

RESEARCH ARTICLE

Seasonal adjustments in body mass and basal thermogenesis in Chinese hwameis (*Garrulax canorus*): the roles of temperature and photoperiod

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ABSTRACT

For small birds to survive during seasonal acclimatization in temperate zones, regulation of body mass and thermogenesis is crucial. To determine the role of temperature and photoperiod in seasonal changes in body mass and thermogenesis in Chinese hwameis (*Garrulax canorus*), we compared body mass, basal metabolic rate (BMR), energy intake and cellular metabolic capacity of the tissue (muscle) and/or organs (liver, kidney, heart and small intestine) in seasonally acclimatized and laboratory-acclimated hwameis. A significant seasonal influence on body mass and BMR (which peaked in winter) was found, and these variations were mirrored by exposing the housed birds to cold temperatures or a short photoperiod. The level of dry matter intake, gross energy intake and digestible energy intake was higher during winter, and in housed animals that were exposed to cold temperatures. These results suggest that by increasing energy intake and thermogenesis, Chinese hwameis can overcome winter thermoregulatory challenges. When compared with warm-acclimated birds, cold-acclimated birds displayed higher mass-specific and whole-organ state 4 respiration in the muscle, liver and kidney, and higher mass-specific and whole-organ cytochrome *c* oxidase activity in the liver. These data demonstrate that cellular thermogenesis partly underpins basal thermoregulation in Chinese hwameis. Cold temperature and short photoperiod can be used as helpful environmental cues during seasonal acclimatization. However, the role of temperature is more significant compared with that of photoperiod in Chinese hwameis; the changes in energy metabolism and thermoregulation induced by temperature appear to be intensified by photoperiod.

KEY WORDS: Basal metabolic rate, Birds, Cytochrome *c* oxidase, Energy intake, State 4 respiration

INTRODUCTION

Phenotypic flexibility as manifested in behavior, physiology and morphology is common in species of small birds because it is essential for adapting to seasonal changes in a living environment (Swanson, 1991; Zheng et al., 2008a; 2008b; Liknes and Swanson, 2011). The concept of phenotypic flexibility describes a phenotypic change that can be reversed and repeated, and is temporary, such as that which is evident during both temperature acclimation and

seasonal acclimatization (Piersma and Drent, 2003; Liknes and Swanson, 2011). Animals living in a temperate zone provide an excellent natural opportunity for studying phenotypic flexibility, responding positively to a seasonal climate change (Liknes and Swanson, 2011; Zheng et al., 2014a). During winter, phenotypic flexibility in metabolic power output is an important component of the thermoregulatory response seen in birds during periods of elevated energy requirements (Swanson, 1991, 1993, 2010; Zheng et al., 2008a,b, 2014a,b; Wu et al., 2015; Li et al., 2017).

The minimum metabolic rate of an animal required to maintain normal physiological function is the basal metabolic rate (BMR), which refers to the rate of energy transformation of an animal at resting and fasting states and in the thermal neutral zones (TNZ). TNZ is the range of ambient temperatures at which temperature regulation is achieved only by the control of sensible heat loss without regulatory changes in metabolic heat production or evaporative heat loss (IUPS Thermal Commission, 1987; McKechnie, 2008; Li et al., 2020). BMR is proof of an elastic trait in small birds that can be regulated seasonally by making modulations at several organizational levels (McKechnie, 2008; Swanson et al., 2017). BMR is among the key physiological criteria used in evaluating the energy cost associated with thermogenesis. Environmental physiologists and comparative physiologists have more recently focused on the use of BMR as a guideline for energy consumption (McNab, 2009; Swanson et al., 2017; Zheng et al., 2014b). Some small birds have been found to display significant seasonal fluctuation in BMR, such as mountain chickadee (*Poecile gambeli*) and Juniper titmouse (*Baeolophus ridgwayi*) (Cooper, 2002), American goldfinch (*Carduelis tristis*) (Dawson and Carey, 1976), downy woodpecker (*Picoides pubescens*) (Liknes and Swanson, 1996), Chinese bulbul (*Pycnonotus sinensis*) (Zheng et al., 2008a, 2014a) and Eurasian tree sparrow (*Passer montanus*) (Zheng et al., 2008b, 2014b). Physiologically, BMR is considered to originate primarily from the metabolically active organs that maintain relatively high activity at rest. Such high activity results from protein turnover, ion transport, maintenance of enzyme activity and proton transport across the mitochondrial membrane (Chappell et al., 1999; Klaassen et al., 2004; Nespolo et al., 2011; Swanson et al., 2017). As the most important regulator of mitochondrial oxidative phosphorylation, cytochrome *c* oxidase (COX) activity in the tissues is frequently used to assess metabolic intensity at the cellular level (Liu et al., 2005; Zheng et al., 2014a; Zhou et al., 2016; Hu et al., 2017). Additionally, state 4 respiration (S4R), defined as the consumption of oxygen by isolated mitochondria on a particular substrate in the absence of ADP or any metabolic poisons or inhibitors, is considered a guideline for resting mitochondrial respiration rates in isolated mitochondria. S4R is an appropriate measure for investigating the effectors in the variation of BMR, because it can reflect resting aerobic flux

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List of abbreviations

BMR	basal metabolic rate
CL	cold and long photoperiod
COX	cytochrome <i>c</i> oxidase
CS	cold and short photoperiod
DEI	digestible energy intake
DMI	dry matter intake
GEI	gross energy intake
S4R	state 4 respiration
WL	warm and long photoperiod
WS	warm and short photoperiod
TNZ	thermal neutral zone

accurately in the cells (Zheng et al., 2014a; Zhou et al., 2016; Hu et al., 2017; Swanson et al., 2017; Li et al., 2020). Therefore, variations in metabolic intensity at the cellular level in some tissues usually correlate with BMR although this is not always the case (Zhou et al., 2016; Swanson et al., 2017; Li et al., 2020).

For small birds, the ability to adjust body mass is thought to be a crucial survival strategy. Birds with stable body mass and body composition can maintain the balance between energy usage and energy intake. However, body mass will change when energy intake is not equal to energy usage (Hammond and Diamond, 1997; Li and Wang, 2005). A number of studies conducted with small birds have reported a higher level of body mass in the winter. Examples include the common redpoll (*Acanthis flammea*) (Pohl and West, 1973; Wu et al., 2015), American goldfinch (Dawson and Carey, 1976), dark-eyed Junco (*Junco hyemalis*) (Swanson, 1991) and white-breasted nuthatch (*Sitta carolinensis*) (Liknes and Swanson, 2011). However, few studies have investigated body mass and energy intake in seasonally acclimatized small bird species (Wu et al., 2014).

In animals, temperature and photoperiod are recognized as the two most significant factors influencing seasonal thermoregulation. Several morphological, physiological and behavioral adaptations are also considered to be driven by changes in temperature and photoperiod (McKechnie and Swanson, 2010; Swanson et al., 2017; Zhang et al., 2018). Several studies have demonstrated the influence of temperature and/or photoperiod on BMR, body mass and the energy balance of birds (McKechnie, 2008; Swanson et al., 2014; Hu et al., 2017). Compared with birds that live in a warm climate and/or under a long photoperiod, those that live in a cold climate and/or under a short photoperiod generally show higher BMR levels (Williams and Tieleman, 2000; Ni et al., 2011; Zheng et al., 2013a; Swanson et al., 2014; Zhou et al., 2016; Hu et al., 2017; Cui et al., 2019; Li et al., 2020). Increased basal thermogenesis and cellular metabolic capacity have also been demonstrated in birds in response to cold and/or short photoperiod conditions (Saarela and Heldmaier, 1987; Ni et al., 2011; Swanson et al., 2014; Hu et al., 2017; Li et al., 2020). However, most studies seem to suggest that temperature is the main cue responsible for changes in metabolic rate (Swanson et al., 2014; Hu et al., 2017; Li et al., 2020).

