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RESEARCH ARTICLE

Temperature and photoperiod as environmental cues affect body mass and thermoregulation in Chinese bulbuls, *Pycnonotus sinensis*

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ABSTRACT

Seasonal changes in temperature and photoperiod are important environmental cues used by small birds to adjust their body mass (M_b) and thermogenesis. However, the relative importance of these cues with respect to seasonal adjustments in M_b and thermogenesis is difficult to distinguish. In particular, the effects of temperature and photoperiod on energy metabolism and thermoregulation are not well known in many passerines. To address this problem, we measured the effects of temperature and photoperiod on M_b, energy intake, resting metabolic rate (RMR), organ mass and physiological and biochemical markers of metabolic activity in the Chinese bulbul (*Pycnonotus sinensis*). Groups of Chinese bulbuls were acclimated in a laboratory to the following conditions: (1) warm and long photoperiod, (2) warm and short photoperiod, (3) cold and long photoperiod, and (4) cold and short photoperiod, for 4 weeks. The results indicate that Chinese bulbuls exhibit adaptive physiological regulation when exposed to different temperatures and photoperiods. M_b, RMR, gross energy intake and digestible energy intake were higher in cold-acclimated than in warm-acclimated bulbuls, and in the short photoperiod than in the long photoperiod. The resultant flexibility in energy intake and RMR allows Chinese bulbuls exposed to different temperatures and photoperiods to adjust their energy balance and thermogenesis accordingly. Cold-acclimated birds had heightened state-4 respiration and cytochrome *c* oxidase activity in their liver and muscle tissue compared with warm-acclimated birds indicating the cellular mechanisms underlying their adaptive thermogenesis. Temperature appears to be a primary cue for adjusting energy budget and thermogenic ability in Chinese bulbuls; photoperiod appears to intensify temperature-induced changes in energy metabolism and thermoregulation.

KEY WORDS: Acclimation, Resting metabolic rate, Cytochrome *c* oxidase, Mitochondria, State-4 respiration

INTRODUCTION

Organisms can generally adjust their morphology, physiology and behavior in response to changing environmental conditions (Swanson, 1991a; Zheng et al., 2008a,b, 2014a), resulting in phenotypic changes that are reversible, temporary and repeatable

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(Piersma and Drent, 2003; McKechnie et al., 2006). In birds, phenotypic flexibility in metabolic power output is an important component of their thermoregulatory responses to the periodically elevated energy requirements they experience in seasonal environments (McKechnie et al., 2007). The capacity for thermogenesis and energy intake is particularly important for the survival of small birds in winter (Swanson, 2010). Temperature and photoperiod are considered to be the most important environmental factors influencing an animal's seasonal thermoregulation and driving the evolution of a suite of morphological, physiological and behavioral adaptations (Zheng et al., 2013a; Swanson et al., 2014; Zhou et al., 2016). Seasonal changes in temperature and photoperiod are important environmental cues that small birds use to adjust morphological and physiological parameters such as body mass (M_b), energy intake and thermogenic capacity (Swanson, 1990; Zheng et al., 2008a; Wu et al., 2014a). Winter is an energetically stressful period for birds living in temperate zones because the cost of thermoregulation increases while food quality and availability are reduced (Yuni and Rose, 2005; Petit et al., 2014). To cope with this, relatively large temperate resident birds, like ptarmigans (*Lagopus* spp.) reduced their thermal conductance in winter, partly through increasing their feather insulation and, in some cases, by increasing subcutaneous fat (Mortensen and Blix, 1986; Lees et al., 2010). However, the size of many bird species limits the effectiveness of such adjustments; rather than reduce thermal conductance, small birds tend to cope with cold environments by increasing their capacity for thermogenesis (Swanson, 1991a,b; Zheng et al., 2008b, 2014a).

Resting metabolic rate (minimum maintenance metabolic rate, RMR) in small birds is one of the fundamental physiological standards for assessing the energy cost of thermoregulation (Clapham, 2012). It has been demonstrated that a bird's M_b, energy balance and RMR are all affected by temperature and photoperiod (McKechnie, 2008; McKechnie and Swanson, 2010; Wu et al., 2014b). Low ambient temperature can increase the RMR of some birds (Tieleman et al., 2003; McKechnie et al., 2007). Short photoperiod, either alone, or in combination with cold, can also increase metabolic thermogenesis in birds (Saarela and Heldmaier, 1987; Ni et al., 2010; Swanson et al., 2014). This suggests that temperature and photoperiod play an important role in mediating the metabolic rates of small birds (Swanson et al., 2014). Under basal metabolic conditions, the liver has been shown to contribute 20–25% of total heat production in animals (Villarin et al., 2003; Zheng et al., 2008b). Skeletal muscle makes up nearly 40% of the total M_b of birds and plays a key role in thermogenesis (Weber and Piersma, 1996; Vézina et al., 2006, 2007). Adjustment of cellular aerobic capacity by modulating the activities of key catabolic enzymes in oxidative pathways may also therefore contribute to the physiological phenotypes of small birds (Liknes

List of symbols and abbreviations	
CL	cold and long photoperiod
COX	cytochrome *c* oxidase
CS	cold and short photoperiod
DEI	digestible energy intake
FE	feces energy
GEI	gross energy intake
M_b	body mass
RMR	resting metabolic rate
WL	warm and long photoperiod
WS	warm and short photoperiod

and Swanson, 2011; Swanson et al., 2014; Zheng et al., 2013b, 2014a). Variation in cellular metabolic intensity is often measured by examining variation in state-4 respiration (reflecting oxidative phosphorylation capacity) or cytochrome *c* oxidase (COX) activity (a key regulatory enzyme of oxidative phosphorylation) (Zheng et al., 2008b, 2014a,b).

Chinese bulbuls, *Pycnonotus sinensis* (Gmelin 1789), are small passerine birds that inhabit vast areas of eastern and southern Asia, including central, southern and eastern China (Zheng and Zhang, 2002). Elevated winter RMR in bulbuls is associated with elevated nutritional and exercise organ masses, and heightened respiratory enzyme activity in liver and muscle (Zheng et al., 2008a, 2010, 2014a). We selected Chinese bulbuls as a study species because they are resident in Zhejiang Province where we are based, because global warming appears to have allowed the species to colonize northeastern and northwestern China, and because previous studies (Zhang et al., 2008; Zheng et al., 2008a, 2010; Ni et al., 2010) provide critical background information required for our research. However, the Chinese bulbul commonly encounters cold temperatures and short photoperiods but the physiological and biochemical mechanisms are unknown. In this study, we compared selected morphological, physiological and biochemical indices of wild-caught Chinese bulbuls to different combinations of temperature (warm and cold) and photoperiod (long day and short day). We hypothesized that cold exposure and short day are key factors driving metabolic flexibility in Chinese bulbuls. We also hypothesized that there would be an interaction between temperature and photoperiod such that the greatest treatment effects would be observed in birds acclimated to both cold temperatures and short days. We predicted that Chinese bulbuls exposed to cold temperatures and short days would increase their RMR and respiratory enzyme activity; by and quantifying these adjustments, we aimed to improve understanding of the morphological, physiological and biochemical responses of small bird species to cold temperatures and short photoperiods.

MATERIALS AND METHODS
Animals
The Chinese bulbul is a common resident bird in Zhejiang Province (MacKinnon and Phillipps, 2000). The Chinese bulbuls used in the experiments were captured with mist nets in Wenzhou city, Zhejiang Province, China, in June 2011. The climate in Wenzhou is warm–temperate with an average annual rainfall of 1700 mm across all months, with slightly more precipitation during winter and spring. Mean daily maximum temperature ranges from 39°C in July to 8°C in January. The mean temperature from June to August is 32°C (Zheng et al., 2008a). We determined bulbul M_b to the nearest 0.1 g immediately upon capture with a Sartorius balance (model BT25S). We transported birds to the laboratory on the day of capture

and kept them in outdoor cages (50×30×20 cm) for 1 or 2 days under natural ambient temperature (28±1°C) and photoperiod conditions before measurements began. Food and water were supplied *ad libitum*. Birds were then kept in individual cages for at least 2 weeks, after which 28 birds were randomly assigned to one of four treatment groups: (1) warm and long photoperiod (WL; 30°C, 16 h light:8 h dark), (2) warm and short photoperiod (WS; 30°C, 8 h light:16 h dark), (3) cold and long photoperiod (CL; 10°C, 16 h light:8 h dark), and (4) cold and short photoperiod (CS; 10°C, 8 h light:16 h dark). Each group of birds was acclimated to its respective treatment for 4 weeks (Ni et al., 2010; Zheng et al., 2013a). All experimental procedures were approved by the Animal Care and Use Committee of Wenzhou City, Zhejiang Province, China.

