Mild spring temperature rising affects the anti-oxidation and immune functions of Asian Short-toed Larks

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Abstract
Background: Predicting the possibility of severe effects of global warming on animals is important for understanding the ecological consequences of climate change on ecosystem. Spring is the season during which birds have to physiologically prepare for the subsequent breeding period, and unusual spring temperature rising probably becomes a heat stress to the birds which have adapted to the low spring temperature. Therefore, it is necessary to understand the physiological effect of spring warming on the temperate birds.

Methods: Using the activities of blood anti-oxidative enzymes (SOD, CAT, GPx) and the concentrations of serum immunogloblins (IgA, IgY, IgM) as indicators, we compared the anti-oxidative and immune functions of Asian Short-toed Larks (Calandrella cheleensis) captured between 10 and 15 March, 2015 and housed under conditions of 21 °C and 16 °C.

Results: The SOD activities of birds in 21 °C group were significantly lower than those in 16 °C group on all the treatment days. The CAT activities of the birds in 21 °C group were significantly lower than those in 16 °C group on the 1st, 5th, 13th, 17 treatment days. The GPx activities of the birds in 21 °C group were significticantly lower than those in 16 °C group on the 1st, 13th and 17th, but significantly higher on the 21st treatment day. The IgA, IgY and IgM concentrations of birds in 21 °C group were significantly lower than those in 16 °C group on all the treatment days.

Conclusions: This study shows that spring temperature rising negatively influences antioxibative and humoral immune functions, which indicates that spring climate warming might reduce the fitness of the temperate passerine birds which have adapted to the low spring temperature.

Keywords: Mild temperature rising, Anti-oxidant capacity, Immunity, Asian Short-toed Larks, Spring

Background
Although physiological responses of ectotherms to rising global temperature have received great amount of attention by biogeographers and physiologists (Somero 2010; Tomanek 2010; Folguera et al. 2011), such responses of endotherms have not been fully understood. Heat-related deaths of birds have been reported in Australia, India, South Africa and the southwestern USA (Marshall and Serventy 1962; Erasmus et al. 2002; Welbergen et al. 2008; McKechnie et al. 2012). Data on southern African desert birds revealed that a suite of physiological variables change rapidly with increasing air temperatures within the comparatively narrow range of 30–40 °C, far below those typically associated with mortality events (McKechnie et al. 2012). Therefore, predicting the possibility of severe effects of global warming on birds is necessary.

Birds that live at middle and high latitudes experience different temperatures in different seasons, therefore
many physiological processes such as energy metabolism, stress response, and reproduction in birds significantly change with environmental temperature in different seasons (Wingfield et al. 1982; Silverin et al. 2008; Swanson 2010; Zheng et al. 2014). Spring is the season during which birds have to physiologically prepare for the subsequent breeding period (Stevenson and Bryant 2000; Nilsson and Raberg 2001; Goutte et al. 2010; Hegemann et al. 2012), hence the ability to maintain homeostasis during this period is important to survival and reproduction of birds. Unusual spring temperature rising probably becomes a heat stress to the birds which have adapted to low spring temperature. Therefore, it is necessary to understand the physiological effect of spring warming on the temperate birds.

High temperature has been found to induce increases in the production of reactive oxygen species (ROS) (Flanagan et al. 1998; Mujahid et al. 2005; Lin et al. 2008; Costantini et al. 2012) and thereby induce oxidative stress (Costantini and Verhulst 2009; Azad et al. 2010), which can cause cell damage even to apoptosis (e.g. Kannan and Jain 2000). The vertebrate cells can eliminate ROS through the activation of antioxidant system such as anti-oxidant enzymes to avoid the damage to the cells (Baxter et al. 2014; Huang et al. 2015). Therefore, anti-oxidation function can reflect the survival ability of birds. In addition, immunal function is another important indicator for survivability of birds. It has been well understood that many environmental stressors especially temperature variation have inhibitory effect on the B-lymphocyte-mediated humoral immunity through hypothalamic–pituitary–adrenal cortex axis (HPA) (Shephard 1998; Sapolsky et al. 2000; Quintana et al. 2011; Habibian et al. 2014; Yang et al. 2015), which is related with the survival of the birds. It has been found in wild birds and chicken that heat stress may reduce the immune function of birds by inhibiting the production of immunoglobulin (Zulkiﬂi et al. 1994; Park et al. 2013). Although there are lots of information about the effects of environment stressors on the antioxidation and immune system such as the effects of spring climate warming on these two functions of wild temperate birds have not been well understood. Therefore, it is necessary to evaluate these effects on the temperate wild birds in order to understand the physiological mechanism of the climate change effects on survival of birds.

