 4B**). SREBF1 acts as an inductor of lipogenesis in liver leading to increased storage of fatty acid as triglycerides for organisms. FASN encodes the enzyme FASN that catalyzes the synthesis of fatty acid from acetyl-CoA and is regulated by SREBF1 (Bhuiyan et al., 2009; Bouchard-Mercier et al., 2012). On the opposite side, ACADVL catalyzes the first step of mitochondrial fatty acid beta-oxidation, which digests fatty acid into acetyl-CoA before the citric acid cycle to produce energy, specifically for long-chain fatty acids (Miller et al., 2015). Among the metabolites within the pathway, L-palmitoylcarnitine is an important ester derivative of carnitine, which plays a core role in fatty acid metabolism by transporting long-chain fatty acids into mitochondria (Wood et al., 1984; Mutomba et al., 2000). In both *P. axillaris* and *P. forsythii*, SREBF1 and FASN showed significant upregulation, while ACADVL and the L-palmitoylcarnitine showed significant downregulation, which strongly suggested increased storage and decreased digestion of fatty acids.

DISCUSSION

The upslope range shifting is one of the primary responses for species under climatic changes (Pecl et al., 2017). Animals are predicted to colonize high-altitude regions following the shifting of suitable climatic conditions (Lenoir and Svenning, 2015). At high altitudes, key environmental factors, such as low oxygen availability, high levels of UV radiation, and their interactions, pose severe challenges to organisms, especially ectothermic species (Li et al., 2018; Sun et al., 2018). Our transplant experiments demonstrate that toad-headed lizards (*P. axillaris* and *P. forsythii*) from low altitudes are capable of acclimating to high-altitude environments without sacrificing their performance. *P. axillaris* showed high plasticity in tail display behavior, with increased tail coil speed and tail lash duration, as well as decreased tail coil duration and tail lash speed, while *P. forsythii* only showed reduced tail coil duration at high altitudes. Genes and metabolites associated with fatty acid metabolism were also identified with significant differentiation in expression when compared to individuals from low-altitude native habitats. Moreover, a large proportion of metabolites showed decreased expression for transplanted groups. Those consistent results imply that toad-headed lizards acclimate to high-altitude environments by reducing the behavioral intensity and increasing energy efficiency in multiple ways. Despite distinct reproductive models, the two lizards have highly concordant plasticity.

Plasticity in behavior plays a crucial role in dealing with the challenges of high-elevation environments from an energy cost perspective (Refsnider et al., 2018; Enriquez-Urzelai et al., 2020). As the direct connection between organisms and their environments, animal behavior is intimately correlated with energy metabolism (Ros et al., 2006; Mowles, 2014). We found that both species showed a trend of decreasing their tail display intensity, either by a specific component (e.g., tail coil in *P. forsythii*) or by a trade-off between different components (e.g., tail coil and tail lash in *P. axillaris*). Tail displays of *Phrynocephalus* lizards play important roles in social conflict alleviation and mate assessment (Wu et al., 2018), but they are energetically costly (Zhu et al., 2021). Consistent with our results, high-altitude *Phrynocephalus* lizards often constrain their activity intensity or reduce the display complexity (Hu et al., 2022). Behavioral traits are intrinsically plastic; lizards and many other ectotherms are well known for adjusting their behavior quickly in a new environment (Refsnider et al., 2018; Enriquez-Urzelai et al., 2020).

Plasticity in nutrient and energy metabolism can balance the requirements of life activities in high-altitude environments (Storz et al., 2010; Zhang et al., 2018). As the primary energy storage for animals, fatty acids play a key role in high-altitude plasticity and adaptation (Cheviron et al., 2012; Lui et al., 2015). Tang et al. (2013) first discovered that high-altitude *Phrynocephalus* species (*Phrynocephalus erythrurus*) had high-fat utility compared to its low-altitude counterpart (*Phrynocephalus przewalskii*). In this study, several core genes and metabolites associated with fatty acid metabolism show concerted patterns of differential expression, suggesting a common plastic response for both *P. axillaris* and *P. forsythii*. The *SREBF1* gene in both species is also significantly upregulated at high altitudes, which functions to facilitate the process of lipogenesis by transforming other nutrients into fatty acids (Bhuiyan et al., 2009; Bouchard-Mercier et al., 2012). The upregulation of FASN is consistent with this process, which directly catalyzes fatty acid synthesis (Bhuiyan et al., 2009). In addition, ACADVL is downregulated at high altitudes, which suppresses the breaking down of long-chain fatty acids during beta-oxidation (Miller et al., 2015). Some endotherms have similar plastic responses at high altitudes. Yang et al. (2006) found upregulated leptin gene at high altitudes for the plateau pika; leptin facilitates the lipolysis process and is a key regulator for fatty acid metabolism (Pan et al., 2014). All these results suggest a common response of fatty acid metabolism, through lipogenesis, synthesis, and lipolysis, at high altitudes.

Global climatic change has increasingly been a leading cause for biodiversity loss (Sala et al., 2000; Thomas et al., 2004; Valtonen et al., 2017). Studying plasticity in response to high-altitude environments will provide useful insights into how organisms cope with new environments after upslope range shifting. In addition to directly buffering the environmental stress, plasticity may also provide direction for adaptive evolution toward high altitudes. The "plasticity-first evolution" model favors phenotypic plasticity that may lead to adaptation and is supported by an increasing number of cases (Moczek et al., 2011; Jones and Robinson, 2018). Environmental changes may first trigger phenotypic plasticity, and the selection on genotypes that influence the plastic expression of phenotypes will follow. Therefore, elucidating the nature of plasticity will help to predict directions of evolution and to manage conservation projects for organisms under the pressure of climatic change. Clearly, more studies are needed to unravel a comprehensive profile for phenotypic plasticity in behavior, performance, and metabolism for upslope range shifting species in high-altitude environments.

DATA AVAILABILITY STATEMENT

The behavioral data that were collected in this study are available in the **Supplementary Material** of this article. All the sequencing data are deposited in National Genomics Data Center with accession number PRJCA008159.

ETHICS STATEMENT

The sampling and experiment in this study were carried out with permission (No. 2017005) from the Ethical Committee for Animal Experiments in Chengdu Institute of Biology, Chinese Academy of Sciences. All animal sample collection protocols complied with the current laws of China. All applicable guidelines for the care and use of animals were strictly followed.

AUTHOR CONTRIBUTIONS

YQ, JF, and WY conceived the ideas and designed the experiments. YW, ZY, PP, and XQ conducted the field collection and transplant experiment. YQ and YW collected the behavior, performance, and RNASeq data. TZ and XT measured the metabolome assay. YQ, TZ, JF, and WY analyzed the data and drafted the manuscript. All authors read, edited, and approved the final submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.845072/full#supplementary-material

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New Insight on Vitality Differences for the Penaeid Shrimp, *Fenneropenaeus chinensis*, in Low Salinity Environment Through Transcriptomics

Jun Liu[1]*, Daizhen Zhang[2], Lei Zhang[1], Zhengfei Wang[2] and Jie Shen[1]*

[1] Lianyungang Key Laboratory of Biotechnology, Lianyungang Normal College, Lianyungang, China, [2] Jiangsu Key Laboratory for Bioresources of Saline Soils, Jiangsu Provincial Key Laboratory of Coastal Wetland Bioresources and Environmental Protection, Jiangsu Synthetic Innovation Center for Coastal Bio-Agriculture, Yancheng Teachers University, Yancheng, China

Edited by:
Lin Zhang,
Hubei University of Chinese Medicine,
China

Reviewed by:
Yunji Xiu,
Qingdao Agricultural University, China
Kai Lyu,
Nanjing Normal University, China

***Correspondence:**
Jun Liu
liuj86@live.cn
Jie Shen
shenjie1969@126.com

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Excessive rainfall changes salinity in shrimp farming ponds in short period and exerts low salinity stress on the outdoor breeding shrimp under global warming. *Fenneropenaeus chinensis* can have different performance on vitality in low salinity environments. To reveal mechanisms of vitality difference in shrimp living in low saline environments. This study based on the normal and moribund *F. chinensis* in 10 ppt salinity environment using high-throughput sequencing identifies 1,429 differentially expressed genes (DEGs), 586 of which are upregulated, while 843 of which are downregulated in the normal group (FCN10) as compared to the moribund group (FCM10). Meanwhile, another transcriptomic analysis is conducted on the normal and moribund shrimp from 25 ppt (FCN25 vs. FCM25) salinity environment as the control, in which 1,311 DEGs (upregulated: 327 genes, downregulated: 984 genes) are identified. In this study, intersective pathways, GO (Gene Ontology) categories and DEGs from the two groups of comparative transcriptome are investigated. The two intersective pathways (Metabolism of xenobiotics by cytochrome P450, Pentose, and glucuronate interconversions) significantly enriched by DEGs are related to detoxification. In these two pathways, there is one vitality regulation-related gene (VRRG), the *Dhdh* (dihydrodiol dehydrogenase), which is upregulated in both the groups of FCN10 and FCN25 as compared to the groups of FCM10 and FCM25, respectively. Similarly, in the 25 top intersective GO categories, four VRRGs are revealed. Three of them are upregulated (*Itgbl*, kielin/chordin-like protein, *Slc2a8*, solute carrier family 2, facilitated glucose transporter member 8-like protein and *Cyp3a30*, cytochrome P450 3A30-like protein); one of them is downregulated (*Slc6a9*, sodium-dependent nutrient amino acid transporter 1-like protein isoform X2). These GO categories are related to transmembrane transporter activity of substance, enzyme inhibitor activity, monooxygenase activity. RT-qPCR analysis further verifies the VRRGs. The study gives new insight into understanding the vitality differences for *F. chinensis*, in low salinity environment. The pathways and DEGs in response to low salinity stress in modulating the vitality of *F. chinensis* that could serve as tools in future genetic studies and molecular breeding.

Keywords: freshwater aquaculture, global warming, moribund, shrimp, vitality

INTRODUCTION

The thermodynamic and kinetic constrained climate system under global warming accelerated the global hydrological cycle. In this case, the salinity in the upper oceans in the northern hemisphere is significantly fresher (Du et al., 2019). In addition, in China from 1961 to 2015, heavy rain and total heavy rainfall showed an increasing trend, with rainfall and rainy day trends of 127.02 and 463.94 mm per year and 7.93 and 4.24 days per year, respectively (Kong, 2019). Excessive rainfall not only changes the pH levels of shrimp farming ponds, but although changes salinity in the ponds in short period of time (He et al., 2019).

In countries such as China, freshwater aquaculture is increasingly important to the sustainable development of the shrimp fishing industry (Cao et al., 2017), the study and breeding of euryhaline marine species with low-salinity resistance is necessary. For example, the Pacific white shrimp (*Litopenaeus vannamei*), a species that is reported to be capable of surviving in a large range of salinities and that has been cultured in freshwater habitats (Yuan et al., 2021). According to Florkin and Schoffeniels (1969), two fundamental physiological mechanisms in marine organisms that are used to cope with changes in environmental salinity are (1) isosmotic intracellular volume regulation (Mechanism 1), and (2) anisosmotic extracellular osmoregulation (Mechanism 2). Based on these mechanisms, shrimp can be classified into two categories, the osmoconformers and osmoregulators; the former *via* Mechanism 1 mainly include marine species that cannot resist the stress of low salinity, while the latter group including some marine species with Mechanism 2 have stronger ability to resist the stress of low salinity (below 26 ppt, Henry et al., 2012). At present, additional mechanisms of salinity variability response have been reported, such as the osmoregulation of ion transporting epithelia and differential expression of some biomolecules in the gill epithelial cells (Henry et al., 2012). These biomolecules include Na^+/K^+-ATPase, K^+ channels, Cl^- channels, carbonic anhydrase (CA), aquaporins (AQPs), and various exchangers ($Na^+/NH_4{}^+$, Na^+/H^+, and $Cl^-/HCO_3{}^-$) (Neufeld and Pritchard, 1979; Henry and Cameron, 1982; Varley and Greenaway, 1994; Henry, 2001; Chung et al., 2012; Henry et al., 2012). These genes could be potential target tools for breeding of high quality stocks better adapted to salinity variability (Abdelrahman et al., 2017).

Fenneropenaeus chinensis, similar to *L. vannamei* that is another economically important penaeid shrimp, naturally distributes in regions of relatively narrow salinity compared to *L. vannamei* and cannot be cultured in freshwater yet (Yuan et al., 2021). A recent research has identified different phenotypes of these two osmoregulators (*L. vannamei* and *F. chinensis*) under low-salinity stress related to their response effectiveness (Yuan et al., 2021). *L. vannamei* seems to have a more rapid response to low-salinity stress, and differentially expressed genes (DEGs) have been found by comparing these penaeid shrimp based on transcriptomic methods. All studies mentioned above will surely be of benefit to understanding osmoregulation mechanisms of adaptation to salinity stress for these penaeid shrimp, but they are insufficient for understanding the mechanisms of vitality differences for shrimp living in a given salinity environment (i.e., some individuals alive and well, but others moribund). The vitality of individuals is associated with the survival of the shrimp and has a significant impact on harvest yield (Whiting et al., 2000).

A previous study used *L. vannamei* with normal and moribund shrimp to investigate the genetic mechanisms associated with the susceptibility to viruses (Yao et al., 2018). *F. chinensis* can also present vitality differences in a given low salt environment, however in the background of breeding by experience, few studies have considered the mechanism of vitality differences in *F. chinensis* (Gao et al., 2014; Li et al., 2019; Meng et al., 2019; Lu et al., 2020). These studies suggest that some key biomolecules exist to modulate the vitality of *F. chinensis* in low salinity environment. To reveal these key biomolecules, the present study aimed to analyze the differences in gene expression between groups of normal and moribund shrimp from two salinity environments (low salinity: 10 ppt, the control: 25 ppt) using transcriptomic methods (RNA-seq). The goal was to explain why some individuals in a certain salinity environment thrive, while others are moribund. The study thus identifies potential genes in pathways or GO terms for further investigation of genetic diversity and breeding programs.

MATERIALS AND METHODS

Sample Collection and Treatment

From July to August 2020, live shrimp were obtained from a farming pool in Lianyungang (N 34°48′52.47″, E 119°12′19.08″) and transported to our laboratory at Lianyungang Normal College. All shrimp were acclimated to a salinity of 25 ppt, the natural isosmotic point of this species (Chen and Lin, 1994; Chen et al., 1995), at 25°C for 24 h. We then randomly divided the shrimp into two groups ($n = 100$ per group): one group was exposed to low salinity levels (10 ppt) for 4 days, while the other group remained at 25 ppt salinity (the control) for acclimation. On the fifth day, the gills of 12 randomly selected individuals per group were harvested (6 normal shrimp from 10 ppt salinity environment, FCN10; 6 moribund shrimp from 10 ppt salinity environment, FCM10; 6 normal shrimp from

25 ppt salinity environment, FCN25; 6 moribund shrimp from 25 ppt salinity environment, FCM25). In our recent project, 48 samples were sequenced, 24 of which were randomly used in this study (25 ppt: mean body length: 10.40 ± 0.60 cm, normal; 10.78 ± 0.70 cm, moribund; 10 ppt: mean body length: 11.30 ± 0.21 cm, normal; 10.46 ± 0.25 cm, moribund). The gills were stored at −70°C for transcriptome and real-time quantitative PCR (RT-qPCR) analysis.

In the study, salinity was maintained using sea salt and pure water and measured by a portable salinity meter (Arcevoos® ST6). In each tank, 50 L of water was used, and one-third was replaced every 12 h (7:00–19:00).

RNA Isolation, Library Construction, and Sequencing

Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, United States). RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States), and RNA integrity was assessed using 1.5% agarose gel electrophoresis. Magnetic oligo (dT) beads were used to isolate mRNA from total RNA. The mRNA was then broken into fragments approximately 200 bps long using fragmentation buffer (Tris-acetate, KOAc, and MgOAc) at 94°C for 35 min. The fragmented mRNA was used to construct the cDNA libraries. At least 5 μL of mRNA solution (≥200 ng/μL) was used to construct each library. Sequencing libraries for each sample were generated using the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA, United States). Libraries were paired-end sequenced using a NovaSeq 6000 platform (Illumina, San Diego, CA, United States). The read length was 300 bps.

Transcriptome Assembly and Unigene Annotation

Raw sequence data were processed using FastqStat.jar V1.0 (Cock et al., 2010) with default parameters. We then used Cutadapt v1.16[1] (Martin, 2011) with parameters -q 20 -m 20 to clean the raw sequence data by deleting adapter sequences, deleting poly-N sequences, trimming low-quality sequence ends (<Q20), deleting sequences with N ratios > 10%, and removing reads less than 25 bps long. We used Trinity[2] (Haas et al., 2013) to assemble the clean reads with default parameters. The longest transcript in each gene cluster defined by Trinity was selected as a unigene for downstream analysis (Leung et al., 2014).

The identified unigenes were annotated against six databases, namely, NCBI non-redundant sequences (NR), Kyoto Encyclopedia of Genes and Genomes Ortholog (KEGG), Gene Ontology (GO), Protein family (PFAM), functional protein association networks (STRING), and a manually annotated and reviewed protein sequence database (SWISS-PROT). We searched the unigenes against these databases using BlastX v2.2.25 (Altschul et al., 1990) with a cutoff E-value of 10^{-5}. Functional unigenes were identified based on GO terms using Blast2GO[3] (Conesa et al., 2005).

Identification and Enrichment of Differentially Expressed Unigenes

We used Kallisto v0.43.1[4] to evaluate the expression levels of the unigenes based on transcripts per kilobase of exon per million reads (TPM) values; higher TPM values reflect higher levels of unigene expression (Li and Dewey, 2011). We used edgeR v3.24 to identify unigenes where | log2 fold change (FC)| was >1 and the false discovery rate (FDR) was <0.05 (Reiner et al., 2003; Trapnell et al., 2013); these unigenes were considered DEGs. We then identified the KEGG pathways and GO terms significantly enriched in the DEGs ($p < 0.05$) using hypergeometric tests (Li et al., 2014).

Verification of Differentially Expressed Unigenes Using Real-Time Quantitative PCR

We conducted RT-qPCR validation to common target DEGs, which were from the two comparisons (the normal vs. moribund in the 10 ppt and 25 ppt salinity groups). We used the *TubA* gene (α-tubulin) as the internal reference gene (Kozera and Rapacz, 2013), and it was stably expressed in the study. Gene-specific primers were designed based on sequences derived from the transcriptome assembly and annotation using Primer Premier 5.0 (Lalitha, 2000) (**Table 1**). For the synthesis of cDNA the HiScript 1st Strand cDNA Synthesis Kit was used. Each RT-qPCR (10 μL) contained 5 μL of 2 × SYBR qPCR Mix, 0.5 μL each of forward and reverse primers, 2 μL of cDNA, and 2 μL of RNase-free H_2O. RT-qPCRs were performed on an Real-time PCR system (Bio-Rad, CFX96, United States), with the following cycling conditions: an initial denaturation step of 5 min at 95°C; 44 cycles of 10 s at 95°C, 30 s at 60°C; and a standard dissociation cycle. Three technical replicates were performed per gene, and the $2^{-\Delta\Delta CT}$ method (Wang et al., 2013) was used to calculate relative expression levels (relative quantification).

RESULTS

Assembling of Transcriptomic Data and Annotation

After raw data filtering, our project yielded a total of 267.074 Gb bases from the transcriptomic sequencing of 48 samples. The 24 samples used in this study with the values of 98.35% and 45.74% for Q20 and GC content, respectively, were investigated for potential genes and pathways in modulating the vitality of *F. chinensis* (**Table 2**). We assembled all the 48 sequences into one transcriptomic map. Assembling results of unigenes and transcripts yielded total sequence numbers of 585,478 and 715,339; the GC content was 42.47 and 42.63%; the N50 values were 686 bps and 1,216 bps with average lengths of 576 bps and 772 bps, respectively (**Table 3**).

Annotation of the 585,478 unigenes using 6 databases showed that the frequency for each database from large to

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[1] http://cutadapt.readthedocs.io

[2] http://trinityrnaseq.github.io

[3] http://www.blast2go.com/b2ghome

[4] http://kallisto.com

TABLE 1 | Primers used for real-time quantitative PCR.

Gene description	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Product length (bp)
α-tubulin (*TubA*)	CTACGAGGAGGTCGGAGTGG	TGCTTCGGAGACGGTTGTT	176
Kielin/chordin-like protein (*Itgbl*)	GTGCAGCAAACCCTCGAA	GAAAATAACGCCGTGGACATA	187
Solute carrier family 2, facilitated glucose transporter member 8-like (*Slc2a8*)	AAGAGGGGAAAGGGAACAAG	TCGAACACGAACGCTGAAA	198
Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase-like (*Dhdh*)	AAGTAGGCTGAGGCAAGGAAA	TGAATAGGAAGCGGTTGAATG	159
Cytochrome P450 3A30-like (*Cyp3a30*)	CCAACCGCCCAAAACTCG	TCCCTGCCATGTGCTTCAT	138
Sodium-dependent nutrient amino acid transporter 1-like isoform X2 (*Slc6a9*)	CAAAGCCGAGCCTCTGAAA	GCATGACCTCCACCACGA	122

small was as follows: NR (68,239, 11.66%), KEGG (35,183, 6.01%), PFAM (34,683, 5.92%), SWISS-PROT (27,089, 4.63%), GO (26,983, 4.61%), and STRING (4,363, 0.75%). In the study, 1,253 of the unigenes were annotated against all of the databases, accounting for only 0.21%. The unigenes accounting for 83.75% were annotated in at least one database. Regarding the species distribution, 26.5% of the distinct sequences showed top matches with sequences from the *Penaeus monodon* and *Penaeus vannamei (synonyms* of *L. vannamei)* (**Figure 1**).

TABLE 2 | Statistics of clean reads.