Measurement of metabolic rate
Metabolic rate of the birds was estimated by measuring their oxygen consumption in an open-circuit respirometry system (S-3A/I, AEI Technologies, Pittsburgh, PA, USA). Metabolic chambers were 1.5 l in volume and made of plastic. A perch was provided for the bird to stand on (Smit and McKechnie, 2010; Zheng et al., 2014a). Chamber temperature was regulated by a temperature-controlled cabinet (BIC-300 artificial climate incubator, Shanghai Boxun Medical Biological Instrument Corp., China) capable of regulating temperature to ±0.5°C. Water vapor and CO_2 were scrubbed from the air passing through the chamber in a silica gel/soda lime/silica column before passing through the oxygen analyzer. We measured the oxygen content of excurrent gas from metabolic chambers with an oxygen sensor (N-22M, AEI Technologies). We used a flow control system (R-1, AEI Technologies) to set the flow of excurrent gas to 300 ml min^{-1} during metabolic rate measurements. This maintained a fractional concentration of O_2 in the respirometry chamber of about 20%, calibrated to ±1% accuracy with a general purpose thermal mass flow-meter (TSI Series 4100, TSI Inc., Shoreview, MN, USA) (McNab, 2006). Oxygen consumption rates were measured at 30±0.5°C, which is within the thermal neutral zone of Chinese bulbuls (Zheng et al., 2008a). Baseline O_2 concentrations were obtained before and after each test (Li et al., 2010). All measurements of gas exchange were obtained during the resting phase of the birds' circadian cycles (between 20:00 h and 24:00 h) in darkened chambers when individual birds could reasonably be expected to be postabsorptive. Resting metabolic rate (RMR) is the energy required to perform vital body functions while the body is at rest. Because it is doubtful that true RMRs can be achieved in the laboratory, the term RMR is often used to refer to such measurements, even when the standard conditions for RMR have been met (Swanson, 2010). Food was removed 4 h before each measurement to minimize the heat increment associated with feeding. We first ensured that birds were perching calmly in the chamber and started recording oxygen consumption at least 1 h later. Each animal was generally in the metabolic chamber for at least 2 h. The data obtained were used to calculate 5 min running means of instantaneous oxygen consumption over the entire test period using eqn 2 of Hill (1972). The lowest 5 min mean recorded over the test period was considered the RMR (Smit and McKechnie, 2010). All values for oxygen consumption were expressed as ml O_2 h^{-1} and corrected to STPD conditions (Schmidt-Nielsen, 1997). Body temperature was measured during metabolic measurements using a lubricated thermocouple inserted into the cloaca to a depth of 1–2 cm, a depth at which a slight withdrawal of the thermocouple did not cause a change in the reading. Thermocouple outputs were digitized using a thermocouple meter (Beijing Normal University Instruments Co.). M_b was measured to

the nearest 0.1 g before and after experiments, and mean M_b was used in calculations. All measurements were made daily between 20:00 h and 24:00 h.

Energy budget

We regarded digestible energy intake as an index of total daily energy expenditure. Food and water were available *ad libitum* throughout the experiment and replenished daily. We collected food residues and feces once for 3 days prior to temperature and photoperiod acclimation (week 0) and thereafter weekly (every 7 days) throughout the 4 week experimental period. We separated the residues manually and oven-dried then at 60°C to constant mass. We then determined their caloric content with a C200 oxygen bomb calorimeter (IKA, Staufen im Breisgau, Germany). We calculated gross energy intake (GEI), feces energy (FE), digestible energy intake (DEI) and energy digestibility according to the methods described in Grodzinski and Wunder (1975) and Ni et al. (2010):

$$GEI = dry\ food\ intake \times caloric\ value\ of\ dry\ food, \quad (1)$$

where GEI is in kJ day^{-1}, dry food intake is in g day^{-1} and the caloric value of dry food is in kJ g^{-1};

$$FE = dry\ mass\ of\ feces \times caloric\ value\ of\ dry\ feces, \quad (2)$$

where FE is in kJ day^{-1}, dry mass of feces is in g day^{-1} and the caloric value of dry feces is in kJ g^{-1};

$$DEI = GEI - FE, \quad (3)$$

$$\%Digestibility = DEI/GEI \times 100. \quad (4)$$

Measurement of organ masses

Birds were killed by cervical dislocation at the end of the 4 week experimental period, and their brain, heart, lungs, liver, kidneys, stomach, small intestine, rectum and pectoral muscle extracted and weighed to the nearest 0.1 mg. Sub-samples of liver and muscle were used for the preparation of mitochondria (Zheng et al., 2014a). We dried internal organs, including the remaining part of the liver and muscle, to a constant mass over 2 days at 75°C, after which these were reweighed to the nearest 0.1 mg (Williams and Tieleman, 2000; Liu and Li, 2006).

Preparation of mitochondria

Liver and pectoral muscle sub-samples were cleaned of any adhering tissue, blotted dry and weighed before being placed in ice-cold sucrose-buffered medium. Both liver and pectoral muscle samples were then coarsely chopped with scissors, after which liver samples were rinsed and resuspended in 5 volumes of ice-cold medium (250 mmol l^{-1} sucrose, 5 mmol l^{-1} Tris/HCl, 1 mmol l^{-1} MgCl$_2$ and 0.5 mmol l^{-1} EDTA, pH 7.4, 4°C) (Rasmussen et al., 2004). Pectoral muscle samples were treated with proteinase for 5–10 min, then resuspended in 10 volumes of ice-cold medium (100 mmol l^{-1} KCl, 50 mmol l^{-1} Tris/HCl, 5 mmol l^{-1} MgSO$_4$ and 1 mmol l^{-1} EDTA, pH 7.4, 4°C). Liver and muscle preparations were then homogenized in a Teflon/glass homogenizer. Homogenates were centrifuged at 600 g for 10 min at 4°C in an Eppendorf centrifuge, and resultant pellets of nuclei and cell debris discarded. The supernatants were then centrifuged at 12,000 g for 10 min at 4°C. The resultant pellets were suspended, respun at 12,000 g, resuspended and the final pellets obtained were placed in ice-cold medium (2:1 w/v for liver and 4:1 w/v for muscle) (Zheng

et al., 2008b, 2013b). We determined the protein content of mitochondria by the Folin phenol method with bovine serum albumin as standard (Lowry et al., 1951).

Mitochondrial respiration and enzyme activity

State-4 respiration in liver and muscle mitochondria was measured at 30°C in 1.96 ml of respiration medium (225 mmol l^{-1} sucrose, 50 mmol l^{-1} Tris/HCl, 5 mmol l^{-1} MgCl$_2$, 1 mmol l^{-1} EDTA and 5 mmol l^{-1} KH$_2$PO$_4$, pH 7.2) with a Clark electrode (DW-1, Hansatech Instruments Ltd, King's Lynn, UK), essentially as described by Estabrook (1967). State-4 respiration was measured over a 1 h period under substrate-dependent conditions, with succinate as the substrate (Zheng et al., 2013b, 2014a). The activity of COX in liver and muscle was measured polarographically at 30°C using a Clark electrode according to Sundin et al. (1987). We express state-4 respiration and COX activity measurements as mean mass-specific values (μmol O$_2$ min^{-1} g^{-1} tissue) (Wiesinger et al., 1989; Zheng et al., 2013b, 2014a).