As one of the endemic species in Asia, Chinese hwameis, *Garrulax canorus* (Linnaeus, 1758) (Passeriformes, Leiothrichidae), are found mainly in central and southern China, as well as in northern and central Vietnam (Li et al., 2006). Hwameis mainly live in the low mountains, hills and plains at the foot of mountains and bushes below an altitude of 1800 m, but they also live in forest margins, farmland, wilderness, small trees,

bamboo forests and gardens near villages and towns (Li et al., 2006). Based on allometric equations, hwameis have a relatively lower BMR compared with the predicted value from its body mass (Liu et al., 2005), as well as a high body temperature, narrow TNZ and high evaporative water production/metabolic water production ratio (Xia et al., 2013). Additionally, as observed in other temperate passerines, Chinese hwameis are capable of daily and seasonal metabolic acclimatization. They have been shown to have an increased level of BMR in the daytime and winter compared with that in the night-time and summer (Wu et al., 2015; Zhao et al., 2015; Wang et al., 2019). Chinese hwameis exhibit significant seasonal (summer and winter) and temperature variation in BMR and cellular aerobic capacity (Zhou et al., 2016; Wang et al., 2019). However, seasonal patterns of thermogenesis and energy demands in Chinese hwameis throughout the entire annual cycle have not been determined previously. Furthermore, very little information is available regarding the combined effect of cold condition and short photoperiod on thermoregulation in Chinese hwameis. The present study is a continuation of an investigation into the seasonal phenotypic flexibility of Chinese hwameis, which have previously been found to exhibit summer to winter variation in thermogenic properties over a range of ambient temperatures (Wu et al., 2015; Wang et al., 2019). We hypothesized that Chinese hwameis could adapt to seasonal changes in temperature and photoperiod by making seasonal adjustments to their body mass and basal thermogenesis, including increasing body mass, BMR, energy intake and cellular metabolic capacity in response to cold temperatures or short photoperiods. We also hypothesized that cold temperature, rather than photoperiod, is the primary factor driving physiological and biochemical changes in the thermoregulatory response of Chinese hwameis.

MATERIALS AND METHODS**Study site**

Our study was conducted in 2019 in Wenzhou City, Zhejiang Province (27°29N, 120°51E, 14 m in elevation), China. Wenzhou has a warm temperate climate with an average annual rainfall of 1500 mm spread across the entire year, with slightly more precipitation during spring and summer (Zheng et al., 2014a). The mean temperature ranges from 29.0°C in August to 9.5°C in January, and mean day length ranges from 13.9 h in July to 10.4 h in December (Fig. 1).

Animals and experimental design

Mist nets were used to capture the adult Chinese hwameis. The captured birds were immediately weighed to the nearest 0.1 g, and then transported to the laboratory and housed in single cages (40×30×30 cm³) at 25±1°C on a 12 h light:12 h dark photoperiod, with lights on at 06:00 h (Zhou et al., 2016; Wang et al., 2019). The birds were provided with *ad libitum* food (Xietong Bioengineering Co. Ltd, Jiangsu, China) and water. They were housed in these conditions for 2 weeks of rearing prior to the following experiments. All experimental procedures were approved by Wenzhou University's Animal Care and Use Committee.

Experiment 1: to confirm the effect of seasonal changes on body mass, basal thermogenesis and energy budget, 15 hwameis were moved from the laboratory and individually kept in cages (40×30×30 cm³) in an outdoor enclosure. Following a 2 week stabilization period, body mass, BMR, dry matter intake (DMI), gross energy intake (GEI), digestible energy intake (DEI) and digestibility were measured monthly for a year.

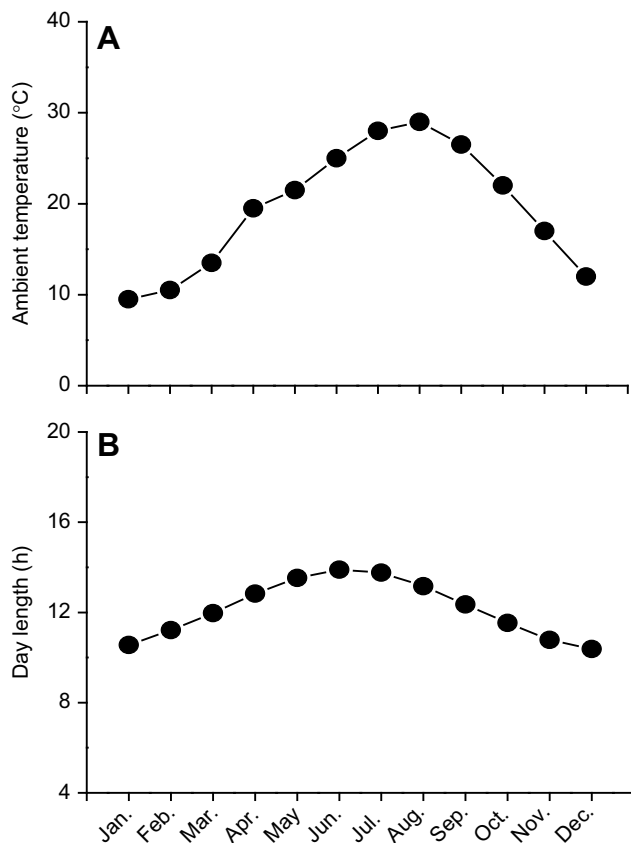


Fig. 1. Annual trends at the capture location for Chinese hwameis (*Garrulax canorus*) in this study. (A) Average ambient temperature and (B) photoperiod in Wenzhou City, Zhejiang Province, China.

Experiment 2: to test for seasonal changes in cellular metabolic capacity in metabolically active tissues and organs, eight birds were captured in spring (March), in summer (June), in autumn (September) and in winter (December) 2019. The birds were euthanized by cervical dislocation and their pectoral muscles, liver, kidneys, heart and small intestine were removed and weighed, and their levels of S4R and cytochrome *c* oxidase (COX) activity were measured (Zheng et al., 2014a; Li et al., 2020).

Experiment 3: to confirm that temperature and photoperiod could lead to seasonal changes in the physiological parameters measured in experiment 1, 32 birds were captured in June of 2019, and after 2 weeks of rearing, they were randomly divided into four 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) (Swanson et al., 2014; Hu et al., 2017; Li et al., 2020). The birds were reared for another 4 weeks, and during this period, their body mass, basal thermogenesis and energy budget were measured once every week. Two birds died during the 4 week acclimation period, one from the WL group and the other from the CL group, and therefore they were eliminated from the data analysis.

Experiment 4: to test for temperature and photoperiod effects on cellular metabolic capacity measured in experiment 3, the birds were euthanized at the end of the 4 week experiment. S4R and COX activity in the pectoral muscles, liver, kidneys, heart and small intestine were measured (Li et al., 2020).

Oxygen consumption

Oxygen consumption was measured with an open-circuit respiration system (TSE Systems, Bad Homburg, Germany) as described by Zhou et al. (2016) and Wen et al. (2019) with some minor modifications. Each bird was placed in a separate 1.5 l plastic metabolic chamber. All gas exchange measurements were taken in the dark during the rest period. To mimic a post-absorptive state, food was withdrawn 4 h before each test. H₂O and CO₂ were scrubbed from the air before it was pumped at a rate of 1000 ml min⁻¹ through the cylindrical sealed Perspex chamber containing silica gel/soda lime/silica. Gases leaving the chamber were also dried using a special drier (TSE Systems) and directed through an oxygen analyzer at a flow rate of 300 ml min⁻¹ in warm conditions and summer, and 600 ml min⁻¹ in cold conditions and winter (Tan et al., 2016; Zhou et al., 2016; Wen et al., 2019). The data were recorded and averaged every 10 s by a computer connected via an analog-to-digital converter (Lab Master, TSE Systems), and then analyzed using standard software (TSE Systems). The Lab Master software can translate this signal into a digital signal (Tan et al., 2016; Wen et al., 2019). The oxygen consumption data were recorded at every minute according to eqn 2 of Hill (1972). Oxygen consumption rate was measured at 30±0.5°C, which is within the TNZ of Chinese hwamei as previously described by Wu et al. (2015) and Zhou et al. (2016). BMR was calculated from the lowest rate of oxygen consumption over 10 min. Metabolic rate was expressed as ml O₂ g⁻¹ h⁻¹ and ml O₂ h⁻¹, and corrected to standard temperature, pressure and dry gas conditions (Zheng et al., 2008b; Zhou et al., 2016; Tang et al., 2022). The average mass of each bird in each experimental group was calculated by measuring the mass of each bird before and after the experiment (Wu et al., 2015; Zhou et al., 2016).

Energy intake

Food residue and feces were collected once for 3 days as described previously (Wu et al., 2014; Li et al., 2020). A C2000 oxygen bomb calorimeter (IKA, Germany) was used to determine the gross energy content of the food and feces (Zhou et al., 2016). DMI (g day⁻¹), GEI (kJ day⁻¹), DEI (kJ day⁻¹) and digestibility (%) were calculated as follows:

$$\text{GEI} = \text{Dry matter intake} \times \text{Food caloric value}, \quad (1)$$

$$\text{Feces energy} = \text{Dry feces} \times \text{Feces caloric value}, \quad (2)$$

$$\text{DEI} = \text{GEI} - \text{Feces energy}, \quad (3)$$

$$\text{Digestibility} = \text{DEI/GEI} \times 100, \quad (4)$$

where food and feces caloric value are in kJ g⁻¹ dry matter, feces energy is in kJ day⁻¹, and dry feces is in g day⁻¹.

