



Experimental warming induces oxidative stress and immunosuppression in a viviparous lizard, *Eremias multiocellata*

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ABSTRACT

Reptiles are especially vulnerable to climate warming because their behavior, physiology, and life history are highly dependent on environmental temperature. In this study, we envisaged new probable mechanisms underlying the high vulnerability of lizards, wherein heat exposure induces oxidative stress and leads to immunosuppression. To test this hypothesis, we conducted a warming experiment on a lizard (*Eremias multiocellata*) from a desert steppe in Inner Mongolia from May to September using open-top chambers set up in their natural habitat and compared the components of oxidative stress (antioxidant ability [Superoxide dismutase (SOD) activity], extent of oxidative damage [malondialdehyde (MDA) content]), and immunocompetence (white blood cells [WBC] counts and immunoglobulin M [IgM] expression) between the warming and control groups. At the end of the experiment, the warming treatment did not affect the survival rate of the lizards. However, MDA content, but not SOD activity, was significantly higher in the warming group than in the control group. The WBC counts and IgM expression were significantly lower in the warming group than in the control group. Our results verified our hypothesis and provided novel cues and methods for the investigation of the mechanisms behind the high probability of extinction of other ectotherms under warming conditions.

1. Introduction

Global climate warming increases the mean and variability of global temperature, resulting in more intense and frequent heat waves (IPCC, 2013; Thornton et al., 2014). For example, the global average surface temperature is expected to increase by 1.8–4.0 °C by the end of this century (IPCC, 2013), with temperatures in some parts of the world increasing 5 °C or more (Voosen, 2019). Global warming significantly affects the life of organisms and thus, is a major threat to biodiversity (Moritz and Agudo, 2013). Under these conditions, ectotherms will suffer an increased extinction risk because of their reliance on external heat sources (Deutsch et al., 2008; Huey et al., 2012; Sinervo et al., 2010). Thus, reptiles are especially vulnerable to climate warming because their behavior, physiology, and life history are highly dependent on environmental temperature (Deutsch et al., 2008; Huey et al., 2012; Sinervo et al., 2010). For example, it is predicted that the expected

increase in the average temperatures could lead to the extinction of almost 40% of total lizard species worldwide by 2080 (Sinervo et al., 2010). Currently, the proximate mechanism underlying the high probability of extinction for lizards includes telomere shortening associated with climate (Dupoué et al., 2017).

Furthermore, climate warming may result in heat stress in organisms, and consequently, induce oxidative stress resulting from an imbalance between the production of pro-oxidant substances and antioxidant defenses (Halliwell, 2007). Oxidative stress can be induced by a wide range of environmental stressors, including extreme temperatures (Voituron et al., 2006). Therefore, higher environmental temperatures caused by global warming could induce oxidative stress. The pro-oxidant substances produced could react with molecular components of cells, such as lipids, proteins, and nucleic acids, and lead to damage of cell machinery (Halliwell, 2007). Simultaneously, antioxidant defenses can maintain reactive oxygen species (ROS) levels at equilibrium and minimize ROS-induced damage to

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protect the cell from oxidative damage (Blokhina et al., 2003). However, recent studies have shown that a temperature challenge can lead to oxidative stress and therefore affect the survival of lizard populations in the field (Dupoué et al., 2019). For example, rising temperatures across altitudinal gradients induced oxidative stress populations of lizards under natural conditions (Reguera et al., 2014, 2015; Reguera et al., 2015; Reguera et al., 2014). Supercooling triggered the activation of the antioxidant system in the common lizard (*Lacerta vivipara*) (Voituron et al., 2006). However, we still know little about how climate warming could affect oxidative stress in lizards.