Statistics

Statistical analyses were performed using the SPSS package (version 12.0). All variables were tested for normality using the Kolmogorov–Smirnov test. Non-normally distributed data were normalized by transforming them to their natural logarithm. Two-way repeated-measures (RM)-ANOVA was used to determine the significance of changes in M_b, GEI, FE, DEI and digestibility over time. Tukey's *post hoc* tests were used to determine the significance of differences among different days of acclimation. The significance of M_b and digestibility on the same day among different groups was evaluated with a two-way ANOVA. Direct comparisons of GEI, FE and DEI on the same day among different groups were made with a two-way ANCOVA with M_b as the covariate. To test whether RMR differed between temperature-acclimated birds, and also between photoperiod-acclimated birds, we performed an ANCOVA, using the Tukey's *post hoc* test for multiple comparisons among groups. This design used the treatment (cold, warm, long photoperiod and short photoperiod) as the independent variable, and log RMR as the dependent variable (Maldonado et al., 2009). Because total RMR was correlated with M_b, the effect of M_b was removed using M_b as the covariate. For the analysis of organ masses, we used M_b minus wet organ mass for the organ in question to avoid statistical problems with part-whole correlations (Christians, 1999). A preliminary model was run to test for homogeneity of slopes of the dependent variable versus the covariate among treatments. The effects of temperature and photoperiod on mitochondrial protein, mitochondrial state-4 respiration and COX activity in liver and muscle were also analyzed with a Tukey's *post hoc* test for multiple comparisons among groups. Allometric and residual correlations were used to evaluate the relationship between RMR and dry organ mass (controlled for M_b minus wet mass of the organ) and least-squares linear regression to evaluate the relationship between log M_b and log RMR, between log M_b, log GEI and log DEI, between log RMR, log GEI and log DEI, and between log RMR, log state-4 respiration and log COX activity. Data are reported as means±s.e.m.

RESULTS

M_b and RMR

Prior to acclimation, no significant difference in M_b was found among the four treatment groups (ANOVA, $F_{3,24}=0.399$, $P=0.755$; Fig. 1A). However, M_b was significantly affected by temperature (RM-ANOVA, $F_{4,96}=4.069$, $P=0.015$) and photoperiod (RM-ANOVA, $F_{4,96}=4.789$, $P=0.007$), but not the interaction between
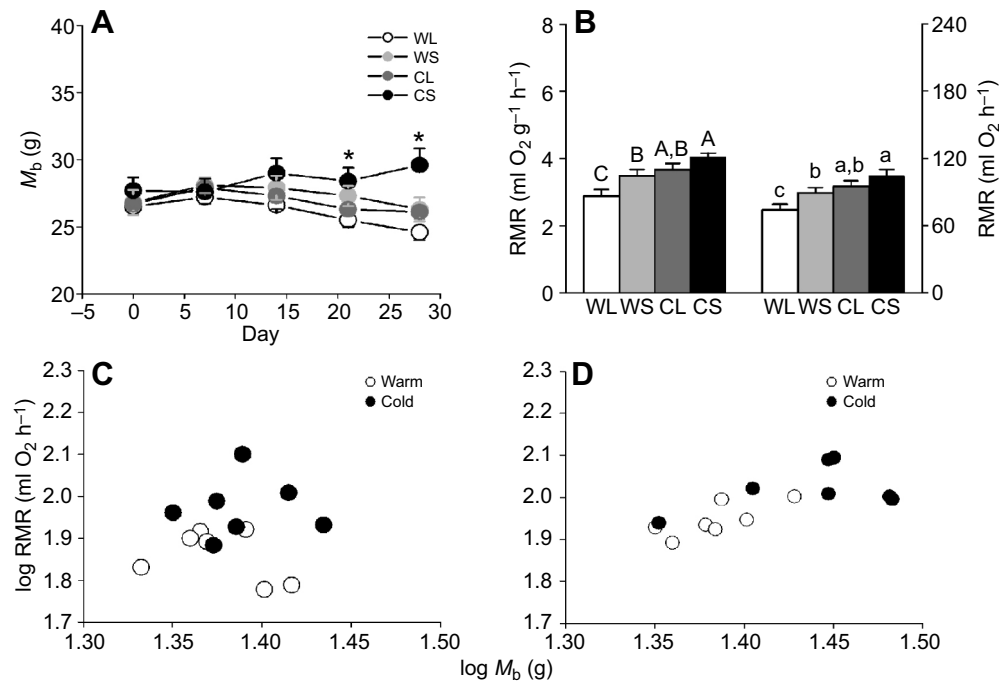
Fig. 1. Comparison of body mass and metabolic rate in Chinese bulbuls (*Pycnonotus sinensis*) acclimated to different temperature and photoperiod for 4 weeks. Birds were acclimated to warm (W) or cold (C) temperature with a long (L) or short (S) photoperiod. (A) Body mass (M_b). (B) Resting metabolic rate (RMR) and RMR as a function of M_b. (C,D) RMR as a function of M_b following acclimation to a long (C) or short (D) photoperiod. The allometric equation representing the linear curve for all birds is RMR=$0.78 M_b^{0.84}$ and RMR=$0.66 M_b^{0.94}$ for temperature acclimation in the short photoperiod. Data are means±s.e.m., bars with different letters indicate significant differences, *$P<0.05$.

temperature and photoperiod (RM-ANOVA, $F_{4,96}=1.478$, $P=0.233$) during acclimation (Fig. 1A). M_b was significantly higher in cold groups than in warm groups ($P=0.021$), and in short photoperiod than in long photoperiod ($P=0.048$). Comparisons of RMR measured at 30°C revealed that means were statistically different among groups (ANOVA, mass-independent, $F_{3,24}=3.056$, $P=0.048$; ANCOVA, total, $F_{3,23}=4.404$, $P=0.014$; Fig. 1B). RMR was significantly higher in the cold group than in the warm group ($P=0.001$), and averaged 18% higher than that of warm-acclimated birds. Homogeneity of slopes test showed that slopes were significantly different for temperature acclimation. An analysis of the temperature acclimation data for the long photoperiod groups indicated that log RMR showed a poor and non-significant association with log M_b ($r^2=0.102$, $P=0.266$; Fig. 1C). When all temperature acclimation data were pooled for the short photoperiod, log RMR showed a positive and significant correlation with log M_b ($r^2=0.503$, $P=0.045$; Fig. 1D).

Energy intake and digestibility
GEI was significantly affected by temperature (RM-ANOVA, $F_{4,96}=23.251$, $P<0.001$), photoperiod (RM-ANOVA, $F_{4,96}=2.979$, $P=0.023$) and the interaction between temperature and photoperiod (RM-ANOVA, $F_{4,96}=5.732$, $P<0.001$; Fig. 2A). No group differences in GEI were found prior to cold acclimation. However, a significant decrease in GEI ($P<0.001$) was apparent in the warm groups after day 7 of acclimation to 30°C and these decreases were sustained for the 28 day duration of the experiment (Fig. 2A). When all temperature acclimation data were pooled for long photoperiod, there was no significant relationship between log GEI and log M_b ($r^2=0.145$, $P=0.179$; Fig. 3A); there was a significant, positive relationship between log GEI and log RMR ($r^2=0.448$, $P=0.009$; Fig. 3C). An analysis of the temperature acclimation data for the short photoperiod indicated that log GEI showed a positive and significant correlation with log M_b ($r^2=0.445$, $P=0.009$; Fig. 3B) and log RMR ($r^2=0.402$, $P=0.014$; Fig. 3D).

FE was also significantly affected by temperature (RM-ANOVA, $F_{4,96}=15.495$, $P<0.001$) and there was a significant interaction

between temperature and photoperiod (RM-ANOVA, $F_{4,96}=4.037$, $P=0.009$; Fig. 2B). Bulbuls acclimated to 30°C had a significantly smaller FE than those acclimated to 10°C (ANCOVA, day 7, 14, 21 and 28, all $P<0.001$; Fig. 2B).