Measurements of tissue S4R and COX activity

S4R in the pectoral muscles, liver, kidneys, heart and small intestine was measured with a Clark electrode (Hansatech Instruments; DW-1) 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 MgCl₂, 1 mmol l⁻¹ EDTA and 5 mmol l⁻¹ KH₂PO₄, pH 7.2). S4R was measured with succinate as the substrate, for 1 h (Zhou et al., 2016; Hu et al., 2017; Li et al., 2020). COX activity in the pectoral muscles, liver, kidneys, heart and small intestine was measured with the Clark electrode at 30°C in respiration medium (100 mmol l⁻¹ KCl, 20 mmol l⁻¹ TES, 1 mmol l⁻¹ EGTA, 2 mmol l⁻¹ MgCl₂, 4 mmol l⁻¹ KH₂PO₄,

60 mmol l⁻¹ BSA, at pH 7.2). A 10 ml aliquot was taken from the tissue extract and 30 ml of cytochrome *c* (37.9 mg ml⁻¹) was added to the electrode, and the activity of COX was measured in a final volume of 2 ml (Zhou et al., 2016; Li et al., 2020). Both measurements were expressed as mean mass-specific values ($\mu\text{mol O}_2 \text{ min}^{-1} \text{ g}^{-1}$ tissue) and as whole-organ activity ($\mu\text{mol O}_2 \text{ min}^{-1}$) (Zhou et al., 2016; Hu et al., 2017; Li et al., 2020).

Data analysis

All data were analyzed by SPSS (version 21.0). The Kolmogorov–Smirnov test was used to check all variables for normality. Non-normally distributed data were normalized by transforming them to natural logarithm (Zheng et al., 2014b; Zhou et al., 2016). Seasonal differences in body mass and digestibility, as well as any changes in DMI, GEI and DEI, were analyzed using repeated measures ANOVA (RM-ANOVA) with body mass as a covariate (Li and Wang, 2005; Zheng et al., 2014a). As digestibility consists of percentage data, which is typically not normally distributed, arcsin-square root transformation was performed prior to statistical analysis. One-way ANOVA was used to determine any seasonal differences in S4R and COX. The effects of temperature and photoperiod on body mass and digestibility were analyzed using RM-ANOVA, and differences in DMI, GEI and DEI over time were analyzed by RM-ANCOVA, with body mass as a covariate. The significance of body mass and digestibility among different groups on the same day was evaluated with two-way ANOVA. Direct comparisons of BMR, DMI, GEI and DEI among different groups on the same day were made using two-way ANCOVA with body mass as the covariate. Differences in S4R and COX among different groups were determined using two-way ANOVA. Preliminary models were used to check whether the slope of the dependent variable was homogeneous with respect to the different treatments. Significant differences among treatment groups were identified with Tukey's *post hoc* tests. Differences between group means were evaluated for significance using Student–Newman–Keul's *post hoc* test. Least-squares linear regression was used to assess the statistical significance of the correlations between different variables. All results are expressed as means \pm s.e.m; $P < 0.05$ was considered statistically significant.

RESULTS

Experiment 1

Seasonal changes of body mass and BMR

The body mass of Chinese hwameis underwent significant seasonal changes in 2019 (RM-ANOVA, $F_{11,154}=19.826$, $P < 0.001$; Fig. 2A). Between January and March, body mass remained relatively stable, but it then decreased from April to July, before increasing to a maximum in December, which was 10% higher than in July. A significant negative linear relationship was found between body mass and ambient temperature ($r = -0.585$, $P = 0.046$), as well as between body mass and photoperiod ($r = -0.848$, $P < 0.001$). Mass-specific BMR varied significantly over the seasons (RM-ANOVA, $F_{11,154}=9.580$, $P < 0.001$; Fig. 2B). It was at a maximum in February and decreased significantly from March to July. Whole-organism BMR also varied substantially over the seasons (RM-ANOVA, $F_{11,154}=17.227$, $P < 0.001$; Fig. 2C). Mass-specific and whole-organism BMR were 35% and 46% higher in February than July, respectively. These results indicated a significant negative correlation between BMR and ambient temperature ($r = -0.696$, $P = 0.012$) and photoperiod ($r = -0.808$, $P = 0.002$). There was also a significant positive linear relationship between log body mass and log BMR ($r = 0.886$, $P < 0.001$).

Seasonal changes of energy intake and digestibility

DMI and GEI varied significantly over the seasons (RM-ANOVA, DMI, $F_{11,154}=11.360$, $P < 0.001$; Fig. 3A; GEI, $F_{11,154}=11.360$, $P < 0.001$; Fig. 3B), with the highest levels in February and the lowest in May. A similar change also occurred in DEI ($F_{11,154}=23.588$, $P < 0.001$; Fig. 3C). Digestibility also showed significant seasonal variation ($F_{11,154}=4.037$, $P < 0.001$; Fig. 3D). However, after adjusting for body mass as the covariate, only DEI was found to differ significantly among seasons (RM-ANCOVA, $F_{11,22}=2.780$, $P = 0.020$). A positive correlation existed between log body mass and log GEI ($r = 0.780$, $P = 0.003$), as well as between log body mass and log DEI ($r = 0.745$, $P = 0.005$). There

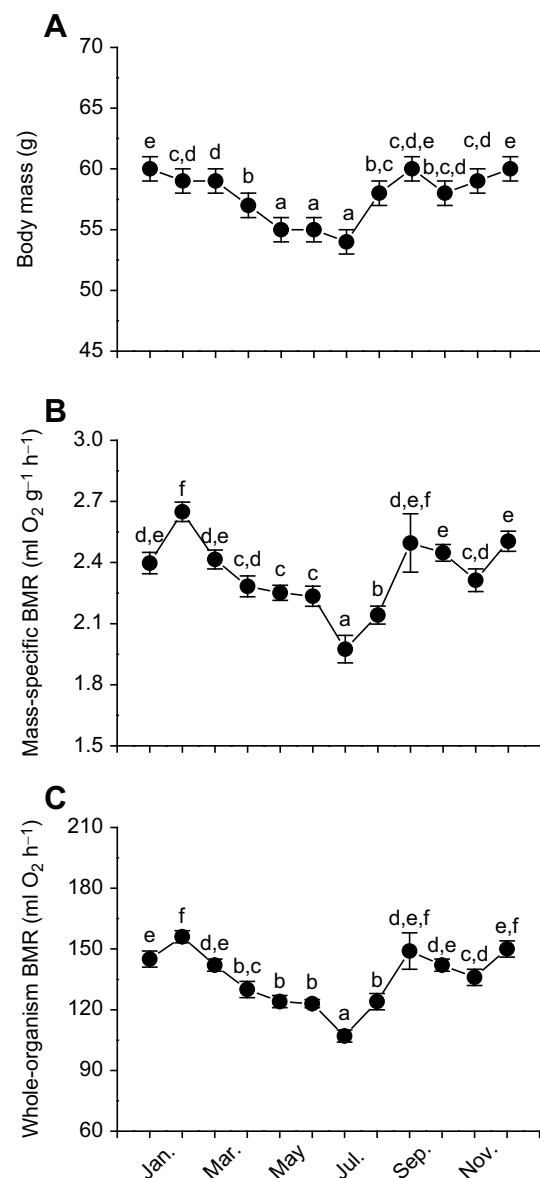


Fig. 2. Seasonal changes in body mass, and mass-specific and whole-organism basal metabolic rate of Chinese hwameis. Body mass (A) and mass-specific basal metabolic rate (BMR; B) were determined by repeated-measures ANOVA (RM-ANOVA). Whole-organism BMR (C) was determined by repeated-measures ANCOVA (RM-ANCOVA) with body mass as a covariate. Data are presented as means \pm s.e.m. Different letters indicate seasonal differences.