Climate warming could also affect the immune system because some immune responses rely on the production of pro-oxidant substances and enhance oxidative stress (Jyoti et al., 2017; Sorci and Faivre, 2009), which is essential to the survival of individuals (Lafferty, 2009; Travers et al., 2009). Similar to other vertebrates, all reptiles possess innate and acquired immune responses (Zimmerman et al., 2017). The innate component is the nonspecific, initial response to pathogens, such as that mounted by white blood cells (also called leukocytes and abbreviated as WBCs). This response may reflect changes in both the specific and nonspecific immune system and can be altered by stress (Espelid et al., 1996). The adaptive component serves as the second line of defense, includes both cell-mediated and humoral responses, and is mounted via T cells, B cells, and many sorts of immunoglobulins (Igs). Studies suggest that reptiles rely more heavily upon their innate immune response, and then follow with a more moderate adaptive response (Zimmerman et al., 2010). Immunoglobulin M (IgM), which is found in all jawed vertebrates, is the first Ig produced in response to an infection. It exists in the serum in a pentameric form, and is highly effective at activating complement (Coico et al., 2003; Zimmerman et al., 2010). The immune system is sensitive to temperature changes in ectotherms. For instance, thermal stress caused by global warming likely induces cascading effects of the immune response, and thus, further reduces the individual fitness (Lafferty, 2009; Travers et al., 2009). Heat waves have reportedly resulted in decreased immunocompetence in some ectotherms, including snails, three-spined sticklebacks (*Gasterosteus aculeatus*), and mesograzers (*Idotea baltica*) (Dittmar et al., 2014; Roth et al., 2010; Seppälä and Jokela, 2011). In contrast, heat waves had no effect on the immune performance of corn snakes (*Pantherophis guttatus*) (Stahlschmidt et al., 2017). Accordingly, more data are needed from a wide range of species to reach a general conclusion regarding the effect of temperature change on the immune system of ectotherms.

Our current understanding of how reptile immunity may respond to climate warming comes mainly from laboratory experiments that used temperature manipulation (Dang et al., 2015; Sacchi et al., 2017). Although the experimental conditions of laboratory warming experiments can be strictly controlled (Viney et al., 2005), these laboratory experiments lack ecological realism and complexity. In the present study, we conducted a warming experiment on a viviparous lizard (*Eremias multiocellata*) from May to September using open-top chambers set up in their natural habitat (a desert steppe in Inner Mongolia). Given the significant effect of high temperatures on oxidative stress and immunocompetence revealed in laboratory experiments and field studies (Dittmar et al., 2014; Reguera et al., 2014; Roth et al., 2010; Seppälä and Jokela, 2011). In this study, we will test unique mechanisms that may contribute to the risk of extinction of lizard populations and species, and we predicted that exposure to warming conditions in outdoor open-top chambers could induce oxidative stress and immunosuppression.

2. Material and methods

2.1. Study site and species

Our study was conducted at the Shierliancheng Field Observation Station, Institute of Grassland Research of the Chinese Academy of Agricultural Sciences (40.2°N, 111.1°E; elevation 1036 m above sea

level). This area is an arid zone with an average annual precipitation of 300–380 mm. The mean monthly temperature reaches its maximum in July (39.1 °C) and minimum in January (-32.8 °C) (Li et al., 2017).

The multiocellated racerunner (*E. multiocellata*) is a viviparous lacertid lizard (adult snout-vent length [SVL] 54.0–67.3 mm, body mass [BM] 4.7–8.2 g) whose natural habitat is the desert steppe in Inner Mongolia, China, which is mainly covered by the shrub *Artemisia ordosica*. The mean selected body temperature (T_{sel}) of *E. multiocellata* is 35.2 ± 0.2 °C (\pm standard error of the mean), ranging from 33.4 ± 0.2 °C to 36.8 ± 0.1 °C; the critical thermal maximum (CT_{max}) is 45.1 ± 0.1 °C (Li et al., 2017).