Sample ID	Total reads	Total bases	Q20 (%)	GC content (%)
FCN25-1	47,808,674	6,885,716,827	98.19	42.91
FCN25-2	44,595,726	6,380,496,290	97.88	44.35
FCN25-3	54,588,066	7,855,462,625	98.14	43.75
FCN25-4	37,594,248	5,373,968,098	98.41	46.95
FCN25-5	39,759,834	5,702,746,383	98.52	47.32
FCN25-6	37,477,000	5,332,358,375	98.09	47.19
FCM25-1	61,167,730	8,786,736,726	98.12	44.90
FCM25-2	62,058,186	8,991,207,687	98.59	41.41
FCM25-3	45,550,974	6,55,6571,475	98.10	43.50
FCM25-4	42,742,186	6,131,663,571	98.56	47.76
FCM25-5	39,083,654	5,607,110,416	98.51	48.27
FCM25-6	37,944,324	5,450,543,459	98.43	48.16
FCN10-1	51,857,090	7,470,240,740	98.12	45.00
FCN10-2	44,181,190	6,378,915,963	98.26	45.24
FCN10-3	50,320,450	7,225,661,567	98.18	45.38
FCN10-4	39,522,388	5,683,903,736	98.52	47.62
FCN10-5	42,763,020	6,120,508,016	98.50	46.68
FCN10-6	39,557,990	5,653,265,111	98.52	46.40
FCM10-1	30,844,710	4,469,453,630	98.66	39.87
FCM10-2	55,095,314	7,937,343,228	98.50	44.09
FCM10-3	41,251,862	5,901,513,074	98.03	46.85
FCM10-4	41,616,956	5,932,371,864	98.47	47.61
FCM10-5	41,391,728	5,927,942,855	98.53	48.46
FCM10-6	42,995,788	6,153,200,765	98.47	48.03
Average	44,657,045.33	6,412,870,937	98.35	45.74

Each group had six replicates. FCN10, Normal F. chinensis in 10 ppt salinity environment; FCM10, Moribund F. chinensis in 10 ppt salinity environment; FCN25, Normal F. chinensis in 25 ppt salinity environment; FCM25, Moribund F. chinensis in 25 ppt salinity environment.

Identification of the Differentially Expressed Unigenes and Enrichment

After quantification of unigenes with TPM values, there were 1,429 DEGs between groups of FCN10 and FCM10 (**Figure 2A**) and 1,311 DEGs between FCN25 and FCM25 based on the criteria of $|\log2FC| > 1$ and FDR < 0.05 (**Figure 2B**). Between the two groups of FCN10 and FCM10, 586 DEGs were upregulated for the group FCN10, while FCN10 had 843 downregulated DGEs as compared to the FCM10 group. Between the two groups of FCN25 and FCM25, 327 DEGs were upregulated for the group FCN25, while FCN25 had 984 downregulated DEGs compared to the FCM25 group.

Enrichment of DEGs based on KEGG and GO showed that 14 of pathways were significantly enriched in the comparison of 10 salinity groups (FCN10 vs. FCM10). Six of pathways were significantly enriched in the comparison of 25 salinity groups (FCN25 vs. FCM25). 335 of GO terms were significantly enriched in the comparison of 10 salinity groups, 216 of GO terms were significantly enriched in the comparison of 25 salinity groups (**Supplementary Table 1**). The main pathways include Ribosome, Pentose and glucuronate interconversions, Metabolism of xenobiotics by cytochrome P450, Cell adhesion molecules (CAMs), ECM-receptor interaction, Arachidonic acid metabolism. The main GO terms include External encapsulating structure, Extracellular matrix, Structural molecule activity, Cuticle development, D-amino acid transport, D-amino acid transmembrane transporter activity, L-amino acid transmembrane transporter activity, Organic acid transmembrane transporter activity, Carboxylic acid transmembrane transporter activity, Monooxygenase activity,

TABLE 3 | Profile of assembling.

Type	Unigenes	Transcripts
Total sequence number	585,478	715,339
Total sequence base	337,187,078	552,241,490
Percent GC (%)	42.47	42.63
Largest (bps)	30,946	30,946
Smallest (bps)	201	183
Average (bps)	575.92	772
N50 (bps)	686	1,216
N90 (bps)	270	307

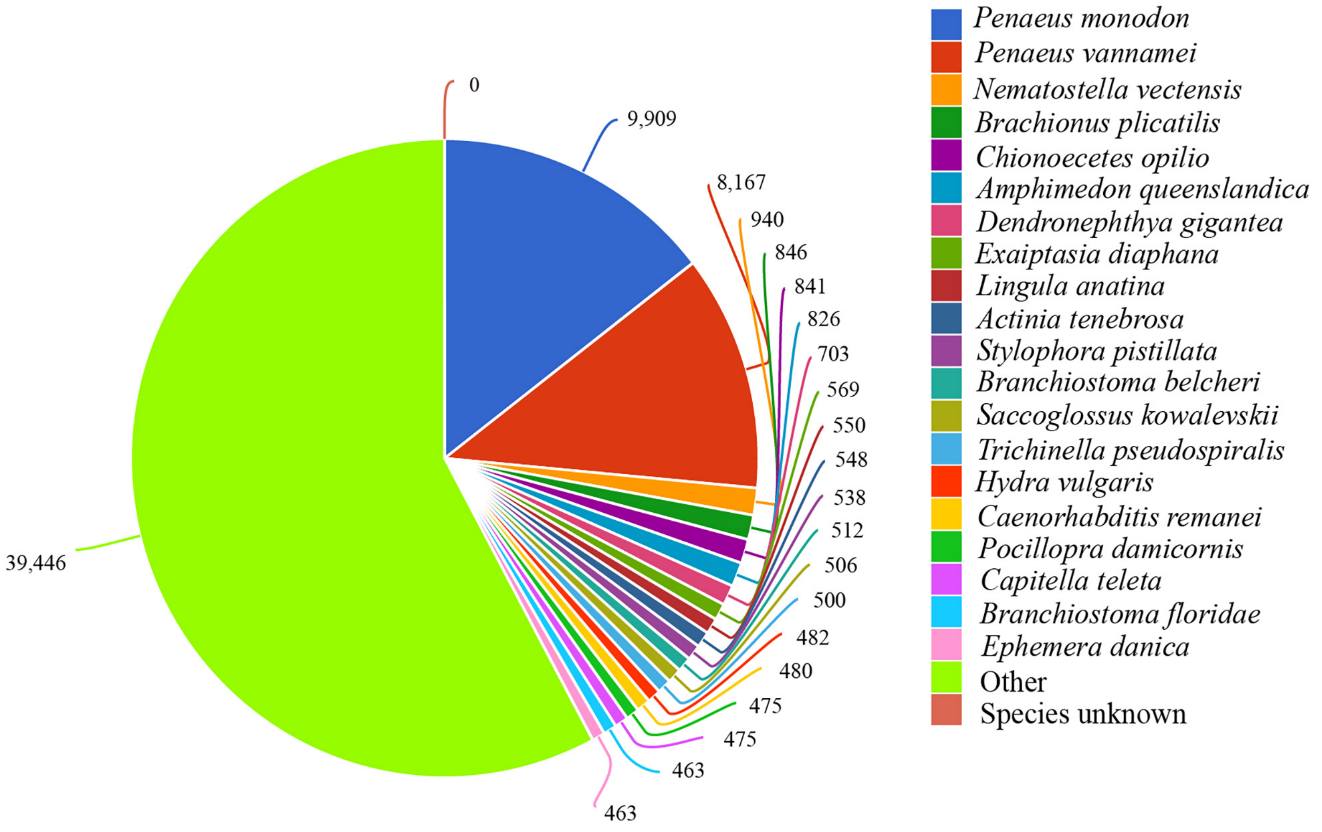

FIGURE 1 | Species distribution of the sequence. Each sector in the figure represents a related species. The larger the area of the sector, the more sequences of the species are aligned. The number represents the amount of aligned sequences.

Amino acid: sodium symporter activity, Organic acid: sodium symporter activity, Amino acid: cation symporter activity, Anion transmembrane transporter activity, Neutral amino acid transmembrane transporter activity, Secondary active transmembrane transporter activity, Solute: sodium symporter activity, Oxidoreductase activity.

Notably, two of the pathways (Metabolism of xenobiotics by cytochrome P450, ko00980; Pentose and glucuronate interconversions, ko00040) were significantly enriched by DEGs from both comparisons, i.e., FCN10 vs. FCM10, and FCN25 vs. FCM25 (**Supplementary Table 2**). In GO databases, 60 terms were significantly enriched by DEGs from both comparisons, and here the detail information of the top 25 GO terms (adjust p-value < 0.05) were shown in **Figure 3** and **Supplementary Table 2**. Most of them were related to transmembrane transporter activity of substance. Others included enzyme inhibitor activity (GO: 0004857), monooxygenase activity (GO: 0004497).

Identification of Potential Vitality-Related Differentially Expressed Unigenes From Pathways and Gene Ontology Terms (Categories)

In the present study, 9 of DEGs for the both groups in 10 ppt (FCN10 vs. FCM10) and 25 ppt (FCN25 vs. FCM25) salinity environments had the same expression pattern (**Table 4**). The results showed that seven of them were upregulated (*Tim2l*, mitochondrial import inner membrane translocase subunit Tim21-like protein; *Itgbl*, kielin/chordin-like protein; *SORD*, sorbitol dehydrogenase-like; *Slc2a8*, solute carrier family 2, facilitated glucose transporter member 8-like; *Dhdh*, trans-1,2-dihydrobenzene-1,2-diol dehydrogenase-like; *Cyp3a30*, cytochrome P450 3A30-like; *Urocl*, urocanate hydratase-like) and two of them were downregulated (*Slc6a9*, sodium-dependent nutrient amino acid transporter 1-like isoform X2; *Rackl*, guanine nucleotide-binding protein subunit beta-like protein isoform X2). Five of the 9 DEGs (*Itgbl*, *Slc2a8*, *Dhdh*, *Cyp3a30*, *Slc6a9*) enriched in the two KEGG pathways (Metabolism of xenobiotics by cytochrome P450; Pentose and glucuronate interconversions) and the top 25 GO terms (**Supplementary Table 2**).

Real-Time Quantitative PCR Verification

We used RT-qPCR to further verify the potential vitality-related DEGs. All these genes had similar expression patterns in both the RT-qPCR and the RNA-Seq analyses (**Figure 4**). Genes of *Itgbl*, *Slc2a8*, *Dhdh*, *Cyp3a30* were upregulated in normal shrimp compared to moribund shrimp, while *Slc6a9* was downregulated.

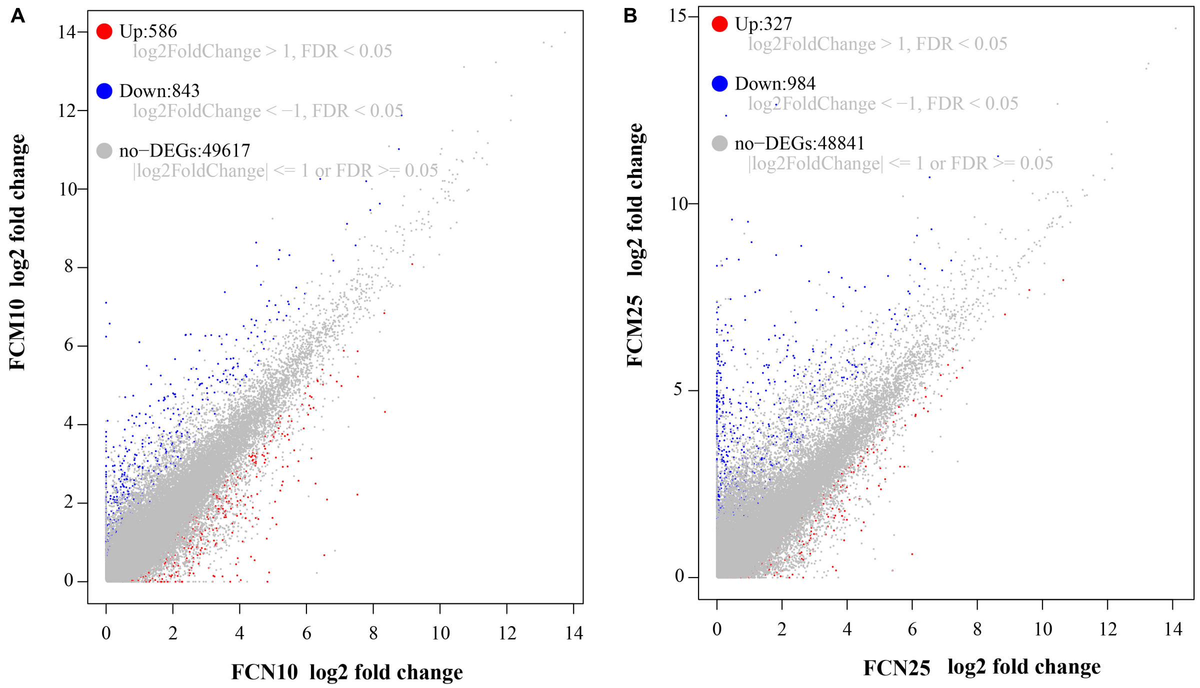

FIGURE 2 | Gene expression patterns. **(A)** Genes of FCN10 vs. FCM10. **(B)** Genes of FCN25 vs. FCM25. FCN10: Normal *Fenneropenaeus chinensis* in 10 ppt salinity environment, FCM10: Moribund *F. chinensis* in 10 ppt salinity environment, and so on. The expression of differentially expressed genes (DEGs) in FCM served as the control. Genes with value | log2FC| ≤ 1 or FDR ≥ 0.05 are considered as not differentially expressed.

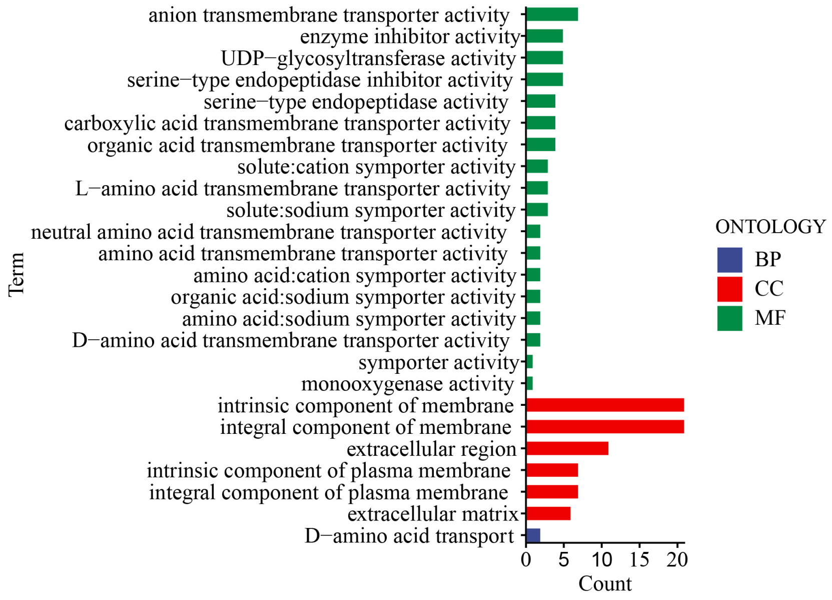

FIGURE 3 | The top 25 of gene ontology (GO) terms significantly enriched by differentially expressed genes (DEGs).

DISCUSSION

Stress of aquatic animals occurs due to physical and physiological disturbances in the certain aquatic environment (Eissa and Wang, 2014; Yu et al., 2016). For example, fishes living in alkaline water, which with the high pH, they will suffer stress from inhibition of ammonia excretion and increasing CO_2 excretion in physiology (Yao et al., 2012). Response ability to stress is closely related to vitality regulation in organisms (Eissa and Wang, 2014). In this study, two pathways and 60 GO terms were

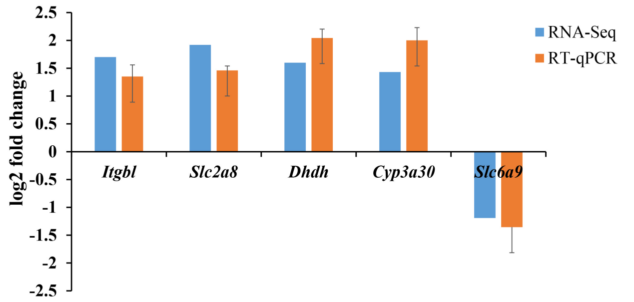

FIGURE 4 | RT-qPCR verification of five target genes identified as differentially expressed between normal and moribund groups of *Fenneropenaeus chinensis* in 10 ppt salinity environment. Four genes (*Itgbl*, *Slc2a8*, *Dhdh*, *Cyp3a30*) were upregulated in normal shrimp compared to moribund shrimp, one gene (*Slc6a9*) was downregulated, as we excepted.

significantly enriched by DEGs from comparisons (the normal shrimp vs. the moribund shrimp) in both salinity environments (10 ppt and 25 ppt). Notably, the two pathways and the top 25 GO terms were enriched by five of DEGs (*Itgbl*, *Slc2a8*, *Dhdh*, *Cyp3a30*, *Slc6a9*), which had the same expression pattern in the comparisons from both the salinities. The expression patterns of the five target genes had also been verified by the results of RT-qPCR. Thus, our study based on two tests of transcriptomic comparisons from two salinity environments identified potential pathways (GO terms) and genes involved in vitality regulation for further investigation of genetic diversity and breeding programs, and these genes may be served as bio-indicator of shrimp health status.

Salt-induced stress is one of the hot issues in stress response induced by environmental factors in aquatic animals. In the crustacean species, the pathway of metabolism of xenobiotics by cytochrome P450 significantly enriched in this study is important in the biotransformation and detoxification of xenobiotics (e.g., hydrocarbons, pesticides, drugs) and endogenous compounds (e.g., fatty acids, eicosenoids, steroids) as it does in bacteria and vertebrates to stress response (James and Boyle, 1998; Snyder, 2000). Similarly, the pathway of pentose and glucuronate interconversions could also involve in stress response (Yao et al., 2012). In the progress of response to toxic substances, whether oxidized, reduced or hydrolyzed, compounds mostly conjugated with toxic substances, such as glucuronide conjugation in the pentose and glucuronate interconversion pathways, which plays an important role in detoxification by masking toxic groups and therefore will change the physical and chemical properties of toxic substances (Sun et al., 2018). These two significant pathways in this study indicated that detoxification of *F. chinensis* contributes to their vitality regulation. In this study, no other xenobiotic substances were added in water of shrimp living, except for sea salt. Shrimp in normal group and moribund group lived in the same condition, thus their physiological difference ought to be attributed to the difference on individual genetic properties. We found that in the two pathways, *Dhdh* was upregulated in the normal shrimp compared to the moribund shrimp from both the salinity environments (**Table 4**). *Dhdh* encodes dihydrodiol dehydrogenases, which have been implicated in the detoxication of carcinogenic metabolites such as polycyclic aromatic hydrocarbons (Penning, 1993). Deng et al. (2002) showed that increased expression of dihydrodiol dehydrogenase could induce resistance to Cisplatin in human ovarian carcinoma cells. Although, the pathway of cytochrome P450 were paid more attention by researchers (Rewitz et al., 2006; Koenig et al., 2012), less information was available for *Dhdh* in crustacean species. Thus, this gene deserves more attention in future for its potential application in vitality regulation.

In this study, genes of *Itgbl*, *Slc2a8*, and *Cyp3a30* were upregulated in the normal shrimp compared to the moribund shrimp from both the salinity environments, while *Slc6a9* was downregulated based on GO terms (**Table 4**). Differential expression (upregulation and downregulation) of all these genes

TABLE 4 | Differentially expressed genes (DEGs) with the same expression pattern in the both comparisons, i.e., FCN10 vs. FCM 10 and FCN25 vs. FCM 25.

No.	Symbol	Genes' description	log2FC[1]	log2FC[2]	Upregulated/ Downregulated
1	TRINITY_DN0_c0_g1	XP_037783062.1 mitochondrial import inner membrane translocase subunit Tim21-like protein, *TIM21*	6.18	5.56	Up
2	TRINITY_DN1160_c0_g1	XP_037802014.1 kielin/chordin-like protein, *Itgbl, CD29*	1.70	2.37	Up
3	TRINITY_DN13256_c0_g1	XP_037798245.1 sorbitol dehydrogenase-like protein, *SORD, gutB*	2.13	2.60	Up
4	TRINITY_DN1806_c0_g1	XP_037779521.1 solute carrier family 2, facilitated glucose transporter member 8-like protein, *Slc2a8, GLUT8*	1.92	3.42	Up
5	TRINITY_DN3998_c0_g1	XP_027227808.1 trans-1,2-dihydrobenzene-1,2-diol dehydrogenase-like protein, *Dhdh*	1.60	2.06	Up
6	TRINITY_DN70493_c0_g1	XP_037798812.1 cytochrome P450 3A30-like protein, *Cyp3a30*	1.43	1.30	Up
7	TRINITY_DN8415_c0_g3	XP_037784188.1 urocanate hydratase-like protein, *UROC1*	1.42	2.40	Up
8	TRINITY_DN1647_c0_g5	XP_037773511.1 sodium-dependent nutrient amino acid transporter 1-like protein isoform X2, *Slc6a9*	−1.19	−1.60	Down
9	TRINITY_DN802_c0_g1	XP_018323985.1 guanine nucleotide-binding protein subunit beta-like protein isoform X2, *RACK1*	−3.58	−2.29	Down

[1] *From the comparison of FCN10 vs. FCM10.*

[2] *From the comparison of FCN25 vs. FCM25.*

in shrimp seem to have effects on their vitality. Previous studies demonstrated that bone morphogenetic proteins (BMPs), the low molecular weight secreted glycoproteins in invertebrates (Lelong et al., 2001), could function in inflammatory responses, cell proliferation and differentiation, angiogenesis and apoptosis (Vinuesa et al., 2015). In this context, it was reported that the kielin/chordin-like protein (KCP, *Itgbl*) could regulate the BMP signaling pathways (Ye et al., 2018). The differential expression of the *Itgbl* in different cell components including extracellular matrix, integral component of membrane, intrinsic component of membrane, integral component of plasma membrane and intrinsic component of plasma membrane (**Supplementary Table 2**), thus we conclude that *Itgbl* regulates vitality of the shrimp *via* the versatility of it. However, as to our knowledge, studies about this gene of crustacean is absent recently.