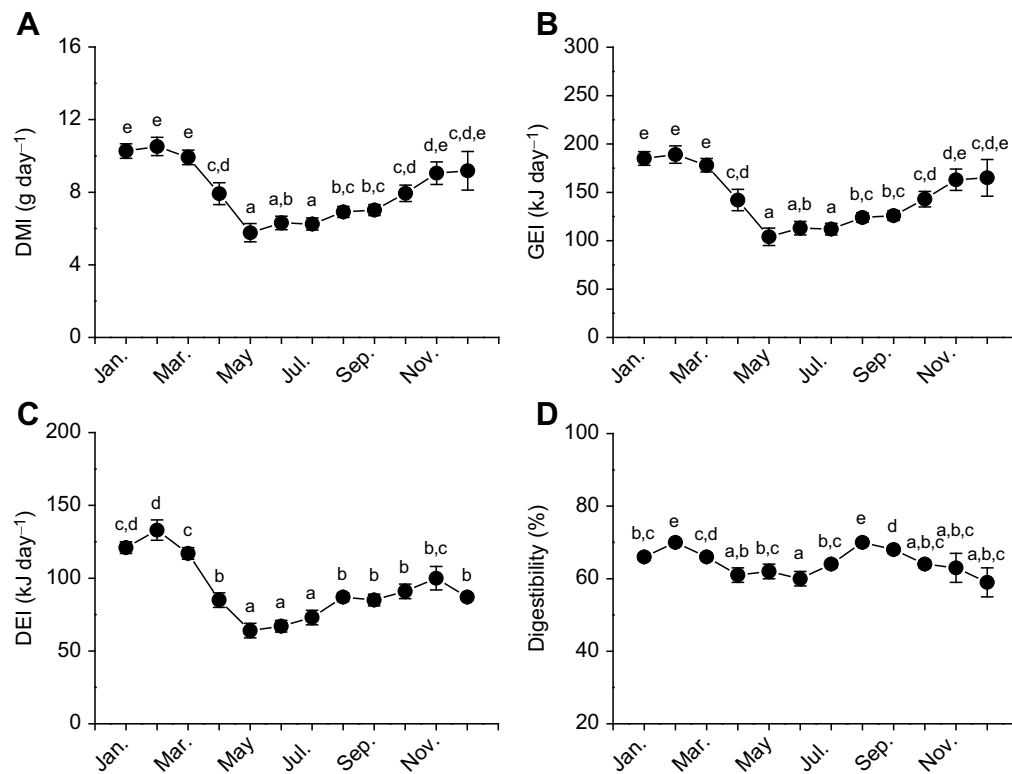


Fig. 3. Seasonal changes in dry matter intake, gross energy intake, digestible energy intake and digestibility in Chinese hwameis. Dry matter intake (DMI; A), gross energy intake (GEI; B) and digestible energy intake (DEI; C) were determined by RM-ANCOVA with body mass as a covariate; digestibility (D) was determined using arcsin-square root transformation prior to RM-ANOVA. Data are presented as means \pm s.e.m. Different letters indicate seasonal differences.

was also a positive relationship between log BMR and log GEI ($r=0.763$, $P=0.004$), and between log BMR and log DEI ($r=0.704$, $P=0.011$).

Experiment 2

Seasonal changes in S4R and COX activity

Mass-specific S4R ($\mu\text{mol O}_2 \text{ min}^{-1} \text{ g}^{-1} \text{ tissue}$; Fig. 4A) in muscle (one-way ANOVA, $F_{3,28}=0.320$, $P=0.811$), liver ($F_{3,28}=0.203$, $P=0.893$), kidney ($F_{3,28}=0.121$, $P=0.947$), heart ($F_{3,28}=0.779$, $P=0.515$) and small intestine ($F_{3,28}=2.421$, $P=0.087$) did not vary significantly among seasons. Similarly, whole-organ S4R ($\mu\text{mol O}_2 \text{ min}^{-1}$; Fig. 4B) in muscle (one-way ANOVA, $F_{3,28}=0.343$, $P=0.794$), liver ($F_{3,28}=1.414$, $P=0.295$), kidney ($F_{3,28}=0.335$, $P=0.800$) and heart ($F_{3,28}=0.592$, $P=0.625$) did not vary significantly among seasons. However, whole-organ S4R in the small intestine varied significantly among seasons ($F_{3,28}=6.230$, $P=0.002$), with a higher level in autumn compared with spring and summer.

Only mass-specific COX activity ($\mu\text{mol O}_2 \text{ min}^{-1} \text{ g}^{-1} \text{ tissue}$; Fig. 5A) of muscle (one-way ANOVA, $F_{3,28}=3.117$, $P=0.042$) showed a significant seasonal variation, with a higher level of activity in spring and autumn compared with summer. Mass-specific COX activity did not differ among seasons for the liver ($F_{3,28}=2.859$, $P=0.055$), kidney ($F_{3,28}=1.155$, $P=0.344$), heart ($F_{3,28}=0.633$, $P=0.600$) or small intestine ($F_{3,28}=0.031$, $P=0.993$). Whole-organ COX activity ($\mu\text{mol O}_2 \text{ min}^{-1}$; Fig. 5B) of muscle (one-way ANOVA, $F_{3,28}=3.750$, $P=0.022$) and liver ($F_{3,28}=5.896$, $P=0.003$) showed a significant seasonal variation. Whole-organ COX activity of muscle was higher in spring and autumn compared with summer. Furthermore, whole-organ COX activity of the liver was higher in autumn compared with that in spring and summer. Whole-organ COX activity did not differ among seasons for the kidney ($F_{3,28}=1.300$, $P=0.294$), heart ($F_{3,28}=0.523$, $P=0.670$) or small intestine ($F_{3,28}=2.161$, $P=0.115$).

Experiment 3

Effect of temperature and photoperiod on body mass and BMR

Prior to acclimation, the body mass of Chinese hwameis did not differ significantly among groups (ANOVA, $F_{3,26}=0.108$, $P=0.955$; Fig. 6A). At the end of the experiment, temperature and photoperiod had a significant influence on body mass (temperature, RM-ANOVA, $F_{4,104}=3.631$, $P=0.008$; photoperiod, RM-ANOVA, $F_{4,104}=3.053$, $P=0.020$); however, the connection between temperature and photoperiod was not significant (RM-ANOVA, $F_{4,104}=2.003$, $P=0.100$) during acclimation. The cold groups had a significantly higher body mass compared with the warm groups ($t_{28}=2.163$, $P=0.039$). Similarly, body mass was higher in the short photoperiod groups than in the long photoperiod groups ($t_{28}=2.110$, $P=0.044$). During acclimatization, mass-specific BMR was significantly affected by temperature (RM-ANOVA, $F_{4,104}=8.088$, $P<0.001$; Fig. 6B) and photoperiod (RM-ANOVA, $F_{4,104}=3.637$, $P=0.008$). However, no significant association between temperature and photoperiod was observed (RM-ANOVA, $F_{4,104}=2.126$, $P=0.083$). Temperature had a substantial effect on mass-specific BMR following acclimation, with cold acclimation conditions causing a 12% increase in mass-specific BMR compared with warm acclimation conditions at the end of the 4 week experimental period ($t_{28}=3.394$, $P=0.002$). However, photoperiod appeared to have a significant effect on the mass-specific BMR of the cold-acclimatized groups on day 7 (two-way ANOVA, $F_{1,26}=12.235$, $P=0.002$; Fig. 6B), in which a significant increase in BMR was detected for the CL group although only a slight increase was observed for the CS group. In contrast, BMR remained essentially constant in the WL group, while decreasing slightly in the WS group on day 7. Thus, the significant photoperiodic effect on the mass-specific BMR on day 7 was mainly manifested by the large BMR increase in the CL group, and this increase was not transient, as it was maintained (or even slightly increased) over the longer acclimation periods, but this was not sustained over the entire

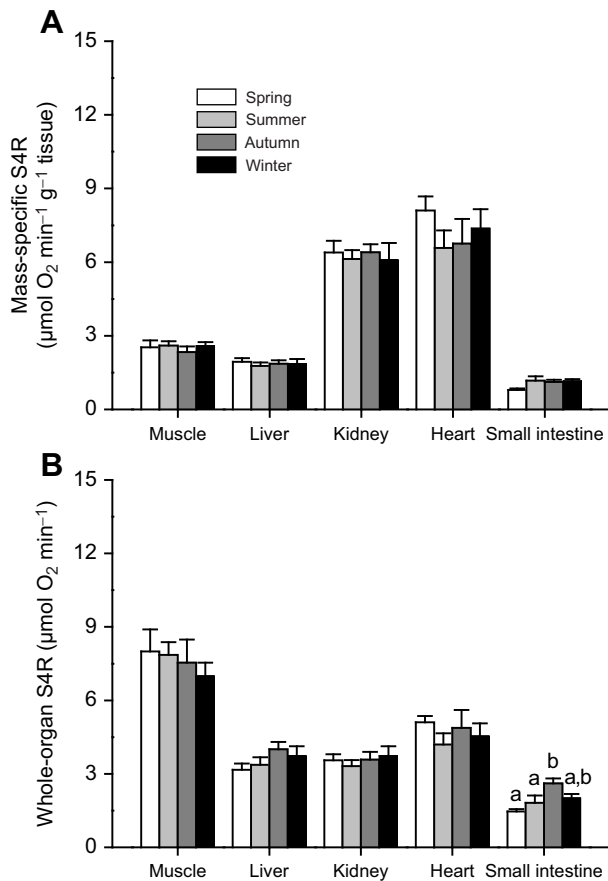


Fig. 4. Seasonal variation in mass-specific and whole-organ state 4 respiration in Chinese hwameis. Mass-specific (A) and whole-organ (B) state 4 respiration (S4R) are shown for the pectoral muscles, liver, kidneys, heart and small intestine in different seasons. Statistical significance was determined by one-way ANOVA. Data are presented as means \pm s.e.m. Different letters indicate seasonal differences.