Whether spring warming affects the antioxidation and immune function of wild temperate birds? To answer this question, we studied antioxidation and immune function in Asian Short-toed Larks (Calandrella cheleensis) distributed in the high-latitude grassland of Inner Mongolia. Asian Short-toed Lark is a resident bird species on the high latitude grassland of China, which initiates breeding in early spring. The species has adapted to the low spring temperature and is vulnerable to the heat stress induced by unusual spring temperature rising (Zhao et al. 2017a). Therefore, we selected this species as a model of this study. We compared activities of anti-oxidative enzymes including super oxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and levels of immunoglobulin IgA, IgY and IgM, in samples of peripheral blood cells of captured Asian Short-toed Larks in normal and higher ambient temperature condition by conducting a controlled laboratory experiment.

Methods
Study site and species
The study site was located within the Hulun Lake National Nature Reserve (47°45′50″N–49°20′20″N; 116°50′10″E–118°10′10″E) situated in the northeastern part of the Inner Mongolian Autonomous Region, China. This reserve is a semiarid, steppe region where the mean annual temperature, precipitation and potential evaporation are −0.6 °C, 283 mm and 1754 mm, respectively. Winter is longer than summer, and the average maximum daytime temperatures in January and July are −20.02 °C and 22.72 °C, respectively. Spring is in March and April. The Asian Short-toed Lark (Calandrella cheleensis, Passeriformes, Alaudidae) is the most common lark species on the grasslands of the study site. The birds used in this study were captured in the study site between 10 and 15 March, 2015.

Experiment design
Two air-conditioned, temperature-controlled chambers were built at the study site. Forty adult Asian Short-toed Larks were randomly assigned to these chambers, with 20 birds to each chamber (sex ratio 1:1). All birds were housed in individual cages (50 cm × 40 cm × 35 cm) within each temperature chamber, and they were fed mixed seeds, boiled eggs and mealworms, and provided with water ad libitum. Considering the physiological status of birds could be influenced by captivity (Li et al. 2019), we initially kept both chambers at 16 °C under a 16:8-h light:dark photoperiod for 10 days to allow the birds to acclimatize. At the end of this 10-day period we increased the temperature of one chamber to 21 °C, while the temperature of the other was kept at 16 °C. This temperature treatment regime was continued for 21 days. The choice of 16 °C as the lower temperature was based on the mean daily maximum temperature recorded at the study site in April 2014. As the mean daily temperature difference between sample days during the field experiment period was about 5 °C, we chose 21 °C as the higher temperature. At least 50 μL of whole blood was collected from each bird at 4-day intervals at 12:00–12:30 h over
the experimental period to measure the levels of anti-oxidation enzymes CAT, SOD, GPx and immunoglobulins IgA, IgY, IgM.

**Anti-oxidation enzyme analysis**

The activities of three enzymes SOD, CAT and GPx were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). SOD activity was measured using the xanthine/xanthine oxidase method based on the production of $O_2^{-}$ anions. GPx activity was measured based on its catalyzation by the oxidation of reduced glutathione in the presence of cumene hydroperoxide. The generation of nicotinamide adenine dinucleotide phosphate was measured spectrophotometrically at 340 nm. CAT activity was measured by analyzing the rate at which it caused the decomposition of $H_2O_2$ at 240 nm.