In shrimp and crab, as the one of the integral components of membrane, the glucose transporter member 8 (GLUT8) encoded by *Slc2a8* is involved in carbohydrate metabolism and glucose transport (Tollefsen et al., 2017). The recent study by Seo et al. (2018) showed that metabolic shift from glycogen to trehalose was facilitated in promoting lifespan and healthspan in *Caenorhabditis elegans*. Actually, Alava and Pascual (1987) had ever pointed that trehalose and sucrose diets can promote higher survival rates of *Penaeus monodon* than glucose diets, they believed that trehalose at the certain level in diets can better meet the energy needs to spare protein for shrimp growth. Moreover, in autophagy-lysosome system, trehalose was found to have the ability to cause autophagy-lysosome biogenesis response, which is important in cellular degradation pathway that recycles dysfunctional organelles and cytotoxic protein aggregates thus make organisms healthy (Jeong et al., 2021). Therefore, differential expression of the *Slc2a8* (downregulated) in moribund shrimp compared to the normal shrimp in this study may imply its disruption to trehalose synthesis, which could support the opinion that trehalose is helpful to promote lifespan, healthspan and survival rates of invertebrates (Seo et al., 2018). The vitality regulation of *Slc2a8* in *F. chinensis* is considered to be associated with trehalose metabolism in this study.

Glycine transporter 1 (*Slc6a9*) belongs to members of the Na^+/Cl^--dependent neurotransmitter transporter superfamily, and it plays a role in response to oxidative challenge (Rees et al., 2006; Howard et al., 2010). Recently a study showed that *Slc6a9* was upregulated in *Procambarus clarkii* from the ammonia stress group (Shen et al., 2021), which implies that this gene has the potential to be served as the one of the bio-indicators of shrimp health status. As the previous study by Shen et al. (2021) mainly focused on the effects of ammonia stress on *Procambarus clarkia* for 24 h, thus the following health status of them with higher expression of *Slc6a9* is unknown. In our study, the differential expression (upregulation) of this gene in the moribund shrimp compared to the normal shrimp demonstrated that this gene is involved in vitality regulation (stress response) which supports the previous studies, but keeping shrimp alive well based on upregulation of this gene still needs more efforts. Notably genetic variants in this gene have been wildly studied in human for health problems (Deng et al., 2008; Koller et al., 2010; Nuzziello et al., 2019), which leads to the direction of future study for shrimp.

As to P450 monooxygenase system (CYPs), previous studies had showed that the induction of CYPs can indicated the exposure of aquatic animals to toxic compounds (Stegeman and Lech, 1991; James and Boyle, 1998). The number of known P450 enzymes exceeds 1000, and more attentions had been paid on the properties of the most important P450 enzymes taking part in metabolism of xenobiotics in organisms (Anzenbacher and Anzenbacherová, 2001). Unlike the *Slc6a9,* upregulation of Cyp3a30 was in the normal shrimp compared to the moribund shrimp in this study, which indicated differential expression (downregulation) of this gene is adverse to health of shrimp. Recently, a study utilized the freshwater shrimp *Caridina nilotica* as indicators of persistent pollutant exposure, in which CYPs activity was significantly higher in shrimp at sites directly adjacent to regions of increased human activity, and the biomarkers of exposure (CYPs) was considered to be suitable to detect effects of stressors, probably persistent pollutants (Rensburg et al., 2020). Thus, CYPs will have the potential to regulate vitality of shrimp. As the members of CYPs, CYP3As are involved in metabolic clearance of numerous chemically diverse compounds, most studies of them have been performed in mammals and fishes (Hegelund and Celander, 2003; Uno et al., 2012). In the future, identification of specific loci on *Cyp3a30* involved in vitality regulation for shrimp and further verifying its function will be the next important work to us as Religia et al. (2021) did, who disrupted *CYP360A8* by coinjecting *CYP360A8* targeting guide RNA and Cas9 proteins into *Daphnia magna* eggs, established one monoallelic *CYP360A8* mutant line. This *CYP360A8* mutant had a higher sensitivity to the herbicide paraquat compared to the wild type.

This study firstly used two tests of transcriptomic comparisons in two salinity environments to identify potential pathways and genes on *F. chinensis*, not only narrowed the search scope of target pathways and genes, but also could improve the accuracy of the results to some extent. Recently, there is little evidence for genes of *Dhdh*, *Itgbl* in crustaceans, and the function of these genes (including other target genes) in vitality regulation of shrimp needs further investigation by more direct methods in the future. In conclusion, differential expression of the five DEGs (*Itgbl*, *Slc2a8*, *Dhdh*, *Cyp3a30*, *Slc6a9*) revealed by this study could well support the previous studies. In addition, the five target DEGs in the key pathways and GO categories have the potential to regulate vitality of shrimp. The study identified potential pathways and genes involved in vitality regulation will be the foundation for further investigation of genetic diversity and breeding programs.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/sra/PRJNA734726.

AUTHOR CONTRIBUTIONS

JS, LZ, and ZW: data curation. JL and DZ: funding acquisition. JS and LZ: resources. JL and JS: writing—original draft. JL, JS, and DZ writing—review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Beneficial Effects of Warming Temperatures on Embryonic and Hatchling Development in a Low-Latitude Margin Population of the High-Latitude Lizard *Lacerta agilis*

Luoxin Cui, Cheng Yang, Decheng Zhang, Shu Lin, Wenge Zhao* and Peng Liu*

College of Life Science and Technology, Harbin Normal University, Harbin, China

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Edited by:
Lin Zhang,
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Yang Wang,
Hebei Normal University, China
Shuran Li,
Wenzhou University, China

***Correspondence:**
Wenge Zhao
zhaowenge311@126.com
Peng Liu
liupeng111111@163.com

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The effects of warming temperatures on embryonic and hatchling development are critical for determining the vulnerability of species to climate warming. However, these effects have rarely been investigated in high-latitude oviparous species, particularly in their low-latitude margin populations. This study investigated the embryonic and hatchling development and fitness-related traits of a low-latitude margin population of a high-latitude lizard (*Lacerta agilis*). These traits were examined under present (24°C), moderate warming (27 and 30°C), and severe warming scenarios (33°C). Based on embryonic and hatchling responses to thermal variation, this study aimed to predict the vulnerability of the early life stages of low-latitude margin population of *Lacerta agilis* to climate warming. The incubation period of the low-latitude margin population of *Lacerta agilis* decreased as the temperature increased from 24 to 33°C. Hatching success was similar at 24, 27, and 30°C but decreased significantly at 33°C. No differences with temperature were observed for hatchling snout-vent length and hatchling body mass. The sprint speed was higher for hatchlings from temperatures of 24 and 33°C. The growth rate of hatchlings was highest at 30°C; however, the survival rate of hatchlings was not affected by the thermal environment. This study demonstrated that even for a low-latitude margin population of the high-latitude lizard, *Lacerta agilis*, moderate warming (i.e., 27 and 30°C) would benefit embryonic and hatchling development. This was indicated by the results showing higher hatching success, growth rate, and survival rate. However, if temperatures increase above 33°C, development and survival would be depressed significantly. Thus, low-latitude margin population of high-latitude species *Lacerta agilis* would benefit from climate warming in the near future but would be under stress if the nest temperature exceeded 30°C.

Keywords: climate warming, embryonic development, hatchling development, temperature, high latitude, lizard, *Lacerta agilis*

INTRODUCTION

Anthropic climate warming has imposed many negative effects on animals, including changes in phenology (e.g., Gibbs and Breisch, 2001; Sherry et al., 2007), inhibition of dispersal (e.g., Massot et al., 2008), contractive distribution (e.g., Thomas et al., 2006), decreasing population growth (e.g., Deutsch et al., 2008), and extinction (e.g., Harte et al., 2004; Jevanandam et al., 2013). Therefore, understanding how animals respond to thermal variations caused by climate warming is critical. It is particularly relevant to understand thermal adaptation for determining the vulnerability of animals to the effects of climate change (Huey et al., 2012). Although it is likely that the effects of climate warming are evidently negative for most animals (Dillon et al., 2010; Barnosky et al., 2011), the vulnerability of species across different latitudes is still controversial. The warming rate has been predicted to increase with higher latitude, supporting the likelihood of increased vulnerability of animals at higher latitudes (e.g., Root et al., 2003; Parmesan, 2006). However, higher sensitivities to warming for low-latitude animals indicated the opposite pattern, that animals from low latitudes, including tropical areas, have a higher vulnerability to warming temperatures from climate change (e.g., Deutsch et al., 2008; Dillon et al., 2010; Sunday et al., 2011). Therefore, the response of animals from different latitudes to climate warming has received increasing attention. In contrast to low latitudes, where animal responses to thermal variations and climate warming have been extensively investigated (e.g., Huey et al., 2009; Laurance and Useche, 2009; Sinervo et al., 2010; Logan et al., 2013; Tao et al., 2021), much less investigations with focus on the responses to climate warming of animals from high latitudes.

Increasing knowledge about physiological and life-history responses to warming temperatures is critical for evaluating potential vulnerabilities (Williams et al., 2008; Huey et al., 2012; Pacifici et al., 2015). As typical ectothermic vertebrates, oviparous reptiles are physiologically sensitive to thermal variations, particularly during the early stages of life (e.g., embryonic development). Therefore, reptiles have an important role in the context of investigating their physiological and life-history responses to thermal variations and climate warming (Huey et al., 2012). Reptiles have been found to be negatively affected by climate warming (e.g., Huey et al., 2010; Sinervo et al., 2010; Barnosky et al., 2011; Diele-Viegas et al., 2020; Taylor et al., 2021). Furthermore, without parental care, most reptile embryos face developmental challenges from the surrounding environment (Du and Shine, 2015; Noble et al., 2018a,b; Du et al., 2019). In addition, many environmental factors can affect embryonic and hatchling development, including hatching success, incubation period, body size and growth of hatchlings, and survival rates (e.g., Sun et al., 2014; Du and Shine, 2015; Zhang et al., 2016; Noble et al., 2018b; Li et al., 2020). Recent research has revealed that reptile embryos are more vulnerable to climate warming than adults because of their lower heat tolerance. Embryonic heat tolerance has thus been predicted to limit the distribution of species under climate warming (Angilletta et al., 2013; Levy et al., 2015; Carlo et al., 2018; Hall and Sun, 2021; Sun et al., 2021).

Reptiles from low latitudes have been predicted to migrate toward high latitudes. This will allow them to escape from the heat stress caused by climate warming (e.g., Massot et al., 2008; Boyle et al., 2016). In addition, the embryos of reptiles at high latitudes may benefit from climate warming by enhancing hatching success, hatchling growth, and survival rates (Andrews et al., 1999; Qualls and Andrews, 1999; Li et al., 2018; Liu et al., 2022). However, for reptile species that have a wide geographic distribution across high latitudes, the response to climate warming by its low-latitude margin population (i.e., population from low-latitude margin of its distribution area) is relatively unknown. Investigating the embryonic and hatchling responses of the low-latitude margin population of high-latitude species to warming temperatures (e.g., Olsson et al., 2011; Ljungström et al., 2015) would increase the understanding of species response to climate warming across latitudes.

This study focused on a low-latitude margin population (i.e., Burqin, Xinjiang, China) of the sand lizard (*Lacerta agilis*), which is a high-latitude species. The eggs were incubated at four different temperatures to mimic the present (i.e., 24°C), moderate warming (27 and 30°C), and severe warming (33°C) scenarios for the population according to predicted warming rate. The hatchlings were then reared at the present, moderate warming, and severe warming scenarios coherently. The increase in egg mass during incubation, hatching success, incubation period, hatchling body size, growth, and survival rates were then determined to reveal the thermal effects of different warming scenarios on embryonic and hatchling development. Furthermore, warming scenarios would benefit the embryonic and hatchling development of *Lacerta agilis* if the low-latitude margin population responded in the same way to the benefits of climate warming as that documented in other species from high latitudes (Andrews et al., 1999; Qualls and Andrews, 1999; Li et al., 2018). Alternatively, it was predicted that the warming scenarios would depress embryonic and hatchling development. This would have been the case if this population followed the consensus that warming temperatures have a negative effect on embryonic and hatchling development.

MATERIALS AND METHODS

Study Species

The sand lizard (*Lacerta agilis*) is a medium-size oviparous lizard of up to 114 mm in snout-vent length (SVL). This species is widely distributed across northern Europe, Russia, Mongolia, and Xinjiang in China (61° N–43°N), which have cold climates with significant thermal fluctuations (Strijbosch and Creemers, 1988; Trakimas, 2005; Ekner et al., 2008). Sand lizard habitats were found in forest, shrubland, and grassland (**Figure 1A**). The sand lizard becomes active in the field in March after the end of hibernation then starts the reproductive season. During the reproductive season, females lay one or two clutches of 3–11 eggs per clutch (IUCN).[1] Previous research on *Lacerta agilis* provided key background information, thereby making it a great study

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[1] https://www.iucnredlist.org/species/157288/5071439

system for investigating the responses of high-latitude reptiles to thermal variations and climate warming (e.g., Olsson, 1994a,b; Olsson and Shine, 1996, 1997a,b; Madsen et al., 2000; Ljungström et al., 2015).

Animal Collection and Environmental Factors

Adult lizards were collected in early May of 2014 from Burqin, Xinjiang, China, (47°22′ N, 88°06′ E), which is almost the lowest latitude at which *Lacerta agilis* is found. The lowest monthly average temperature was –16°C in January, and the highest monthly average temperature was 21°C in July in Burqin.[2] All the lizards were collected either by hand or using a lizard noose. The active body temperature of a subset of lizards ($n = 16$) was measured randomly during collection. The average body temperature for lizards in the field was 32.97 ± 0.62°C (28.2–36.3°C). After collection, the lizards were transferred to the laboratory at the Harbin Normal University. Six to eight lizards were housed in each terrarium (950 × 450 × 500 mm, length × width × height). These were placed in a temperature-controlled room at 18°C, with a photoperiod of 12 L: 12D (0700–1,900). A 100 W UVA + UVB heating lamb was suspended 50 mm above one end of each terrarium to provide a thermal gradient of 18–40°C for behavioral thermoregulation from 0800 to 1,600. Food (Larva of *Tenebrio molitor* and crickets dusted with mixed vitamins) and water were provided *ad libitum*.

Embryonic Development

The female lizards were palpated every other day to determine their gravid status. The female lizards with oviduct eggs were reared individually in plastic terraria (35 × 30 × 40 mm, length × width × height). The terraria were lined with 100 mm of moist soil, where the females laid their eggs. The terraria were checked three times per day for freshly laid eggs. Once found, the eggs were weighed, numbered, and randomly assigned to one of the four incubation temperatures. A total of 160 eggs from 43 females were incubated. A split clutch design was used for incubation, with eggs from each female being incubated at different temperatures. The present temperature scenario was set according to soil temperatures during June and July (**Figure 1B**). Moderate warming scenarios were set to mimic the maximum temperature increasing at RCP 4.5 and RCP 6.0 scenarios, while the severe warming scenario was set to that of RCP 8.5 (IPCC, 2013, 2021). The incubation temperatures were set at 24, 27, 30, and 33°C, which mimicked the thermal environments under present climates (24°C), moderate warming scenarios (27 and 30°C), and severe warming scenario (33°C). The eggs were half-buried in plastic boxes (160 × 115 × 40 mm) which were filled with moist vermiculite (–220 kPa). A subset of eggs (7, 10, 9, and 8 eggs for 24, 27, 30, and 33°C) were measured every 5 days, to determine the increase in egg mass during incubation. Water was added to the boxes every 5 days to maintain the moisture of the vermiculite until the hatchlings emerged. After hatching, the incubation period was recorded. The hatching success was calculated by the ratio of the number of hatchlings to the number of eggs incubated.

Hatchling Husbandry

The hatchlings were measured for SVL (± 0.001 mm) and weighed for body mass (BM, ± 0.001 g). Hatchlings were individually marked and reared under different thermal regimes. To detect the coherent effects of temperature on embryonic and hatchling development, the hatchlings were reared at the same average temperatures as those under incubation. These were 24 ± 3°C, 27 ± 3°C, 30 ± 3°C, and 33 ± 3°C for hatchings that incubated at 24, 27, 30, and 33°C, respectively. A total of 8–10 hatchlings were reared in each temperature-controlled chamber (450 L), with a light cycle of 12 L:12D (0700–1,900). Food and water were provided *ad libitum*.

Locomotion

One week after hatching, the locomotor performance of the hatchlings was started to be determined. Using the average body temperatures recorded in the field as a baseline (32.97°C), the locomotor performance tests were conducted at 33°C. Prior to the test, the hatchlings were acclimated to a temperature at 33°C for at least 1 h. Hatchlings were encouraged to run across a 1 m racetrack by touching their tails with a soft paintbrush. The process was recorded using a digital video camera (DCR-SR220E, Sony, Japan). Each hatchling was tested twice with an interval of 1 h between each test for resting. The videos were then analyzed using AVS Video Editor software (Online Media Technologies Ltd., United Kingdom). The fastest speed at 20 cm intervals was recorded. The average of the fastest speed out of the two tests was calculated as being the sprint speed. The maximum distance for each burst and the number of stops during each test were also recorded, and the average of two records were calculated as the maximum distance and number of stops, respectively.

Growth and Survival

Husbandry of all the hatchlings lasted for at least 40 days. The hatchlings were weighed and measured, with their survival status being checked every 10 days after hatching. SVL and BM were recorded to determine the growth rate. The growth rate was calculated as the difference in SVL and BM per day, expressed as mm/day and g/day, respectively. Survival rates were determined by the number of live individuals after 40 days of husbandry against the total number of hatchlings.

Statistical Analysis

IBM SPSS 21.0 was used for statistical analysis. Before the analysis, the normality of distributions and homogeneity was tested using the *Kolmogorov–Smirnov* test and *Levene*'s test, respectively. As the initial egg mass being the same but the embryonic processes were different on the same day across the incubation temperatures, the egg mass increase was analyzed using the final egg mass before hatching by one-way analysis of covariance (ANCOVA), with incubation temperature was the main factor, and initial egg mass was a covariate. The incubation period for the embryos, hatchling sprint speed, and

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[2] http://data.cma.cn/en

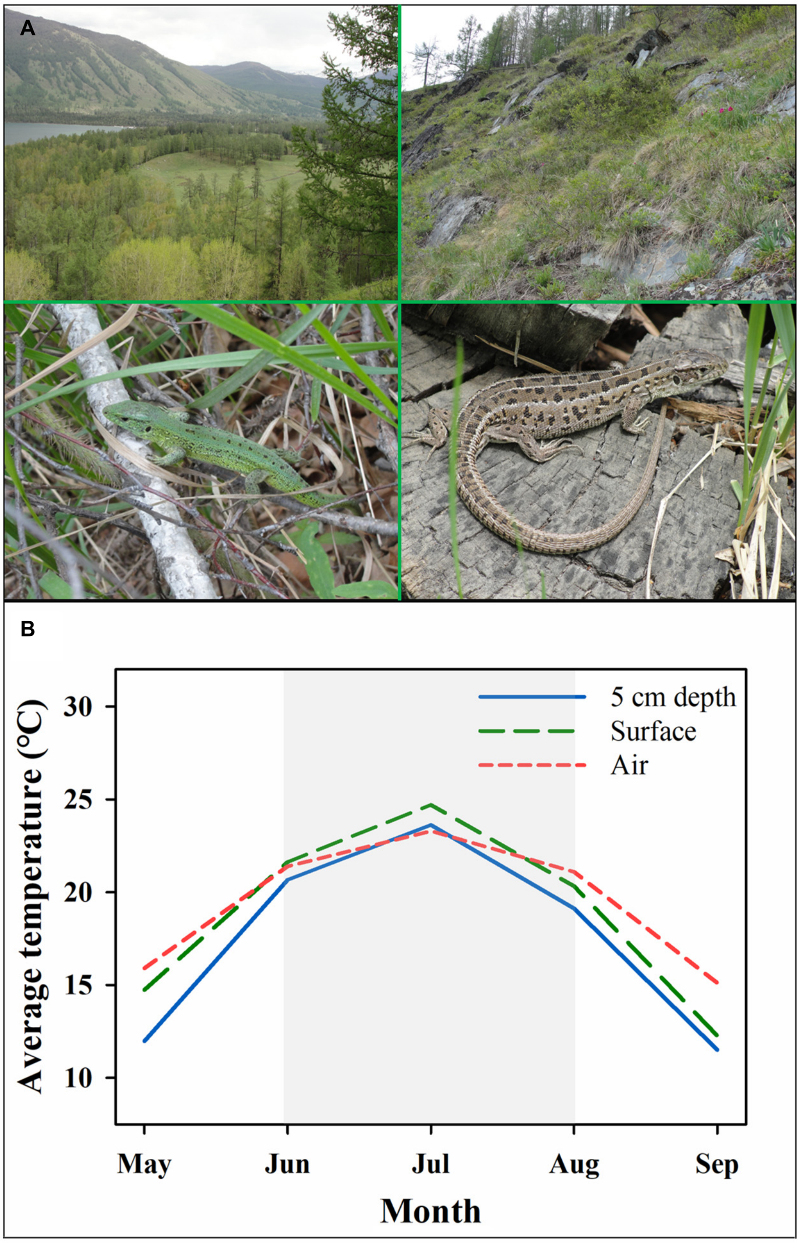

FIGURE 1 | Natural habitats of **(A)** and thermal environments **(B)** for sand lizards (*Lacerta agilis*) from Burqin population. **(A)** The photographs indicate the natural habitats and demographic photographs for *Lacerta agilis*. **(B)** The blue solid, green long dash and red short dash lines indicate the temperatures of nest at depth of 5 cm underground, surface and air. The shade column indicates the reproductive season mainly for embryonic development. The data of temperatures were from China Meteorological Data Service Centre (http://data.cma.cn/en).

the number of stops were analyzed by one-way ANOVA, with incubation temperature as the main factor. The hatchling SVL and BM and growth rate of hatchlings SVL and BM were analyzed by one-way ANCOVA with incubation temperatures, and husbandry temperature as the main factors, and egg mass, initial SVL, and BM as covariates, respectively. The hatchling SVL and hatchling BM before winter were analyzed by one-way ANOVA. The hatching success and hatchling survival rates were analyzed by the *Chi-square* test. The *Tukey post hoc* comparison was conducted once significant difference was detected across temperature regimes.

RESULTS

Embryonic Development

The egg mass increase was affected by incubation temperatures across the incubation duration, with egg mass increasing the most at 24°C than at other incubation temperatures [$F_{(3,\ 29)} = 10.858$, $P < 0.0001$]. The other incubation temperatures did not influence the egg mass increase (min $P = 0.054$) (**Figure 2**). The incubation period was influenced by the incubation temperature, which decreased from 24 to 33°C [$F_{(3,\ 63)} = 557.55$, $P < 0.0001$] (**Figure 3A**). The hatching success was significantly different among incubation temperatures, which was lower at 33°C (8/40) than at 24°C (14/40), 27°C (21/40), and 30°C (24/40) ($\chi^2 = 15.89$, $P < 0.01$). However, the incubation temperatures of 24–30°C did not affect hatching success (all $P > 0.05$) (**Figure 3B**).