experiment. The whole-organism BMR was also significantly affected by temperature (RM-ANCOVA, $F_{4,84}=5.924$, $P<0.001$) and photoperiod (RM-ANCOVA, $F_{4,84}=3.335$, $P=0.014$), but not the interaction between temperature and photoperiod (RM-ANCOVA, $F_{4,84}=1.630$, $P=0.174$) during acclimation (Fig. 6C). The whole-organism BMR was significantly higher in the cold groups than in the warm groups ($t_{28}=4.392$, $P<0.001$), and averaged 20% higher than that of warm-acclimated birds at the end of the experiments. The effect of photoperiod on whole-organism BMR was similar to that on mass-specific BMR, as whole-organism BMR also underwent a significant increase on day 7 (two-way ANCOVA, $F_{1,21}=5.389$, $P=0.030$; Fig. 6C). The slopes for temperature acclimatization were considerably varied, according to a homogeneity of slopes test. There was a significant, positive correlation between log body mass and log BMR for both the long photoperiod ($r=0.549$, $P=0.042$) and short photoperiod ($r=0.672$, $P=0.004$) groups.

Effect of temperature and photoperiod on energy intake and digestibility

There were no significant differences among temperature and photoperiod treatment groups for DMI, GEI, DEI or digestibility before acclimation ($P>0.05$). However, DMI, GEI and DEI were significantly affected by temperature (DMI, RM-ANCOVA,

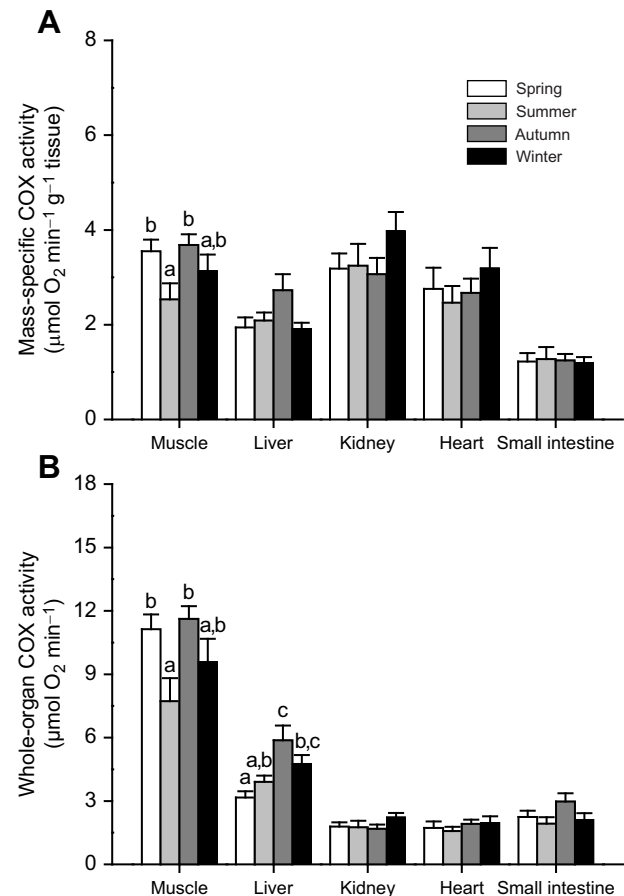


Fig. 5. Seasonal variation in mass-specific and whole-organ cytochrome c oxidase activity in Chinese hwameis. Mass-specific (A) and whole-organ (B) cytochrome c oxidase (COX) activity of the pectoral muscles, liver, kidneys, heart and small intestine in different seasons. Statistical significance was determined by one-way ANOVA. Data are presented as means \pm s.e.m. Different letters indicate seasonal differences.

$F_{4,84}=21.984$, $P<0.001$; Fig. 7A; GEI, RM-ANCOVA, $F_{4,84}=21.984$, $P<0.001$; Fig. 7B; DEI, RM-ANCOVA, $F_{4,84}=16.702$, $P<0.001$; Fig. 7C) but not photoperiod (DMI, RM-ANCOVA, $F_{4,84}=1.227$, $P=0.285$; GEI, RM-ANCOVA, $F_{4,84}=1.227$, $P=0.285$; DEI, RM-ANCOVA, $F_{4,84}=1.625$, $P=0.176$), and there was a significant interaction between temperature and photoperiod (DMI, RM-ANCOVA, $F_{4,84}=0.767$, $P=0.549$; GEI, RM-ANCOVA, $F_{4,84}=0.767$, $P=0.549$; DEI, RM-ANCOVA, $F_{4,84}=1.797$, $P=0.137$). After 7 days of acclimation, DMI, GEI and DEI increased more under cold conditions than under warm conditions (DMI, two-way ANCOVA, $F_{1,21}=52.587$, $P<0.001$; GEI, two-way ANCOVA, $F_{1,21}=52.587$, $P<0.001$; DEI, two-way ANCOVA, $F_{1,21}=21.977$, $P<0.001$), and this trend remained until the end of the acclimation period. Digestibility was not affected by temperature (RM-ANOVA, $F_{4,104}=3.25$, $P=0.861$; Fig. 7D) or photoperiod (RM-ANOVA, $F_{4,104}=1.015$, $P=0.403$) and the interaction between temperature and photoperiod was not significant (RM-ANOVA, $F_{4,104}=0.542$, $P=0.705$). When all the temperature acclimation data were pooled for the long photoperiod, no significant relationship was observed between log GEI and log body mass ($r=0.461$, $P=0.097$) or between log DEI and log body mass ($r=0.438$, $P=0.117$). However, when all the temperature acclimation data were pooled for short photoperiod, a significant relationship was observed between log GEI and log body mass

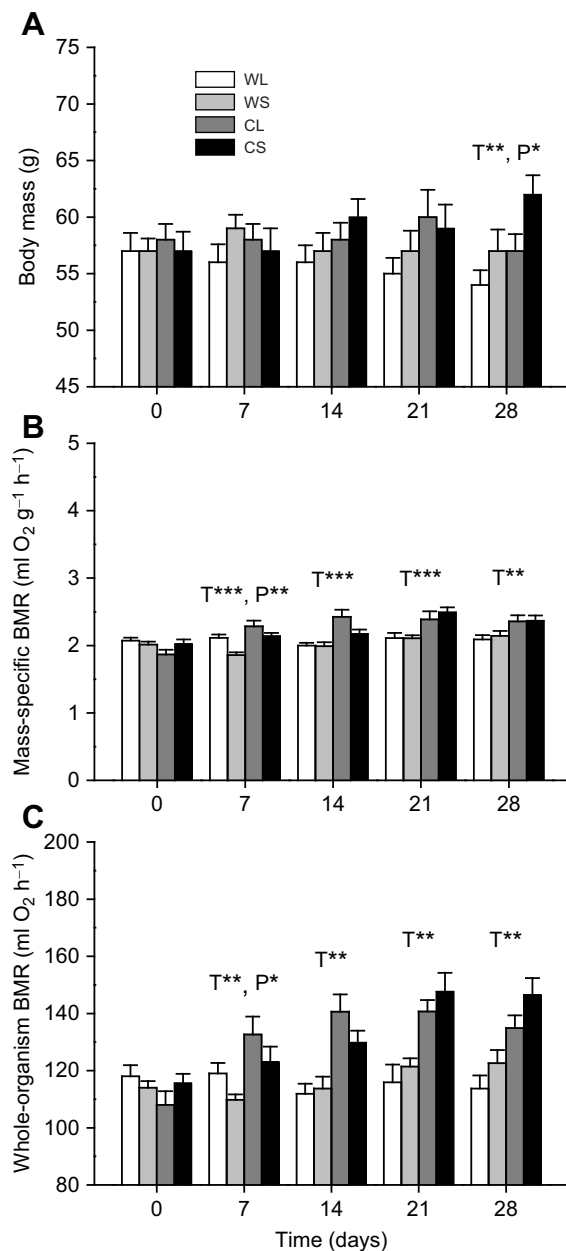


Fig. 6. Effects of temperature and photoperiod on body mass, and mass-specific and whole-organism BMR in Chinese hwameis. Data are for the warm and long photoperiod group (WL), the warm and short photoperiod group (WS), the cold and long photoperiod (CL) group and the cold and short photoperiod group (CS). Body mass (A) and mass-specific BMR (B) were determined by RM-ANOVA, and whole-organism BMR (C) was analyzed by RM-ANCOVA with body mass as a covariate. Differences in body mass (A) and mass-specific BMR (B) on the same day among different groups were evaluated with a two-way ANOVA, and comparisons of whole-organism BMR (C) on the same day among different groups were done using a two-way ANCOVA with body mass as the covariate. Data are presented as means \pm s.e.m. Body mass and mass-specific BMR were significantly affected by temperature (T) and photoperiod (P); whole-organism BMR was significantly affected by temperature (T) and photoperiod (P) (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