**Immunoglobulin analysis**

IgA, IgY and IgM concentrations were determined using chicken enzyme immunoassay (ELISA) kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Briefly, all serum samples were 1:5 diluted (10 μL of the sample and 40 μL of the sample dilution) and added to sample wells in triplicates. The standard wells and the sample wells were added with 100 μL of detection antibody which were marked by horseradish peroxidase (HRP) and incubated for 60 min at 37 °C. Then the plates were washed five times with PBS. Some 50 μL of substrate A and B were added to plates and incubated for 15 min at 37 °C. Finally, 50 μL of stop solution was added to each well, and the OD value of each well at a wavelength of 450 nm was measured within 15 min by microplate reader (Thermo company, USA). The respective inter- and intra-plate coefficients of variation for IgA, IgY and IgM were < 12%.

**Data analysis**

We used linear mixed models (LMMs) to analyze the effects of temperature, sex, body mass and their interactions on plasma SOD, CAT, GPx activities and IgA, IgY, and IgM concentrations. Temperature, sex, body mass, and the interactions between these factors were modeled as fixed factors, with individual as a random factor. All data were log transformed to correct for departures from normality and homogeneity of variance. All data analyses were performed using SPSS version 18.0 and $\alpha = 0.05$ in all tests.

**Results**

**The activities of plasma SOD, CAT and GPx in Asian Short-toed Larks**

The LMM results indicated that temperature significantly influenced the blood SOD, CAT and GPx activities of Asian Short-toed Larks (Table 1). The SOD activity of birds in 21 °C group was significantly lower than that in 16 °C group on all the treatment days (Independent sample $t$ test, $n = 20$, $P < 0.05$, Fig. 1a). The CAT activity of the birds in 21 °C group was significantly lower than that in 16 °C group on the 1st, 5th, 13th, 17th treatment days (Independent sample $t$-test, $n = 20$, $P < 0.05$, Fig. 1b). The GPx activity of the birds in 21 °C group was significantly lower than that in 16 °C group on the 1st, 13th and 17th, but significantly higher on the 21st treatment day (Independent sample $t$-test, $n = 20$, $P < 0.05$, Fig. 1c).

**The concentrations of serum IgA, IgY and IgM in Asian Short-toed Larks**

The LMM results indicated that temperature significantly influenced the plasma IgA, IgY and IgM concentrations of Asian Short-toed Larks (Table 2). The serum IgA, IgY and IgM concentrations of birds in 21 °C group were significantly lower than those in 16 °C group on all the treatment days (Independent $t$-test, $n = 20$, $P < 0.05$, Fig. 2).

**Table 1 Results of a linear mixed model for the effects of temperature, sex, body mass, on blood CAT, SOD, GPx activities in wild Asian Short-toed Larks (Calandrella cheleensis) captured in Hulun Lake Nature Reserve, Inner Mongolia, China**

| Response variable | Explanatory variable | $F$   | $P$   |
|-------------------|----------------------|-------|-------|
| CAT               | Temperature          | 14.481| < 0.001|
|                   | Sex                  | 1.701 | 0.196 |
|                   | Body mass            | 0.081 | 0.776 |
|                   | Temperature x sex    | 1.779 | 0.141 |
|                   | Temperature x body mass| 0.703 | 0.404 |
|                   | Sex x body mass      | 0.702 | 0.593 |
| SOD               | Temperature          | 136.056| < 0.001|
|                   | Sex                  | 3.816 | 0.057 |
|                   | Body mass            | 3.816 | 0.057 |
|                   | Temperature x sex    | 3.020 | 0.058 |
|                   | Temperature x body mass| 1.401 | 0.242 |
|                   | Sex x body mass      | 2.650 | 0.081 |
| GPx               | Temperature          | 5.992 | 0.040 |
|                   | Sex                  | 0.082 | 0.776 |
|                   | Body mass            | 0.414 | 0.523 |
|                   | Temperature x sex    | 0.055 | 0.815 |
|                   | Temperature x body mass| 1.015 | 0.370 |
|                   | Sex x body mass      | 0.178 | 0.837 |