Hatchling Body Size and Locomotion

Hatchling snout-vent length (HSVL) [$F_{(3,\ 62)} = 1.010$, $P = 0.394$] and body mass (HBM) [$F_{(3,\ 62)} = 2.050$, $P = 0.116$] were not affected by incubation temperatures (**Table 1**). The sprint speed of hatchlings was significantly affected by incubation temperature [$F_{(3,\ 61)} = 6.827$, $P < 0.001$]. Hatchlings incubated at 24 and 33°C had higher sprint speeds than siblings at 27 and 30°C (**Figure 4A**). The number of stops for hatchlings at 30°C were significantly higher than the siblings at 27°C, along with those at 24 and 33°C [$F_{(3,\ 61)} = 3.838$, $P = 0.014$] (**Figure 4B**). However, the maximum distance for each sprint test was not influenced by incubation temperature [$F_{(3,\ 61)} = 1.886$, $P = 0.141$].

Hatchling Development

The growth rates of HSVL and HBM were significantly affected by the thermal environment of the embryos and hatchlings. The growth rate of HSVL in hatchlings at 24°C was lower than siblings from temperatures of 27, 30, and 33°C [$F_{(3,\ 49)} = 7.234$, $P < 0.001$; 30°C ^{a} > 33°C ^{a} > 27°C ^{a} > 24°C ^{b}] (**Table 1**). The growth rate of HBM was the lowest for hatchlings from 24°C, highest for hatchlings from 30°C, and in between for hatchlings from 27 and 33°C [$F_{(3,\ 49)} = 7.234$, $P < 0.001$; 30°C ^{a} > 33°C ^{ab} > 27°C ^{b} > 24°C^{c}] (**Table 1**). Consequently, the SVL [$F_{(3,\ 50)} = 11.484$, $P < 0.0001$; 30°C ^{a} > 33°C ^{ab} > 27°C ^{b} > 24°C ^{c}] and BM before winter [$F_{(3,\ 50)} = 11.834$, $P < 0.0001$; 30°C ^{a} > 33°C ^{ab} > 27°C ^{b} >24°C ^{c}] were the highest for hatchlings at 30°C, and the lowest at 24°C, with those at 27 and 33°C in between (**Table 1**). However, the survival rates of hatchlings from 24 (10/14, 71.4%), 27 (17/21, 80.9%), 30 (17/24, 70.8%), and 33°C (7/8, 87.5%) were similar after 40 days of husbandry before winter ($\chi^2 = 1.38$, $P > 0.05$).

DISCUSSION

The effects of climate warming on animals have been extensively investigated over the previous decades (Pacifici et al., 2015). Although the current consensus is that climate warming would have negative effects on animals at many biological hierarchies, additional research is required to comprehensively determine the latitudinal pattern of vulnerabilities (e.g., Root et al., 2003; Parmesan, 2006; Huey et al., 2009, 2012; Dillon et al., 2010; Sun et al., 2021). This study focuses on how the low-latitude margin population of a high-latitude species, *Lacerta agilis*, would respond to climate warming.

Although in this study, low incubation temperature (i.e., 24°C) enhanced the egg mass during incubation (**Figure 2**), they did not affect the hatchling body size (**Table 1**). In contrast, with shorter incubation periods and enhanced hatching success, embryonic development was found to be enhanced under moderate warming scenarios at 27 and 30°C (**Figure 3**). Furthermore, higher growth rates in SVL and BM indicated that moderate (30°C) and severe (33°C) warming temperatures were beneficial for hatchling development (**Figure 4** and **Table 1**). In combination, similar to other high-latitude reptiles (e.g., Andrews et al., 1999; Qualls and Andrews, 1999; Li et al., 2018; Liu et al., 2022), moderate warming (i.e., 30°C) would benefit embryonic and hatchling development in the low-latitude margin population of a high-latitude lizard, *Lacerta agilis*.

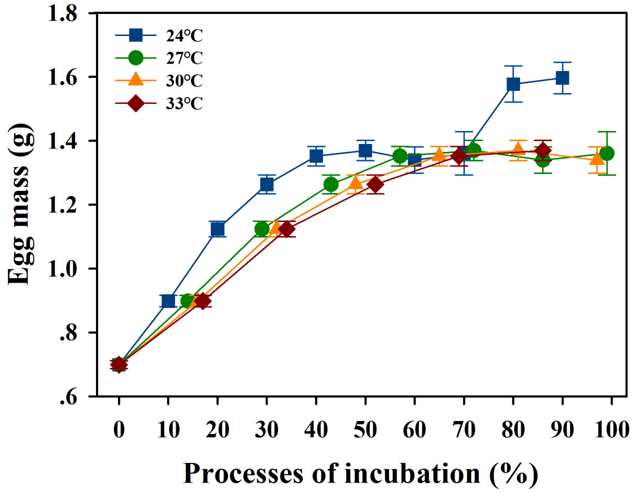

FIGURE 2 | Egg mass increase during incubation. The blue, green, orange and red spot and lines indicate the eggs incubated under 24, 27, 30, and 33°C, respectively. The data are shown as mean ± *SE*.

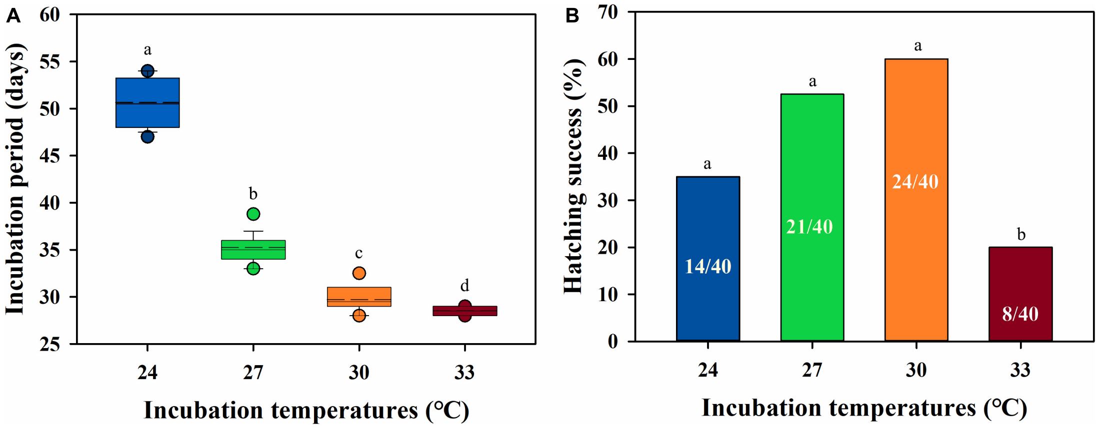

FIGURE 3 | Incubation period **(A)** and hatching success **(B)** of embryos under 24, 27, 30, and 33°C. **(A)** The dash lines indicate the mean value of incubation period; the data are shown as mean ± *SE*. **(B)** The number in the columns indicate the sample size for each temperature. The different letters above the error bars indicate the significant difference between incubation temperatures.

TABLE 1 | Initial body size (SVL and BM), growth rate of hatchlings (SVL and BM), and hatchlings body size (SVL and BM) before winter under different temperatures.

	N	24°C	*N*	27°C	*N*	30°C	*N*	33°C
Initial-SVL	14	31.923 ± 0.470	21	31.092 ± 0.383	24	31.282 ± 0.361	8	32.057 ± 0.622
(mm)		30.983–32.863		30.327–31.856		30.561–32.002		30.813–3.301
Initial-BM	14	0.819 ± 0.030	21	0.727 ± 0.024	24	0.758 ± 0.023	8	0.792 ± 0.040
(g)		0.759–0.878		0.678–0.776		0.712–0.804		0.713–0.872
Growth rate-HSVL	13	–0.007 ± 0.065	17	0.273 ± 0.057	17	0.380 ± 0.057	7	0.319 ± 0.089
(mm/day)		–0.137-0.123		0.159–0.387		0.266–0.495		0.140–0.498
Growth rate-HBM	13	0.013 ± 0.004	17	0.034 ± 0.004	17	0.050 ± 0.004	7	0.045 ± 0.006
(g/day)		0.004–0.021		0.026–0.041		0.043–0.057		0.033–0.056
HSVL before winter	13	40.834 ± 0.849	17	43.535 ± 0.757	17	47.174 ± 0.711	7	45.201 ± 1.058
(mm)		38.985–42.683		41.930–45.139		45.665–48.682		42.612–47.791
HBM before winter	13	1.776 ± 0.101	17	2.177 ± 0.141	17	2.818 ± 0.127	7	2.611 ± 0.174
(g)		1.555–1.996		1.878–2.477		2.550–3.086		2.185–3.037

The data for initial body size and growth rates are shown as least square mean ± SE, and –95 to 95 confidence intervals. The data for body size of hatchlings before winter are shown as mean ± SE, and –95 to 95 confidence intervals.

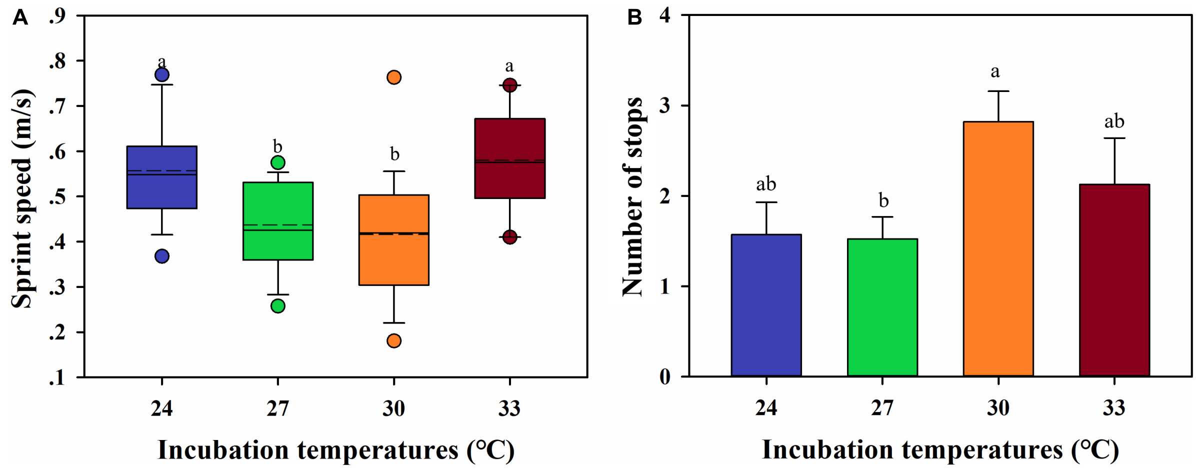

FIGURE 4 | Sprint speed **(A)** and the number of stops **(B)** of hatchlings incubated at 24, 27, 30, and 33°C. **(A)** The dash lines indicate the mean value of sprint speed; the data are shown as mean ± *SE*. **(A,B)** The different letters above the bars indicate the significant differences between incubation temperatures.

Moderate Warming Enhanced the Embryonic Development

For oviparous species, a shortened incubation period has been considered an advantage for hatchlings. In this study, the moderate warming temperatures and severe warming temperature shortened the incubation period, potentially decreasing the possibility of embryos being preyed upon by predators (e.g., Torralvo et al., 2017) or having reduced survival rates due to extreme heat events (Levy et al., 2015; Ma et al., 2018b; Hall and Sun, 2021; Sun et al., 2021). Meanwhile, a shorter incubation period can allow a relatively long period for hatchling development. This potentially increases the chance of hatchlings having an increased body size to respond to the challenges of lower temperatures during the autumn and winter months (e.g., Sun et al., 2018a; Liu et al., 2022). The physiological and biochemical mechanisms underlying the shortened incubation period may be associated with enhanced mitochondrial functions and metabolic enzyme activities in embryos at high temperatures (e.g., Sun et al., 2015). By enhancing the mitochondrial metabolism, embryonic metabolism and thus embryonic development can be significantly increased. The incubation period can therefore be shortened (e.g., Du and Shine, 2010, 2015; Du et al., 2010a,b, 2011; Sun et al., 2013; Liu et al., 2022).

Hatching success is an indicator of the survival rate of embryos during development, which is a critical proxy for evaluating the effects of incubation environments on embryonic development (e.g., Du and Shine, 2015; Noble et al., 2018b). In this study, moderate warming temperatures increased hatching success, without any depress on hatchling SVL and BM (**Table 1**), indicating the presence of a higher hatchling recruitment for the population (e.g., Ma et al., 2018a,b; Liu et al., 2022). However, a severe warming incubation temperature of 33°C significantly decreased hatching success, with only 20% (8/40) of embryos hatched successfully. In combination, moderate warming temperatures of 27 and 30°C, respectively, led to higher hatching success and shortened incubation periods, both of which are advantages for embryonic development.

Hatchling Growths Are Enhanced by Warming Husbandry

For hatchling husbandry in this study, the same thermal regimes were consistently used for incubation temperatures, with a ± 3°C fluctuation. Although this limited the interpretation of the interactive effects of incubation and husbandry temperatures, which are normally employed in factory-manipulated investigations (e.g., Deeming and Ferguson, 1991; Taylor et al., 2021). The current thermal regime still met the need to evaluate the thermal effects on the development of hatchlings. With higher growth (**Table 1**) and equal survival rates, the hatchlings from moderate warming at 30°C and severe warming at 33°C both benefited in comparison with hatchlings from the present thermal environments of 24°C in this study. The higher growth rate of hatchlings allowed faster mass accumulation before winter, which improved resistance to cold weather during the winter months, which is a widespread adaptive strategy employed at high latitudes by many animals, including fish, reptile, and mammal (e.g., Festa-Bianchet et al., 1997; Larivée et al., 2010; Eto et al., 2018; Sun et al., 2018b; Lu et al., 2019). As a high-latitude species, *Lacerta agilis* faces the challenge of cold climatic conditions for a long time from October to May each year. Owing to their large body sizes, the hatchlings are predicted to have enhanced survival rates during winter (e.g., Litvak and Leggett, 1992; Steiger, 2013). In addition, larger body size for *Lacerta agilis* is a critical proxy for mating choice, as larger adults may have priority in mating, thereby enhancing reproduction (Olsson, 1993).

Implication for Vulnerability to Climate Warming

At the most sensitive life-history stage, understanding the role of physiological proxies of embryos and hatchlings, such as their hatching success, incubation period, hatchling growth, and body size, to thermal variations are fundamental and necessary to evaluate the vulnerabilities of species and their populations (e.g., Pike, 2014; Howard et al., 2015; Ma et al., 2018a,b). In this study, we mimicked the present climate and moderate and severe warming scenarios to reveal the responses of embryos and hatchlings according to different RCP scenarios (IPCC, 2013, 2021). Based on the responses of embryos and hatchlings, it was predicted that the low-latitude margin population of *Lacerta agilis* would not be vulnerable to moderate climate warming but would be severely vulnerable to severe warming scenarios.

This study revealed that moderate warming temperatures of 27 and 30°C enhanced embryonic development. Moderate warming at 30°C and severe warming temperatures of 33°C facilitated hatchling development. Based on the present findings, it is predicted that moderate warming scenarios for embryonic and hatchling development would be beneficial for *Lacerta agilis*. In contrast, severe warming temperatures also enhanced the growth of hatchlings but reduced hatching success at 33°C. This would be harmful to population recruitment (e.g., Spear and Nur, 1994; Tang et al., 1998). Similarly, extreme warming temperatures for embryonic development would also depress the hatchling body size, growth rates, and even survival rates in the brown anoles (*Anolis sagrei*) and fence lizards (Carlo et al., 2018; Hall and Warner, 2021).

In summary, with the low-latitude margin population of a high-altitude species, *Lacerta agilis*, we found that embryonic and hatchling development can be enhanced under moderate warming temperatures. The responses follow the increasingly demonstrated benefits of climate warming at high latitudes. This has indicated that *Lacerta agilis* would not be vulnerable to climate warming in the near future. Nonetheless, this study was limited by its failure to reveal the interactive effects of incubation and hatchling temperatures on embryonic and hatchling development (e.g., Sun et al., 2018a,b). Moreover, with a focus on embryonic development, the nesting behavior and maternal effects of females in this study were excluded, that can significantly affect embryonic and hatchling development (e.g., Refsnider and Janzen, 2010a,b; Meylan et al., 2012; Li et al., 2018). Further research is required to reveal the interactive effects of embryonic and hatchling developmental temperatures, as well as the effects of nesting behavior and maternal modifications.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committees of Harbin Normal University.

AUTHOR CONTRIBUTIONS

LC, CY, WZ, and PL designed the studies. CY, LC, DZ, SL, and PL collected the data. LC, WZ, and PL analyzed the data and led the draft writing. All authors contributed critically to the drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.845309/full#supplementary-material

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Heat wave induces oxidative damage in the Chinese pond turtle (*Mauremys reevesii*) from low latitudes

Weijie Tao[1†], Junwei Ou[1†], Danyang Wu[2,3†], Qiong Zhang[3], Xingzhi Han[3], Lei Xie[1], Shuran Li[1*] and Yongpu Zhang[1*]

[1]College of Life and Environmental Science, Wenzhou University, Wenzhou, Zhejiang, China, [2]College of Life and Environment Sciences, Engineering Research Center of Food Environment and Public Health, Minzu University of China, Beijing, China, [3]Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

Introduction: Global warming has led to frequent heat waves, causing global organisms to face severe survival challenges. However, the way in which heat waves threaten the fitness and survival of animals remains largely unclear. Oxidative damage and immunity are widely considered the link between heat waves and threats to animals.

Methods: To evaluate the oxidative damage caused by heat waves and to reveal the physiological resistance to heat waves by the antioxidant defense of animals from different latitudes, we exposed both high-latitude (Zhejiang) and low-latitude (Hainan) populations of Chinese pond turtle (Mauremys reevesii) to simulate heat waves and a moderate thermal environment for 1 week, respectively. Next, we compared the oxidative damage by malondialdehyde (MDA) and antioxidant capacity by superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and total antioxidant capacity (T-AOC) in the liver tissues and evaluated the innate immunity by serum complement protein levels (C3, C4) and lysozyme activity in plasma of turtles.

Results and discussion: We found that heat waves significantly increased the content of MDA and the activity of CAT, whereas it decreased the activity of SOD, T-AOC, and GSH/GSSG in turtles from low latitudes. Furthermore, heat waves increased CAT activity but decreased GSH/GSSG in turtles from high latitudes. Although the turtles from high latitudes had higher levels of innate immunity, the heat waves did not affect the innate immunity of C3, C4, or lysozyme in either population. These results indicate that the low-latitude population suffered higher oxidative damage with lower antioxidant capacities. Therefore, we predict that Chinese pond turtles from low latitudes may be more vulnerable to heat waves caused by climate warming. This study reveals the physiological and biochemical resistance to heat waves in Chinese pond turtles from different latitudes and highlights the importance of integrative determination of fitness-related responses in evaluating the vulnerability of ectotherms from different latitudes to climate warming.

KEYWORDS

climate warming, heat waves, oxidative stress, antioxidant defense, innate immunity, turtle

EDITED BY
Lin Zhang,
Hubei University of Chinese Medicine,
China

REVIEWED BY
Wenyi Zhang,
Nanjing Normal University, China
Hong-Liang Lu,
Hangzhou Normal University, China
Li Ding,
Hainan Normal University, China

*CORRESPONDENCE
Shuran Li
lishuran@wzu.edu.cn
Yongpu Zhang
zhangyp@wzu.edu.cn

†These authors have contributed equally to this work

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Introduction

A serious consequence of climate warming is the occurrence of more frequent and extreme heat waves. In recent years, the warmest summers and continuous heat events have been recorded, even in polar areas (Hansen et al., 2012; Witze, 2022). Therefore, understanding the effects of heat waves on species has been more critical and urgent than ever (Stillman, 2019). Heat events (e.g., summer heat waves) make summer a terrible season for animals, during which they face the challenges of stressful heat (Burggren, 2018; Vinagre et al., 2018). Both terrestrial and marine ecosystems experience biodiversity loss due to heat waves (Rogers-Bennett and Catton, 2019). Heat waves can have disastrous effects on animals, including direct mortality, depressing population growth, and declining individual fitness (see details in Stillman, 2019). For example, mortality has been widely observed in individuals or populations that are unable to escape or resist heat waves (Morignat et al., 2018). Some birds cannot maintain their body temperatures under heat waves by cool evaporating, and thus die of dehydration (McKechnie and Wolf, 2010; Xie et al., 2017). Furthermore, heat waves are associated with functional decline and infectious disease outbreaks in some species, leading to large-scale population collapse (Harvell et al., 2019; Rogers-Bennett and Catton, 2019). For instance, the innate immunity of common musk turtles (*Sternotherus odoratus*) is modified by heat events, thus modulating their resistance to heat waves (Goessling et al., 2019). Extremely high air temperatures can also cause a decline in cognitive and locomotor performance and decreased fitness (Danner et al., 2021). However, the mechanism by which animals resist heat waves is largely unclear (Stillman, 2019; Logan and Cox, 2020).

The antioxidant defense may be the most important physiological adjustment when animals are exposed to heat waves (Troschinski et al., 2014; Baker et al., 2020). Extremely high temperatures would seriously increase the metabolic rates and synchronously produce reactive oxygen species (ROS; Ben Ameur et al., 2012), which would react with the molecular components of cells, such as lipids, proteins, and nucleic acids, leading to oxidative damage in cell machinery, indicated by the content of malondialdehyde (MDA), carbonylated protein, and DNA injury (Li et al., 2021). The accumulation of oxidative damage then induces the collapse of functions such as immunity, and even leads to mortality of the individuals; hence, depressed immunity can serve as a proxy for oxidative damage. For instance, a simulated heat wave induced oxidative damage in the corn snake (*Pantherophis guttatus*) and depressed its innate immunity (Stahlschmidt et al., 2017). Heat waves also decrease body mass, lysozyme activity, and individual fitness in tropical butterflies (*Bicyclus anynana*; Fischer et al., 2014). In animals, the antioxidant defense can eliminate ROS production through a series of reactions catalyzed by antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx; Munro and Treberg, 2017). In summary, heat waves induce oxidative damage (e.g., MDA) and affect immunity; however, individuals can enhance their antioxidant defense to reduce the potential threat of heat waves (Speakman and Garratt, 2014). For example, our previous study demonstrated that when yellow pond turtles (*Mauremys mutica*) faced heat waves, enhanced GPx facilitated antioxidant defense and plausibly reduced the production of MDA (i.e., oxidative damage), thereby sustaining innate immunity, including increased lysozyme activity and serum complement C4 levels (Li et al., 2021).