($r = 0.504$, $P = 0.046$) and between log DEI and log body mass ($r = 0.507$, $P = 0.045$). When all the temperature acclimation data were pooled for the long photoperiod, no significant relationship was detected between log GEI and log BMR ($r = 0.771$, $P = 0.001$) and

between log DEI and log BMR ($r = 0.746$, $P = 0.002$). A significant and positive linear relationship was found between log GEI and log BMR ($r = 0.704$, $P = 0.002$) and between log DEI and log BMR ($r = 0.684$, $P = 0.003$) for the short photoperiod.

Experiment 4

Effects of temperature and photoperiod on S4R and COX activity

Mass-specific S4R ($\mu\text{mol O}_2 \text{ min}^{-1} \text{ g}^{-1}$ tissue; Fig. 8A) was significantly affected by temperature in muscle (two-way ANOVA, $F_{1,26} = 4.666$, $P = 0.040$), liver ($F_{1,26} = 9.839$, $P = 0.004$) and kidney (two-way ANOVA, $F_{1,26} = 7.430$, $P = 0.011$). However, temperature did not appear to have an effect on mass-specific S4R in the heart (two-way ANOVA, $F_{1,26} = 2.317$, $P = 0.140$) and small intestine (two-way ANOVA, $F_{1,26} = 0.025$, $P = 0.876$). Whole-organ S4R ($\mu\text{mol O}_2 \text{ min}^{-1}$; Fig. 8B) was also significantly affected by temperature in muscle (two-way ANOVA, $F_{1,26} = 6.935$, $P = 0.014$), liver ($F_{1,26} = 20.769$, $P < 0.001$), kidney ($F_{1,26} = 41.441$, $P < 0.001$) and heart ($F_{1,26} = 4.198$, $P = 0.050$). No difference in whole-organ S4R was found between the two groups in the small intestine ($F_{1,26} = 2.403$, $P = 0.133$) in response to temperature. *Post hoc* analysis suggested that S4R of birds adapted to the cold environment was higher than that of birds adapted to the warm environment. Photoperiod exerted no significant effect on S4R in the muscle (mass-specific, $F_{1,26} = 1.836$, $P = 0.187$; whole-organ, $F_{1,26} = 1.804$, $P = 0.191$), liver (mass-specific, $F_{1,26} = 0.492$, $P = 0.489$; whole-organ, $F_{1,26} = 2.328$, $P = 0.139$), kidney (mass-specific, $F_{1,26} = 0.007$, $P = 0.932$; whole-organ, $F_{1,26} = 2.403$, $P = 0.133$), heart (mass-specific, $F_{1,26} = 0.000$, $P = 0.998$; whole-organ, $F_{1,26} = 0.013$, $P = 0.911$) or small intestine (mass-specific, $F_{1,26} = 0.758$, $P = 0.392$; whole-organ, $F_{1,26} = 0.728$, $P = 0.401$). None of the interaction terms were statistically significant. After summarizing all the temperature acclimation data for the long photoperiod groups, a significant and positive correlation was observed between log BMR and log S4R for the liver ($r = 0.660$, $P = 0.010$) and kidney ($r = 0.839$, $P < 0.001$). Similarly, for the short photoperiod groups, a significant correlation was observed between log BMR and log mitochondrial S4R in the liver ($r = 0.670$, $P = 0.005$) and kidney ($r = 0.607$, $P = 0.013$) during temperature acclimation.

Mass-specific COX activity ($\mu\text{mol O}_2 \text{ min}^{-1} \text{ g}^{-1}$ tissue; Fig. 9A) was significantly affected by temperature only in the case of the liver (two-way ANOVA, $F_{1,26} = 6.628$, $P = 0.016$), while the muscle (two-way ANOVA, $F_{1,26} = 0.131$, $P = 0.720$), kidney ($F_{1,26} = 0.524$, $P = 0.476$), heart ($F_{1,26} = 0.308$, $P = 0.584$) and small intestine (two-way ANOVA, $F_{1,26} = 1.231$, $P = 0.277$) all showed no such effect. Whole-organ COX activity ($\mu\text{mol O}_2 \text{ min}^{-1}$; Fig. 9B) was also significantly affected by temperature in the liver (two-way ANOVA, $F_{1,26} = 29.507$, $P < 0.001$), kidney ($F_{1,26} = 17.464$, $P < 0.001$) and small intestine ($F_{1,26} = 6.421$, $P = 0.018$) but not in the muscle ($F_{1,26} = 0.034$, $P = 0.855$) and heart ($F_{1,26} = 1.409$, $P = 0.2467$). *Post hoc* analysis revealed that cold-acclimated birds had heightened activity of respiratory enzymes compared with warm-acclimated birds. No significant effect of photoperiod on COX activity was found in the muscle (mass-specific, $F_{1,26} = 2.120$, $P = 0.157$; whole-organ, $F_{1,26} = 1.890$, $P = 0.181$), liver (mass-specific, $F_{1,26} = 1.381$, $P = 0.251$; whole-organ, $F_{1,26} = 3.889$, $P = 0.059$), kidney (mass-specific, $F_{1,26} = 0.000$, $P = 0.987$; whole-organ, $F_{1,26} = 0.412$, $P = 0.527$), heart (mass-specific, $F_{1,26} = 0.001$, $P = 0.973$; whole-organ, $F_{1,26} = 0.139$, $P = 0.712$) or small intestine (mass-specific, $F_{1,26} = 0.027$, $P = 0.871$; whole-organ, $F_{1,26} = 0.007$, $P = 0.933$). All the interactions were not statistically significant. Analysis of the temperature acclimation data for the long photoperiod indicated a significant correlation between log BMR and log COX activity in

