Effects of air temperatures on antioxidant defense and immunity in Mongolian gerbils

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ABSTRACT

Temperature influences many physiological processes including antioxidant defense and immunity. The hypothesis that air temperatures has no effects on antioxidant defense and innate immunity in Mongolian gerbils (*Meriones unguiculatus*) was tested. Thirty-three male gerbils were randomly divided into the 4 °C (n = 11), 23 °C (n = 11) and 32 °C groups (n = 11), in which the treatment course lasted for 27 days. We found that air temperatures had no effects on body mass. At lower temperature, gross energy intake and the masses of most organs were higher, whereas fat free dry carcass and body fat were lower. H_2O_2 titres increased in liver but decreased in small intestine, and remained unchanged in heart, kidney and testis upon cold exposure. At lower temperature, malonaldehyde (MDA) content was higher in the liver, lower in kidneys and testis, and did not differ in the heart and small intestine. The activities of superoxide dismutase (SOD), total antioxidant capacity (T-AOC) in liver were higher in 4 °C group than 23 °C group, while liver catalase (CAT) activity was lower in the 4 °C group than in the 23 °C group. No significant difference was observed in the activities of SOD, CAT and T-AOC in the heart, kidney, testis and small intestine among the 4 °C, 23 °C and 32 °C groups. As expected, bacteria killing capacity indicating innate immunity, white blood cells and thymus mass were all not affected by air temperatures. Similarly, air temperatures had no effect on the levels of testosterone and corticosterone, both of which were not correlated with innate immunity, H_2O_2 and MDA levels, the activity of SOD, CAT, and T-AOC in all the detected tissues. In conclusion, air temperature affected antioxidant capacity, but not immune responses or serum concentrations of corticosterone and testosterone. Overall, up-regulation or maintenance of antioxidant defenses and immunity might be an important mechanism for gerbils to survive highly variable temperature.

1. Introduction

Environmental temperature is an important ecological factor influencing many physiological processes including antioxidant defense and immune responses in animals (King, 2004; Marnila and Lilius, 2015). Antioxidant defense could get rid of reactive oxygen species (ROS), and oxidative stress occurs when ROS production exceeds the antioxidant capacity (Finkel and Holbrook, 2000; Costantini, 2008; Selman et al., 2013; Hulbert et al., 2007; Dickinson and Chang, 2011). Oxidative stress could result in oxidative damage to biomolecules such as lipids, proteins and DNA and hence harms the structure and function of the cell and tissues (Burton and Jauniaux, 2011; Raut et al., 2012; Marri

and Richner, 2015). Immune system protects animals from the infection and attack of pathogens, which plays an important role in the survival and fitness (Sheldon and Verhulst, 1996; Owens and Wilson, 1999). Consequently, antioxidant defense and immunity, which is affected by air temperatures, is crucial for the health of animals (Metcalfe and Alonso-Alvarez, 2010; Carroll et al., 2012; Boonstra, 2013).

The influences of temperature on antioxidant defense have been examined in some researches. For example, the activity of superoxide dismutase (SOD) was enhanced in the heart and kidney, but suppressed in the lungs or pancreas in rats under cold exposure (Yuksel et al., 2008; Vasilijević et al., 2007). Lipid peroxidation increased in the brain while decreased in the liver in rats in cold temperature (Lomakina, 1980).

Additionally, protein oxidation increased in the liver and muscle, but remained stable in the brown adipose tissue (BAT) in cold-adapted short-tailed field voles (*Microtus agrestis*) (Selman et al., 2002). Compared with the warm controls, lipid peroxidation, total antioxidant capacity (T-AOC) and glutathione peroxidase activity in BAT were improved by low temperature but depressed by high temperature in striped hamsters (*Cricetulus barabensis*) (Zhou et al., 2015). Moreover, high temperature either caused oxidative damage in broiler chickens (Tan et al., 2010), or suppressed SOD activity in the testis in rats (Kanter et al., 2013).

Bacteria killing capacity, which involves that action of phagocytes, opsonizing proteins and natural antibodies against a specific pathogen, has been used to evaluate innate immunity in mammals (Demas et al., 2011; Xu et al., 2017). Immune organs (i.e., thymus and spleen) and total white blood cells (WBC) are also indirect immunological indices (Calder and Kew, 2002), and a larger spleen represents a stronger immune response (Smith and Hunt, 2004). Besides acting as energy depots, adipose tissues have recently been regarded as an important endocrine and immune organ (Ahima and Flier, 2000; Fantuzzi, 2005). Effect of temperature on immunity has been investigated in laboratory rodents such as mice (Xu et al., 1992) and rats (Kozyreva and Eliseeva, 2000, 2004). For example, the intensity of genital infection was increased in the cold-adapted mice (Belay and Woart, 2013). Similarly, WBC also increased in cold water swimmers (Lombardi et al., 2011). However cold temperature had no effect on the proliferation of lymphocytes and the ability of macrophages to bind bacterial lipopolysaccharide in golden-mantled ground squirrels (*Spermophilus lateralis*) (Maniero, 2002, 2005). Bacteria killing capacity was not affected by cold exposure in female striped hamsters (*Cricetulus barabensis*) (Xu et al., 2017), but was increased in male hamsters (Xu and Hu, 2017), indicating sex plays an important role in innate immunity in response to low temperature. However, research about the effects of air temperatures on antioxidant defense and innate immunity in combination is scarce.

Stressors including low or high air temperature usually activate the hypothalamic-pituitary-adrenal axis, and hence the secretion of stress hormones such as corticosterone increases, which is related with oxidative damage and immunity (Sapolsky et al., 2000; Kim et al., 2013). Testosterone has oxidative costs such as the increase of ROS production in light of the oxidation handicap hypothesis (Alonso-Alvarez et al., 2007, 2008; Taff and Freeman-Gallant, 2014). It can also suppress immune responses in many species including mammals and birds (Trigunaite et al., 2015).

Mongolian gerbils (*Meriones unguiculatus*), which are small seasonally breeding, non-hibernating, granivorous rodents, are distributed mainly in semi-arid, typical steppes, and desert grasslands of the southeast of the Bakal area in Russia, Mongolia, and Northern China (Zhang and Wang, 1998; Wang et al., 2003). The climate is arid and characterized by warm and dry summer (extreme maximum temperatures is 42.6 °C) and cold winter lasting for more than 5 months (the lowest temperature is −47.5 °C), indicating fluctuations in temperature is common to this species (Chen, 1998). Therefore this species is a good model to study their special adaptive strategies to environments (Xia et al., 1982; Zhang and Wang, 2007). Previous studies have shown that cold exposure had no influence on body mass, body fat mass and uncoupling protein 1 content in gerbils (Wang, 2007). Similarly, humoral immunity was not affected by a low protein diet (Chen et al., 2007), photoperiod, low temperature and housing density (Li, 2005). We also found that food restriction and refeeding did not affect cellular and humoral immunity in gerbils (Xu et al., 2011). Similarly, air temperatures had no influences on cellular immunity and innate immunity in female gerbils (Yang et al., 2013). It appears that gerbils are insensitive to the changes of the surroundings, which might be important for their survival in the highly fluctuating environment. Immune responses (Demas, 2004; Martin et al., 2003) and oxidative stress are costly (Dowling and Simmons, 2009) and life-history trade-offs might occur

(Hall et al., 2010; Martin et al., 2007; Monaghan et al., 2009). In the present study, we tested the hypothesis that air temperatures would have no effects on antioxidant defense and innate immunity in gerbils.