2.2. Experimental design and thermal treatments

We set up six circular enclosures (diameter 1.5 m, height 0.7 m) in our study site. These enclosures were constructed using iron sheeting in the natural habitat of the lizard. The vegetation in the enclosures was maintained intact. The types and the coverage of vegetation in each enclosure were similar. The vegetation was used as shelter for the lizards. There was no variation in prey availability existed among enclosures. To evaluate the influence of climate warming on lizards, we designed three of the six enclosures as warming treatments and the other three as the control group. The warming enclosures were covered with a transparent plastic cloth to facilitate an increase in temperature. A circular hole with a diameter of 0.5 m was made at the top of the enclosure forming an open-top chamber into which rain could go. Both the warming and control enclosures were covered with fish nets to avoid avian predation. The mean temperature difference between warming and control enclosures was designed to be 3 °C.

This experiment proceeded for approximately 5 months (from the beginning of May to the end of September). At the beginning of May, we collected 60 adult lizards locally and randomly assigned them to the six enclosures with 10 lizards in each enclosure (M:F = 4:6). The sex ratio was similar to that of the lizards in the field (Han, per. obs.). For all individuals, SVL was measured to the nearest 0.01 mm, and BM was assessed to the nearest 0.001 g using a Vernier caliper (Kanon Instruments, Japan) and an electronic balance (Mettler-Toledo GmbH, Greifensee, Switzerland), respectively. Each enclosure was checked every 2 days. Food (crickets, *Acheta domestica*, dusted with mixed vitamins and minerals) and water were provided *ad libitum*. We recorded ambient temperatures (air temperatures at 50 cm above the ground) and humidity every hour using HOBO data loggers (HOBO U12-012, Onset Computer Corporation, Bourne, MA, USA). At the end of September, we recaptured the lizards in enclosures to calculate the survival rate. A total of 18 lizards (3 from each enclosure, M: F = 1:1) were randomly selected and euthanized with an injection of sodium pentobarbital to determine oxidative stress levels and immunocompetence; the measurements were made simultaneously for the test subjects. We collected the blood and livers from the euthanized lizards. After the livers were washed with ice-cold saline solution to remove residual blood, they were snap-frozen in liquid nitrogen and immediately stored at -80 °C until subsequent assays.

2.3. MDA and antioxidant assay

Malondialdehyde (MDA) is one of the final products of lipid peroxidation as a result of cell damage induced by ROS (Del Rio et al., 2005). Superoxide dismutase (SOD) catalyzes the dismutation of superoxide radicals to H_2O_2 and therefore plays a central role in antioxidant defenses (Ames, 1983). MDA levels and SOD activity are commonly used as indicators of oxidative damage and antioxidant status, respectively (Ames, 1983; Azad et al., 2010; Gawel et al., 2004; Voituron et al., 2006; Xu et al., 2014). MDA content was assessed in livers using a TBARS assay kit (Nanjing Jiancheng, Nanjing, China) following the manufacturer's instructions. The absorbance of the eluent was monitored spectrophotometrically at 532 nm (Bio Tek SynergyTM 4 Hybrid Microplate

Reader; Bio Tek, Vermont, USA). Lipid peroxidation was expressed as nanomoles of MDA per milligram of protein and MDA content (nmol/mg) = $(OD_{\text{Measured}} - OD_{\text{Control}}) / (OD_{\text{Standard}} - OD_{\text{Blank}}) \times \text{Standard concentration} \div \text{Sample protein concentration}$.

To evaluate the antioxidant capacity of the lizards, we measured the SOD activity of livers using specific kits (SOD Assay Kit-WST; Nanjing Jiancheng, Nanjing, China) according to the manufacturer's instructions. One unit of SOD was defined as the amount of enzyme that caused 50% inhibition of the superoxide radical produced by the reaction between xanthine and xanthine oxidase at 37 °C. SOD activity = SOD inhibition % / (1 - SOD inhibition %) unit, in which SOD inhibition % = $(A_{1\text{Blank control}} - A_{\text{Standard or Sample}}) / (A_{1\text{Blank control}} - A_{2\text{Blank control}}) \times 100\%$, in which the absorbance (A) was read at 450 nm using a microplate reader.