$P$ values in italics indicate that the explanatory variables significantly influence the response variables.
The results that activities of antioxidative enzymes SOD, CAT and GPx in the blood of Asian Short-toed Larks (Calandrella cheleensis) decreased significantly at 21 °C indicate that mild temperature rising can inhibit the antioxidative function of Asian Short-toed Larks distributed in high latitude grassland which have adapted to relatively low temperature in spring. Antioxidant enzymes play a vital role in protecting cellular damage from harmful effects of ROS (Baxter et al. 2014; Huang et al. 2015) and it has been found that heat stress can increase lipid peroxidation (Altan et al. 2003; Lin et al. 2008; Altan et al. 2010), therefore reduced oxidative protection can result in increased oxidative damage and fitness costs.

In addition, Asian Short-toed Larks start to breed in early spring (April), while breeding is an energy consuming process which will produce more oxygen free radicals than non-breeding period (Wiersma et al. 2004). Moreover, the negative relationships between brood size and activity of antioxidative enzymes have been found in bird species (Alonso-Álvarez et al. 2010). Therefore, the spring temperature rising together with breeding efforts will aggravate oxidative damage on birds. Our results implicate that birds which have adapted to the low spring

### Table 2 Results of a linear mixed model for the effects of temperature, sex, body mass, on plasma IgA, IgY and IgM activities in wild Asian Short-toed Larks (Calandrella cheleensis) captured in Hulun Lake Nature Reserve, Inner Mongolia, China

| Response variable | Explanatory variable | F     | P       |
|-------------------|----------------------|-------|---------|
| IgA               | Temperature          | 10.948| <0.001  |
|                   | Sex                  | 0.009 | 0.924   |
|                   | Body mass            | 0.927 | 0.403   |
|                   | Temperature × sex    | 0.000 | 0.997   |
|                   | Temperature × body mass | 0.958 | 0.391   |
|                   | Sex × body mass      | 0.515 | 0.600   |
| IgY               | Temperature          | 40.853| <0.001  |
|                   | Sex                  | 0.635 | 0.429   |
|                   | Body mass            | 0.603 | 0.551   |
|                   | Temperature × sex    | 0.104 | 0.748   |
|                   | Temperature × body mass | 0.192 | 0.826   |
|                   | Sex × body mass      | 1.045 | 0.359   |
| IgM               | Temperature          | 17.386| <0.001  |
|                   | Sex                  | 2.471 | 0.123   |
|                   | Body mass            | 1.381 | 0.261   |
|                   | Temperature × sex    | 0.097 | 0.757   |
|                   | Temperature × body mass | 0.253 | 0.777   |
|                   | Sex × body mass      | 0.198 | 0.821   |

*P* values in italics indicate that the explanatory variables significantly influence the response variables.

## Discussion

The results that activities of antioxidative enzymes SOD, CAT and GPx in the blood of Asian Short-toed Larks decreased significantly at 21 °C indicate that mild temperature rising can inhibit the antioxidative function of Asian Short-toed Larks distributed in high latitude grassland which have adapted to relatively low temperature in spring. Antioxidant enzymes play a vital role in protecting cellular damage from harmful effects of ROS (Baxter et al. 2014; Huang et al. 2015) and it has been found that heat stress can increase lipid peroxidation (Altan et al. 2003; Lin et al. 2008; Altan et al. 2010), therefore reduced oxidative protection can result in increased oxidative damage and fitness costs.

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temperature will be susceptible to the spring climate warming. Meanwhile, we cannot neglect the result that GPx at 21 °C is significantly higher than that at 16 °C on the 21st treatment day, which implicates that the antioxidative function of the cells may recover partially after long time acclimation. A previous study on Asian Short-toed Larks showed that marked daily variations in ambient temperature in spring can activate apoptosis protein Caspase-3 expression in the cells of Asian Short-toed Larks while the Bcl-2 and HSP60 can maintain cellular homeostasis (Qin et al. 2017). Therefore, the HSPs’ protection on the cell might be related with the variation of GPx, and the mechanism should be verified in the future.