2. Material and methods

2.1. Animals and experimental design

Animals used in this study were the offspring of a captive colony that was trapped in Inner Mongolian grasslands in May 1999 and brought to the animal facility in the Institute of Zoology, Chinese Academy of Sciences in Beijing, China. Body mass of wild-captured animals including gerbils usually increases after laboratory raising (Stuermer et al., 2003). The reason may be due to the good raising environments in laboratory, for instance the food and water are sufficient, and there are no predators or other dangerous conditions (Stuermer et al., 2003). All animal procedures were carried out according to EU Directive 2010/63/EU for animalexperiments (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm). The present study was licensed under the Institutional Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences the Institute of Zoology, Chinese Academy of Sciences (approval number: QFNUDW2012016; approval date: approval date: 20120628). The gerbils were housed individually after weaning in plastic cages (30 cm × 15 cm × 20 cm) with sawdust as bedding under a constant photoperiod of 16 L:8D (16 h:8 h light-dark cycle) and temperature of 23 ± 1 °C. Gerbils had free access to water and commercial standard rat pellets (Beijing KeAo Feed Co.). Thirty-three male gerbils (age: 4–6 months) were randomly assigned into the cold (4 ± 1 °C) (n = 11), the warm (23 ± 1 °C) (n = 11) and the hot group (32 ± 1 °C) (n = 11) (hereafter referred to as the 4 °C, 23 °C and 32 °C groups). The treatment course lasted for 27 days.

2.2. Energy intake

Body mass was recorded every three days, and energy budgets were determined at 3-day intervals during the course of study. Food intake was measured in metabolic cages as previously described (Xu et al., 2011). Food was sufficient supply in quantity and food residues and feces were collected from each gerbil at 3-day intervals during the experimental course, and then they were separated manually after being dried at 60 °C to constant mass (Liu et al., 2003). Energy contents of the food and feces were determined by a Parr 1281 oxygen bomb calorimeter (Parr Instrument, USA). Gross energy intake (GEI) and metabolizable energy intake (MEI) were calculated according to Grodzinski and Wunder (1975) and Liu et al. (2003) as follows:

GEI (kJ/d) = dry matter intake (DMI; g/d) × energy content of food (kJ/g DMI)
MEI (kJ/d) = GEI - dry fecal mass × energy content of feces (kJ/g)/day.

2.3. Organs and body composition

At the end of the experiment, gerbils were sacrificed by means of CO_2 asphyxiation and trunk blood was collected for later measurements of WBC. Blood samples were allowed to clot for 1 h and centrifuged at 4000 rpm for 30 min at 4 °C. Sera were collected and stored in polypropylene microcentrifuge tubes at −80 °C for later assays of hormones (i.e., corticosterone and testosterone) and bacterial killing capacity. Organ mass was measured as described previously (Xu and Wang, 2010; Xu et al., 2011). In brief, after interscapular brown adipose tissue (IBAT) was removed, the visceral organs, including heart, thymus, lung, liver, spleen, kidneys, paired adrenal glands and testes, epididymis, seminal vesical and the digestive organs with contents (i.e., stomach, small intestine, caecum and colon) were dissected on ice box and

weighed (± 1 mg) quickly. The lengths of small intestine, caecum and colon were measured by extending the organ to its unstressed length along a ruler (± 1 mm). The stomach, small intestine, caecum and colon were opened and then were rinsed with saline to remove all the gut content, blotted dry on tissue paper, and weighed. Five tissues including heart, liver, kidneys, testes and small intestine were stored at −80 °C for later assays of antioxidant enzymes. The carcass was dried in an oven at 60 °C to constant mass, and then weighed again to obtain the dry mass. The difference between the wet carcass mass and dry carcass mass was the water mass of carcass. Total body fat was extracted from the dried carcass by petroleum ether extraction in a Soxhlet apparatus, and water content, body fat content were calculated as the proportion of water mass and total body fat mass divided by wet carcass mass (Xu and Wang, 2010), respectively.

2.4. Oxidative stress markers assays

Lipid peroxidation indicative of oxidative damage was measured as previously described (Yang et al., 2013). Specifically, lipid peroxidation was evaluated by quantifying MDA content (Del Rio et al., 2005) using a thiobarbituric acid reactive substances (TBARS) assay kit (Nanjing Jiancheng, Nanjing, China) following the manufacturer's instructions. The absorbance of the eluent was monitored spectrophotometrically at 532 nm (BioTek Synergy 4 Hybrid Microplate Reader, BioTek, Winooski, Vermont, United States). The intra- and inter-assay coefficients of variations for this assay were < 1.5% and < 3.32%, respectively. Lipid peroxidation was expressed as nmol MDA mg^{-1} protein.

ROS levels were measured in the tissues by determining peroxide (H_2O_2) levels (Zhou et al., 2015). H_2O_2 levels were analyzed using a commercial kit (Nanjing Jiancheng, Nanjing, China) in accordance with the manufacturer's instructions and guidelines. Levels of H_2O_2 were expressed as µmol/g protein.

2.5. Antioxidant enzymes

The activities of antioxidant enzymes, including SOD and CAT, total antioxidant capacity (T-AOC) were also determined using commercial kits (Nanjing Jiancheng, Nanjing, China) according to the manufacturer's instructions. One unit of SOD was defined as the amount of enzyme that causes 50% inhibition of superoxide radical produced by the reaction between xanthine and xanthine oxidase at 37 °C; one unit of CAT activity was defined as the decomposition of 1 µmol H_2O_2 per min; one unit of T-AOC was defined as the extent to which optical density is increased by 0.01 per milligram protein per minute (Chen et al., 2014).

2.6. Immunological parameters

WBC was determined as described previously (Xu and Wang, 2010). In brief, 20 µl whole blood was diluted immediately in 0.38 ml solution containing 1.5% glacial acetic acid, 1% crystal violet (Sigma) and the leukocytes were counted in an improved Neubauer chamber under a microscope. The total number of WBC was determined by counting all leucocytes in the four corner large-squares of the Nebauer chamber, and multiplying the number leucocytes by 5×10^7 to obtain the final values (10^9 cells/L) (Xu and Wang, 2010).

