Phenotypic flexibility of thermogenesis in the Hwamei (Garrulax canorus): responses to cold acclimation

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Abstract:

Cold acclimation in birds involves a comprehensive array of physiological and morphological adjustment ranging from changes in aerobic enzyme activity, to metabolic rate and organ mass. In the present study, we investigated phenotypic variation in thermogenic activity in the Hwamei (*Garrulax canorus*) under normal (35°C) or cold (15°C) ambient temperature conditions. Acclimation to an ambient temperature of 15°C for 4 weeks significantly increased the body mass, basal metabolic rate (BMR), and energy intake, including both gross energy intake (GEI) and digestible energy intake (DEI), compared to birds kept at 35°C. Furthermore, birds acclimated to 15°C increased the dry mass of their liver and kidneys, but not their heart and pectoral muscles, and displayed higher state-4 respiration in the liver, kidneys, heart and pectoral muscles, and higher cytochrome C oxidase (COX) activity in liver, kidney and pectoral muscle, compared to those kept at 35°C. There was a positive correlation between BMR and state-4 respiration in all of the above organs except the liver, and between BMR and COX activity in all of the above organs. Taken together, these data illustrate the morphological, physiological, and enzymatic changes associated with cold acclimation, and support the notion that the Hwamei is a bird species from temperate climates that exhibits high phenotypic flexibility of thermogenic capacity.
**Introduction**

Decreased food availability, shortened day length for foraging, and increased energy demands due to decreased ambient temperature mean that winter is an energetically challenging time for homoeothermic animals (7, 13, 29, 31, 50). Ambient temperature is considered to be one of the most important environmental factors influencing an animal’s seasonal thermoregulation and driving the evolution of a suite of morphological, physiological, and behavioral, adaptations (1, 5, 9, 33, 34). Therefore, substantial efforts have been made to examine the effects of temperature on animal adaptation, survival, and reproductive success (6, 14, 31).

Cold ambient temperatures have been shown to be particularly challenging to small birds (22, 35, 43). During seasonal cold acclimatization, birds typically improve their heat production and cold resistance by increasing shivering and thermogenic capacity (35, 37). This is achieved by a series of physiological transformations, ranging from biochemical adjustments to changes in internal and whole organ mass (1, 4, 43, 53). The basal metabolic rate (BMR) measures the minimum maintenance metabolic rate of a normothermic resting endotherm and is one of the fundamental physiological standards for assessing the energy cost of thermoregulation (23, 24, 43). Winter- and cold-acclimated animals often, though not always, exhibit higher BMR than summer- and warm-acclimated ones (17, 20). This has been demonstrated in a variety of avian species such as hoopoe larks (*Alaemon alaudipes*) (43), red knots (*Calidris canutus*) (40), laughing doves (*Streptopela senegalensis*) (22), Chinese bulbuls (*Pycnonotus sinensis*) (52, 53), and Eurasian tree sparrows (*Passer montanus*) (50, 51). The coincidence of enhanced cold resistance and increased BMR in winter reveals a possible functional link between maximum sustained energy expenditure and BMR (17).

Increased body mass is thought to be associated with cold resistance because this reduces heat loss by decreasing an animal’s surface to volume ratio (8, 17, 53). Cold
acclimated birds also show increases in internal organ mass. For example, an increase in pectoral muscle mass which is an important source of heat produced by shivering under cold stress was found (34). Cold-associated increases in mass have also been found in other internal organs, including the liver and kidneys (3, 10, 41, 52). It has been hypothesized that such changes are an adaption to different energy requirements (43) and relate to an increase in metabolic activity leading to elevated BMR (28). At the cellular level, mitochondrial respiration produces heat from its imperfect coupling to ADP phosphorylation (8). Furthermore, improved catabolic enzyme activity in oxidative pathways may lead to high cellular aerobic capacity and therefore improved heat production (19, 52). Finally, levels of state-4 respiration and cytochrome C oxidase (COX) activity have been commonly used as enzymatic markers indicative of altered BMR at the cellular level (34, 39, 50, 53).