Although the latitudinal vulnerability of animals to heat waves under climate warming is largely unclear, heat waves are predicted to increase toward low latitudes (IPCC, 2021), indicating that species from these areas would face more challenges. Nonetheless, heat tolerance increases toward low latitudes (i.e., tropical areas) in insects, fish, turtles, and birds (Sunday et al., 2011, 2012). The latitudinal vulnerability of animals to heat waves is determined by the interaction between heat waves and how local animals physiologically resist them (Hall and Sun, 2021). Therefore, revealing the physiological resistance to heat waves across latitudes is critical for understanding the thermal adaptation to environments and for evaluating the vulnerability of animals to climate warming geographically (Carlo et al., 2018; Dahlke et al., 2020; Li et al., 2021).

As ectotherms, the physiological functions and fitness of turtles are significantly affected by their thermal environment (Ackerman, 1981; Attaway et al., 1998; Binckley et al., 1998; Ashmore and Janzen, 2003; Bostrom et al., 2010). Turtles are becoming increasingly vulnerable to ongoing climate warming (Taylor et al., 2020). The Chinese three-keeled pond turtle (*Mauremys reevesii*) is an endangered and unique species that is widespread in mainland China (IUCN, 2022). The species biology has been widely investigated, including life-history cycle, sex determination, and thermal biological traits (Du et al., 2009; Ye et al., 2009; Zhang et al., 2019; Wu et al., 2022), making it an appropriate system to study physiological resistance to heat waves.

In this study, we used *M. reevesii* from low latitudes (Hainan) and high latitudes (Zhejiang) as the study system. We first exposed the two populations of turtles to a simulated heat wave and then determined oxidative damage with MDA; anti-oxidant ability with the activity of SOD, CAT, GPx; and total antioxidant capacity (T-AOC) in the liver. We also evaluated the functional damage by determining the innate immunity of serum complement protein levels (C3, C4) and lysozyme activity in plasma. By comparing the traits of each population between the control and heat treatments, we aimed to reveal the effects of heat waves on the antioxidant responses and innate immune function of *M. reevesii* across latitudes. Based on the heat tolerance pattern in turtles across latitudes (Sunday et al., 2011), we predicted that heat waves would induce oxidative stress and depress immune function in *M. reevesii* at high latitudes rather than at low latitudes.

Materials and methods

Ethics statement

We experimented with animals under the supervision of the Animal Ethical and Welfare Committee of Wenzhou University (Approval No. WZU-049).

Experimental design

We used 16 yearling turtles for each population, which were purchased from the turtle farm in Jiaxing, Zhejiang Province (120°53′E, 30°46′N) for the high-latitude population and from Haikou, Hainan province (110°19′E, 20°2′N) for the low-latitude population in 2019. The phylogenetic relationship of these two populations with CytB indicated that they were from one species without differentiation (Supplementary Figure S1). The turtles were transferred to the laboratory at Wenzhou University by train in 2 days, where they were reared in individual terraria (length × width × height = 45 × 35 × 10 cm) in temperature-controlled incubators (KB400, Binder GmbH, Tuttlingen, Germany). The temperature was modified daily, mimicking the field thermal environment of July and August in in Jiaxing and Haikou (28 ± 4°C, Supplementary Figure S2; China Meteorological Data Service Center, http://data.cma.cn). After 1 week, we used a 2 × 2 experimental design to acclimate the turtles: population origin (high latitude vs. low latitude) × thermal treatment (control vs. heat waves). Accordingly, turtles from each population were randomly and evenly assigned to two treatments: control and heat wave. Animals under the control treatment were reared at 28 ± 4°C continuously, and their counterparts under the heat wave treatment were reared under a fluctuating thermal regime of 35 ± 4°C, which mimicked the heat waves of Jiaxing in July and August from 2011 to 2017 (China Meteorological Data Service Center, http://data.cma.cn; Figure 1). Accordingly, we acclimated eight turtles to each treatment for each population. During acclimation, we provided commercial food to the turtles daily. The light cycle inside the incubator was set at 14 h light:10 h dark (6 a.m.–8 p.m.) using fluorescent lamps. The water was changed daily when the light was turned off.

Oxidative damage, antioxidant defense, and immunity indices

After 1 week of acclimation, the turtles were used to determine oxidative damage, antioxidant ability, and innate immunity. Following an established method (Li et al., 2021), we sampled the liver tissues for oxidative damage and antioxidant ability determination, and the blood for innate immunity determination. Eight turtles from each population in each treatment were used in this study. In brief, the turtles were first sacrificed by decapitation, followed by pithing using heavy shears according to the AVMA Guidelines for the Euthanasia of Animals (2013 Edition). Then, we collected the liver tissue on ice. Blood-free livers were weighed immediately (±0.0001 g) and frozen in liquid nitrogen. Blood was collected from the carotid artery and centrifuged at 3,000 rpm for 15 min (Fresco 21, Thermo Fisher Scientific, Waltham, MA, United States) to obtain serum and plasma.

Following the protocols of commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and our previously established methods, we determined the MDA content and the activity of the antioxidant enzymes SOD, CAT, GPx, and T-AOC in the liver tissue (Li et al., 2021). In brief, the MDA content was determined by measuring the absorbance of the compound produced by MDA and thiobarbituric acid at 532 nm. SOD activity was then determined at 550 nm in the xanthine-xanthine oxidase system, and the rate of cytochrome *c* reduction by superoxide ions was monitored and calculated. CAT activity was estimated at 405 nm by monitoring the consumption of H_2O_2. The GSH consumption rate at 412 nm was used to determine GPx. Total antioxidant capacity (T-AOC) was measured based on the absorbance of the Fe^{2+}-o-phenanthroline complex at 520 nm.

Similar to oxidative damage and antioxidant defense, we followed established methods and enzyme-linked immunosorbent assay (ELISA) kits (Shanghai MLBIO Biotechnology Co., LTD, Shanghai, China) to determine the concentrations of serum C3, C4, and plasma lysozyme, as part of innate immunity (Li et al., 2021). Samples were incubated with anti-fish horseradish peroxidase-linked antibodies and after thorough washing, and color was developed using 3,3′,5,5′-tetramethylbenzidine. Sulfuric acid was added to terminate the reaction and absorbance was measured at 450 nm.

Statistical analysis

Data were analyzed using Statistica 6.0. Before the analysis, the residuals of the variance were checked for normality and homogeneity using *Shapiro–Wilk* and *Levene's* tests, respectively. Data were normalized by exponential or Ln-transformation if necessary. Two-way ANOVAs were used to test the differences between treatments and populations in MDA, SOD, CAT, GPx, T-AOC, C3, C4, and lysozyme levels, followed by the *post hoc* Tukey's HSD test. Data were expressed as the mean ± SE. The significance level was set at $\alpha = 0.05$.

Results

Oxidative damage and antioxidant defense

The MDA content was significantly higher in the turtles from low latitudes than in those from high latitudes ($F_{1,\,28} = 32.307$, $p < 0.001$), and was higher in animals exposed to heat waves than in the controls ($F_{1,\,28} = 25.541$, $p = 0.002$). However, there was no interaction in determining the MDA content between the population origin and thermal treatments ($F_{1,\,28} = 2.728$, $p = 0.110$; Figure 2A).

The activity of SOD was affected by population origin ($F_{1,\,28} = 40.330$, $p < 0.001$) and the interaction between population origin and thermal treatment ($F_{1,\,28} = 10.960$, $p = 0.003$). Thermal treatment did not affect the SOD activity ($F_{1,\,28} = 2.010$, $p = 0.168$). The turtles from low latitudes had higher SOD activity than those from high latitudes, mainly induced by the

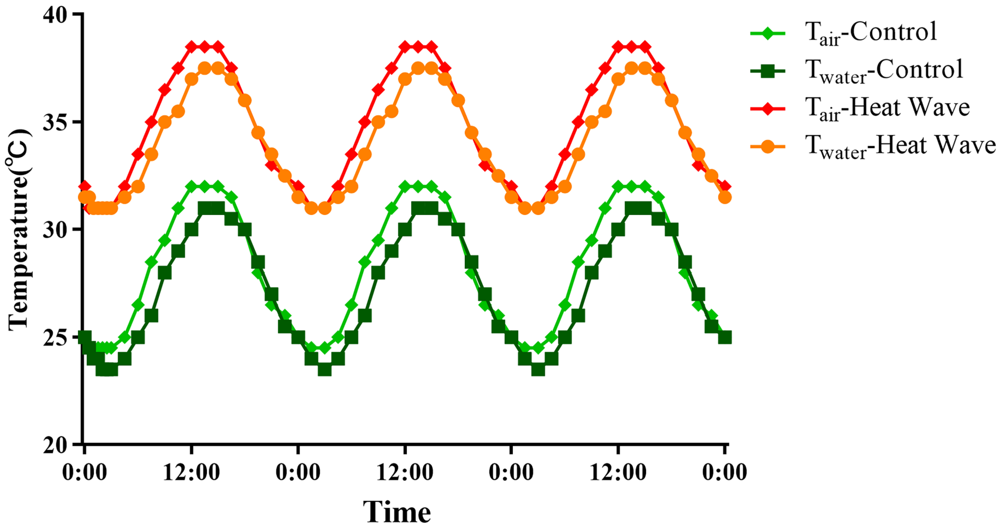

FIGURE 1
Thermal treatments for turtles under control and heat waves, respectively. The red and light green lines indicate the air temperatures under heat waves and control treatments, while orange and dark green lines indicate the water temperatures under heat waves and control treatments, respectively.

significantly higher activity of SOD in turtles under the control treatment. However, SOD activity in turtles from different populations did not differ under the heat wave treatment (Figure 2B).

CAT activity was enhanced by heat waves in both populations ($F_{1,28} = 7.354$, $p = 0.011$). However, neither population origin ($F_{1,28} = 0.058$, $p = 0.812$) nor the interaction between population origin and thermal treatment ($F_{1,28} = 0.121$, $p = 0.730$) affected CAT activity (Figure 2C). Similarly, neither population origin ($F_{1,28} = 0.127$, $p = 0.724$) nor the interaction between population and treatment ($F_{1,28} = 0.244$, $p = 0.625$) affected the GSH/GSSG; however, the turtles from both populations had lower GSH/GSSG if they were under heat wave treatment ($F_{1,28} = 12.953$, $p = 0.001$; Figure 2D). The total antioxidant capacity (T-AOC) was affected by population origin ($F_{1,28} = 13.305$, $p = 0.001$) and the interaction between population origin and thermal treatment ($F_{1,28} = 5.555$, $p = 0.026$). However, heat waves did not affect the T-AOC ($F_{1,28} = 2.791$, $p = 0.106$). Turtles from low latitudes had higher T-AOC than those from high latitudes, mainly induced by significantly higher T-AOC under the control treatment. Interestingly, the T-AOC of the two populations did not differ under the heat-wave treatment (Figure 2E).

Innate immunity responses

Levels of C3 ($F_{1,28} = 13.683$, $p < 0.001$) and C4 ($F_{1,28} = 5.324$, $p = 0.029$) were significantly higher in turtles from high latitudes than in turtles from low latitudes. However, neither the thermal treatment (C3: $F_{1,28} = 0.260$, $p = 0.614$; C4: $F_{1,28} = 1.443$, $p = 0.240$) nor the interaction between population origin and thermal treatment (C3: $F_{1,28} = 2.354$, $p = 0.136$; C4: $F_{1,28} = 4.008$, $p = 0.056$) affected the C3 or C4 levels (Figures 3A,B).

In contrast, lysozyme activity was not affected by population origin ($F_{1,28} = 0.155$, $p = 0.697$), thermal treatment ($F_{1,28} = 0.243$, $p = 0.626$), or their interactions ($F_{1,28} = 470$, $p = 0.499$; Figure 3C).

Discussion

Summer heatwaves caused by climate warming have been demonstrated to impose increasing threats to animals (Stillman, 2019; Witze, 2022). However, how heat waves affect animals is still largely unknown, especially at the physiological and biochemical levels (Somero, 2011; Campbell-Staton et al., 2020). This study demonstrated that the effects of simulated heat waves on the biochemistry and physiology of the Chinese pond turtle, *M. reevesii,* were complex and varied across latitudes. We found that 1-week heat waves caused significant damage at the cellular level by increasing MDA levels, together with a depressed antioxidant defense in turtles from low latitudes, but not affecting the turtles from high latitudes (Figure 2). Although innate immunity-related traits were not depressed by heat waves, the lower daily level of immunity combined with higher oxidative damage may still predict vulnerability to climate warming in turtles from low latitudes.

Higher oxidative damage by heat waves in low-latitude turtles

When ROS are not quenched by antioxidant defenses, they cause oxidative damage such as lipid peroxidation, protein

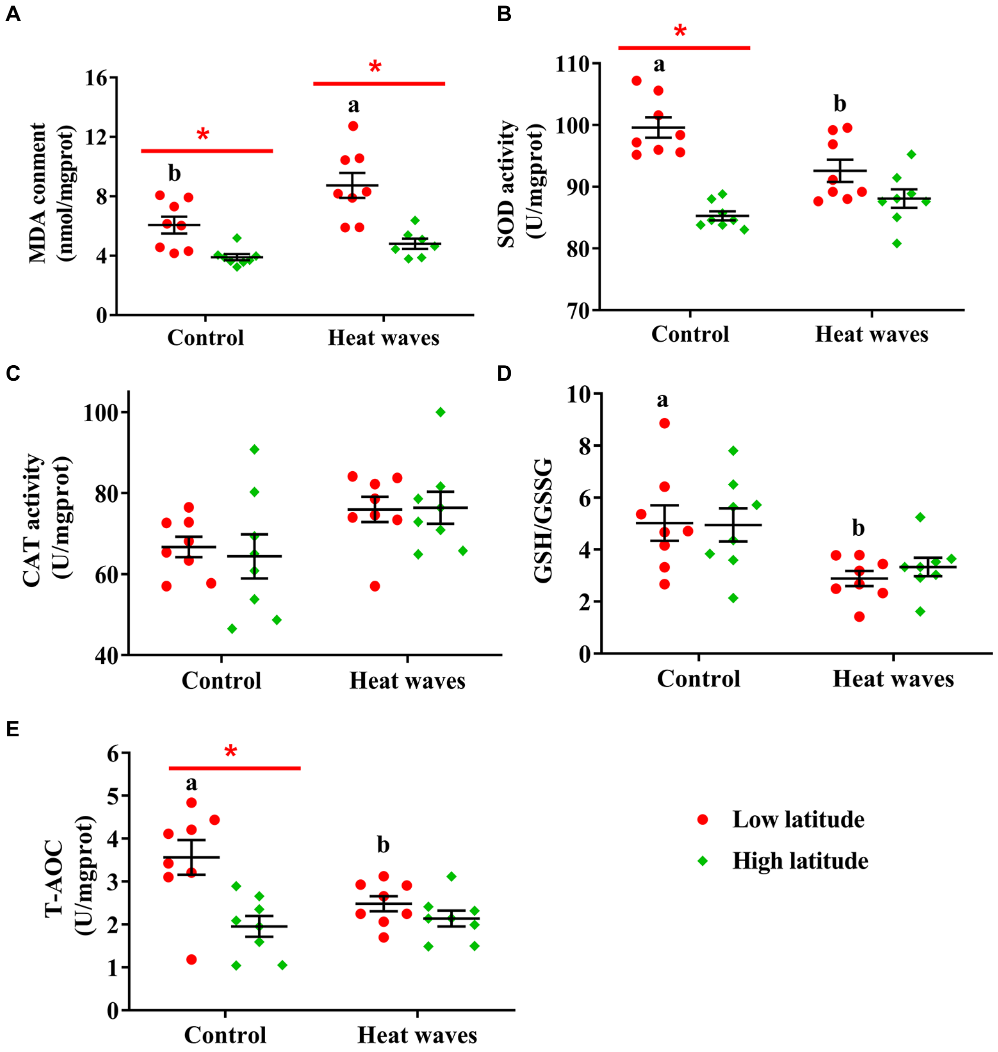

FIGURE 2
The content of MDA **(A)**, and the activity of SOD **(B)**, CAT **(C)**, GPx **(D)**, and T-AOC **(E)** in liver tissues of the turtles from low and high latitudes. The red spots and green diamonds indicate the turtles from low and high latitudes, respectively. Each dot indicates an individual, and the error bars indicate the mean±SE. Red asterisks indicate significant differences between latitudes under the same treatment. Lowercases indicate significant differences between treatments in turtles from low latitude.

carbonylation, and DNA damage (Ben Ameur et al., 2012). As one of the final products of lipid peroxidation, MDA content reflects the degree of oxidative damage suffered by important biomacromolecules, including phospholipids, glycolipids, and cholesterol (Parrish, 2013; Ayala et al., 2014). This damage leads to functional loss and disruption of homeostasis (Dong et al., 2021).

In this study, we found that the MDA content in the liver of turtles from low latitudes (tropical areas) increased significantly after 1 week of exposure to heat waves (Figure 2A), indicating significant negative effects. Heat waves could be increasing the metabolism by which ROS are produced, and consequently, oxidative damage (e.g., MDA) would accumulate (Jena et al., 2013). This result is similar to the adverse effects observed on a lizard (*Eremias multiocellata*; Han et al., 2020). However, heat waves did not induce significant oxidative damage in the Chinese soft-shell turtle (*Pelodiscus sinensis*; Zhang et al., 2019), or the yellow pond turtle (*Mauremys mutica*; Li et al., 2021). This discrepancy may be due to the population origin of the studied individuals. Previous studies on *P. sinensis* (Hebei, 39°58′N) and *M. mutica* (Zhejiang, 30°46′N) have been conducted on turtles from high latitudes (Zhang et al., 2019; Li et al., 2021). As it may be that high-latitude reptiles live at temperatures lower than their physiological optimum (Hao et al., 2020; Liu et al., 2022), higher environmental temperatures would be beneficial for their functions (Huey et al., 2009), and heat waves may not lead to significant oxidative damage. Similarly, in the present study, the MDA content of high latitude specimens did not increase after exposure to heat waves.

Alternatively, the MDA content is also regulated by the balance between oxidative stress and antioxidant defense

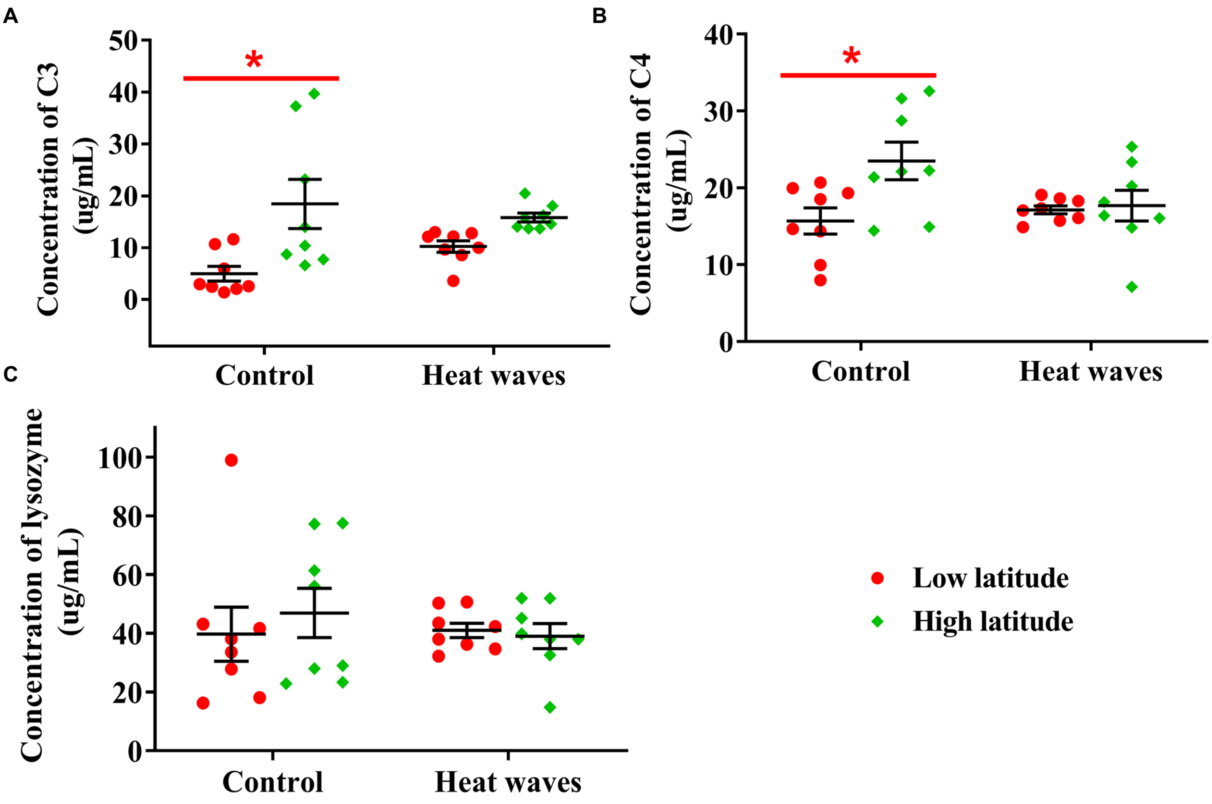

FIGURE 3
The concentration of serum C3 **(A)**, C4 **(B)**, and lysozyme **(C)** in plasma of the turtles from low and high latitudes. The red spots and green diamonds indicate the turtles from low and high latitudes, respectively. Each dot indicates an individual, and the error bars indicate the mean±SE. Red asterisks indicate significant differences between latitudes under the same treatment. Lowercases indicate significant differences between treatments in turtles from low latitude.