Serum bacterial killing capacity indicative of innate immunity was determined in a sterile laminar flow cabinet to assess the functional response by the animal's innate immune system against a relevant pathogen, *Escherichia coli* (Tieleman et al., 2005; Demas et al., 2011; Yang et al., 2013). Briefly, serum samples were diluted 1:20 in CO_2-independent medium (Gibco no. 18045, Carlsbad, GA, USA). A standard number of colony-forming units (CFUs) of *E. coli* (ATCC no. 8739, Microbial Culture Collection Center of Guangdong Institute of Microbiology, China) was added to each sample in a ratio of 1:10, and the mixture was allowed to incubate at 37 °C for 30 min to induce bacterial
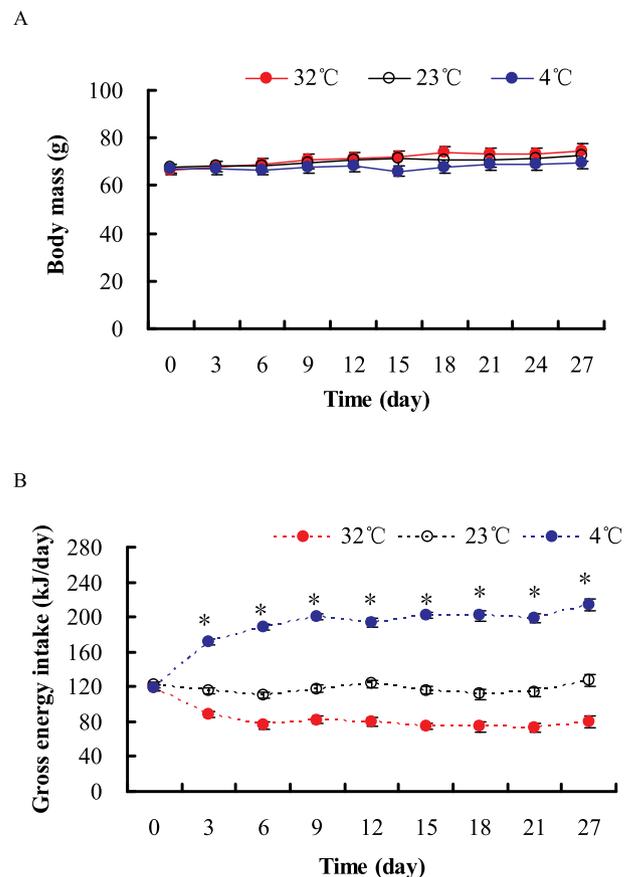

Fig. 1. Effect of air temperatures on body mass (A), and gross energy intake (B) in Mongolian gerbils acclimated to 4 °C, 23 °C and 32 °C. Data are means ± SE. *P<0.05.

killing. After incubation, 50 µl of each sample was added to tryptic soy agar plates in duplicate. All plates were covered and left to incubate upside down at 37 °C for 24 h, and then total CFUs were counted and bactericidal capacity was calculated as 100% minus the mean number of CFUs for each sample divided by the mean number of CFUs for the positive controls (containing only medium and standard bacterial solution), i.e. the percentage of bacteria killed relative to the positive control.

2.7. Serum testosterone and corticosterone assays

The levels of serum testosterone and corticosterone were determined by ELISA kits for rats (Cat. No. HR083, RapidBio Lab. Calabasas, California, USA), respectively. The range tested of testosterone was 0.13–25.6 ng/ml and its intra- and inter-assay variability were < 9.0% and 11.0%. The lowest level of corticosterone that could be detected by this assay was 1.0 nmol/L. Its inter- and intra-assay variability were < 1.1% and 7.5%, respectively. The detailed procedure followed the manufacturer's instructions of these kits.

2.8. Statistical analysis

Data were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Prior to all statistical analyses, data were examined for normality and homogeneity of variance, using Kolmogorov–Smirnov and Levene tests, respectively. The differences of body mass at any time point, the levels of MDA and H_2O_2, the activity of SOD, CAT and T-AOC, WBC and innate immunity, corticosterone and testosterone concentrations in different groups were analyzed by one-way analysis of variance (ANOVA) followed by Turkey's *post hoc* tests. The changes of GEI and
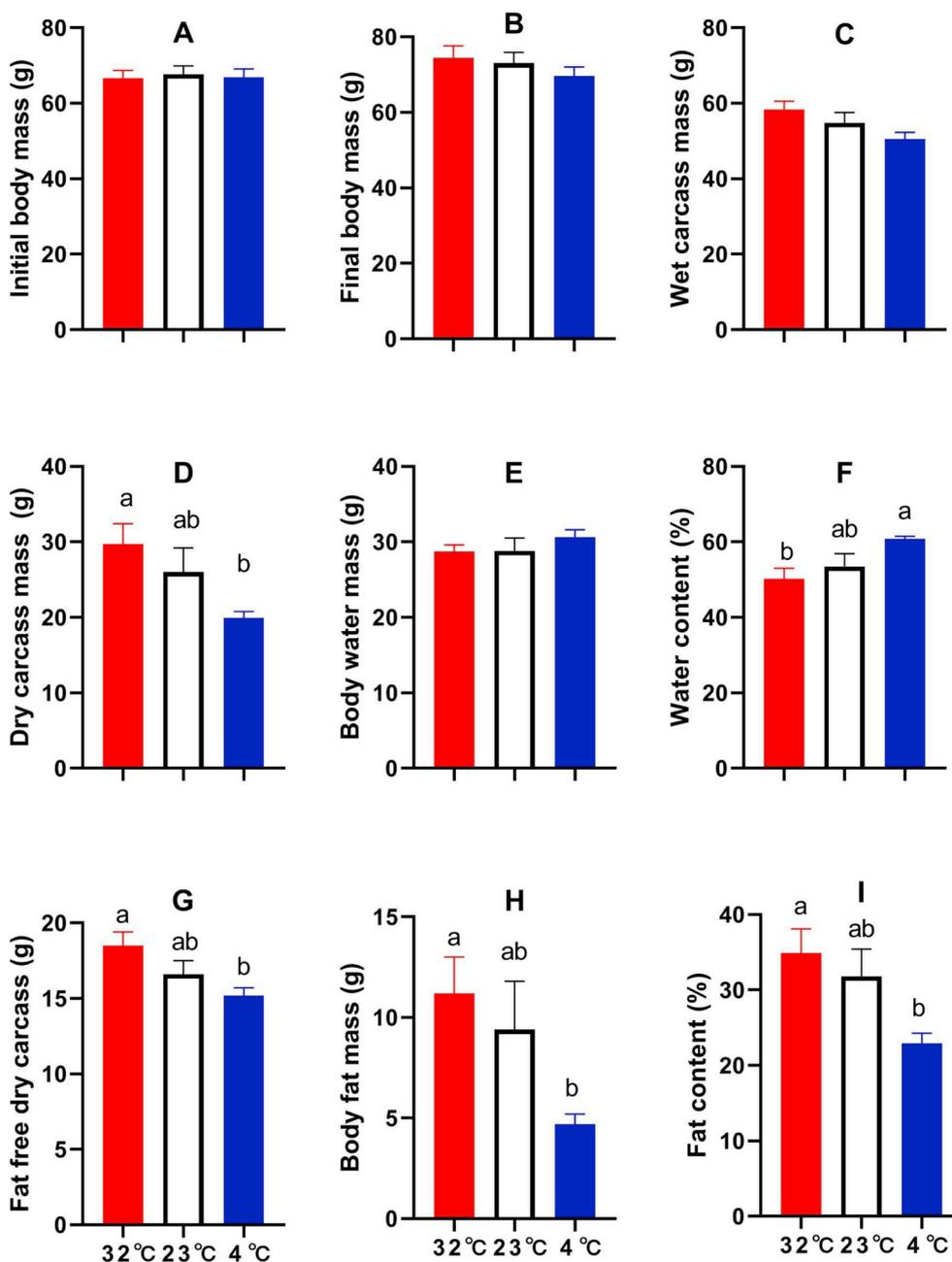
Fig. 2. Effect of air temperatures on initial body mass (A), final body mass (B), wet carcass mass (C), dry carcass mass (D), body water mass (E), water content (F), fat free dry carcass (G), body fat mass (H), and fat content (I) in Mongolian gerbils acclimated to 4 °C, 23 °C and 32 °C. Data are means ± SE. Different letters above the columns indicate significant differences at the level of P<0.05.