2.4. Immunity measurements

To investigate the effects of high temperature on the immunity of lizards, we compared the WBC counts between the warming and the control groups. In total, 5 µl blood was collected from the lizards and diluted 40 times with acetic acid in a clean test tube, which was shaken well. Cell counts were performed using a Neubauer's counting chamber.

Furthermore, target genes were chosen as representatives of acquired humoral immunity (IgM). Total RNA was extracted from 10 µl blood leukocytes using the TRIZOL reagent (Invitrogen, USA) in accordance with the manufacturer's protocol. RNA quantification was performed using an ND-1000-Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Then 2 µg of total RNA was used for first-strand cDNA synthesis using M-MLV Reverse Transcriptase (Promega, USA). Quantitative PCR was performed using the SYBR Select Master Mix (Takara, Dalian, China) with an ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). The primer information is listed in Table 1. The reference gene was 18S rRNA. The total volume was 10 µl (9 µl of master mix + 1 µl of cDNA). The master mix contained 2 µl of each primer and 5 µl of Applied Biosystems SYBR Green PCR Master Mix. PCR conditions for IgM and 18S were 5 min at 95 °C, followed by 40 cycles for 15 s at 95 °C, 15 s at 60 °C, and 15 s at 72 °C. Both reactions ended with a dissociation program of 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C.

2.5. Statistical analysis

We tested the normality of distributions and homogeneity of all variances in the data using the Shapiro-Wilks test and Levene's tests before the analysis, respectively. The data were all square-root transformed. We used the Kruskal-Wallis tests for traits that still violated the normality of distribution and homogeneity of variance assumptions after transformation. No significant sex or sex × temperature effects were present for any variables assessed during this study (all $P > 0.05$). Therefore, male and female samples were pooled in subsequent analyses. One-way ANOVAs, with the thermal treatments as fixed factors, were used to determine the effect of the increasing temperature in the enclosures and how the thermal treatments influenced the SVL, BM, SOD activity, MDA content, WBC, and IgM expression of the lizards. We used the Kruskal-Wallis tests for traits that violated the normality of distribution and homogeneity of variance assumptions. Pearson's chi-squared test was used to compare between-treatment differences in the survival rate of lizards. The clutch sizes were not statistically different among

enclosures. We randomly rearranged the lizards after each recapture to average out the differences of the thermal treatment and other conditions among enclosures. Therefore, the random effect of the enclosure was largely controlled. The data are presented as mean ± standard error of the mean (SEM), except for survival. The results were considered statistically significant at $P < 0.05$. All of the analyses were performed using SPSS ver. 13.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Temperature and humidity regimes in outdoor enclosures

The temperature regimes in outdoor enclosures were showed in Table 2 and Fig. 1. The relative humidity (RH) in the warming group was $58.7 \pm 4.81\%$, and in the control group was $57.0 \pm 4.64\%$. The difference between two groups was significant ($t = -5.259$, $df = 23$, $P = 0.000$).

3.2. Oxidative stress and immunity

Liver MDA content was significantly higher in the warming group than that of the control group ($Z = -2.340$, $P = 0.019$) for *E. multiocellata* (Fig. 2A). However, SOD activity did not differ between the warming and control groups ($F_{1,16} = 1.168$, $P = 0.296$, Fig. 2B). Both the WBC concentration and IgM expression were significantly lower in the warming group than in the control group (WBC: $F_{1,16} = 12.566$, $P = 0.003$; IgM: $Z = -2.557$, $P = 0.011$; Fig. 3A, Fig. 3B).

3.3. Body size and survival rates

At the end of the experiment, we did not observe any significant difference in SVL ($t = -1.453$ $df = 44$, $P = 0.153$) or BM ($Z = -0.264$, $P = 0.792$). The survival rate of the warming group (61.9%) was lower than that of the control group (71.4%), but this difference was not statistically significant ($\chi^2 = 0.429$, $P = 0.513$).