(Halliwell, 2007). In this study, all the antioxidant defense-related traits, except CAT, were downregulated (i.e., SOD, GSH/GSSG, and T-AOC) in turtles from low latitudes after being exposed to heat waves (Figure 2). Widely depressed antioxidant defenses (i.e., SOD, GPx, and T-AOC) in low-latitude turtles may limit their ability to eliminate ROS and prevent oxidative damage, such as the production of MDA (Došenović et al., 2021; Pinya et al., 2021). The effects of SOD, CAT, and GPx activity on the elimination of MDA have been widely demonstrated in vertebrates, including fishes (Fakhereddin and Doğan, 2021), reptiles (Han et al., 2020; Kou et al., 2021; Pinya et al., 2021), birds (Altan et al., 2003), and mammals (Wei et al., 2016). For instance, oxidative damage (i.e., MDA content) caused by seawater in rats is higher in individuals with lower SOD activity (Wei et al., 2016). In this study, the high-latitude population of pond turtles showed conflicting ability in antioxidant defense, exhibiting decreased GSH/GSSG but increased CAT after exposure to heat waves (Figures 2C,D).

Another important finding in this study was that under the control treatment, the MDA content, and the activity of SOD and T-AOC, in low-latitude turtles were higher than those in their counterparts from high latitudes (Figures 2A,B,E). The MDA content can be increased by chronic warming as reported in the mussel *Mytilus coruscus* (Khan et al., 2021), and the octocoral *Veretillum cynomorium* (Lopes et al., 2018). As the low-latitude population of pond turtles in this study experienced higher ambient temperatures than the high-latitude population, the higher MDA content may reflect a chronic accumulation of oxidative damage induced by average warmer temperatures similar to other reptiles (Reguera et al., 2014). Accordingly, the higher activities of SOD and T-AOC may also reflect that the antioxidant defense was chronically triggered by oxidative stress in low-latitude pond turtles.

Heat waves did not depress innate immunity

As an initial response to pathogens, innate immunity is nonspecific and essential for individuals to survive in stressful surroundings (Lafferty, 2009). In addition, the immune response of organisms relies on the production of pro-oxidant substances and is regulated by oxidative stress (Espelid et al., 1996; Sorci and Faivre, 2009). Therefore, innate immunity is a robust index for evaluating the fitness consequences of oxidative damage caused by heat waves (Han et al., 2020). Innate immunity, universal to reptiles, has been demonstrated to be thermally sensitive in turtles (Zimmerman et al., 2017).

In this study, the concentrations of C3, C4, and lysozyme did not differ among thermal treatments in either high- or low-latitude turtles, implying that innate immunity was not adjusted by heat waves. These results contradict our assumption that oxidative damage would induce decreased immunity. Although innate immunity is widely demonstrated to be inhibited by heat waves in ectotherms, including snails (Leicht et al., 2013), fish (Dittmar et al., 2014), and reptiles (Stahlschmidt et al., 2017), it was also found to be enhanced (Adamo and Lovett, 2011; Li et al., 2021), or unaltered (Zimmerman et al., 2017). This study provides further evidence that the effects of heat waves on innate immunity are complex in ectotherms. Although we did not find decreased C3, C4, or lysozyme levels, heat waves might decline some other traits of innate immunity. For example, heat waves induce oxidative stress and depress innate immunity by low IgM and white blood cells (WBC) in racerunners (*Eremias multiocellata*; Han et al., 2020).

Interestingly, this study found higher concentrations of C3 and C4 in high-latitude pond turtles under the control treatment, indicating a higher ability to kill bacteria or digest pathogens than those in low-latitude turtles under moderate temperatures (Baker et al., 2019). Although heat waves did not depress the innate immunity of turtles from low latitudes, their lower C3 and C4 concentrations under moderate temperatures may imply weaker immunity.

In summary, this study revealed oxidative damage in the liver of low-latitude pond turtles (*M. reeviesii*) after experiencing 1-week heat waves, relative to their high-latitude counterparts. The innate immunity of C3, C4, and lysozyme were not affected in either latitudinal population; however, we found lower daily C3 and C4 levels in the low-latitude turtles. By integrating the above physiological and biochemical responses, we predicted that pond turtles would be more vulnerable to climate warming. Nonetheless, it is notable that in this study, we only focused on oxidative damage and related immunity at physiological and biochemical levels. As the frequency of heat waves has increased in recent years (Stillman, 2019; Witze, 2022), the consequences on fitness-related traits of individuals and populations, including reproduction, survival, and population dynamics, should be given more attention (Deutsch et al., 2008; Stahlschmidt et al., 2017; Sun et al., 2021). In addition, oxidative stress, damage, and antioxidative defense are complex networks, and before we can draw a comprehensive picture, more indices should be added in future research, such as carbonylated protein, 8-hydroxy-2-deoxy Guanosine (8-OH-dG), IgA, IgM, and WBC, to evaluate the damage to biomacromolecules and immunities (Espelid et al., 1996; Zimmerman et al., 2010, 2017). Furthermore, most current studies have evaluated the effects of heat waves under climate warming by immediate or short-term responses of the species, as in this study. Aging and longevity should be included in future research to provide a chronic and long-lasting view for investigating the effects of climate warming on animals (Zhang et al., 2018; Zhu et al., 2019; Dupoué et al., 2022).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Animal Ethical and Welfare Committee of Wenzhou University (Approval No. WZU-049).

Author contributions

YZ and SL designed the study, analyzed the data, and wrote the manuscript with comments from XH, QZ, and LX. SL, WT, JO, and DW conducted the experiments. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.1053260/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Identification of phylogenetic relationship between the turtles from Zhejiang and Hainan populations using CytB gene as marker, and *Pelodiscus sinensis* as the outgroup. 'S' indicates low latitude population and 'N' indicates high latitude population.

SUPPLEMENTARY FIGURE S2
Mean monthly air temperatures from Zhejiang and Hainan populations (1981-2010). Data were obtained from China Meteorological Data Service Center.

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EDITED BY
Lin Zhang,
Hubei University of Chinese
Medicine, China

REVIEWED BY
Jingguang Wei,
South China Agricultural University, China
Biswajit Maiti,
Nitte University, India
Simin Chai,
Southern Marine Science and Engineering
Guangdong Laboratory
(Guangzhou), China

*CORRESPONDENCE
Zhengfei Wang
✉ wangzf01@yctu.edu.cn

†These authors have contributed equally to this work

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Molecular characterization, adaptive evolution, and expression analysis of the *Toll-like receptor* gene family in *Fenneropenaeus chinensis*

Lulu Chen[1,2†], Dan Tang[1,2,3†], Yuyan Hua[1,2], Xinyu Wang[1,2], Yanxuan Li[1,2] and Zhengfei Wang[1,2*]

[1]Jiangsu Key Laboratory for Bioresources of Saline Soils, Jiangsu Synthetic Innovation Center for Coastal Bio-Agriculture, School of Wetlands, Yancheng Teachers University, Yancheng, Jiangsu, China, [2]Jiangsu Provincial Key Laboratory of Coastal Wetland Bioresources and Environmental Protection, School of Wetlands, Yancheng Teachers University, Yancheng, Jiangsu, China, [3]Senior High School Teaching Department, Shazhou Middle School, Zhangjiagang, Jiangsu, China

Global warming is a challenge to animal health because of the increased environmental temperature, with subsequent induction of immune suppression and increased susceptibility to disease during summer. The Toll-like receptor (TLR) family is an essential pattern recognition receptor (PRR) that initiates the innate immune response by sensing conserved molecular patterns of pathogens. However, research on the *TLR* gene family in decapod crustaceans has been conducted sporadically, without systematic naming, and the relationship between pathogen immunity adaptation and adaptive evolution of immune-related genes is unclear. In this study, various *TLR* gene sequences in decapod crustaceans were collected, and the unified name of *Fenneropenaeus chinensis* was confirmed using sequence alignment. Structural characteristics and evolutionary analyses of *TLR* genes in decapod crustaceans were performed, and ten *FcTLR* genes were identified in *F. chinensis*. Protein domain analysis revealed that FcTLR proteins contain 4–25 LRR domains used to recognize different pathogens. Selection pressure analysis revealed that *TLR1* and *TLR9* were subjected to positive selection pressure in decapod crustaceans, which may be related to their resistance to environmental changes. Furthermore, the expression of ten *TLR* genes was detected in *F. chinensis* following white spot syndrome virus (WSSV) infection. The results demonstrated that *FcTLR1*, *FcTLR7*, and *FcTLR9* responded positively, which was also consistent with the results of the protein domain and selection pressure analyses. This study provides new insights into the immune response and adaptive evolution of *TLRs* in decapod crustaceans to prevent environmental damage, such as pathogens and high temperature.

KEYWORDS

***Fenneropenaeus chinensis*, innate immunity, TLR, WSSV infection, adaptive evolution, expression analysis**

1 Introduction

Global warming is a huge challenge to animal health, causing a decline in animal production and the suppression of immune function (Li et al., 2021). Reports on climate change predicted that the global temperature may rise between 1.4°C and 4.8°C by the end of this century (IPCC, 2014; Savitha et al., 2021). Shrimps and crabs can be found in almost any ecosystem, including shallow coral reefs and hydrothermal vents in the ocean, as well as freshwater and terrestrial habitats (Ma et al., 2019; Wang et al., 2021). As a kind of ectotherm, species of Decapoda are more susceptible to changes in body temperature than endotherms with changes in environmental temperature (Chen et al., 2020). Temperature is considered as a major factor that regulates the development and response of the immune system in decapod crustaceans especially aquaculture organisms (Miest et al., 2019). During periods of elevated temperatures in summer, aquaculture organisms enter a rapid growth period, particularly with respect to the growth and reproduction of microorganisms. Due to the decrease in oxygen content and deterioration of aquaculture environment, pathogen infection is the main disease at this stage.

The innate immunity of decapod crustaceans serves as the main barrier against infections by many pathogens. Innate immunity relies on unique pattern recognition receptors (PRRs) that recognize the unique pathogen-associated molecular patterns (PAMPs) in external pathogenic microorganisms (Habib and Zhang, 2020). After receiving the signal, it is transmitted to the cell through a series of cascade reactions to regulate the transcription and expression of immune effector factors, which are quickly induced to prevent foreign substances from causing harm to the body (Hughes and Piontkivska, 2008). The Toll-like receptor (TLR) family is an essential PRR family that initiates the innate immune response by sensing the conserved molecular patterns of pathogens. They are the first line of defense against pathogen invasion and play key roles in inflammation, immune cell regulation, survival, and proliferation (Habib and Zhang, 2020; Wan et al., 2022).

TLRs are transmembrane proteins that are involved in signal transduction at the surface of the cell membrane. They are remarkably conserved in the evolutionary process and have three specific domains: interleukin-1 receptor (TIR), transmembrane domain structure, and leucine-rich repeats (LRR) (Wan et al., 2022). TLRs can be roughly divided into two categories based on their location and respective PAMP ligands within the cell. On the surface of cells, a class of TLRs (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11) mainly recognizes microbial membrane components, such as lipids, proteins, and lipoproteins (Kawai and Akira, 2010; Xing et al., 2017). Intracellular vesicles, such as the endoplasmic reticulum (ER), endosomes, lysosomes, and endolysosomes, contain other intracellular TLRs (TLR3, TLR7, TLR8, and TLR9) that mainly recognize nucleic acids of microorganisms or viruses, such as dsRNA, ssRNA, siRNA, shRNA, and viral DNA (Barton and Kagan, 2009; Li et al., 2019).

In 1980, TLRs were first discovered in *Drosophila melanogaster* embryos (Nusslein-Volhard et al., 1980), and homologous TLRs were found in mice and adult *D. melanogaster* in the 1990s (Chiang and Beachy, 1994; Hoshino et al., 1999). The immune response mechanism mediated by TLRs to recognize antigens invading the body has also been uncovered. Subsequently, 10 and 13 *TLRs* (*TLR1–13*) were found in vertebrates, such as humans and mice (Roach et al., 2005), whereas nine *TLRs* (*Toll1–9*) were identified in *D. melanogaster* (Ooi, 2001). Among aquatic organisms, many *TLRs* have been identified in fish, among which 27 *TLRs* have been found in *Cyprinus carpio* (Gong et al., 2017) and 16 *TLRs* in *Lateolabrax maculatus* (Fan et al., 2019). The number and function of *TLRs* vary greatly among fish species.

In decapod crustaceans, Li et al. (2018) discovered a relatively complete family of nine *TLRs* (*Toll1–9*) in *Litopenaeus vannamei* (Li et al., 2018). In a subsequent study by Habib et al. (2021), 11 *TLRs* in *L. vannamei* were identified through the genome-wide screening. Furthermore, *LvToll4* was a crucial receptor for sensing white spot syndrome virus (WSSV) and therefore activated the downstream pathway to induce the production of specific antimicrobial peptides (AMP) (Habib et al., 2021). Moreover, ten *TLRs* were identified in *Marsupenaeus japonicus* (Zheng et al., 2020), and seven *TLRs* were identified in *Procambarus clarkii* (Wan et al., 2022). However, only scattered genes have been cloned into other shrimp and crab species. For instance, five *TLRs* have been found in *P. clarkii* (Wang et al., 2015; Lan et al., 2016a; Lan et al., 2016b; Huang et al., 2017) and two in *M. japonicas* (Mekata et al., 2008). Three *TLRs* have been found in *Penaeus monodon* (Arts et al., 2007; Assavalapsakul and Panyim, 2012; Liu et al., 2018), two *TLRs* in *Macrobrachium rosenbergii* (Srisuk et al., 2014; Feng et al., 2016), two *TLRs* in *Scylla paramamosain* (Chen et al., 2018), and only one *TLR* in *F. chinensis* (Yang et al., 2008); however, the number and structure of *TLRs* are not completely understood.

WSSV is a common pathogen in aquatic organisms. Shrimp and crabs are highly infectious and have a very high mortality rate. It is considered the most serious threat to the aquaculture industry, leading to severe annual economic losses to the aquaculture industry. WSSV is a large enveloped double-stranded DNA (dsDNA) virus, which is highly pathogenic and especially virulent for prawns; hence, it is also known as prawn WSSV (Sun and Zhang, 2022). The interaction between WSSV and its shrimp host has received increasing attention, but the expression patterns of *TLR* and Toll-pathway-related genes involved in viral infection remain unclear. *Toll1*, *Toll2*, and *Toll3* were upregulated after WSSV attack in *L. vannamei* experiments, but their function in WSSV infection is poorly understood. During WSSV infection, the expression of antimicrobial peptides (AMPs) was induced by TLR proteins in *Cherax quadricarinatus* (Li et al., 2017), *P. clarkii* (Lan et al., 2016a; Lan et al., 2016b), and *M. rosenbergii* (Feng et al., 2016), suggesting that these AMPs may have antiviral effects. Furthermore, WSSV infection activated Toll pathway genes in *P. monodon*, implying that the entire Toll pathway plays a crucial role in the immune response during WSSV infection (Arts et al., 2007). Overall, some shrimp TLR proteins are involved in innate immune responses against viral infection; however, their antiviral function remains unknown, and their potential antiviral mechanisms require further investigation.

More than 90% of aquaculture production comes from Asia, including but not limited to China, Thailand, Bangladesh, and India (Bondad-Reantaso et al., 2005; Piamsomboon et al., 2016; Macusi et al., 2022). *Fenneropenaeus chinensis* is an important aquaculture shrimp in China, with endemic aquaculture species in the Yellow Sea and the Bohai Sea of China and the highest annual production of 200,000 tons (Gao et al., 2023). During 1988–1993, aquaculture production of prawns ranked first worldwide for several years. However, after the WSSV attack in 1993, the *F. chinensis* aquaculture industry suffered a huge blow, and its production and output value declined significantly. The Toll signaling pathway plays an important role in the resistance of shrimp to antigens, such as viruses. Therefore, it is essential to investigate the expression pattern of the *TLR* gene under WSSV stress in healthy shrimp and crab breeding, and it is also of great significance to further explore the functional differentiation of members of the *TLR* gene family and the responses of *TLR* genes under the long-term evolution pressure.

In this study, the *TLR* gene family was identified in *F. chinensis* at the genome scale, and a *TLR* gene tree was constructed using available *TLR* gene sequences from Decapoda in the NCBI genome database and transcriptome databases to better characterize these *FcTLR* genes. To elucidate the functional differentiation of *TLR* genes in *F. chinensis*, selective pressure analysis was employed to examine the evolutionary adaptation of *TLR* genes to the environment in the decapod crustaceans. Additionally, qRT-PCR was used to determine the relative expression level of crucial *TLR* genes in *F. chinensis* in response to WSSV infection.

2 Materials and methods

2.1 Experimental materials

F. chinensis for the WSSV infection experiment was obtained from the Huaguoshan Market in Lianyungang City, Jiangsu Province, China. Samples with a complete body, full vitality, and similar size were selected as test animals. The average body length of *F. chinensis* was nearly 120 mm, and the average weight was between 15 g and 20 g. The other species sequenced in the transcriptome were sampled from the Renmin Road Market and coastal beach of Yancheng City, Jiangsu Province, China.

2.2 Acquirement of sample data

The *TLR* genes analyzed in this study were extracted from five public genomic datasets (*Eriocheir sinensis*, *Portunus trituberculatus*, *Paralihodes platypus*, *L. vannamei*, and *F. chinensis*) from the NCBI database, and nine transcriptome datasets (*P. clarkii*, *Cardisoma armatum*, *E. sinensis*, *Macrophthalmus pacificus*, *Metopograpsus quadrident*, *Sesarma plicata*, *Parasesarma pictum*, *S. sinensis*, and *Alpheus bellulus*) obtained from previous research of our team (Wang et al., 2020; Shen et al., 2021; Wu et al., 2021; Wang et al., 2022; Zhu et al., 2022).

2.3 Identification and bioinformatics analysis of TLRs

The reference species was *L. vannamei*, which contains the most prominent *TLR* family genes in decapod crustaceans, but the names of *TLRs* were not unified. Therefore, the accurate number of *TLRs* in *L. vannamei* should be determined initially. The *TLRs* reported by Li et al. (2018) and Habib et al. (2021) in *L. vannamei* (Li et al., 2018; Habib et al., 2021), as well as the coding sequences (CDS) of other species (*Carcinus maenas*, *Daphnia pulex, D. melanogaster, E. sinensis, F. chinensis, M. rosenbergii, M. japonicas, P. monodon, P. trituberculatus, P. clarkii* and *S. serrata*) obtained from the NCBI database, were placed into the same FASTA file for sequence alignment using MEGA7.0 software (Kumar et al., 2016) (Table S1, Supplementary file 1). In this process, all nucleotide sequences of the CDS region were initially translated into the amino acid sequence, and then the sequence alignment was performed using Muscle v5 software (Edgar, 2004).

The phylogenetic tree of *L. vannamei* was constructed using the neighbor-joining (NJ) method in MEGA7.0 software by comparing the amino acid sequences. The Kimura 2-parameter model (K2P) was selected, and an optimal tree was obtained using the bootstrap method. The number of copies was set at 1000.

After confirming the accurate number of *TLR* genes in *L. vannamei*, the genomes of *E. sinensis*, *P. trituberculatus*, *P. platypus*, and *F. chinensis* were searched for using BLASTP. The number of *TLR* genes in the other four genomic species was initially determined, and the number of *TLR* genes from the transcriptome data was then determined. The obtained *TLR* sequences from the genome and transcriptome data were verified using MEGA7.0. The BLASTP program was used to search for homologous TLR protein sequences, and the results were compared with the nucleotide sequences. *TLR* genes with identical sequences were merged, and various *TLRs* were considered as single *TLR* genes. The integrity of *TLR* genes obtained from the transcriptome data was verified using cDNA libraries. If *TLR* sequences were incomplete, those larger than 300 bp were retained for subsequent analysis. The evolutionary relationships among different species of TLR proteins in decapods were analyzed using MEGA7.0 and FigTree v1.4.4 (Rambaut et al., 2018). Conserved protein functional domains were analyzed and annotated using the Simple Modular Structure Research Tool (SMART) program (https://smart.embl.de/smart/set_mode.cgi?NORMAL=1) (Letunic et al., 2021).

2.4 Selective pressure analysis of *TLRs* in decapod crustaceans

To investigate whether members of the *TLR* family in decapod crustaceans were affected by positive selection, selective pressure analysis was performed using PAML software (Yang, 2007). The CodeML program was used to estimate the synonymous replacement rates (*dS*) and non-synonymous replacement rates (*dN*). The ratio of ω (*dS* to *dN*) represents the change in selection pressure; $\omega > 1$, $\omega < 1$, and $\omega = 1$ denote positive, purified, and neutral selection, respectively (Zhou et al., 2013). The site model (M8 and M8a site models) was used to detect the selection pressure on *TLR* genes in decapod crustaceans, and the branch-site model was evaluated for the main branches of shrimp and crabs (Brachyura B, *L. vannamei* and *F. chinensis*) (Yang and Nielsen, 2000). All the positive selected sites in both the site and branch-site models were identified by using Bayes Empirical Bayes (BEB) analysis with posterior probabilities greater than or equal to 0.80 (Yang, 2007). The significance of differences between the two nested models was conducted *via* likelihood ratio tests (LRTs) by calculating twice the log-likelihood (2InL) of the difference following a Chi-square distribution. The degrees of freedom were the difference in the number of free parameters between models (Guo et al., 2018).

2.5 WSSV challenges in *F. chinensis*

According to the method described by Sun et al. (2013), WSSV detection had been taken by One-step PCR before the infection experiment, and the sample was free of WSSV. Healthy *F. chinensis* were temporarily reared for a week after being returned to a temperature of 25°C and a salinity of 25%. During the temporary rearing process, oxygenation was continued to ensure an adequate oxygen supply. The water was changed daily, and the prawns were fed twice daily.