The Hwamei (*Garrulax canorus*) is a small, non-migratory passerine found primarily in central and southern China and northern Indochina (44). Within its natural habitats, the Hwamei preferentially lives in scrubland, open woodland, secondary forest, parks and gardens up to 1800 m above the sea level. These birds are omnivorous and mainly feed on arthropods (insects and spiders) in the breeding season, but also eat plants (fruits and seeds) in autumn and winter (44). It has been found that the Hwamei has a relatively low BMR (93% of the expected value based on body mass), a high body temperature, a narrow thermal neutral zone (TNZ), and high evaporative water loss that exceeds metabolic water production at ambient temperatures up to 14°C (46). A narrow TNZ is characteristic of avian species living in warm climates (48). However, the Hwamei commonly encounters cold temperatures in winter and early spring but the physiological mechanisms by which this species copes with cold are unknown. To test the hypothesis that the Hwamei copes with cold by having high levels of phenotypic flexibility, we compared selected morphological, physiological, and biochemical indices.
of a group of Hwamei acclimated to 15˚C to those of another group maintained at the ambient temperature of 35˚C. Our data illustrate the species-specific adaptation by the Hwamei to cold temperature. In addition, these results will enhance our understanding of the morphological, physiological, and biochemical adaptations by small bird species in their thermogenic adjustments to cold environment conditions.

**Materials and Methods**

**Subjects**

This study was carried out in Wenzhou City, Zhejiang Province (27˚29'N, 120˚51'E, 14 m in elevation), China. The climate is warm–temperate with an average annual rainfall of 1500 mm spread across all months with slightly more precipitation during spring and summer. Mean daily temperatures range from 28˚-39˚C in July to 3˚-8˚C in January (51).

Hwameis (*Garrulax canorus*) were captured by mist nets in Wenzhou City in July 2013, then transported to the laboratory and maintained in individual enclosures (50×30×20 cm³) at 35±1˚C on a 12:12 h light: dark photoperiod with lights on at 0600. Food (commercial Hwamei pellets: 20 % crude protein, 6 % crude fat, 4 % crude fiber, 1 % calcium, 0.5 %lysine, and 0.5 % methionine+cysteine; Jiangsu Xie Tong Bioengineering Co) and water were provided *ad libitum*. Subjects were kept under these conditions for two weeks, then randomly assigned to one of two treatment groups; a control group (n=8), which continued to be kept at 35˚C, and a low temperature group (LTG) (n=8), which was kept at 15˚C for 4 weeks. Two birds in the LTG group died during the 4-weeks acclimation, and thus they were excluded from data analyses. All experimental procedures were approved by the Animal Care and Use Committee of the Wenzhou City, Zhejiang Province, China.
Metabolic trials

Oxygen consumption was measured using an open–circuit respirometry system (S–3A/I, AEI technologies, Pittsburgh, PA, USA) (44, 53). Individual birds were allowed to rest in the 1.5-L plastic metabolic chamber housed in a temperature–controlled cabinet capable of regulating temperature to ±0.5˚C (Artificial Climatic Engine BIC–300, Shanghai, China). Air was scrubbed of H₂O and CO₂ with a silica gel/soda lime/silica column, before and after passing through the metabolic chamber. Dry CO₂-free air was pumped through the chamber at 300 ml min⁻¹ with a flow control system (AEI technologies R–1, USA). The fractional concentrations of oxygen in inlet and outlet chamber air were determined by an oxygen sensor (AEI technologies N–22M, USA). Oxygen consumption rates were measured at 35 ± 0.5˚C, which is within the thermal neutral zone of the Hwamei (46). All measurements of gas exchange were obtained during the rest-phase of birds’ circadian cycles (between 2000 and 0400) in dark chambers. Food was removed 4 h before each test to create post-absorptive conditions. Measurement of oxygen consumption commenced when birds were observed perching calmly in the chamber and each animal was placed in the metabolic chamber for at least 2 h. The oxygen consumption data were recorded at every minute according to the equation 2 of Hill (15). We took the lowest 5 min mean oxygen consumption data over the test period to calculate basal metabolic rate (44). Metabolic rates were expressed as ml O₂ h⁻¹ and corrected to standard temperature, pressure, and dry gas (STPD) conditions. Body temperature (Tb) was measured during metabolic measurements using a lubricated thermocouple inserted in the cloaca, and the output was digitized using an Oakton thermocouple meter (Eutech Instruments, Singapore). Mean body mass of each experimental group was calculated by measuring the body mass of each bird before and after the experiment.