Although immunoglobulin decreasing induced by heat stress has been found in domestic chicken (Chin et al. 2013), the effects of mild temperature rising on the immunity on wild birds in spring have not been well known. Our results that the concentration of immunoglobulins IgA, IgY and IgM in the serum of Asian Short-toed Larks decreased significantly at 21 °C indicate that temperature rising can reduce the B-lymphocyte-mediated humoral immunity of Asian Short-toed Larks in spring. A wild bird study on Eurasian Tree Sparrow (Passer montanus) found that the plasma IgA level is higher in winter than in breeding season (Zhao et al. 2017b), which supports the above deduction from our results. The concentration of immunoglobulins in serum can reflect the disease resistance of the body (Peppas et al. 2019). IgA, IgY and IgM are three important immunoglobulins in birds. IgA is an important barrier of respiratory mucosa (Rose et al. 1974; Kaspers et al. 1996; Bencina et al. 2005; Bar-Shira et al. 2014). IgY is a functional homolog of mammalian IgG and has been found to efficiently opsonize pathogens for engulfment by phagocytes (Huang et al. 2016). IgM has many functions such as precipitation and agglutination (Díaz-Zaragoza et al. 2015; Atif et al. 2018; Peppas et al. 2019). Therefore, reduction of these immunoglobulins can lead to the decrease or inhibition of humoral immunity. To the wild birds, there is an trade-off between innate immunity and acquired immunity in different ecological conditions (Zhao et al. 2017b), therefore the innate immunity should be combined to evaluate the immunity vulnerability of birds to temperature in natural condition.

The immunoglobulin reduction in the birds treated by 21 °C may be related with the HSPs expression (Qin et al. 2017). The HSPs can maintain the homeostasis of the cells, overexpression of HSPs, however, is known to have deleterious consequences (Feder and Hofmann 1999). Synthesis of HSPs represents a significant energetic cost (Hamdoun et al. 2003), therefore their response usually results in a concomitant reduction in the synthesis of antibodies. Synthesizing more HSPs to mitigate stress

![Fig. 2](image)

**Fig. 2** Serum IgA (a), IgY (b) and IgM (c) concentrations of Asian Short-toed Larks in 21 °C and 16 °C treatment groups
has been found in passerine birds to be traded-off against mounting humoral and cell-mediated immune responses (Morales et al. 2006). The available results jointly indicate that mild temperature rising in spring can induce cell stress response, which could subsequently induce the immune function reduction.

Our results suggest that mild spring temperature rising can lead to the reduction of antioxidative and immune functions of temperate passerine birds. Under the climate warming scenario, discriminating the climate susceptible species is urgent (Glover 2018). The species with narrow environmental tolerances or thresholds are likely to be susceptible to the climate warming at any stage in the life cycle. It is important, therefore, to investigate physiological responses to global warming in more terrestrial vertebrates in different thermal environments to assess the potential threat of global warming-induced heat stress to biodiversity.

Conclusion

In summary, this study shows that spring temperature rising negatively influences antioxidative and humoral immune functions, which indicates that spring climate warming might reduce the fitness of the temperate passerine birds which have adapted to the low spring temperature.

Acknowledgements

We are grateful to Manquan Gui, Muren Wu, Songtao Liu in Hulun Lake National Nature Reserve for their help on the field study.

Authors’ contributions

SZ and WL conceived the study and designed the experiments. NZ, FW and TL conducted the experiments. NZ wrote the first draft of the article. SZ supervised the research and revised the draft. All authors read and approved final manuscript.

Funding

This study was supported by the National Natural Science Foundation of China (No. 31872246).

Availability of data and materials

The data used in the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Our experimental procedures complied with the current laws on animal welfare and research in China and had the approval of the Animal Research Ethics Committee of Hainan Normal University. In addition, all procedures followed standard protocols, such as the ARRIVE guidelines for reporting animal research.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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