WSSV extracted from a WSSV-infected dying prawn was provided by Professor Fuhua Li at the Institute of Oceanology, Chinese Academy of Sciences. According to the method described by Sun et al. (2013), the DNA fragment of *VP28* gene encoding the extra-cellular part of the WSSV envelope protein VP28 was cloned into the pMD19T vector (TAKARA, Japan) and transfected into *E. coli* competent cells. The recombinant plasmids were extracted and quantified, and the copy number of WSSV was calculated. The plasmids were gradient diluted by ddH_2O to generate the standard curve of WSSV copy numbers ranging from 10^8 to 10^3. qRT-PCR was used to determine the concentration of virus particles and performed with primers qVP28F and qVP28R (Table 1) (Sun et al., 2013). The reaction system was as follows: 10 μL 2 × SuperReal PreMix Plus (TIANGEN, China), 2 μL diluted DNA template, 0.6 μL forward primer (10 μmol/L), 0.6 μL reverse primer (10 μmol/L), and 6.8 μL ddH_2O. The mixture was introduced into the ABI QuantStudio 3 quantitative PCR instrument (Applied Biosystems, USA), and the following procedure was procedures were conducted: pre-denaturation at 95°C °C for 15 min; denaturation at 95°C for 10 s, annealing at 55°Cfor 20 s, elongation at 72°C for 20 s, 45 cycles; dissolution curves were 95°C for 10 s, 65°C for 60 s, and 97°C for 1 s. The WSSV copy number per μL was calculated based on the standard curve. WSSV was diluted with PBS for further study. The experiment was divided into two groups: the WSSV group (experiment group) and the PBS group (control group), with 20 healthy *F. chinensis* in each group. The prawn in the experimental group was injected with 0.1 mL of WSSV virus (10^6 CFU/mL) in the third or fourth segments of their tails. The tails of prawns in the control group were injected with 0.1 mL of PBS solution. After 1 h and 24 h of injection, *F. chinensis* from the experimental and control groups were dissected separately, and hepatopancreas tissues were quickly frozen in liquid nitrogen.

2.6 Total RNA extraction and *TLRs* expression analysis

RNA was extracted from hepatopancreas tissues of *F. chinensis* using TRIzol reagent (TAKARA, Japan). cDNA libraries of different tissues were constructed according to the instructions of the PrimeScript^{TM} Master Mix (TAKARA, Japan). Quantitative primers were designed using Primer 5.0 (Table 1). The expression levels of *TLR* genes in the WSSV and PBS groups at different infection time points were detected and analyzed using qRT-PCR.

TABLE 1 Primers used for quantitative real-time PCR.

Gene Name	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
FcTLR1	TGGCAAACCTTACCATTCCCTA	CTCCTCACCATCACTGGCACA
FcTLR2	GTTTCTTCTATGGTGGCG	CAGTTTGGGCGTGTATTT
FcTLR3	CTCCGACGACCGAGATAA	TGTCACTGGCGTAGATGC
FcTLR4	GAACTTAGCCTTGAACCC	GACCCATCATACCTGTGC
FcTLR5	TCTGGTAGTGATGGCGATGC	TGTCCTTTAGCCCATCAACGT
FcTLR6	CGCACAAAGTGAACATACGCC	GAGACGCCTACCTCGGACA
FcTLR7	GCTGCGTGAGGAGCTTGTCGT	TGAGTCCGCTGGCATCGTTTG
FcTLR8	CTCCATTCCCTTCACATC	GTAGTCTCGGTCTCGTTGT
FcTLR9	TTCAAGTGCGACTGTAGCCT	TACTTGTCCGCTTCATCGTG
FcTLR10	CGAGCACCACAGAGCCAAGA	CACAGCCACCACGACGAACAT
ACTIN	GCGAGAAATCGTGCGTGAC	AGGGTGCGAGGGCAGTGAT
VP28	AAACCTCCGCATTCCTGTGA	TCCGCATCTTCTTCCTTCAT

The reaction system was as follows: 10 μL 2 × SuperReal PreMix Plus (TIANGEN, China), 2 μL cDNA template (50-fold dilution), 0.6 μL forward primer (10 μmol/L), 0.6 μL reverse primer (10 μmol/L), and 6.8 μL ddH_2O. The mixture was introduced into an ABI QuantStudio 3 quantitative PCR instrument (Applied Biosystems, USA), and the following procedures were conducted: pre-denaturation at 95°C for 15 min; denaturation at 95°C for 10 s, annealing at 55°C for 20 s, elongation at 72°C for 20 s, 45 cycles; dissolution curves were 95°C for 10 s, 65°C for 60 s, and 97°C for 1 s. The $2^{-\Delta CT}$ method (Visser et al., 2011) was used to calculate the relative expression level of the target gene relative to the internal reference *ACTIN*. The calculation method is as follows: $\Delta CT=CT^T-CT^G$; relative transcript level = $2^{-\Delta CT}$(CT, cycle threshold; CT^T is CT value of target gene; CT^G is CT value of internal reference gene *ACTIN*).

Significance was conducted using Independent-Samples T Test by SPSS Statistics 23 (Field, 2013). Different experiment groups (1 h PBS vs. 1 h WSSV group, or 1 h WSSV group vs. 24 h WSSV group) were set as Grouping Variable, whereas the relative expression level of *FcTLRs* was set as Test Variable. * and ** indicated statistically significant differences at $P<0.05$ and $P<0.01$, respectively. Histogram drawing was performed using GraphPad Prism 7.0 (Swift, 1997).

3 Result

3.1 Identification of *TLR* genes

Nine *TLR* genes reported by Li et al. (2018) and 11 genes reported by Habib et al. (2021) were aligned to construct phylogenetic trees for *L. vannamei* (Li et al., 2018; Habib et al., 2021). A total of eight *TLRs* genes were conserved between the two study groups (*PTL-2* and *Lv-Toll2*, *PTL-1* and *Lv-Toll1*, *TLR-13x* and *Lv-Toll9*, *TLR-Tollo4* and *Lv-Toll5*, *TLR-6* and *Lv-Toll6*, *TLR-Tollo2* and *Lv-Toll4*, *TLr-Tollo3* and *Lv-Toll8*, *TLR* and *Lv-Toll3*) and clustered in the near branch. The remaining *Lv-Toll7*, *TLR-13*, *TLR-3*, and *TLR-4* were grouped dispersedly in separate branches. Therefore, 12 *TLR* genes were identified in *L. vannamei* (Figure 1, Table S2). In the present study, *Toll1–9*, reported by Li et al. (2018), were named *LvTLR1–9*, whereas *TLR-3*, *TLR-4*, and *TLR-13* in the Habib et al. (2021) were named as *LvTLR10*, *LvTLR11*, and *LvTLR12*, respectively (Figure 1, Table S2). Ten *TLR* genes were identified using a BLASTP search with genomic and transcriptome data in both *F. chinensis* (*FcTLR1–10*) and *P. trituberculatus* (*PtTLR1–9, PtTLR11*), seven complete *TLRs* genes were identified in *E. sinensis* (*EsTLR1–5 and EsTLR7–8*), and seven *TLRs* genes were identified in *P. gigas* (*PgTLR1–6 and PgTLR9*). There were relatively

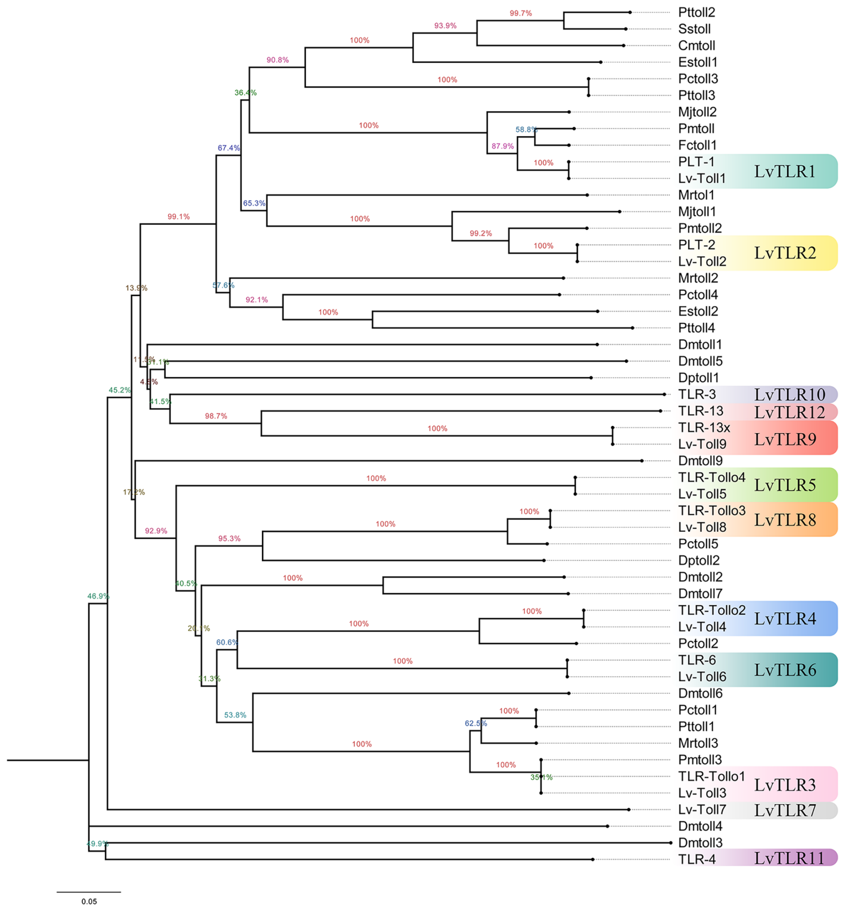

FIGURE 1
Construction of a unified naming system of *TLR* genes in decapods according to the previous two naming systems in *L.vannamei*.

few complete *TLR* genes from the other transcriptome data, and most of the sequences were incomplete (Table 2). Eight *TLR* gene sequences were found in *P. clarkii*, and only four had complete CDS (*PcTLR1–3* and *PcTLR6*). Only the intermediate sequences of *PcTLR4*, *PcTLR5* and the 3'UTR of *PcTLR7* and *PcTLR8* were obtained. Seven *TLR* gene sequences were found in *C. armatum*, and *CaTLR2*, *CaTLR4*, *CaTLR7*, and *CaTLR8* had complete CDS sequences, *CaTLR1* and *CaTLR3* had incomplete 5'UTR, and *CaTLR6* had only partial intermediate sequences. Transcripts longer than 300 bp were retained for subsequent analysis. Phylogenetic analysis displayed that different TLR proteins were clustered in similar clusters, which were divided into 10 categories, and each type of TLR protein was concentrated in the same category. Prawns were further aggregated, and the internal genetic distances were relatively close (Figure 2).

3.2 Prediction of the functional domain of the TLR proteins

SMART was used for protein domain prediction to determine whether the nucleotide sequence obtained from BLASTP represented *TLR* genes. Most typical TLRs contain a signal peptide, LRR domains, a transmembrane domain, and an intracellular toll-interleukin receptor (TIR) domain; similar characteristics were found in *L. vannamei* and *F. chinensis*. The number of LRR domain repeats in *L. vannamei* (Figure 3), *F. chinensis* (Figure 4), and *E. sinensis* (Figure 5) ranged from 5 (*LvTLR12* to 25 (*LvTLR8*), 4 (*FcTLR7*) to 25 (*FcTLR8*), and 9 (*EsTLR1*) to 25 (*EsTLR3*), respectively.

3.3 Selective pressure analysis of *TLRs* in decapod crustaceans

M8 and M8a of the site model are used to represent the selection pressure received by each site. In the present study, the positive selection model M8 of the *TLR1*, *TLR2*, *TLR7*, and *TLR9* genes was better than the neutral model M8a, and the ω values of the four genes were 5.16, 8.07, 1.53, and 5.14 (ω>1), indicating a strong positive selection. There were two positively selected sites in *TLR1* (325 0.812, 505 0.838), one positively selected site in *TLR2* (599 0.816), 11 positively selected sites in *TLR7* (172 0.932, 175 0.899, 186 0.838, 359 0.922, 361 0.803, 388 0.894, 391 0.870, 856 0.839, 959 0.923, 965 0.848, and 975 0.918), and four positively selected sites in *TLR9* (239 0.803, 616 0.813, 699 0.957, and 904 0.821) (Table 3).

We then used the branch-site model to analyze the branches of Brachyura (B), *L. vannamei* (*Lv*), and *F. chinensis* (*Fc*) in the *TLR* gene dataset of decapod crustaceans. Four (*TLR1*, *TLR6*, *TLR7*, and *TLR9*) of nine *TLR* family genes were also found to be under positive selection in decapod crustaceans, where the LRTs of the branch-site model were statistically significant (Table 4). *TLR1* and *TLR9* detected positive selection signals in Brachyura (B), which had three (153, 218, and 25) and seven (368, 378, 380, 390, 412, 415, and 859) positively selected sites, respectively. *TLR9* also detected positive selection signals in *L. vannamei* (*Lv*) with two (605 and 760) positively selected sites. *TLR7* did not detect positive selection signals in Brachyura (B), *F. chinensis* (*Fc*), and *L. vannamei* (*Lv*), but detected positive selection signals in *F. chinensis* (*Fc*) with seven (368, 378, 380, 381, 383, 412, and 859) positively selected sites. In addition, *TLR6* detected a positive selection signal in *L. vannamei* (*Lv*) with one (545) positively selected site (Table 4).

TABLE 2 Identification of *TLR* genes in decapod crustaceans*.

	TLR1	*TLR2*	*TLR3*	*TLR4*	*TLR5*	*TLR6*	*TLR7*	*TLR8*	*TLR9*	*TLR10*	*TLR11*	*TLR12*
L. Vannamei	●	●	●	●	●	●	●	●	●	●	●	●
F. chinensis	●	●	●	●	●	●	●	●	●	●		
E. sinensis	●	●	●	●	●			●	●			
P. trituberculatus	●	●	●	●	●	●	●	●	●		●	
P. gigas	●	●	●	●	●	●			●			
P. clarkii [a]	●	●	●	▲	▲	●	3'	3'				
C. Armatum [a]	5'	●	5'	●		▲	●	●				
E. sinensis [a]		▲	3'		▲	▲			●			
M. pacificus [a]		▲				▲						
M. quadridentatus [a]		▲		3'					●			
S. plicata [a]									5'			
P. Pictum [a]			▲						5'			
S. sinensis [a]		▲										
A. bellulus [a]							▲					

* ● represents complete coding sequence; ▲ represents the middle part of the sequence; 5' and 3' represent the 5' and 3' end of CDS region, respectively. [a] indicates the data was obtained from transcriptome.

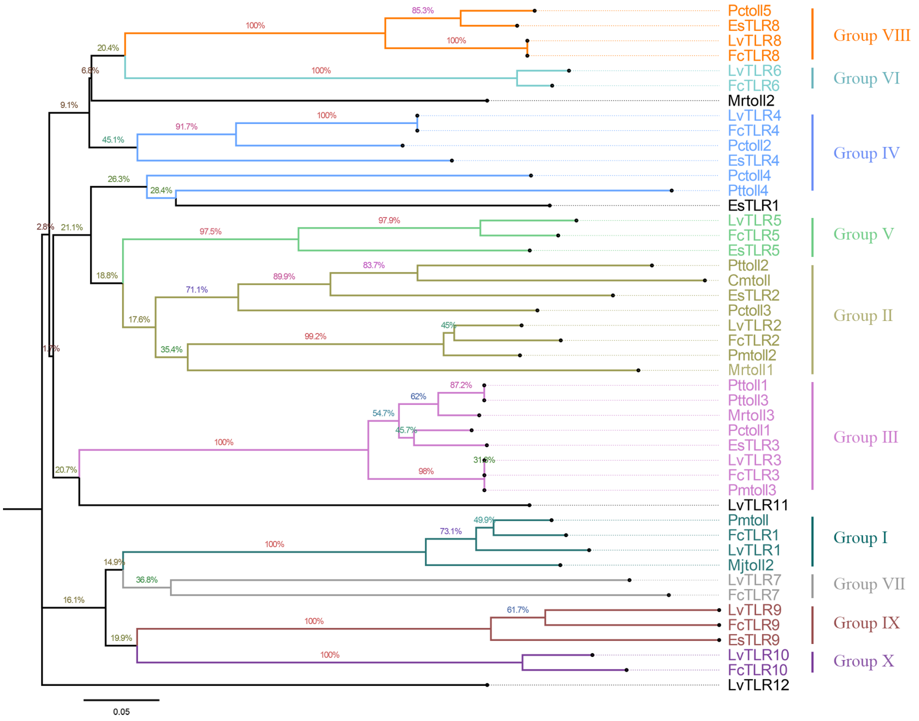

FIGURE 2
Phylogenic analysis of TLR protein sequences in decapods (neighbor-joining tree).

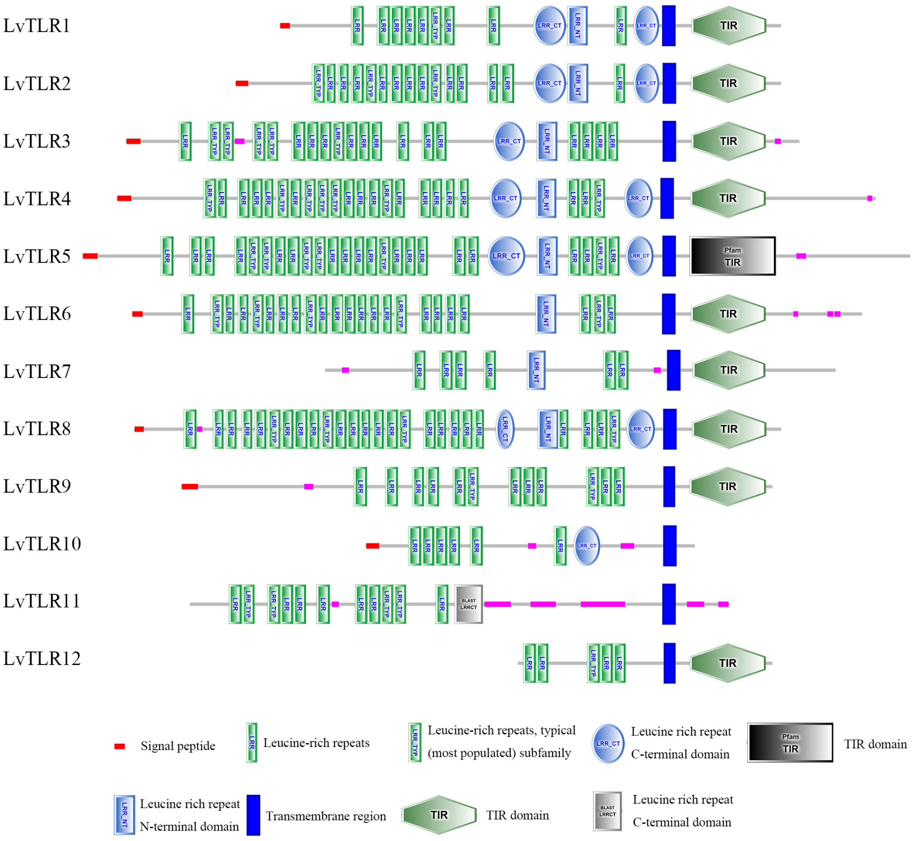

FIGURE 3
Protein domain analysis of the 12 LvTLR proteins in *L. vannamei*.

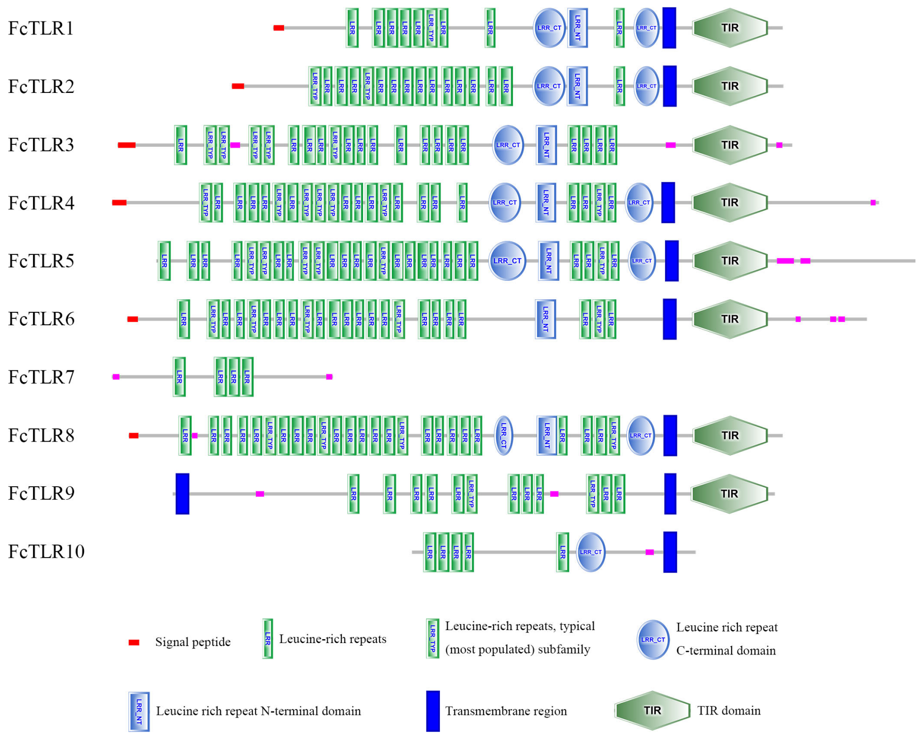

FIGURE 4
Protein domain analysis of the 10 FcTLR proteins in *F. chinensis*.

3.4 Expression analysis of *FcTLRs* in *F. chinensis* under WSSV challenge

The expression levels of *FcTLR1–10* in *F. chinensis* were detected by 1-h short-term infection and 24-h long-term WSSV infection in the experimental group, and PBS solution was injected in the PBS group as a control. The expression levels of *FcTLR7* and *FcTLR9* significantly increased in the 1-h short-term WSSV infection, while their expression levels decreased following 24-h WSSV infection. The expression level of *FcTLR1* had no significant difference in the 1-h WSSV infection, compared with 1-h PBS, but *FcTLR1* represented a significant response in the 24-h infection

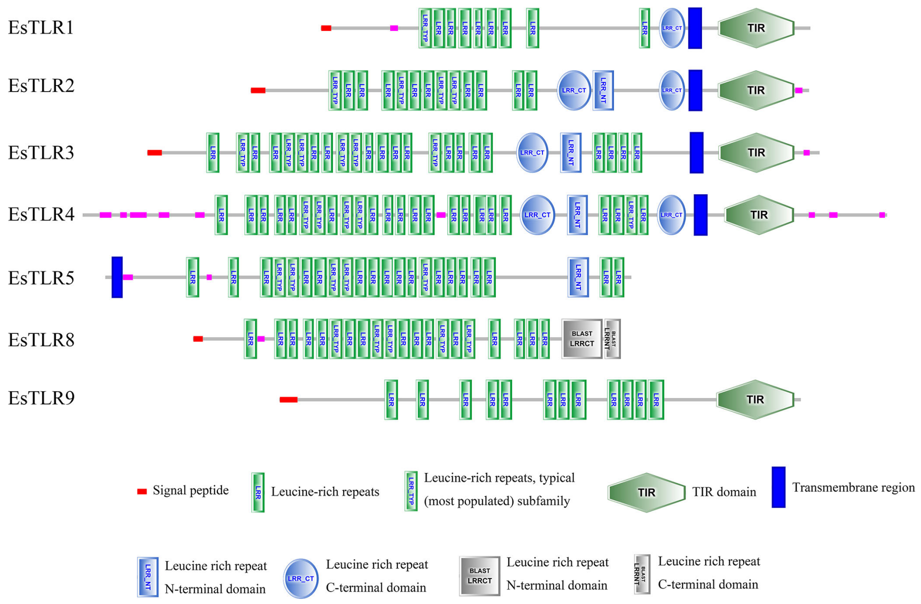

FIGURE 5
Protein domain analysis of the 7 EsTLR proteins in *E. sinensis*.