4. Discussion

Our outdoor warming treatments did not lead to a decreased survival rate or growth difference of the viviparous lacertid lizard, *E. multiocellata*, but did induce oxidative stress and immunosuppression in this species. This suggests that temperature rising under global warming could influence the oxidative stress and immune system of

Table 2
Thermal regimes of the warming and control outdoor enclosures.

		Control group (°C)	Warming group(°C)	Statistics
Whole day (0:00–24:00)	mean	26.37±2.02	29.28 ± 2.66	$Z = -0.845$, $P = 0.398$
	maximum	36.88 ± 2.36	41.68 ± 3.43	$Z = -0.949$, $P = 0.343$
	minimum	8.33 ± 1.30	9.41 ± 1.19	$Z = -1.113$, $P = 0.265$
Day time (8:00–18:00)	mean	36.21±0.96	42.36 ±1.41	$F_{1,20} = 12.931$, $P = 0.002$
	maximum	48.18 ±1.19	58.32 ±1.78	$F_{1,20} = 22.442$, $P = 0.000$
	minimum	14.79 ±0.20	15.12±0.21	$F_{1,20} = 1.340$, $P = 0.261$
Night time (19:00–7:00)	mean	18.05 ±1.06	18.22±1.18	$F_{1,24} = 0.012$, $P = 0.915$
	maximum	27.33 ±1.44	27.60 ±1.81	$F_{1,24} = 0.014$, $P = 0.907$
	minimum	2.86 ±0.72	4.57 ±0.84	$F_{1,24} = 2.376$, $P = 0.136$

Table 1
Primer information for IgM and 18S rRNA genes.

Primer	Sequence (5'-3')
IgMF	TCCAACAACAAGAAGTCCG
IgMR	CCATTGTAGGTGAAGCCAGAG
18S rRNAF	ACTCAACACGGGAAACCTCA
18S rRNAR	AACCAGACAAATCGC TCCAC