Temperature (RM-ANOVA, $F_{4,96}=21.267$, $P<0.001$) and photoperiod (RM-ANOVA, $F_{4,96}=4.208$, $P=0.004$) also significantly affected DEI and there was a significant interaction between temperature and photoperiod (RM-ANOVA, $F_{4,96}=5.769$, $P<0.001$, Fig. 2C). A significant difference in DEI ($P<0.001$) was also apparent in the warm groups after day 7 of acclimation to 30°C and these differences were sustained for the 28 day duration of the experiment (Fig. 2C). An analysis of the temperature acclimation data for the long photoperiod indicated that log DEI had a non-significant association with log M_b ($r^2=0.058$, $P=0.408$; Fig. 4A), but a significant association with log RMR ($r^2=0.325$, $P=0.033$; Fig. 4C). There was a significant, positive linear relationship between log DEI and log M_b ($r^2=0.426$, $P=0.011$; Fig. 4B), and between log DEI and log RMR ($r^2=0.423$, $P=0.012$; Fig. 4D) for the short photoperiod.

Digestibility was significantly affected by temperature (RM-ANOVA, $F_{4,96}=2.208$, $P<0.001$), photoperiod (RM-ANOVA, $F_{4,96}=4.478$, $P<0.001$) and the interaction between temperature and photoperiod (RM-ANOVA, $F_{4,96}=2.648$, $P=0.038$; Fig. 2D). Bulbuls acclimated to the warm showed a greater energy digestibility than those under cold conditions after day 14 ($P<0.001$) and these increases were sustained ($P<0.001$) for the 28 day duration of the experiment (Fig. 2D).

Organ and muscle mass
The ANCOVA revealed that organ masses were affected differentially by the experimental treatment: liver mass was significantly affected (wet mass, $F_{3,23}=4.341$, $P=0.015$; dry mass, $F_{3,23}=6.061$, $P=0.003$), and the *post hoc* analysis revealed that birds in the CS treatment had the heaviest livers among the groups (Table 1). Stomach mass was also affected by the experimental treatment (wet mass, $F_{3,23}=7.988$, $P=0.001$; dry mass, $F_{3,23}=5.454$, $P=0.006$). Here, the *post hoc* analysis revealed that cold-acclimated birds had larger stomachs than
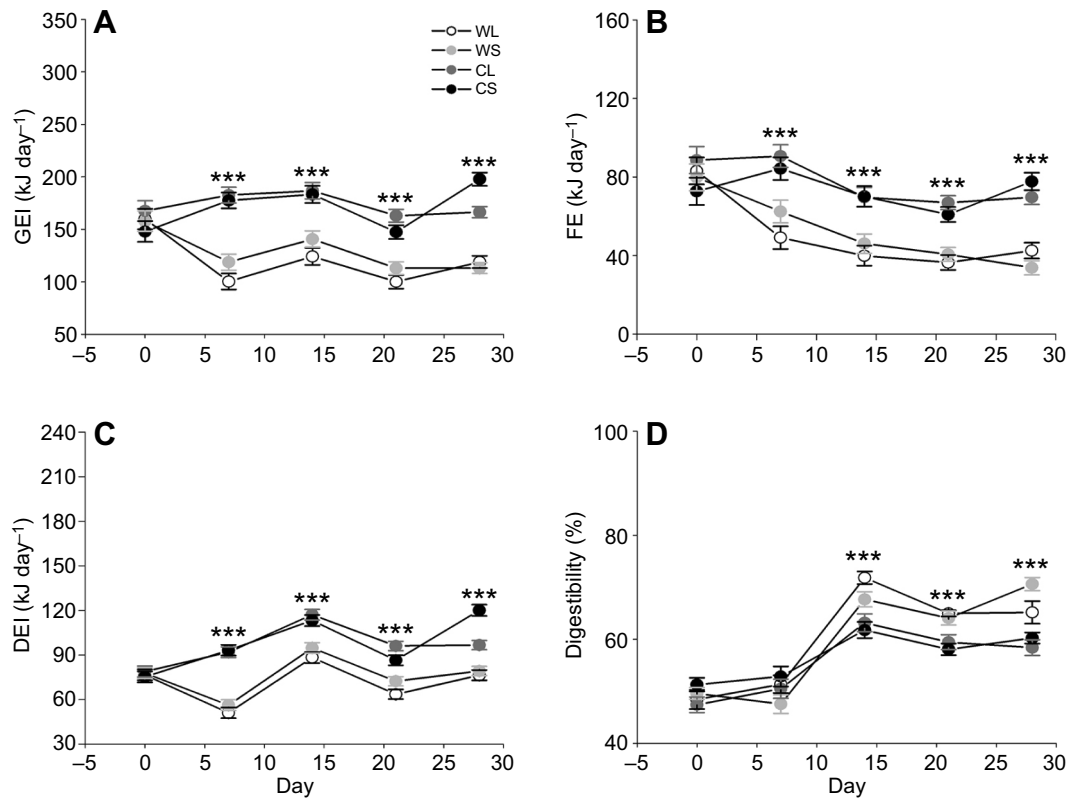
Fig. 2. Comparison of gross energy intake, feces energy and digestible energy intake in Chinese bulbuls acclimated to different temperature and photoperiod for 4 weeks. (A) Gross energy intake (GEI). (B) Feces energy (FE). (C) Digestible energy intake (DEI). (D) Digestibility. Data are means±s.e.m., ***$P<0.001$. Treatment abbreviations as for Fig. 1.

warm-acclimated birds (Table 1). The mass of both the small intestine and the total digestive tract was affected by the experimental treatment (small intestine wet mass, $F_{3,23}=5.253$, $P=0.007$; dry mass, $F_{3,23}=6.900$, $P=0.002$; total digestive tract

wet mass, $F_{3,23}=6.837$, $P=0.002$; dry mass, $F_{3,23}=7.889$, $P=0.001$), and the *post hoc* analysis showed that cold-acclimated birds had heavier small intestines and total digestive tracts than warm-acclimated birds (Table 1). Muscle, heart, lung,
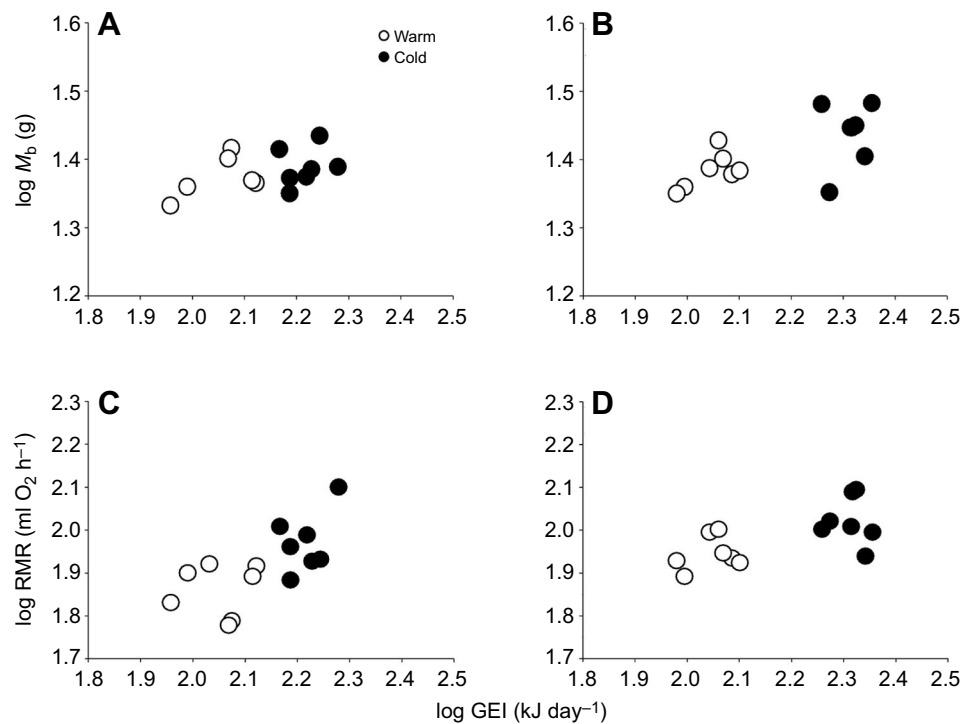