Table 1
Effect of air temperatures on body composition in Mongolian gerbils.

Parameters	32 °C	23 °C	4 °C	Statistical summary	
				$F_{2,30}$	P
Sample size	11	11	11		
Initial body mass (g)	66.7 ± 2.0	67.7 ± 2.2	66.8 ± 2.3	0.056	0.946
Final body mass (g)	74.5 ± 3.1	73.0 ± 2.9	69.6 ± 2.4	0.777	0.469
Wet carcass mass (g)	58.3 ± 2.3	54.8 ± 2.8	50.5 ± 1.8	2.785	0.078
Dry carcass mass (g)	29.7 ± 2.7[a]	26.0 ± 3.2[ab]	19.9 ± 0.9[b]	4.090	0.027
Body water mass (g)	28.7 ± 0.9	28.8 ± 1.7	30.6 ± 1.0	0.778	0.468
Water content (%)	50.2 ± 2.8[b]	53.5 ± 3.4[ab]	60.8 ± 0.7[a]	4.446	0.020
Fat free dry carcass (g)	18.5 ± 0.9[a]	16.6 ± 0.9[ab]	15.2 ± 0.5[b]	4.381	0.021
Body fat mass (g)	11.2 ± 1.8[a]	9.4 ± 2.4[ab]	4.7 ± 0.5[b]	3.690	0.037
Fat content (%)	34.9 ± 3.2[a]	31.8 ± 3.6[ab]	22.9 ± 1.4[b]	4.622	0.018

Values are means ± SE. Values for a specific parameter that share different superscripts are significantly different at $P < 0.05$. Body composition was analyzed by One Way ANOVA, followed by Tukey's *post hoc* tests.

Table 2
Effect of air temperatures on organ mass in Mongolian gerbils.

Parameters	32 °C	23 °C	4 °C	Statistical summary	
				$F_{2,29}$	P
Sample size	11	11	11		
IBAT (mg)	157 ± 19[b]	219 ± 18[ab]	281 ± 19[a]	9.932	0.001
Hypothalamus (mg)	18 ± 1	19 ± 1	17 ± 1	0.563	0.576
Brain (mg)	1089 ± 21	1141 ± 20	1127 ± 21	1.741	0.193
Heart (mg)	235 ± 8[c]	297 ± 7[b]	390 ± 8[a]	92.233	< 0.001
Thymus (mg)	59 ± 18	94 ± 17	58 ± 18	1.438	0.254
Lungs (mg)	451 ± 30[b]	502 ± 29[ab]	601 ± 30[a]	6.016	0.007
Liver (mg)	2562 ± 90[b]	2793 ± 86[b]	3202 ± 90[a]	11.995	< 0.001
Spleen (mg)	40 ± 4[b]	56 ± 4[a]	52 ± 4[ab]	4.765	0.016
Kidneys (mg)	519 ± 20[c]	663 ± 19[b]	811 ± 20[a]	50.453	< 0.001
Adrenal glands (mg)	43 ± 4	43 ± 4	58 ± 5	3.490	0.044
Stomach with contents (mg)	1174 ± 176	1236 ± 169	1774 ± 177	3.288	0.052
Stomach (mg)	338 ± 16[b]	383 ± 15[b]	453 ± 16[a]	12.453	< 0.001
Small intestine length (cm)	36.3 ± 0.7[c]	39.1 ± 0.7[b]	42.9 ± 0.7[a]	18.896	< 0.001
Small intestine with contents (mg)	1470 ± 92[c]	1985 ± 89[b]	2882 ± 93[a]	55.132	< 0.001
Small intestine (mg)	380 ± 52[b]	495 ± 49[b]	769 ± 52[a]	13.866	< 0.001
Caecum length (cm)	8.6 ± 0.5[b]	8.7 ± 0.5[b]	11.2 ± 0.5[a]	7.377	0.003
Caecum with contents (mg)	1356 ± 122[b]	1526 ± 117[b]	2180 ± 122[a]	11.879	< 0.001
Caecum (mg)	315 ± 16[b]	324 ± 15[b]	493 ± 16[a]	37.392	< 0.001
Colon length (cm)	13.0 ± 0.5	13.9 ± 0.5	14.6 ± 0.5	2.211	0.128
Colon with contents (mg)	757 ± 122	791 ± 117	1035 ± 122	1.452	0.251
Colon (mg)	193 ± 22	254 ± 21	239 ± 22	2.113	0.139
Total digestive tract (mg)	1225 ± 67[b]	1456 ± 64[b]	1954 ± 67[a]	28.606	< 0.001
Total digestive tract length (cm)	57.9 ± 1.2[b]	61.7 ± 1.1[b]	68.6 ± 1.2[a]	19.384	< 0.001
Testes (mg)	723 ± 45	724 ± 43	655 ± 45	0.756	0.478
Epididymis (mg)	104 ± 17	131 ± 17	104 ± 17	0.836	0.444
Seminal vesical (mg)	163 ± 36	124 ± 35	70 ± 37	1.514	0.237

Values are means ± SE. Values for a specific parameter that share different superscripts are significantly different at $P < 0.05$, determined by General Linear Model multivariate analysis followed by Bonferroni *post hoc* tests with body mass as the covariate.