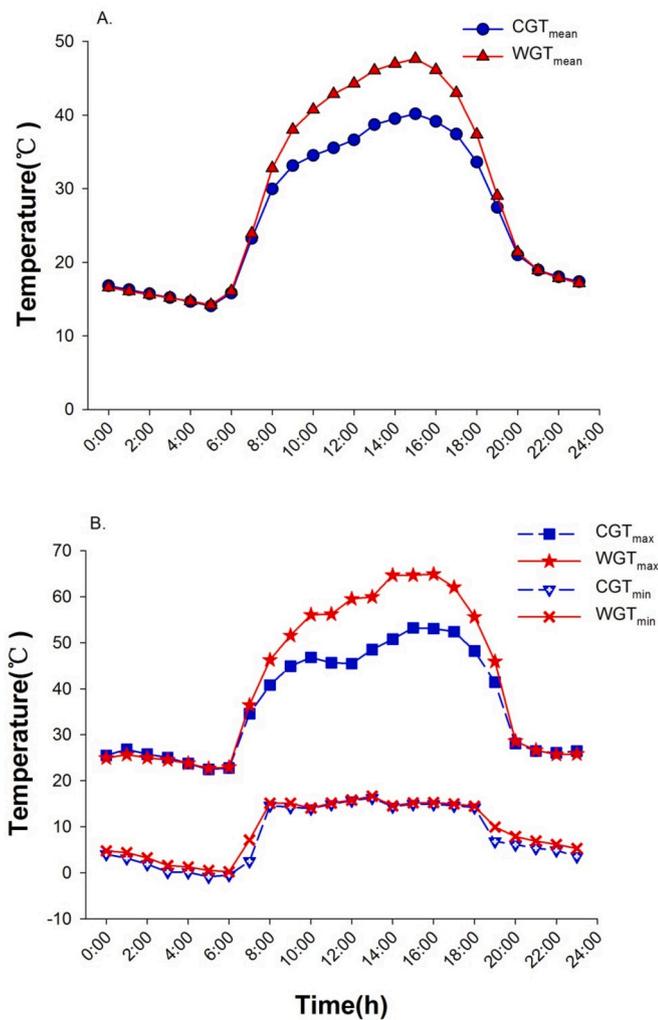


Fig. 1. Thermal regimes of the warming and control outdoor enclosures. CGT_{mean} and WGT_{mean} represent the mean temperature in the control and warming groups, respectively (A). CGT_{max} and WGT_{max} represent the maximum temperatures in the control and warming groups, and CGT_{min} and WGT_{min} represent the minimum temperature in the control and warming groups, respectively (B).

ectothermic species. Nonetheless, our study could not completely exclude the potential effect of humidity differences between the thermal treatments, although this difference was small (1.7% RH) and likely could cause only minor effects.

In our study, higher MDA content in the warming group suggested that climate warming could induce oxidative damage in lizards. Similar results have also been observed in other ectotherms, such as some fish (*Dicentrarchus labrax*) (Vinagre et al., 2012) and bivalves (Alberts et al., 2002). Additionally, Reguera et al. (2014, 2015) reported that in the lizard, *Psammodromus algirus*, oxidative stress was higher under higher temperatures when examined across an altitudinal gradient. One possible explanation for this result is that temperature affects metabolism (and thus ROS production); rising temperature increases metabolism, and consequently, the production of oxidant molecules (Karmabeer et al., 2013). However, some individuals with higher metabolic rates have lower levels of ROS, such as brown trout (*Salmo trutta*) (Salin et al., 2015) and corn snakes (*Pantherophis guttatus*) (Stahlschmidt et al., 2017). This inconsistent pattern indicates that climate warming will likely have complex and perhaps opposite effects on oxidative stress across taxa (Stahlschmidt et al., 2017). Thus, the mechanism behind this response requires further investigation. Moreover, in our study, high temperature did not induce a significant increase