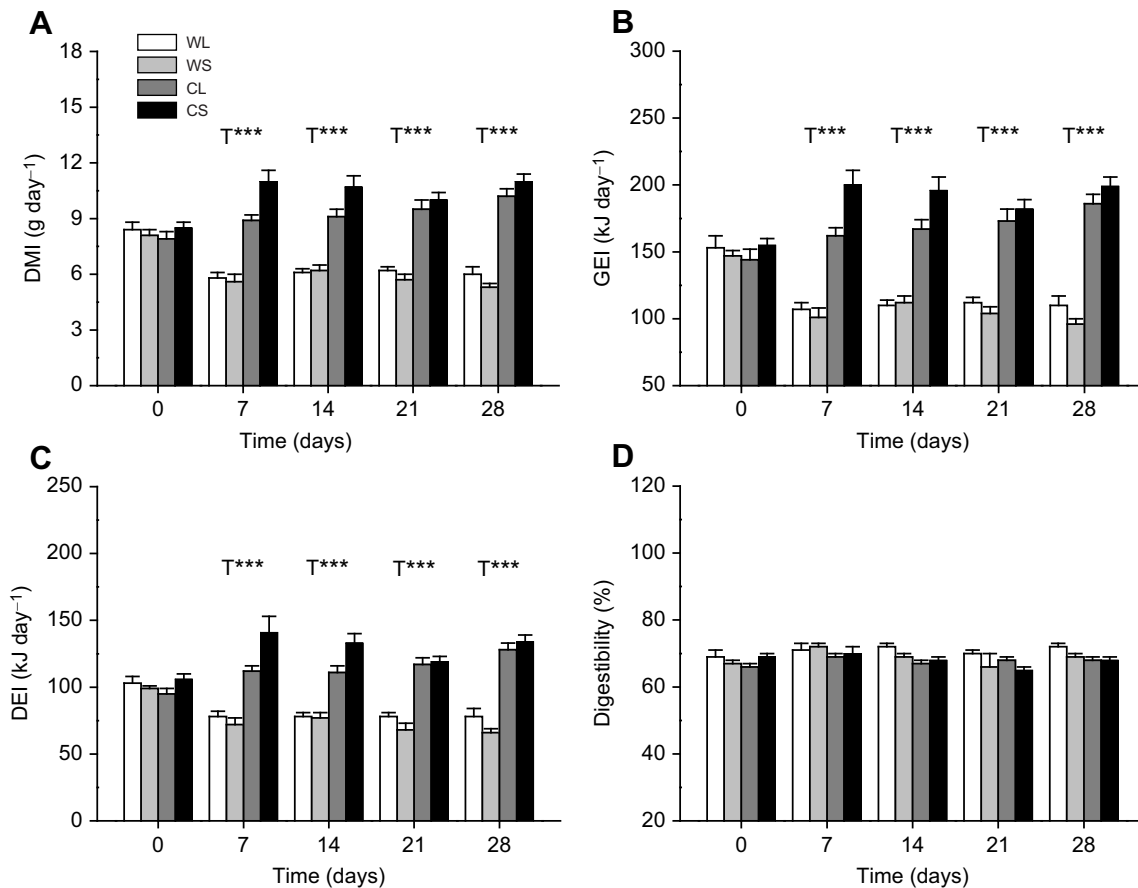


Fig. 7. Effects of temperature and photoperiod on DMI, GEI, DEI and digestibility in Chinese hwameis. Data are for the warm and long photoperiod group (WL), the warm and short photoperiod group (WS), the cold and long photoperiod (CL) group and the cold and short photoperiod group (CS). DMI (A), GEI (B) and DEI (C) were determined by RM-ANCOVA with body mass as a covariate; digestibility (D) was determined using arcsin-square root transformation prior to RM-ANOVA. Comparisons of DMI (A), GEI (B) and DEI (C) on the same day among different groups were done using a two-way ANCOVA with body mass as the covariate; differences in digestibility (D) on the same day among different groups were evaluated with a two-way ANOVA. Data are presented as means \pm s.e.m. DMI, GEI and DEI were significantly affected by temperature (T) (** P <0.001).

the liver ($r=0.728$, $P=0.003$) and kidney ($r=0.517$, $P=0.050$). There was also a significant and positive correlation between log BMR and log COX activity in the kidney ($r=0.632$, $P=0.009$) and small intestine ($r=0.550$, $P=0.027$) for the short photoperiod groups during temperature acclimation.

DISCUSSION

A key component of seasonal adaptation in animals is the ability to adjust body mass (Swanson et al., 2017; Li et al., 2017). Based on the prediction that maximal annual body mass is linked to maximal energy requirement, body mass is often used as an index of an animal's overall condition (Kelly and Weathers, 2002; Swanson et al., 2017). Coldness and/or short photoperiod have been shown to increase the body mass of several small bird species in temperate and subtropical zones (Cooper, 2007; Chamane and Downs, 2009; Zheng et al., 2014a; Li et al., 2017). Our study with Chinese hwameis also revealed a similar trend in seasonal variation in body mass, with a higher body mass detected for birds in winter than in summer. In addition to the morphological adjustments in terms of body mass, the seasonally acclimatized birds also showed a significant variation in BMR. For example, in winter (February), mass-specific and whole-organism BMR increased by 35% and 46%, respectively, over those recorded in summer (July).

Several factors, including temperature and photoperiod, have been found to exert an effect on the BMR of small bird species. Increased BMR during seasonal acclimation appears to be primarily induced by cold temperatures (Williams and Tieleman, 2000; Zheng et al., 2013b; Zhou et al., 2016; Cui et al., 2019); however, short photoperiod may also have a role (Ni et al., 2011; Wang et al., 2016; Hu et al., 2017; Li et al., 2020). A significant effect on BMR was apparent after 1 week of temperature acclimation, which lasted for the remaining 4 weeks. However, a significant effect on BMR was only found after 1 week of photoperiod acclimation. These results could indicate that temperature exerts a greater influence on BMR than photoperiod, consistent with previous findings where the effect of cold was found to be a primary cue that takes part in the regulation of thermogenesis in small bird species, such as Chinese hwameis, in winter (Swanson and Olmstead, 1999; Williams and Tieleman, 2000; McKechnie et al., 2007; McKechnie and Swanson, 2010; Zhao et al., 2015; Wang et al., 2019).

Adjustment of energy intake is essential for survival as it can compensate for the energy expended via thermogenesis (Hegemann et al., 2012). Increased energy consumption linked to thermogenesis in cold conditions can be compensated for by adjusting the energy requirement (Williams and Tieleman, 2000). The response in energy intake and utilization is adaptive to cold temperatures, and it

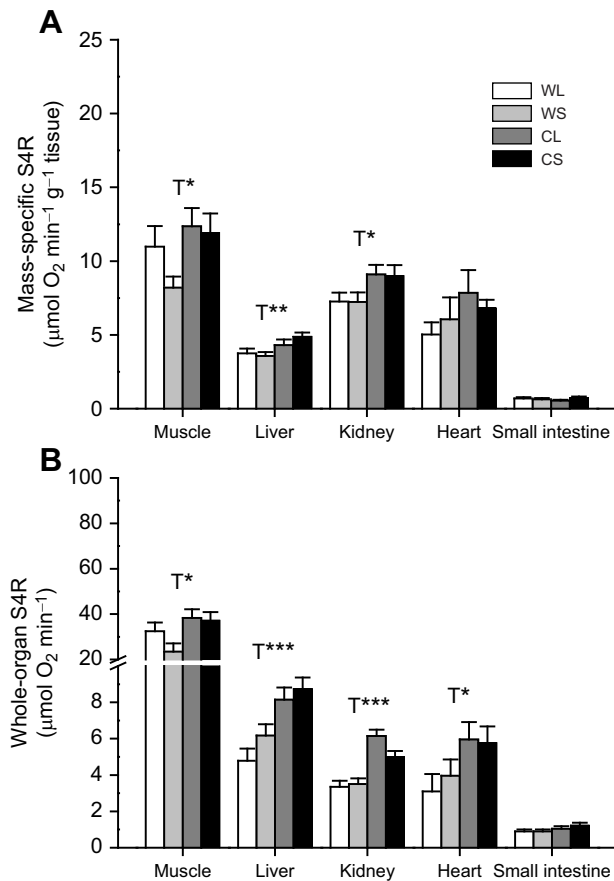


Fig. 8. Effects of temperature and photoperiod on mass-specific and whole-organ S4R in Chinese hwameis. Mass-specific (A) and whole-organ (B) S4R are shown for the pectoral muscles, liver, kidneys, heart and small intestine of Chinese hwameis in the warm and long photoperiod group (WL), the warm and short photoperiod group (WS), the cold and long photoperiod (CL) group and the cold and short photoperiod group (CS). Statistical significance was determined by two-way ANOVA. Data are presented as means \pm s.e.m. Mass-specific S4R and whole-organ S4R were significantly affected by temperature (T) (* P <0.05, ** P <0.01 and *** P <0.001).

is often shown as a significant increment in GEI and DEI during winter (Wu et al., 2014; Lou et al., 2013). Our findings indicate that the energy intake of Chinese hwameis increased during winter and that this was partly in response to the corresponding increases in BMR. Cold temperature and/or short photoperiod can also lead to an increase in food intake in a few small birds (Williams and Tieleman, 2000; Salvante et al., 2010; Zhou et al., 2016). Increases in GEI and DEI have been observed in Chinese bulbuls when these birds were acclimated to cold and a short photoperiod compared with those acclimated to warm and a long photoperiod (Hu et al., 2017). Previous studies have concluded that when Chinese hwameis are exposed to cold alone or in combination with a short photoperiod, increasing food intake is one of the ways to compensate for the increase in thermogenesis (Hammond and Diamond, 1997; Zhou et al., 2016). It is worth noting that significant increases in body mass were apparent after 4 weeks of acclimation. Moreover, significant increases in BMR, DMI, GEI and DEI were found under cold conditions compared with warm conditions following 7 days of acclimation. Our results enrich the increasing body of data that suggests physiological responses to cold ambient temperature can occur rapidly. Similarly, Chinese hwameis were found to eat more