A

B
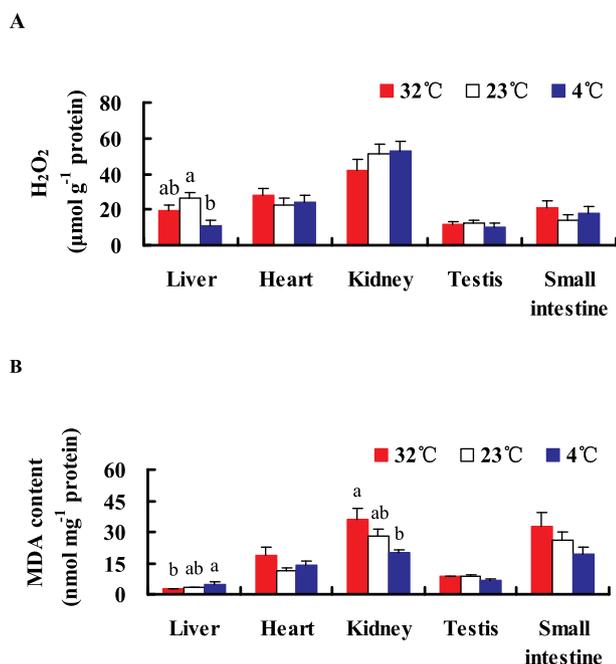
Fig. 3. Effect of air temperatures on the levels of H_2O_2 (A) and MDA (B) in Mongolian gerbils acclimated to 4 °C, 23 °C and 32 °C. Different letters above the columns indicate significant differences at the level of $P<0.05$.

MEI among the three groups with experimental time were analyzed by Repeated Measures of General Linear Model (GLM). Group differences in wet organ mass and GEI, MEI with body mass as the covariate at any time point were analyzed by a one-way analysis of covariance (ANCOVA) followed by Bonferroni *post-hoc* tests. Significant group differences were further evaluated by GLM multivariate analysis followed by Bonferroni *post hoc* tests. Pearson correlation analysis was performed to determine the correlations of body fat mass,

corticosterone and testosterone with antioxidant parameters and innate immunity. Results were expressed as mean ± SE, and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Body mass and energy intake

There was no difference in body mass among the 4 °C, 23 °C and 32 °C groups at any time point from day 0 ($F_{2,30} = 0.056$, $P = 0.946$) to day 27 ($F_{2,30} = 0.777$, $P = 0.469$) (Fig. 1 A). GEI changed with experimental time ($F_{8,240} = 5.432$, $P < 0.001$) and was affected by the interaction of time × groups ($F_{16,240} = 22.066$, $P < 0.001$). Similarly, MEI was also influenced by experimental time ($F_{8,240} = 7.354$, $P < 0.001$) and the interaction of time × groups ($F_{16,240} = 20.747$, $P < 0.001$). No significant differences were observed in gross energy intake (GEI) ($F_{2,29} = 0.447$, $P = 0.644$) and metabolizable energy intake (MEI) ($F_{2,29} = 0.224$, $P = 0.801$) among the 4 °C, 23 °C and 32 °C groups on day 0, yet high temperature suppressed whereas low temperature increased GEI and MEI in gerbils from day 3 (GEI: $F_{2,29} = 168.3$, $P < 0.001$; MEI: $F_{2,29} = 167.7$, $P < 0.001$) to day 27 (GEI: $F_{2,29} = 105.9$, $P < 0.001$; MEI: $F_{2,29} = 105.6$, $P < 0.001$) in contrast with the warm controls (Fig. 1 B).

3.2. Body composition and organs

At lower air temperature, the masses of dry carcass, fat free dry carcass, body fat and fat content were lower, whereas water content was higher, and wet carcass mass and water of carcass did not change (Fig. 2; Table 1). However, at lower temperature, the masses of most organs including IBAT, heart, lungs, liver, spleen, kidney, adrenal glands, stomach, small intestine with contents, small intestine, caecum with contents, caecum, total digestive tract and the lengths of small intestine, caecum and total digestive tract were all higher, while hypothalamus, brain, thymus, stomach with contents, colon with contents, colon, testes, epididymis, seminal vesical remained unchanged (Table 2).
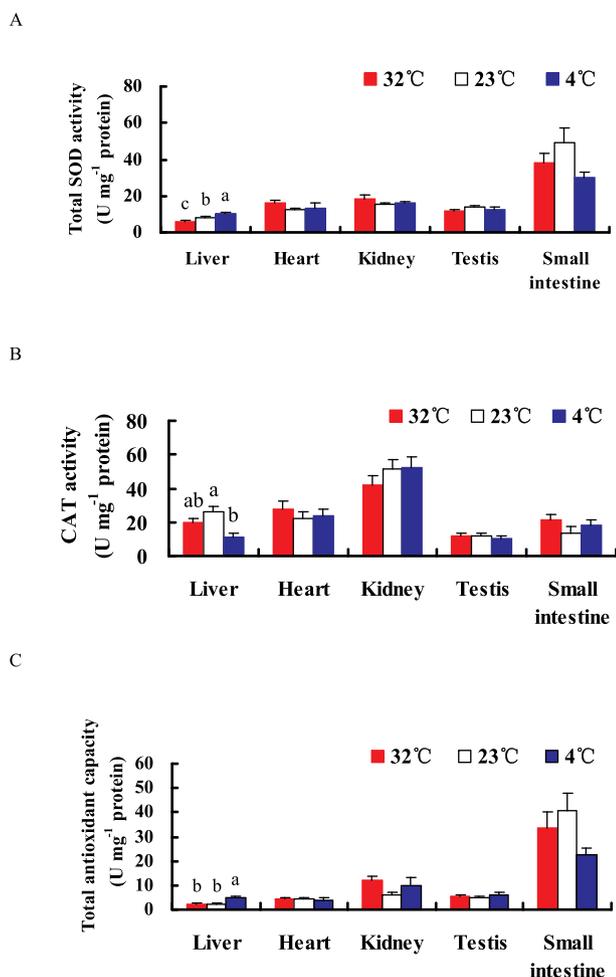
A

B

C

Fig. 4. Effect of air temperatures on total SOD activity (A), CAT activity (B), total antioxidant capacity (C) in Mongolian gerbils. Different letters above the columns indicate significant differences at the level of *P*<0.05.