Fig. 3. Correlations between M_b and GEI and RMR and GEI in Chinese bulbuls acclimated to different temperature and photoperiod for 4 weeks. (A,B) M_b and GEI in the long (A) and short (B) photoperiod. (C,D) RMR and GEI in the long (C) and short (D) photoperiod. The allometric equations representing the linear curve for all birds are $M_b=0.98GEI^{0.19}$ and $RMR=1.07GEI^{0.41}$, $RMR=0.86GEI^{0.58}$ for temperature acclimation in the long photoperiod, and $M_b=0.95GEI^{0.21}$ and $RMR=1.40GEI^{0.27}$ for temperature acclimation in the short photoperiod. Treatment abbreviations as for Fig. 1.
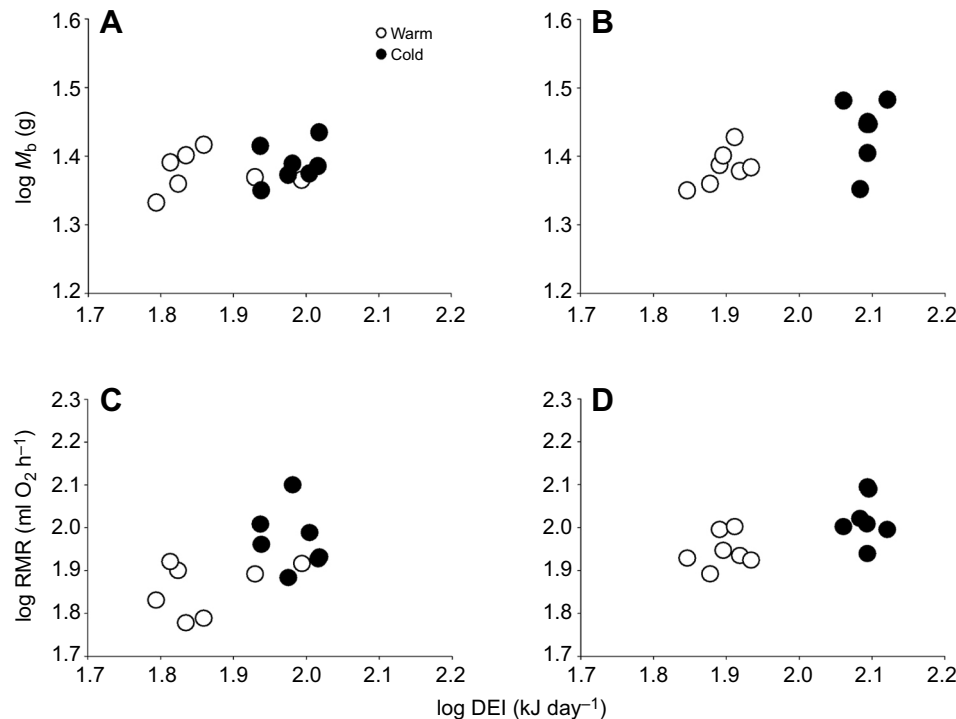
Fig. 4. Correlations between M_b and DEI and RMR and DEI in Chinese bulbuls acclimated to different temperature and photoperiod for 4 weeks. (A,B) M_b and DEI in the long (A) and short (B) photoperiod. (C,D) RMR and DEI in the long (C) and short (D) photoperiod. The allometric equations representing the linear curve for all birds are $M_b=0.94DEI^{0.24}$ and $RMR=0.92DEI^{0.53}$, $RMR=0.77DEI^{0.60}$ for temperature acclimation in the long photoperiod, and $M_b=0.84DEI^{0.21}$ and $RMR=1.23DEI^{0.38}$ for temperature acclimation in the short photoperiod. Treatment abbreviations as for Fig. 1.

kidney and rectum mass were not affected by temperature and photoperiod (Table 1). Allometric relationships for the all-birds pooled data indicated that log dry organ mass was positively correlated with log RMR in the case of the heart, liver, small intestine and total digestive tract (Table 2). The correlation analysis between log dry organ mass and log RMR of the temperature acclimation data for the long photoperiod showed a positive and significant association only for the small intestine and the total digestive tract. These analyses also revealed a significant association between these variables in the lung, liver, small intestine and total digestive tract in temperature acclimation data for the short photoperiod (Table 2). The residuals of dry organ and RMR against M_b minus wet mass of the organ only showed a positive and significant association in lung and small intestine in the all-birds pooled data (Table 2).

Protein content, mitochondrial respiration and COX activity in liver

Although mitochondrial protein content of the liver was not affected by the experimental treatment ($F_{3,24}=0.741$; $P=0.538$; Fig. 5A), state-4 respiration was significantly affected ($F_{3,24}=14.378$; $P<0.001$; Fig. 5B), and the *post hoc* analysis revealed that CS-acclimated birds had the highest mitochondrial respiration. COX activity was also significantly affected by the experimental treatment ($F_{3,24}=5.750$; $P=0.004$; Fig. 5C), and the *post hoc* analysis showed that the cold-acclimated birds had heightened COX activity compared with warm-acclimated birds. When all temperature acclimation data were pooled for the long photoperiod, log RMR showed a positive and significant correlation with log mitochondrial state-4 respiration ($r^2=0.482$, $P=0.006$; Fig. 6A), but not with log COX activity ($r^2=0.127$, $P=0.210$; Fig. 6C). During temperature acclimation in the short photoperiod, there was no significant relationship between log RMR and log mitochondrial state-4 respiration ($r^2=0.094$, $P=0.287$; Fig. 6B), but there was a significant, positive relationship between log RMR and log COX activity ($r^2=0.457$, $P=0.008$; Fig. 6D).

Protein content, mitochondrial respiration and COX activity in muscle

The mitochondrial protein content of skeletal muscle was not affected by the experimental treatment ($F_{3,24}=0.550$; $P=0.653$; Fig. 5A). However, state-4 respiration and COX activity in muscle were significantly affected by the experimental treatment (state-4 respiration, $F_{3,24}=4602$, $P=0.011$; COX activity, $F_{3,24}=3.478$, $P=0.032$; Fig. 5B,C), and *post hoc* analysis revealed that cold-acclimated birds had heightened activity of respiratory enzymes compared with warm-acclimated birds. An analysis of the temperature acclimation data for the long photoperiod indicated that log RMR had a non-significant association with log state-4 respiration ($r^2=0.080$, $P=0.328$; Fig. 7A) and log COX activity ($r^2=0.136$, $P=0.195$; Fig. 7C). There was a significant, positive relationship between log RMR and log state-4 respiration ($r^2=0.456$, $P=0.008$; Fig. 7B), but not with log COX activity ($r^2=0.127$, $P=0.044$; Fig. 7D) during temperature acclimation for the short photoperiod.

DISCUSSION

Temperature and photoperiod have been shown to affect a wide variety of morphological, physiological and behavioral functions in birds (Swanson et al., 2014; Zhou et al., 2016). In the present study in Chinese bulbuls, we found that temperature and photoperiod had significant effects on the M_b, energy budget, RMR and organ mass of Chinese bulbuls, all of which increased significantly in birds acclimated to a colder ambient temperature and shorter day length. These birds also underwent a significant increase in mitochondrial respiration and COX activity in liver and muscle following cold acclimation.