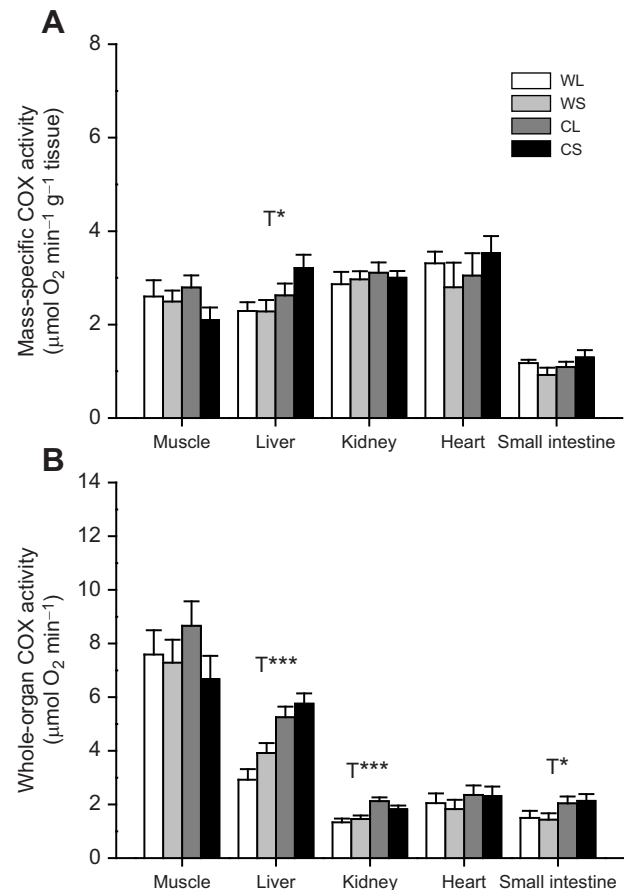


Fig. 9. Effects of temperature and photoperiod on mass-specific and whole-organ COX activity in Chinese hwameis. Mass-specific (A) and whole-organ (B) COX activity are shown for the pectoral muscles, liver, kidneys, heart and small intestine of Chinese hwameis in the warm and long photoperiod group (WL), the warm and short photoperiod group (WS), the cold and long photoperiod (CL) group and the cold and short photoperiod group (CS). Statistical significance was determined by two-way ANOVA. Data are presented as means \pm s.e.m. Mass-specific COX activity and whole-organ COX activity were significantly affected by temperature (T) (* P <0.05 and ** P <0.01).

food under cold conditions, suggesting that rapid physiological adjustments to sudden changes in ambient temperature may be advantageous for small birds (Zhou et al., 2016).

Regulation of the activity of key catabolic enzymes, as well as enzymes and transporters which are involved in the substrate delivery pathways, may be used to adjust the intensity of cellular metabolism in tissues and/or organs (Swanson, 2010; Liknes and Swanson, 2011; Zheng et al., 2014a; Zhou et al., 2016). Many bird studies have found positive correlations between BMR and S4R and/or COX activity in various tissues under seasonal, temperature or photoperiod variation (Zhou et al., 2016; Hu et al., 2017; Cui et al., 2019; Li et al., 2020). For instance, Zheng et al. (2008b, 2014a) found that S4R and COX activity in the muscle and liver of Eurasian tree sparrows and Chinese bulbuls increased significantly in winter compared with summer, indicating that the whole respiratory capacity of the muscle and liver of sparrows and bulbuls increases during winter. Cellular metabolic capacity in birds can also be increased by cold alone or together with short photoperiod (Swanson et al., 2014; Zhou et al., 2016; Li et al., 2020). For example, leiothrix were found to display increased S4R and COX activity in the muscle, liver, kidney and liver in response

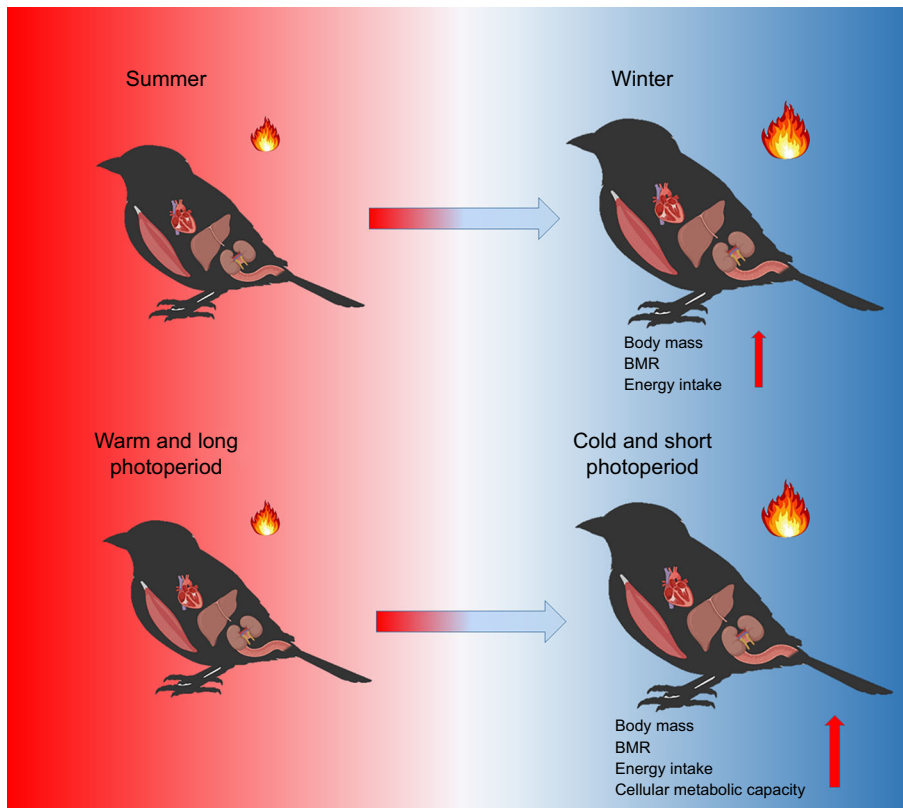


Fig. 10. Schematic model of seasonal acclimatization and temperature-photoperiod acclimation in Chinese hwameis. For seasonal acclimatization, Chinese hwameis can improve their chances of surviving in winter by increasing body mass, BMR and energy intake. For temperature-photoperiod acclimation, Hwameis will have greater body mass and a higher BMR after cold acclimation and short-photoperiod acclimation than after warm acclimation and long-photoperiod acclimation. Cellular metabolic capacity in selected tissue or organs will also increase significantly in cold-acclimated birds compared with warm-acclimated birds.

to cold acclimation (Cui et al., 2019). Another example is the higher S4R and COX activity in the organs of Eurasian tree sparrows acclimated at 10°C compared with those at 30°C (Li et al., 2020). In contrast, only mass-specific COX activity in the pectoral muscle showed seasonal changes for the Chinese hwameis. Furthermore, whole-organ S4R in the small intestine and whole-organ COX activity in the pectoral muscle and liver also showed seasonal changes, whereas only mass-specific COX activity in the liver and mass-specific S4R in the pectoral muscle, liver and kidney were significantly affected by temperature. Similarly, whole-organ S4R in the pectoral muscle, liver, kidney and heart was significantly affected by temperature. Additionally, whole-organ COX activity in the liver, kidney and small intestine was markedly affected by temperature. These data suggest that the differences in organismal metabolic capacity are partly due to changes in cellular metabolic intensity and partly to differences in organ mass resulting from different treatment conditions. Despite our expectations, S4R and COX activity in hwameis did not increase after short photoperiod acclimation; hence, this result did not support the prediction that photoperiod affects cellular metabolic capacity.

In summary, Chinese hwameis increased body mass, BMR, energy intake and cellular metabolic capacity during winter, supporting the prediction that Chinese hwameis can enhance their chance of survival in winter by increasing body mass, thermogenesis and energy intake, as well as cellular metabolic capacity. Low ambient temperature and short photoperiod appeared to be the primary environmental factors governing this process. Hwameis had greater body mass and a higher BMR after cold acclimation and short-photoperiod acclimation than after warm acclimation and long-photoperiod acclimation (Fig. 10). These findings suggest that winter survival may be enhanced by increasing the intake of energy and thermogenesis in a cold environment. S4R and COX activity in several tissues were also

significantly higher in cold-acclimated birds compared with warm-acclimated birds. Thus, instead of photoperiod, ambient temperature was found to be the primary driving force of energy metabolism and thermoregulation in Chinese hwameis, with increased BMR associated with increased body mass, mass of energetically active organs, energy intake and cellular metabolic capacity.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C. Li, C. Liu, M.L., J.L.; Methodology: J.L.; Software: C. Li, P.H., X.Z.; Validation: C. Li, C. Liu; Formal analysis: C. Li, X.Z.; Investigation: C. Liu, P.H.; Resources: J.L.; Data curation: C. Liu; Writing - original draft: C. Li; Writing - review & editing: C. Li, M.L., J.L.; Supervision: M.L., J.L.; Project administration: M.L., J.L.; Funding acquisition: M.L., J.L.

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