3.3. Oxidative stress

H$_2$O$_2$ levels showed significant differences among various tissues in all three treatment groups (F$_{4,155}$ = 61.70, P < 0.001, Fig. 3 A). In the 4 °C group, H$_2$O$_2$ levels were significantly higher in the small intestine than that in the liver, heart, kidney and testis (post hoc, P < 0.05). Temperature had a significant effect on H$_2$O$_2$ levels in the liver, which was 62.4% higher in the 4 °C group and similar in the 32 °C group, compared to the 23 °C group (F$_{2,30}$ = 3.574, P = 0.041). H$_2$O$_2$ levels in the small intestine were also affected significantly by temperature, which was 44.9% and 26.8% lower in the 4 °C and 32 °C groups, respectively, compared to the 23 °C group (F$_{2,30}$ = 3.842, P = 0.033). However, temperature had no effect on H$_2$O$_2$ levels in either kidney (F$_{2,30}$ = 3.256, P = 0.054), or in testes (F$_{2,30}$ = 0.176, P = 0.839), heart (F$_{2,30}$ = 2.852, P = 0.075) (Fig. 3 A).

MDA levels differed significantly among various tissues (F$_{4,155}$ = 39.46, P < 0.001, Fig. 2B), and were significantly higher in kidney and small intestine than that in the liver, heart and testis in the 4 °C group (post hoc, P < 0.05). Temperature had no influence on MDA content in either heart (F$_{2,30}$ = 2.301, P = 0.119), or small intestine (F$_{2,30}$ = 2.126, P = 0.137). At lower temperature, MDA content in liver (F$_{2,30}$ = 3.796, P = 0.034) was higher, while it was lower in kidney (F$_{2,27}$ = 3.561, P = 0.042) and testes (F$_{2,30}$ = 3.442, P = 0.045) (Fig. 3 B).

3.4. Antioxidant enzymes

A significant difference in SOD activity was found among the five tissues (F$_{4,155}$ = 50.86, P < 0.001, Fig. 3A), and its activity was higher in small intestine than other four tissues (post hoc, P < 0.05). SOD activity in liver was influenced significantly by temperature, which was 24.7% higher in the 4 °C group and 26.0% lower in the 32 °C group, compared to the 23 °C group (F$_{2,30}$ = 19.759, P < 0.001). There was no significant difference of total SOD activity in heart (F$_{2,30}$ = 0.745, P = 0.484), kidney (F$_{2,30}$ = 0.919, P = 0.411), testis (F$_{2,30}$ = 2.670, P = 0.086) and small intestine (F$_{2,30}$ = 2.763, P = 0.079) among the 4 °C, 23 °C and 32 °C groups (Fig. 4 A).

CAT activity exhibited significant differences among the five tissues (F$_{4,155}$ = 40.96, P < 0.001, Fig. 4 B), which was higher in the kidney than the other four tissues in 4°C group (post hoc, P < 0.05). CAT activity in liver was significantly lower by 58.7% and 26.4% in the 4 °C and 32 °C groups, respectively, compared to the 23 °C group. However, temperature had no significant impact on CAT activity in heart, kidney, testis and small intestine (heart, F$_{2,30}$ = 0.462, P = 0.635; kidney, F$_{2,30}$ = 0.985, P = 0.385; testis, F$_{2,30}$ = 0.283, P = 0.756; small intestine, F$_{2,30}$ = 0.993, P = 0.382) (Fig. 4 B).

T-AOC activity differed significantly among the five tissues (F$_{4,155}$ = 51.90, P < 0.001, Fig. 3C), and its activity was higher in small intestine than that in the other four tissues in 5 °C group (post hoc, P < 0.05). T-AOC activity in liver was affected significantly by temperature, which was 110.3% higher in the 4 °C group and 0.05% lower in the 32 °C group, compared to the 23 °C group (F$_{2,30}$ = 10.075, P < 0.001). No significant differences of T-AOC activity was observed in heart (F$_{2,30}$ = 0.166, P = 0.848), kidney (F$_{2,30}$ = 2.371, P = 0.113), testes (F$_{2,30}$ = 0.220, P = 0.804) and small intestine (F$_{2,30}$ = 2.524, P = 0.097) among the 4 °C, 23 °C and 32 °C groups (Fig. 4 C). Significant correlations between H$_2$O$_2$ and MDA levels and SOD, CAT, T-AOC activities in small intestine and/or heart, kidney, but only a few significant correlations were found in liver and testis (Table 3).

3.5. Immunological parameters

No statistical significant differences were observed in thymus wet mass (Fig. 5 A, Table 2), WBC (F$_{2,30}$ = 0.869, P = 0.430) (Fig. 5 C), and bacteria killing capacity (F$_{2,30}$ = 0.147, P = 0.864) (Fig. 5 D) among the 4 °C, 23 °C and 32 °C groups. However, spleen mass were lower in 32 °C group compared with 23 °C group (Fig. 5 D). Innate immunity was not correlated with body fat mass (Table 3).

3.6. Hormone profiles

The levels of testosterone (F$_{2,30}$ = 0.775, P = 0.470) and corticosterone (F$_{2,30}$ = 0.323, P = 0.727) did not differ among the 4 °C, 23 °C and 32 °C groups (Fig. 6 A, B). Neither testosterone nor corticosterone was correlated with innate immunity, H$_2$O$_2$ and MDA levels, SOD, CAT and T-AOC activities in liver, heart, kidney, testis and small intestine (Table 3). However, testosterone titres was negatively correlated with titres (Table 3).

4. Discussion

Environmental temperature is a crucial factor influencing physiological functions in animals. Unexpectedly, we found that air temperatures had different effects on oxidative stress, antioxidant enzymes, and immunity depending on the tissues and parameters tested. ROS levels indicated by H$_2$O$_2$ levels were higher in liver but decreased in small intestine, and still remained stable in heart, kidney and testis upon cold exposure. Immunological parameters (i.e., bacteria killing capacity, thymus and WBC) except spleen were all not responsive to air temperatures.

Table 3
Pearson's correlation coefficients of the relationship between H_2O_2 and MDA levels and of SOD, CAT, T-AOC activity, body fat mass, innate immunity, corticosterone, testosterone in Mongolian gerbils.