TABLE 3 Selective pressure analyses (M8 and M8a site models) of nine *TLR* genes in decapod crustaceans[a].

Gene	ln_M8a	ln_M8	2lnL	*P* value	ω value	Positive selected sites
TLR1	−12745.23326	−12741.82251	6.821496	0.009006	5.15682	325 0.812 505 0.838
TLR 2	−15435.04939	−15430.52841	9.041974	0.002638	8.07432	599 0.816
TLR 3	−12946.60537	−12946.60537	0.000	1.000	1.000000	
TLR 4	−15656.20063	−15656.20063	0.000	1.000	1.000000	
TLR 5	−14041.98676	−14041.98676	0.000	1.000	1.000000	
TLR 6	−16747.38272	−16747.38272	−4E−06	1.000	1.000000	
TLR 7	−10645.07466	−10644.57474	0.99984	0.317349	1.53089	172 0.932 359 0.922 391 0.870 965 0.848 175 0.899 361 0.803 856 0.839 186 0.838 959 0.923 975 0.918 388 0.894
TLR 8	−12977.70037	−12977.70037	0.000	1.000	1.000000	
TLR 9	−15635.05141	−15628.85063	12.40155	0.000429	5.14341	239 0.803 616 0.813 699 0.957 904 0.821

[a] **In M8:** Assume all sites 0 < ω <1 or ω>1, with beta distribution; **InM8a,** Assume all sites 0 < ω <1 or ω=1, with beta distribution; **2InL,** twice the logarithmic likelihood. **ω value:** The ratio (ω = dN/dS) of nonsynonymous to synonymous substitutions rate was estimated for selective constraint, where ω > 1, = 1, and <1 respectively indicate positive, neutral, and purifying selection.

TABLE 4 Selective pressure analyses (branch-site model) of nine *TLR* genes in decapod crustaceans[a].

Gene	Model	lnL	2lnL	*P* level	Parameters	Positive selected sites
TLR1	**B**					
	ma	−12784.846			ω0 = 0.093 ω1 = 1.0 ω2 = 2.715	153 0.950* 218 0.954* 25 0.953*
	ma0	−12787.896	6.100142	0.013517	ω0 = 0.09 ω1 = 1.0 ω2 = 1.0	
	Fc					
	ma	−12798.47			ω0 = 0.098 ω1 = 1.0 ω2 = 1.0	
	ma0	−12798.47	0	1	ω0 = 0.098 ω1 = 1.0 ω2 = 1.0	
	Lv					
	ma	−12797.514			ω0 = 0.096 ω1 = 1.0 ω2 = 1.0	
	ma0	−12797.514	0	1	ω0 = 0.096 ω1 = 1.0 ω2 = 1.0	
TLR2	**B**					
	ma	−15485.769			ω0 = 0.11 ω1 = 1.0 ω2 = 1.0	
	ma0	−15485.769	0	1	ω0 = 0.11 ω1 = 1.0 ω2 = 1.0	
	Fc					
	ma	−15480.564			ω0 = 0.111 ω1 = 1.0 ω2 = 8.791	
	ma0	−15483.362	5.595322	0.018009	ω0 = 0.11 ω1 = 1.0 ω2 = 1.0	
	Lv					
	ma	−15486.948			ω0 = 0.113 ω1 = 1.0 ω2 = 1.0	
	ma0	−15486.948	0	1	ω0 = 0.113 ω1 = 1.0 ω2 = 1.0	
TLR3	**B**					
	ma	−13010.772			ω0 = 0.037 ω1 = 1.0 ω2 = 998.984	

(Continued)

TABLE 4 Continued

Gene	Model	lnL	2lnL	*P* level	Parameters	Positive selected sites
	ma0	−13014.628	7.712554	0.005484	ω0 = 0.036 ω1 = 1.0 ω2 = 1.0	
	Fc					
	ma	−13029.506			ω0 = 0.039 ω1 = 1.0 ω2 = 1.0	
	ma0	−13029.506	0	1	ω0 = 0.039 ω1 = 1.0 ω2 = 1.0	
	Lv					
	ma	−13029.506			ω0 = 0.039 ω1 = 1.0 ω2 = 1.0	
	ma0	−13029.506	0	1	ω0 = 0.039 ω1 = 1.0 ω2 = 1.0	
TLR4	**B**					
	ma	−15664.312			ω0 = 0.03 ω1 = 1.0 ω2 = 65.709	
	ma0	−15666.895	5.167332	0.023016	ω0 = 0.028 ω1 = 1.0 ω2 = 1.0	
	Fc					
	ma	−15689.899			ω0 = 0.03 ω1 = 1.0 ω2 = 1.0	
	ma0	−15689.899	0	1	ω0 = 0.03 ω1 = 1.0 ω2 = 1.0	
	Lv					
	ma	−15689.401			ω0 = 0.03 ω1 = 1.0 ω2 = 1.0	
	ma0	−15689.401	0	1	ω0 = 0.03 ω1 = 1.0 ω2 = 1.0	
TLR5	**B**					
	ma	−14049.918			ω0 = 0.036 ω1 = 1.0 ω2 = 999.0	
	ma0	−14065.634	31.43218	0	ω0 = 0.031 ω1 = 1.0 ω2 = 1.0	
	Fc					
	ma	−14090.91			ω0 = 0.037 ω1 = 1.0 ω2 = 1.0	
	ma0	−14090.91	0	1	ω0 = 0.037 ω1 = 1.0 ω2 = 1.0	
	Lv					
	ma	−14091.749			ω0 = 0.038 ω1 = 1.0 ω2 = 1.0	
	ma0	−14091.561	−0.37605	1	ω0 = 0.037 ω1 = 1.0 ω2 = 1.0	
TLR6	**B**					
	ma	−16823.765			ω0 = 0.029 ω1 = 1.0 ω2 = 44.54	
	ma0	−16794.793	−57.9425	1	ω0 = 0.026 ω1 = 1.0 ω2 = 1.0	
	Fc					
	ma	−16823.765			ω0 = 0.029 ω1 = 1.0 ω2 = 1.0	
	ma0	−16823.765	0	1	ω0 = 0.029 ω1 = 1.0 ω2 = 1.0	
	Lv					
	ma	−16821.402			ω0 = 0.029 ω1 = 1.0 ω2 = 13.521	545 0.957*
	ma0	−16823.765	4.726142	0.029707	ω0 = 0.029 ω1 = 1.0 ω2 = 1.0	
TLR7	**B**					
	ma	−10643.226			ω0 = 0.049 ω1 = 1.0 ω2 = 999.0	

(Continued)

TABLE 4 Continued

Gene	Model	lnL	2lnL	*P* level	Parameters	Positive selected sites
	ma0	−10646.594	6.73536	0.009452	ω0 = 0.042 ω1 = 1.0 ω2 = 1.0	
	Fc					
	ma	−10635			ω0 = 0.042 ω1 = 1.0 ω2 = 19.648	368 0.994** 378 0.996** 380 0.998** 381 0.983* 383 0.988* 412 0.991** 859 0.992**
	ma0	−10635.599	1.381402	0.239862	ω0 = 0.036 ω1 = 1.0 ω2 = 1.0	
	Lv					
	ma	−10645.018			ω0 = 0.047 ω1 = 1.0 ω2 = 6.474	
	ma0	−10644.915	−0.20504	1	ω0 = 0.049 ω1 = 1.0 ω2 = 1.0	
TLR8	**B**					
	ma	−13082.354			ω0 = 0.023 ω1 = 1.0 ω2 = 14.801	
	ma0	−13082.353	−0.00147	1	ω0 = 0.023 ω1 = 1.0 ω2 = 1.0	
	Fc					
	ma	−13082.231			ω0 = 0.023 ω1 = 1.0 ω2 = 1.0	
	ma0	−13082.231	0	1	ω0 = 0.023 ω1 = 1.0 ω2 = 1.0	
	Lv					
	ma	−13082.353			ω0 = 0.023 ω1 = 1.0 ω2 = 1.0	
	ma0	−13082.353	0	1	ω0 = 0.023 ω1 = 1.0 ω2 = 1.0	
TLR9	**B**					
	ma	−15701.442			ω0 = 0.075 ω1 = 1.0 ω2 = 999.0	368 0.994** 378 0.996** 380 0.998** 390 0.990** 412 0.991** 415 0.994** 859 0.992**
	ma0	−15726.22	49.55767	0	ω0 = 0.07 ω1 = 1.0 ω2 = 1.0	
	Fc					
	ma	−15735.486			ω0 = 0.07 ω1 = 1.0 ω2 = 1.0	
	ma0	−15735.486	0	1	ω0 = 0.07 ω1 = 1.0 ω2 = 1.0	
	Lv					
	ma	−15726			ω0 = 0.065 ω1 = 1.0 ω2 = 1.0	605 0.991** 760 0.965*
	ma0	−15725.985	0	1	ω0 = 0.065 ω1 = 1.0 ω2 = 1.0	

[a] **InL,** logarithmic likelihood; **2InL,** twice the logarithmic likelihood; **B**, Brachyura; **Fc**, F. chinensis; **Lv**, L. vannamei; The alternative (**ma**, positive selection: 0<ω0<1, ω1 = 1, ω2≥1) and the null model (**ma0**, neutral evolution with ω2 = 1 fixed) in the branch-site test were used to detect selective pressure on each branch.

group. The expression levels of *FcTLR2* and *FcTLR8* decreased in the 1-h WSSV infection, but their expression levels had no significant difference in the 24-h WSSV infection. Besides, the expression levels of *FcTLR3*, *FcTLR4*, *FcTLR5*, *FcTLR6*, and *FcTLR10* have no significant difference following WSSV infection (Figure 6).

4 Discussion

TLRs are essential membrane receptors in the TLR signaling pathway and play an important role in innate immunity against pathogens. To date, *TLR* genes have been extensively acknowledged in vertebrates, such as humans, mice, common carp, and spotted sea bass, but are relatively rare in decapod crustaceans (Roach et al., 2005; Gong et al., 2017; Fan et al., 2019; Habib et al., 2021). Decapod crustaceans exhibit a broad distribution across diverse habitats, and their growth characteristics are influenced by a range of ecological factors, including temperature. The *TLR* gene is a crucial component of innate immunity in invertebrate, and its reaction to environmental stimuli may vary among distinct crustacean species. This study compared *TLR* gene sequences of decapod crustaceans from NCBI and our laboratory, and a unified naming system was developed. A total of nine *TLR* genes from Li et al. (2018) and 11

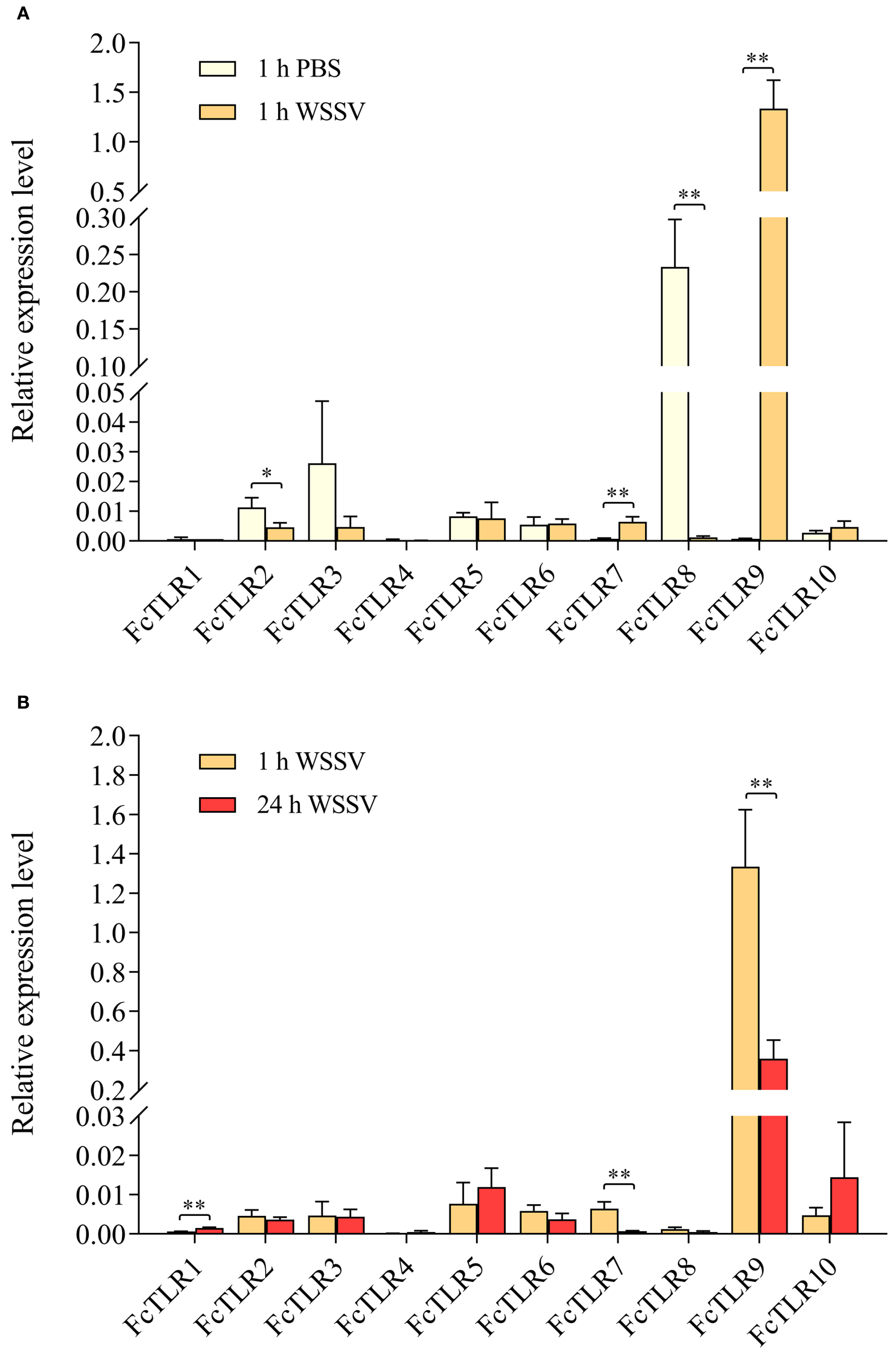

FIGURE 6
The relative expression level of 10 *FcTLRs* in *F. chinensis* infected with WSSV. **(A)** the relative expression level of *FcTLRs* between the 1 h PBS group and 1 h WSSV group at the 1-h time point; **(B)** the relative expression level of *FcTLRs* in the WSSV group between the 1-h time point and the 24-h time point. The data are the mean± SD of three independent biological replicates, n=3 hepatopancreas. * and ** indicate statistical significance at *P*<0.05 and *P*<0.01 by Student's *t*-test, respectively.

TLR genes from Habib et al. (2021) in *L. vannamei* were renamed as 12 *TLR* genes (Li et al., 2018; Habib et al., 2021). In the present study, two relatively complete *TLRs* gene families in *F. chinensis* (10 *TLRs*) and *E. sinensis* (7 *TLRs*) were annotated using the BLASTP program to BLAST other available RNA and genome databases in decapod crustaceans. Because of some limitations in the genome and transcriptome data quality, some *TLR* genes obtained contained only the 5'UTR, 3'UTR, or intermediate cDNA fragments. Complete *TLR9* was annotated in *E. sinensis* and *M. quadridentatus* in the RNA database of our lab, and partial sequences of the *TLR* genes were obtained for the remaining species (*P. clarkia*, *C. armatum*, *M. pacificus*, *M. quadridentatus*, *S. plicata*, *P. pictum*, *S. sinensis*, *A. bellulus*) in our databases. This may be related to the tissue-specific expression of the *Toll* gene and the size restriction of the transcriptome. In the future, high-quality publications of shrimp and crab genomes will allow for the discovery of *TLR* genes. According to multiple sequence alignment and NJ phylogenetic tree construction, different species of TLR proteins were clustered in one group. *F. chinensis* and *L. vannamei* had the highest TLR homology and were clustered in the near branch. They have higher homology with shrimp families and a greater relationship with carbs, such as *E. sinensis* (Figure 2). This suggests that more closely related species have a higher sequence homology.

Analysis of the gene-conserved protein domain revealed domains typical of TLRs in mammals, bony fish, and crustaceans, including signaling peptides, extracellular LRR domains, transmembrane domains, and intracellular toll-interleukin receptors (TIR). LRRs are associated with pathogen recognition, and the intracellular TIR domain acts as a connector to initiate downstream signaling. Furthermore, TLR domain analysis revealed the existence of multiple extracellular LRR domains in PtToll1, ranging from 5 to 25, similar to the 13–25 LRR domains reported in previous studies (Zhang et al., 2018). In the present study, 4–25 LRR domains of FcTLRs were also found in *F. chinensis*, indicating that the TLRs were highly conserved and that various TLRs might be used to respond to different pathogens. This result was similar to that of protein domain analysis of TLRs in *L. vannamei*. TLRs, an important pattern recognition protein, are essential for innate immunity. The analysis of protein domains revealed that they had different repeats of LRR domains, indicating that TLRs may have changed differently in response to various pathogen invasion environments during the long evolutionary process.

The adaptation of gene function is based on the long-term adaptive evolution of the genome sequence and the rapid adaptation of changes in gene expression to environmental changes. There are few systematic descriptions of the interspecific genomic changes in TLR pathway genes in decapod crustaceans, especially in specific variation sites, functional analysis at the domain level, and the study of gene evolution rate. The phylogenetic relationship tree of the *TLR* gene family can help us to understand the functional differentiation of family genes. This provides substantial evidence for determining *TLR* gene function. In general, the ω values of most genes are equal to one, that is, neutral selection. When $\omega > 1$ and the posterior probabilities at the positive selection site is greater than 0.8, the site is considered under positive selection pressure (Zhou et al., 2013). Positive selection signals were detected in the *TLR1* (5.16), *TLR2* (8.07), *TLR7* (1.53), and *TLR9* (5.14) genes of decapod crustaceans by site model analysis. Moreover, positive selection signals were detected in *TLR1* ($P<0.05$, $\omega2 = 2.72$) and *TLR9* ($P<0.01$, $\omega2 = 999.00$) in Brachyura (B), *TLR6* ($P<0.05$, $\omega2 = 13.52$) in *L. vannamei* (*Lv*) using a branch-site model. By combining the site and branch-site model analysis results, *TLR1* and *TLR9* were subjected to positive selection during the evolutionary process in decapod crustaceans.

RNAi of *Toll4* in *L. vannamei* showed that prawns became more sensitive to WSSV infection as the virus load increased (Li et al., 2018). *TLRs* in *S. serrata* (*SsToll*), *P. clarkia* (*PcToll3*, *PcToll5*, and *PcToll6*), *S. paramamosain* (*SpToll2*) exhibited positive responses to the WSSV challenge (Vidya et al., 2014; Lan et al., 2016; Chen et al., 2018; Huang et al., 2018). qRT-PCR was used to detect *TLR* genes in the hepatopancreas of *F. chinensis* infected with WSSV, and the results indicated that *FcTLR1*, *FcTLR7*, and *FcTLR9* exhibited positive responses, suggesting that they may play an essential role in WSSV stress adaptation. This study also provides a reference for other health aquaculture systems. Positive selection signals were detected for *TLR1*, *TLR7*, and *TLR9*, suggesting that different *TLR* genes in decapod crustaceans may respond differently to various pathogens.

This study identified 10 *FcTLR* family genes and analyzed their functional domains in *F. chinensis*. The expression level of *FcTLR* family genes under WSSV challenge was detected in *F. chinensis*, and it was examined whether the *TLR* genes in decapod crustaceans were driven by positive selection to explore the gene adaptation and functional differentiation of *TLRs*. Discussing these issues provides new insights into the immune defense mechanism and cultivation of healthy decapod crustaceans to prevent environmental damage, such as pathogens and temperature.

5 Conclusion

This study comprehensively studied the molecular characterization, adaptive evolution, and expression analysis of *TLR* genes in *F. chinensis*. Ten *TLR* genes in *F. chinensis* and seven *TLR* genes in *E. sinensis* were identified using genome-wide analysis, and their protein domains were relatively conserved. Most TLR proteins contain a typical leucine repeat domain, a transmembrane region, an interleukin receptor domain, and other structures, but the number of LRR in different TLRs varies from 4 (*FcTLR7*) to 25 (*FcTLR8*), which might be related to their response to various pathogens. In the selection pressure analysis, M8 and M8a of the site model and branch-site models were used to determine the selection pressure of the currently available *TLR* genes in decapod crustaceans. Positive selection signals were detected in some genes, such as *TLR1* and *TLR9*, indicating that these genes were subjected to positive selection during the evolutionary process. Finally, the expression of the *FcTLR* genes after WSSV infection was detected in *F. chinensis*. The stress time was detected at 1 h and 24 h. *FcTLR7* and *FcTLR9* revealed strong positive responses at the 1-h time point, whereas *FcTLR1* revealed strong positive responses at the 24-h time point. These results indicate that *FcTLR1*, *FcTLR7*, and *FcTLR9* could be essential in response to WSSV infection in *F. chinensis*, consistent with protein domain and selective pressure analyses.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

LC and ZW conceived and designed the experiments. LC analyzed the data and wrote the manuscript. DT performed the main experimental work. YH was involved in the drawing of phylogenetic trees and protein domain analysis. XW and YL participated in the feeding and anatomy of samples. All authors participated in editing the manuscript and have approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1175220/full#supplementary-material

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