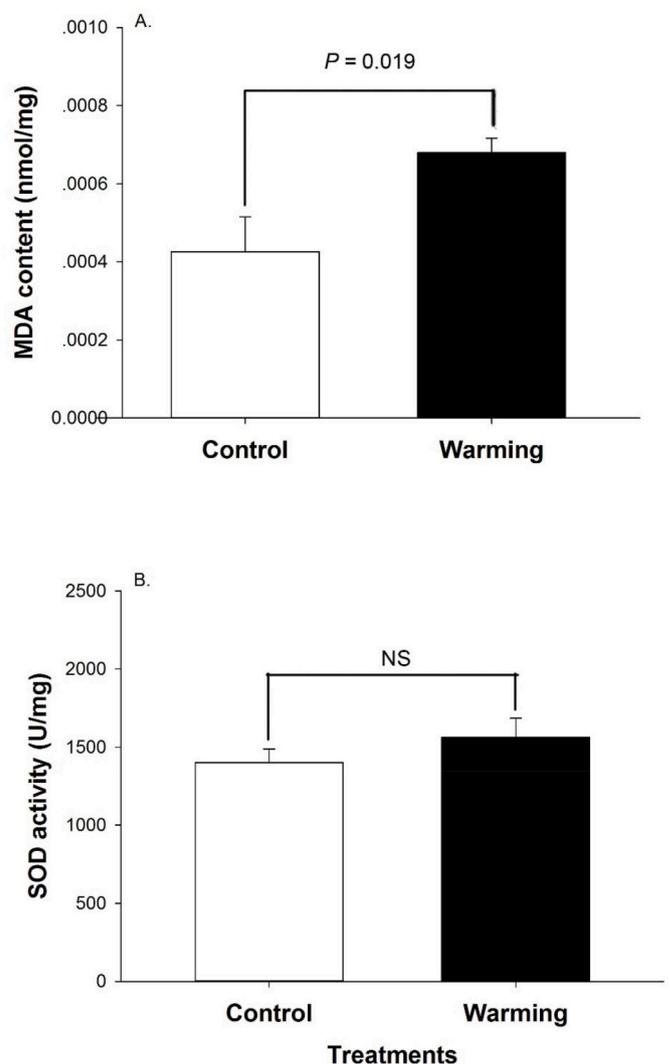


Fig. 2. Comparisons of liver malonaldehyde (MDA) content and superoxide dismutase (SOD) activity between two thermal treatment groups in the multi-ocellated racerunner (*Eremias multiocellata*). MDA content was significantly higher in the warming group than in the control group (A). SOD activity did not differ between the warming and control groups (B). Values are means \pm standard error of the mean (SEM).

in SOD activity; similarly, high temperature treatment in the laboratory did not affect SOD activity in the desert toad-headed agama (*Phrynocephalus przewalskii*) (Zhang et al., 2018) and the lizard, *Psammodromus algirus* (Reguera et al., 2014). A possible reason is that an increased SOD activity response may occur in the first few days after exposure to sustained stress, but later this response gradually decreases (Mober and Mench, 2000). Our warming experiment lasted for approximately 4 months (from May to September). SOD activity possibly decreased when we collected the tissue (at the end of September). Further studies are needed to examine oxidative damage using more indicators, including protein carbonylation and DNA damage [i.e. 8-hydroxy-2-deoxy Guanosine (8-OH-dG)]. Additionally, future studies should determine whether climate warming induces antioxidant availability (including several additional enzymatic antioxidants, such as catalase and glutathione peroxidase, and non-enzymatic antioxidants, such as vitamin E) in various tissues.

Our results revealed that high temperatures elicited immunosuppression in lizards, as indicated by the decrease in WBC counts and IgM expression. This negative effect of high temperature on the immune response is consistent with that observed in other lizards species (Sacchi