		H_2O_2	MDA	SOD	CAT	T-AOC	Immunity	Fat	CORT	T
Liver	H_2O_2	1								
	MDA	0.208	1							
	SOD	0.247	0.357*	1						
	CAT	−0.083	−0.195	0.097	1					
	T-AOC	0.641**	0.603**	0.379*	−0.215	1				
	Immunity	0.246	0.192	0.050	0.018	0.273	1			
	Fat	−0.164	−0.115	−0.427*	0.181	−0.127	−0.042	1		
	CORT	−0.084	0.164	−0.013	−0.319	0.069	0.205	−0.267	1	
	T	0.038	−0.233	0.097	0.263	−0.093	−0.103	0.375*	−0.486**	1
		H_2O_2	MDA	SOD	CAT	T-AOC	Immunity	Fat	CORT	T
Heart	H_2O_2	1								
	MDA	0.215	1							
	SOD	0.154	0.691**	1						
	CAT	−0.215	0.589**	0.775**	1					
	T-AOC	0.206	0.412	0.593**	0.287	1				
	Immunity	0.064	−0.136	−0.071	−0.178	−0.074	1			
	Fat	0.040	−0.234	−0.119	−0.095	−0.032	−0.042	1		
	CORT	0.073	0.047	−0.183	−0.290	−0.308	0.205	−0.267	1	
	T	−0.114	−0.171	0.019	0.142	0.051	−0.103	0.375*	−0.486**	1
		H_2O_2	MDA	SOD	CAT	T-AOC	Immunity	Fat	CORT	T
Kidney	H_2O_2	1								
	MDA	0.713**	1							
	SOD	0.757**	0.762**	1						
	CAT	0.333	0.269	0.496**	1					
	T-AOC	0.201	0.368*	0.328	0.172	1				
	Immunity	0.077	0.119	0.210	0.478**	0.080	1			
	Fat	0.238	−0.030	0.066	0.025	−0.105	−0.042	1		
	CORT	0.036	−0.101	0.115	−0.082	−0.178	0.205	−0.267	1	
	T	0.027	0.015	0.055	0.172	0.042	−0.103	0.375*	−0.486**	1
Testis	H_2O_2	1								
	MDA	0.266	1							
	SOD	0.299	0.190	1						
	CAT	0.334	0.456**	0.369*	1					
	T-AOC	0.028	0.054	0.133	0.066	1				
	Immunity	−0.318	0.275	0.108	0.145	0.164	1			
	Fat	−0.035	0.227	−0.057	0.151	0.070	−0.042	1		
	CORT	0.000	0.046	−0.047	−0.003	0.010	0.205	−0.267	1	
	T	−0.034	0.054	0.026	0.057	0.096	−0.103	0.375*	−0.486**	1
		H_2O_2	MDA	SOD	CAT	T-AOC	Immunity	Fat	CORT	T
SI	H_2O_2	1								
	MDA	0.584**	1							
	SOD	0.899**	0.476**	1						
	CAT	0.149	0.423*	0.273	1					
	T-AOC	0.817**	0.517**	0.804**	0.244	1				
	Immunity	−0.494**	−0.063	−0.451**	0.159	−0.413*	1			
	Fat	0.366*	0.243	0.279	−0.152	0.133	−0.042	1		
	CORT	−0.154	0.150	−0.162	0.070	−0.083	0.205	−0.267	1	
	T	0.156	−0.035	0.056	−0.200	−0.102	−0.103	0.375*	−0.486**	1

SI = small intestine, CORT = corticosterone, T = Testosterone. *$P < 0.05$, **$P < 0.01$.

4.1. Body mass, body composition and organs

Body mass did not differ among the 4 °C, 23 °C and 32 °C groups throughout the experiment, being consistent with previous researches (Wang, 2007). To satisfy the increase of energy requirement at lower temperature, gerbils elevated gross energy intake and metabolizable energy intake and mobilized energy reserves including fat free carcass and body fat mass. These results were compatible with the increase in energy expenditure upon cold exposure (Chi and Wang, 2011; Hammond and Wunder, 1995). At lower temperature, expensive metabolic organs such as lungs, heart, liver, kidneys and small intestine were higher in gerbils, which was an adaptive response to elevated energy requirement during cold or winter-like conditions (Zhang and Wang, 2006; Chi and Wang, 2011). In the current study, the masses of digestive organs including stomach, small intestine with contents, small intestine, caecum with contents, caecum, total digestive tract and the lengths of small intestine, caecum and total digestive tract were all higher at lower temperature, indicating the enhancement of food processing capability to satisfy the increased energy requirement under cold temperature (Konarzewski and Diamond, 1995; Hammond and Wunder, 1995).

4.2. Oxidative stress and gross energy intake

The energy requirement increases across many species at cold temperature (Liu et al., 2009; Chi and Wang, 2011; Zhou et al., 2015). To satisfy this end, animals usually increase energy intake, which was also seen in gerbils in our study. Metabolic rate would also increase across many species including gerbils upon cold exposure (Li et al., 2001; Zhang and Wang, 2006; Liu et al., 2009; Chi and Wang, 2011). Although we did not measure the metabolic rate in our study, the increase of GEI in gerbils may indicate the elevation of metabolic rate with the decline of temperature. In terms of the rate of living-free radical hypothesis, higher metabolic rates which is achieved by enhancing mitochondrial oxidative phosphorylation, should increase the production of free radicals (i.e., ROS) (Pearl, 1928; Harman, 1956; Speakman et al., 2004; Selman et al., 2013). At lower temperature, higher ROS production in liver in gerbils agreed with this hypothesis,
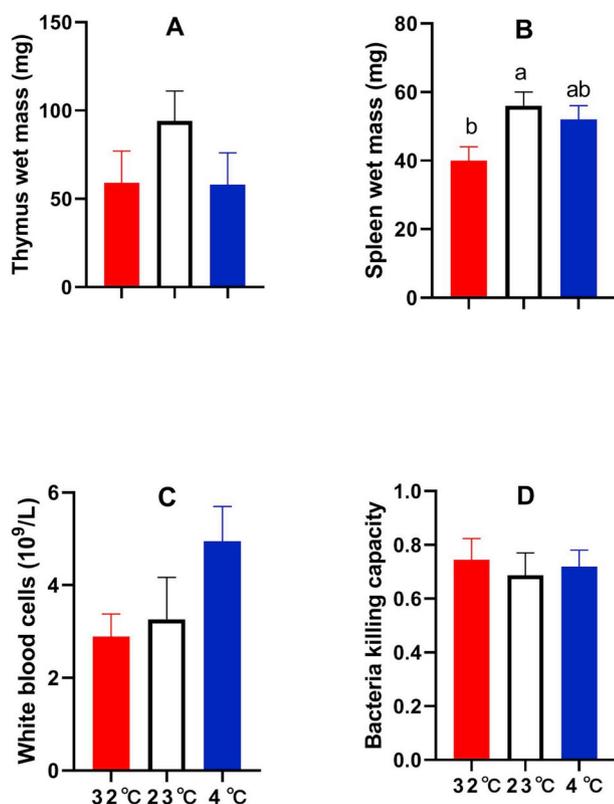
Fig. 5. Effect of air temperatures on thymus (A) and spleen wet mass (B), white blood cells (C) and bacteria killing capacity (D) in Mongolian gerbils. Different letters above the columns indicate significant differences at the level of $P < 0.05$.

while lower ROS in small intestine and the unchanged ROS production in heart, kidney and testis did not support this hypothesis. That elevated metabolic rate during cold acclimation does not necessarily result in greater ROS production might account for these different findings. According to the 'uncoupling to survive' hypothesis, cold temperatures increased uncoupling proteins (UCP) and hence reduce ROS production by lowering the potential of the inner mitochondrial membrane (Brand, 2000; Speakman et al., 2004). Some investigators had not found associations between increased metabolic rate and elevated ROS levels in skeletal muscle, and in certain organs, including the liver, heart, lungs, spleen, kidneys and digestive tract (Selman et al., 2002; Chen et al., 2014; Stier et al., 2014). Previous findings that cold exposure had on effect on UCP1 content in gerbils might account for the results of ROS in our experiment (Li et al., 2001; Wang, 2007).