Effects of temperature and photoperiod on morphology and physiology in Chinese bulbuls

Colder temperatures or shorter photoperiods can cause increased surface heat loss in birds (Saarela and Heldmaier, 1987; Tieleman et al., 2003). Proper adjustment of the morphology, physiology and behavior of small birds helps to ensure their survival in seasonal environments (Swanson, 2010; Zheng et al., 2014a). Changes in

Table 1. Effects of temperature and photoperiod acclimation on organ mass in Chinese bulbuls (*Pycnonotus sinensis*)

	Long day		Short day	
	Warm	Cold	Warm	Cold
Sample size	7	7	7	7
Organ wet mass (g)				
Muscle	3.69±0.26	3.57±0.25	3.18±0.25	3.75±0.28
Brain	0.90±0.02	0.96±0.02	0.93±0.02	0.88±0.03
Heart	0.35±0.02	0.30±0.02	0.33±0.02	0.35±0.02
Lung	0.26±0.01	0.24±0.01	0.24±0.01	0.25±0.01
Liver	0.95±0.10[a]	1.29±0.10[b,c]	1.15±0.10[a,b]	1.48±0.11[c]
Kidney	0.30±0.02	0.33±0.02	0.30±0.02	0.37±0.02
Stomach	0.37±0.02[a]	0.52±0.02[c]	0.41±0.02[a,b]	0.47±0.03[b,c]
Small intestine	0.85±0.11[a]	1.21±0.10[b,c]	1.07±0.10[a,b]	1.49±0.12[c]
Rectum	0.11±0.01[a]	0.14±0.01[a,b]	0.12±0.01[a]	0.16±0.01[b]
Total digestive tract	1.33±0.12[a]	1.87±0.12[b,c]	1.60±0.12[a,b]	2.11±0.13[c]
Organ dry mass (g)				
Muscle	1.01±0.08	0.95±0.08	0.89±0.07	1.04±0.08
Brain	0.20±0.01	0.20±0.00	0.20±0.01	0.19±0.01
Heart	0.09±0.01	0.08±0.01	0.08±0.01	0.09±0.01
Lung	0.06±0.00	0.05±0.00	0.05±0.00	0.06±00
Liver	0.28±0.03[a]	0.38±0.03[a,b]	0.37±0.03[b]	0.49±0.03[c]
Kidney	0.08±0.01	0.09±0.01	0.08±0.01	0.09±0.01
Stomach	0.11±0.01[a]	0.15±0.01[a,b]	0.13±0.01[c]	0.14±0.01[b,c]
Small intestine	0.19±0.03[a]	0.27±0.02[b]	0.24±0.02[a,b]	0.36±0.03[c]
Rectum	0.03±0.00	0.04±0.00	0.04±0.00	0.05±0.00
Total digestive tract	0.33±0.03[a]	0.45±0.03[b]	0.41±0.03[a,b]	0.55±0.03[c]

Long day: 16 h light:8 h dark; short day: 8 h light:16 h dark; warm, 30°C; cold, 10°C. Organ values were corrected by body mass minus wet organ mass resulting from the regression for all birds. Different letters indicate significant differences among treatments after ANCOVA, at $P<0.05$. Data are presented as means±s.e.m.

M_b, especially in small birds, are considered an adaptive strategy essential for survival (Cooper, 2000). M_b can be influenced by a number of environmental factors, including temperature, photoperiod, the quality and abundance of food, and physiological status (Chamane and Downs, 2009; Ni et al., 2010; Zheng et al., 2014a). Some small birds that live in seasonal environments increase their M_b in winter (McKechnie, 2008; McKechnie and Swanson, 2010), by increasing their fat deposits and/or lean mass (Swanson, 1991a; Piersma et al., 1996). There is evidence to suggest that seasonal variation in the M_b of Chinese bulbuls is due, at least in part, to seasonal variation in fat deposits and lean mass (Zheng et al., 2014a; Wu et al., 2014a). Our results are consistent with those of previous reports on the response of Chinese bulbuls to seasonal change (Zheng et al., 2008a, 2014a), and the M_b of bulbuls was higher in cold groups than in warm groups, and in short photoperiod than in long photoperiod. Increased M_b is thought to be associated with cold resistance because this reduces heat loss by decreasing an animal's surface area to volume ratio (Christians, 1999; Zheng et al., 2008a; Chamane and Downs, 2009; Swanson, 2010). Increased M_b may influence thermogenic demands and contribute to the observed increase in RMR, as indicated by the positive correlation between these two variables (see below). The increase in RMR commonly observed under such conditions is thought to be an adaptive response to cold (Swanson, 2001; Vézina et al., 2006; McKechnie et al., 2007; Wiersma et al., 2007). Although we attempted to minimize the potential confounding effects of circadian rhythms by testing experimental subjects simultaneously, we cannot exclude the possibility that our results were confounded by variation in the time of testing, which was conducted between 20:00 h and 24:00 h. Nonetheless, the fact that we detected significant differences among the four treatment groups suggests that the effects of temperature and photoperiod on thermogenesis were unaffected by between-bird differences in circadian rhythm. Our results show that both temperature and photoperiod are important environmental cues

inducing thermogenesis in Chinese bulbuls. Mass-independent and total RMR in the cold-acclimated treatment groups were 16% and 18% higher, respectively, than in the warm-acclimated groups. Mass-independent and total RMR in the short photoperiod treatment groups were 6% and 7% higher, respectively, than in the long photoperiod groups. However, the fact that temperature had a more pronounced effect than photoperiod on RMR is consistent with the documented role of winter temperature as a proximate cue for regulating thermogenic capacity in small birds, including Chinese bulbuls, in cold winter climates (Zheng et al., 2008a, 2010, 2014a). The ability to adjust energy intake to compensate for the energy expended in thermogenesis is essential for survival (Hegemann et al., 2012). Environmental temperature and photoperiod can, however, alter birds' energy intake (Kendeigh, 1945; Stokkan et al., 1986; Lou et al., 2013; Wu et al., 2014b). We found that Chinese bulbuls in the CS group had the highest M_b and that this was consistent with changes in GEI and DEI. These results suggest that acclimation to colder temperatures and shorter photoperiods increases energy consumption because of the increased energy required to maintain body temperature (Cain, 1973; Syafwan et al., 2012). If different physiological systems compete for energy, this would be expected to affect heat production. This is exactly what we found. Chinese bulbuls acclimated to a colder temperature and shorter photoperiod for 4 weeks increased their RMR, liver, stomach and small intestine mass, and liver and muscle mitochondrial state-4 respiration and COX activity, compared with those acclimated to a warmer temperature and longer photoperiod. In view of the mass-specific energy metabolism of these organs and/or tissues, the observed increases in GEI and DEI are not surprising.

McKechnie (2008) and Swanson (2010) found that metabolic rates are regulated via three major physiological and morphological pathways, one of which is changes in internal organ and muscle mass. Organs such as the liver, brain, heart and kidney collectively consume about 60% of an endotherm's total energy expenditure and

Table 2. Allometric correlation and residual correlation for RMR versus dry organ mass (controlled for M_b minus wet organ mass) in Chinese bulbul

Correlation		Muscle	Brain	Heart	Lung	Liver	Kidney	Gizzard	Small intestine	Rectum	Digestive tract
Allometric											
R^2	All birds	0.063	0.004	0.185	0.018	0.306	0.132	0.106	0.496	0.081	0.446
	Temperature, LP	0.001	0.028	0.008	0.003	0.225	0.146	0.208	0.473	0.002	0.383
	Temperature, SP	0.233	0.025	0.258	0.447	0.312	0.204	0.003	0.416	0.114	0.365
P	All birds	0.199	0.760	**0.025**	0.489	**0.002**	0.057	0.091	**0.000**	0.143	**0.000**
	Temperature, LP	0.942	0.572	0.760	0.860	0.087	0.177	0.101	**0.007**	0.893	**0.018**
	Temperature, SP	0.080	0.590	0.064	**0.006**	**0.047**	0.105	0.852	**0.013**	0.238	**0.022**
Slope	All birds	0.137	0.172	0.452	0.218	0.367	0.398	0.339	0.409	0.181	0.514
	Temperature, LP	0.017	0.524	0.112	0.091	0.323	0.687	0.449	0.514	0.029	0.556
	Temperature, SP	0.162	-0.116	0.347	0.814	0.387	0.289	0.048	0.275	0.165	0.363
Residual											
R^2	All birds	0.001	0.001	0.101	0.242	0.007	0.002	0.051	0.189	0.001	0.097
	Temperature, LP	0.001	0.012	0.078	0.152	0.001	0.001	0.069	0.185	0.000	0.073
	Temperature, SP	0.006	0.044	0.190	0.442	0.003	0.036	0.135	0.169	0.000	0.076
P	All birds	0.994	0.861	0.101	**0.008**	0.662	0.829	0.247	**0.025**	0.919	0.106
	Temperature, LP	0.937	0.706	0.332	0.168	0.828	0.962	0.365	0.125	0.920	0.350
	Temperature, SP	0.800	0.481	0.119	**0.009**	0.863	0.512	0.195	0.144	0.972	0.339
Slope	All birds	0.10	37.14	267.79	144.53	13.44	39.99	150.18	88.21	23.46	53.33
	Temperature, LP	1.91	137.31	288.34	952.14	11.39	23.94	215.04	177.95	48.53	57.69
	Temperature, SP	3.64	176.12	287.56	1359.36	7.15	124.13	200.74	63.59	9.73	38.37