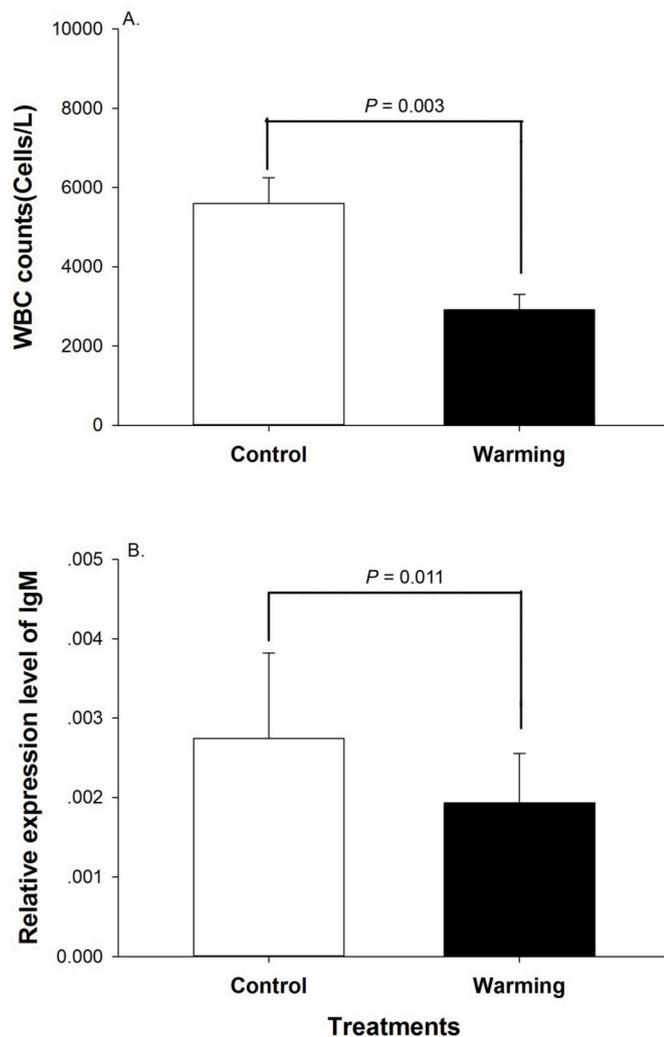


Fig. 3. Comparisons of blood white cell (WBC) counts and immunoglobulin M (IgM) expression between different thermal treatment groups in the multi-ocellated racerunner (*Eremias multiocellata*). The white cell counts (A) and IgM expression (B) were significantly lower in the warming group than in the control group. Values are means \pm standard error of the mean (SEM).

et al., 2017) and squamate reptiles (Butler et al., 2013; Stahlschmidt et al., 2017). Low temperature facilitates an adaptive response in regards to tick infestations in the western fence lizard (*Sceloporus occidntalis*) (Megía-Palma et al., 2020). Warmer temperatures also lead to a decline in some immune functions of insects, such as the cricket (*Gryllus texensis*) (Adamo and Lovett, 2011). In addition, heat stress can affect passively acquired and antibody-mediated immunity and decrease immune capability by reducing the number of peripheral blood leukocytes (Kelley, 1980). The mechanism behind the effects of increasing temperature on the immune system involves the energy available to it. High temperature may increase the energy demands of metabolism, and consequently, reduce the energy available for immunity when other activities are more important for total well-being (Segerstrom, 2007; Yang et al., 2013). In contrast, some studies have suggested that high temperature enhances immunity, such as in the red-eared slider turtle (*Trachemys scripta*) (Engelsma et al., 2003; Houston et al., 1996; Martins et al., 2011; Zimmerman et al., 2017) and that low temperature causes immunosuppression in ectotherms (Martins et al., 2011). Moreover, temperature oscillation also causes increased susceptibility to infection (Raffel et al., 2006). Therefore, the immune activity of ectothermic species is likely differentially affected by increased temperature, because the adaptive range varies across species (Zimmerman et al.,

2017).

Our results revealed that high temperature induced oxidative stress and led to low immune functions. However, the interaction between oxidative stress and immune capability was not addressed in the present study. Several studies have shown the interdependency of oxidative stress and the immune system because the immune defense mechanism uses the lethal effects of oxidants in a beneficial manner with ROS playing a pivotal role in killing pathogens. Simultaneously, the use of these oxygen radicals in the cytotoxic response of the immune system also leads to increased oxidative stress and injury to host tissues (Cannizzo et al., 2011; Rahal et al., 2014). For example, the immune response is accompanied by oxidative damage in many animal taxa, including reptiles (Tobler et al., 2015). There is a positive correlation between the indicators of oxidative state (levels of ROS) and immune resistance (Stahlschmidt et al., 2015). However, the causal relationship between oxidative stress and immune function is unclear. Some studies have indicated that oxidative stress might be a major underlying cause of inflammatory and immune dysfunction (Cannizzo et al., 2011; Sordillo and Aitken, 2009) because oxidative stress decreases the function of immune cells and contributes to the chronic inflammatory process (Cannizzo et al., 2011; Rahal et al., 2014). Conversely, some studies have shown that oxidative stress is a consequence of the immune response because part of the immune response relies on immune cells that kill pathogens by releasing pro-oxidant compounds (Costantini and Møller, 2009; Sadd and Siva-Jothy, 2006). Therefore, it will be of great interest to study the interaction between oxidative stress and immunity response in the future.

In conclusion, we used warming experiments in the field to demonstrate that increasing temperature, mimicking global warming, induced oxidative stress and immunosuppression in a viviparous lizard (*E. multiocellata*). Our results provide an interesting connection between global warming and oxidative stress and immunology. Future studies should focus on understanding the interaction between oxidative stress and the immune system. Such information will help to predict the evolutionary potential of reptiles under the globe climate warming.

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Declaration of competing interest

The authors declare no conflicts of interest.

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