4.3. Antioxidant defense and hormone profiles

Stressful conditions such as high or low ambient temperature often lead to the increase of stress hormones such as corticosterone (Bligh-Tynan et al., 1993; Sapolsky et al., 2000; Kim et al., 2013). We found no change of corticosterone levels in gerbils in response to air temperatures, being not compatible with other researches in which corticosterone titres increased upon cold exposure (Adels et al., 1986; Shu et al., 1993). The different duration of cold exposure might account for this discrepancy. Acute cold exposure usually increases corticosterone titres, whereas its levels would restore to the baseline levels under chronic cold exposure (Bligh-Tynan et al., 1993).

Oxidative stress increased or SOD activity decreased in rats (Dhanabalan et al., 2010) and birds (Lin et al., 2004; Alonso-Alvarez et al., 2007) after exogenous corticosterone supplement. Here, no correlation was observed between corticosterone titres and the levels of ROS and MDA, the activities of SOD, CAT and T-AOC in liver, heart, kidney, testis and small intestine. Therefore, it seems that
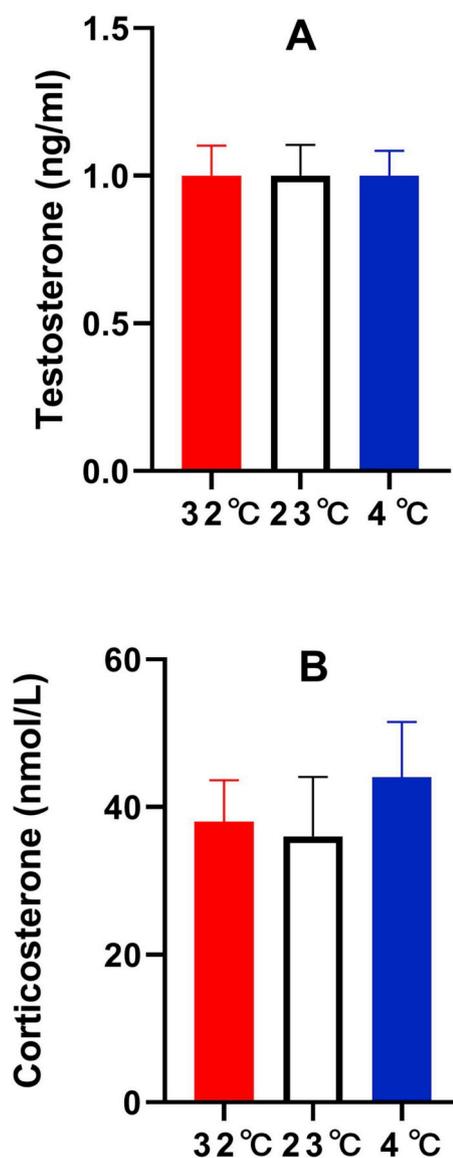

Fig. 6. Effect of air temperatures on the levels of testosterone (A) and corticosterone (B) in Mongolian gerbils acclimated to 4 °C, 23 °C and 32 °C. Different letters above the columns indicate significant differences at the level of $P < 0.05$.

corticosterone could not fully explain the influence of air temperatures on oxidative stress and antioxidant capacity in gerbils.

Testosterone incurs oxidative costs in light of the oxidation handicap hypothesis (Alonso-Alvarez et al., 2007, 2008). In our study, its concentration was not affected by air temperatures, and it was also not correlated with H_2O_2 levels, MDA content, the activities of SOD, CAT, T-AOC in the five tissues (i.e., liver, heart, kidney, testis and small intestine). Because detection of oxidative costs is both tissue and assay-dependent (Yang et al., 2013), additional parameters in more tissues might have detected some direct oxidative costs of testosterone in the present study.

4.4. Immunity, body fat and hormone profiles

Bacteria killing capacity did not respond to air temperatures in gerbils, which was consistent with cold-adapted female hamsters but was not compatible with cold-adapted male hamsters (Xu et al., 2017; Xu and Wang, 2011). Adipose tissues not only provide energy reserves for expensive physiological processes including immune responses (Demas et al., 1997; Moret and Schmid-Hempel, 2000; Demas, 2004),

but also are regarded as endocrine and immune organs (Ahima and Flier, 2000; Trayhurn, 2005). Animals with low energy reserves usually allocate less energy to immune defense than those with higher reserves (Houston et al., 2007). Thus, decrease in body fat can harm immunity (Chandra, 1996; Demas et al., 2003). Although body fat mass were lower in gerbils at lower temperature, it was not correlated with innate immunity. Further researches are required to clarify the role of body fat in immunity in gerbils. Immune function is usually suppressed by corticosterone and testosterone (Sapolsky et al., 2000; Marketon and Glaser, 2008; Trigunaite et al., 2015). However, these two hormones were not correlated with innate immunity in gerbils.

Total WBC usually increases due to stress (Dudzinski et al., 1962; Bubenik and Brownlee, 1987), or disease and infection (Bush et al., 1981; Bubenik and Brownlee, 1987). Enhanced WBC numbers may benefit overall health by increasing the ability of fighting pathogens (Sams et al., 1996; McMurry et al., 1999). Because gerbils used in the present study were healthy and free of infection, no difference of WBC and thymus mass among the 4 °C, 23 °C and 32 °C groups indicated they were not influenced by air temperatures. However, high temperature decreased spleen mass compared with warm temperature, implying its suppressive role on the function of spleen.

In summary, air temperature affected antioxidant capacity, but not immune responses or serum concentrations of corticosterone and testosterone in gerbils. This species adopted several adaptive strategies to cope with air temperatures. At lower temperature, gerbils increased gross energy intake, the masses of most active metabolic organs such as lungs, heart, liver, kidneys, stomach and small intestine, and the lengths of digestive tract including small intestine, total digestive tract, and mobilized energy reserves to satisfy the increased energy requirements in cold temperature. SOD and total antioxidant capacity were higher in the liver but remain stable in other four tissues (i.e., heart, small intestine, kidneys and testis) in gerbils at lower temperature. Thus, up-regulation or maintenance of antioxidant defenses and immunity might be important for gerbils to survive the highly fluctuating ambient temperatures.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.06.008.

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