RMR, resting metabolic rate; M_b, body mass; LP, long photoperiod; SP, short photoperiod.
P-values in bold type are statistically significant.

consequently are major contributors to overall RMR (Daan et al., 1990; Vermorel et al., 2005). The elevation in RMR of the cold group was presumably related to metabolic and/or morphological adjustments, including changes in organ mass, required to meet the energy demands of acclimation to colder temperature conditions. The dry mass of the liver, stomach, small intestine and total digestive tract all increased significantly with cold acclimation but,

with the exception of the liver, photoperiod did not significantly influence internal organ mass. Similar winter increases in the mass of the liver, stomach, small intestine and total digestive tract in Chinese bulbuls in temperate parts of their range suggest that winter increments in internal organ mass are an important and general metabolic adjustment to cold in this species (Starck and Rahmaan, 2003; Zhang et al., 2008; Zheng et al., 2010, 2014a). Moreover,
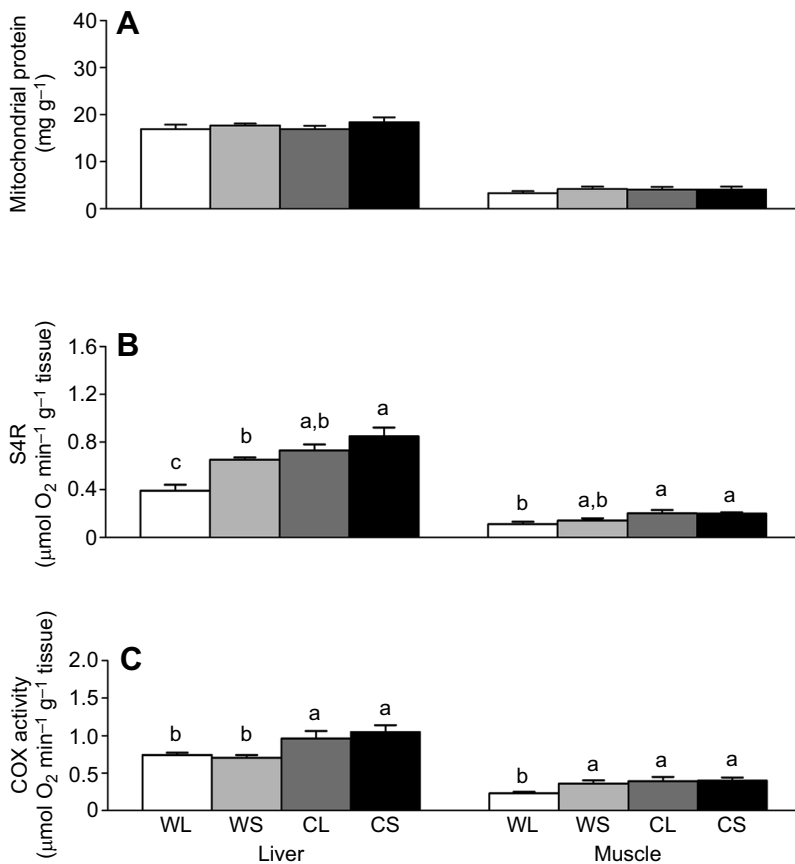

Fig. 5. Differences in mitochondrial protein, state-4 respiration and cytochrome *c* oxidase activity in the liver and pectoral muscle of Chinese bulbuls acclimated to different temperature and photoperiod for 4 weeks.
(A) Mitochondrial protein. (B) State-4 respiration (S4R). (C) Cytochrome *c* oxidase (COX) activity. Data are means± s.e.m., bars with different letters indicate significant differences. Treatment abbreviations as for Fig. 1.
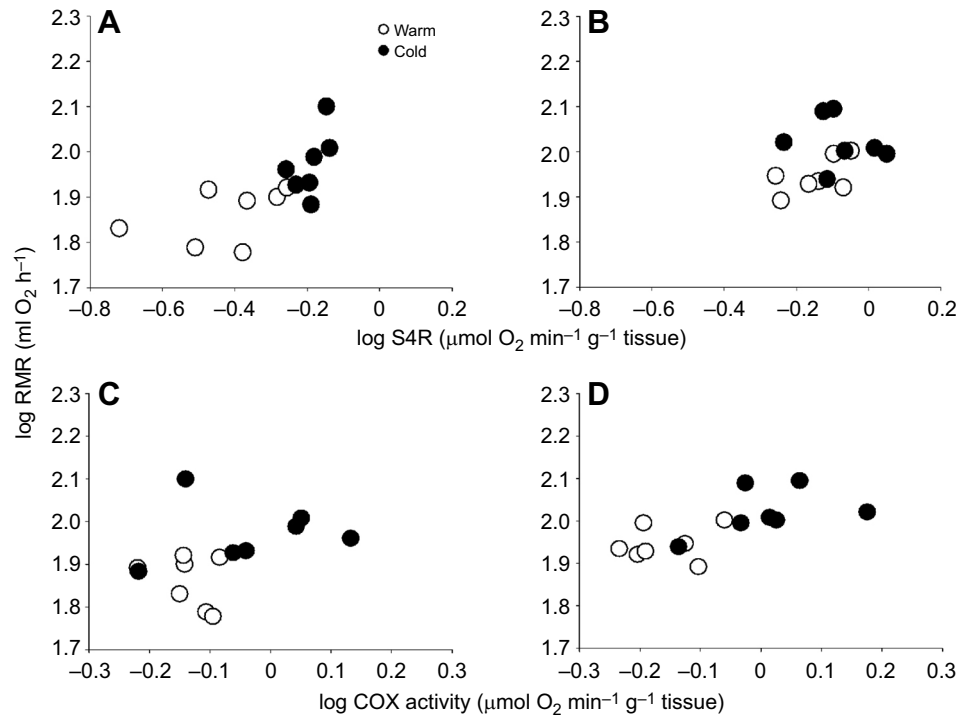
Fig. 6. Correlations between RMR and S4R and RMR and COX activity in the liver of Chinese bulbuls acclimated to different temperature and photoperiod for 4 weeks. (A,B) RMR and S4R in the long (A) and short (B) photoperiod. (C,D) RMR and COX activity in the long (C) and short (D) photoperiod. The allometric equations representing the linear curve for all birds are RMR=2.02S4R$^{0.33}$ and RMR=1.98COX$^{0.34}$, RMR=2.03S4R$^{0.36}$ for temperature acclimation in the long photoperiod, and RMR=2.01COX$^{0.35}$ for temperature acclimation in the short photoperiod. Treatment abbreviations as for Fig. 1.

these results suggest that temperature is the main factor influencing seasonal variation in liver, stomach and small intestine mass in birds, and that increasing the mass of these organs increases the thermogenic capacity of small birds in winter.

Effects of temperature and photoperiod on biochemical responses in liver and muscle

In addition to RMR, cold-acclimated bulbuls in the present study also differed in several physiological and biochemical markers indicative of differences in thermogenic capacity. The dominant role of the liver in RMR is well established (Villarin et al., 2003; Else et al., 2004; Zheng et al., 2008b, 2010). Cellular metabolic intensity in the liver, inferred from mitochondrial respiration or COX activity, is higher in winter than in summer in several bird species (Zheng et al., 2008b, 2013b, 2014a,b). The liver can generate heat by uncoupling oxidative phosphorylation, futile cycling of substrates and high mass-specific metabolic intensity (Else et al., 2004; Zheng et al., 2008b, 2014a; Zhou et al., 2016).
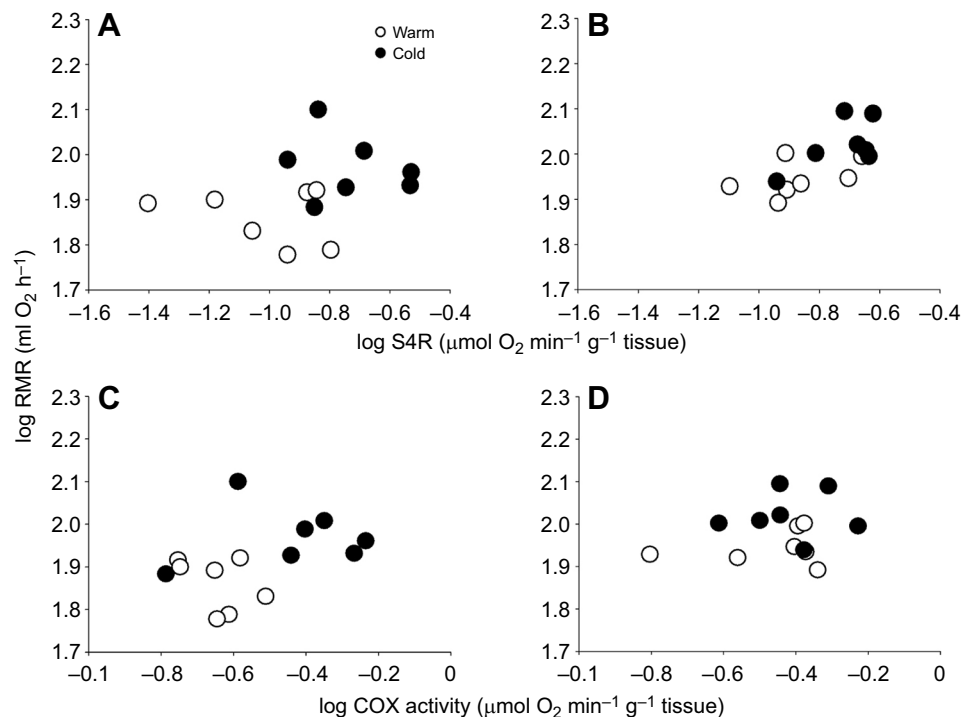

Fig. 7. Correlations between RMR and S4R and RMR and COX activity in the pectoral muscle of Chinese bulbuls acclimated to different temperature and photoperiod for 4 weeks. (A,B) RMR and S4R in the long (A) and short (B) photoperiod. (C,D) RMR and COX activity in the long (C) and short (D) photoperiod. The allometric equations representing the linear curve for all birds are RMR=2.10S4R$^{0.18}$ and RMR=2.05COX$^{0.20}$, and RMR=2.20S4R$^{0.27}$ for temperature acclimation in the short photoperiod. Treatment abbreviations as for Fig. 1.

Muscle is a major organ involved in thermogenesis (Weber and Piersma, 1996; Petit and Vézina, 2014; Swanson et al., 2014), and the observed increase in state-4 respiration and COX activity in muscle indicate enhanced basal thermogenic capacity (Zheng et al., 2008b, 2014a). Previous studies have shown that acclimation to cold temperatures or short photoperiods can induce an increase in mitochondrial state-4 respiration, and citrate synthase and COX activity in muscle, accompanied by enhanced thermogenic capacity, in several passerine species (Vézina and Williams, 2005; Liknes and Swanson, 2011; Zheng et al., 2013a; Swanson et al., 2014). Mitochondrial respiration and enzyme activity in this study were significantly correlated with RMR in either liver or pectoral muscle, which suggests that variation in cellular oxidative phosphorylation capacity (state-4 respiration and COX activity) is a prominent mediator of RMR variation in Chinese bulbuls. Some studies have examined correlations of cellular metabolic capacity with metabolic output in birds, and our results are consistent with data from Eurasian tree sparrow (*Passer montanus*), where variation in state-4 respiration and COX activity was correlated with variation in RMR (Zheng et al., 2008b, 2014b). In the present study, cold temperature induced an increase in liver and muscle state-4 respiration and COX activity in Chinese bulbuls, which also displayed enhanced thermogenic capacity. Consistent with the results of previous studies, cold-acclimated birds had higher levels of state-4 respiration and COX activity in liver and muscle compared with those acclimated to 30°C. Many small birds are known to adjust their cellular metabolic capacity during seasonal activities such as reproduction, migration and winter acclimatization (Swanson, 2010). For example, winter-acclimatized Chinese bulbuls increase their state-4 respiration and COX activity in liver and muscle tissue, which suggests that these adjustments could play an important role in winter thermogenesis (Villarin et al., 2003; Zheng et al., 2014a). State-4 respiration and COX activity in the liver and muscle of birds may either increase or remain seasonally stable during winter acclimatization or migration (Swanson, 2010; Zheng et al., 2008b, 2013b, 2014b). In hwamei, cold acclimation increased the oxidative capacity of the pectoral muscles and liver, which collectively could make a significant contribution to non-shivering thermogenesis (Zhou et al., 2016). High levels of state-4 respiration and COX activity are related to elevated RMR (Zheng et al., 2013a,b), a finding that is supported by the significant correlations between state-4 respiration, COX activity and RMR in this study. Thus, temperature is an important factor affecting thermogenesis in Chinese bulbuls. These results are also consistent with those obtained for other avian species, including the Eurasian tree sparrow (Zheng et al., 2008b, 2014b) and little buntings (*Emberiza pusilla*) (Zheng et al., 2013b). It would be interesting to examine, in further studies, whether increased state-4 respiration and COX activity in bulbuls is also due to their responses to chronic shivering (Bicudo et al., 2001; Zhou et al., 2016). Although we expected state-4 respiration and COX activity in bulbuls to increase with short photoperiod acclimation in this study, this was not the case. We therefore found no evidence to support the hypothesis that photoperiod influences the biochemical markers we measured in this study.

Conclusions

Chinese bulbuls mainly live in habitats with marked seasonal variation in temperature and photoperiod (Zheng et al., 2008a, 2014a). In winter, these animals show enhanced thermogenic capacity that is considered to be an important adaptation for their survival (Zheng et al., 2008a, 2014a). Bulbuls acclimated to a cold temperature and short day length attained higher M_b, internal organ mass, thermogenic capacity and energy intake than those acclimated to a warmer temperature and longer day length. These results support our hypothesis that temperature and photoperiod are important environmental cues for adaptive adjustments in M_b, energy metabolism and thermogenesis in birds. The activation of liver and muscle mitochondrial respiration and the elevation of COX activity appear to be cellular mechanisms underlying the elevation of RMR. The observed physiological and biochemical changes observed under different temperature and photoperiod treatments would allow Chinese bulbuls to overcome the physiological challenges of the extreme seasonal variation in temperature that they encounter in much of their natural range (Zhang et al., 2008; Zheng et al., 2010, 2014a). The morphological, physiological and biochemical changes induced by cold temperature and short photoperiod may lead to energy expenditure, thereby enhancing the survival of Chinese bulbuls in winter, the most energetically challenging time of the year. Further work is required to determine the maximum metabolic rate and to resolve apparent anomalies in the geographical distribution of Chinese bulbuls (Swanson and Garland, 2009; Zheng et al., 2014a). In addition, better molecular data are required to improve our understanding of the complex trade-offs between competing physiological processes that take place during seasonal acclimatization (Teulier et al., 2010).

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