Energy budget
Food residues and feces were collected once for 3 days prior to temperature acclimation (week 0) and thereafter weekly (every 7 days) throughout the 4–week experimental period. The residues were separated manually and then oven-dried at 60°C to constant mass. The caloric values of the residual food and feces were determined using a C200 oxygen bomb calorimeter (IKA Instrument, Germany). Gross energy intake (GEI), feces energy (FE), digestible energy intake (DEI), and energy digestive efficiency were calculated as follows: GEI (kJ/day) = dry food intake (g/day) × caloric value of dry food (kJ/g); FE (kJ/day) = dry mass of feces (g/day) × caloric value of dry feces (kJ/g); DEI (kJ/day) = GEI (kJ/day) – FE (kJ/day); and Digestive efficiency (%) = DEI (kJ/day)/GEI (kJ/day) × 100% (45, 47).

Measurements of organ mass, tissue state-4 respiration and cytochrome C oxidase (COX) activity

Birds were euthanized by cervical dislocation at the end of the 4 week experimental period, and their liver, kidneys, heart, and pectoral muscles were extracted and weighed (19, 52). State-4 respiration in liver, kidney, heart, and pectoral muscle was measured at 30°C in 1.96 mL of respiration medium (225 mM sucrose, 50 mM Tris/HCl, 5 mM MgCl2, 1 mM EDTA and 5 mM KH2PO4, pH 7.2) with a Clark electrode (Hansatech Instruments LTD., England, DW–1). State-4 respiration was measured over a 1 h period under substrate dependent conditions, with succinate as the substrate (43, 50). The activities of COX in liver, kidney, heart, and pectoral muscle were measured polarographically at 30°C using a Clark electrode. The respiration medium contained 100mM KCl, 20mM TES, 1mM EGTA, 2mM MgCl2, 4mM KH2PO4, 60mM BSA, pH 7.2. Ten ml aliquot taken from the supernatant and 30 ml cytochrome c (37.9 mg/ml) were added to the electrode and the activity of COX were measured in a final volume of 2 ml (52, 53). Both measurements were expressed as whole organ activity, mmol
Data analysis

Data were analyzed using SPSS (version 19.0). The normality of all variables was examined using the Kolmogorov–Smirnov test, and non–normally distributed data were log_{10}-transformed prior to analyses. Differences in body mass, body temperature, GEI, FE, DEI, and digestive efficiency over time were analyzed by repeated measures analysis of variance (RM-ANOV A) and changes in metabolic rate over time were analyzed by repeated measures ANCOVA with tarsus length as a covariate. The significance of any differences between group means was evaluated with the Student Newman Keul’s (SNK) post hoc test. Partial and residual correlations were used to evaluate the relationship between BMR and dry organ mass and least–squares linear regression to evaluate the relationship between log BMR and log body mass, and between log BMR, log state-4 respiration and log COX. Alpha for all statistical tests was set at p<0.05.

Results

Body mass, body temperature, and BMR

Overall, birds acclimated at 15°C for 4 weeks underwent a significant increase in body mass ($F_{(1,12)}=12.043, p<0.01$; Figure 1A) and BMR ($F_{(1,12)}=63.228, p<0.001$; Figure 1C), compared to control ones under 35°C conditions. Significant group-by-time interactions were also found for body mass ($F_{(4,48)}=10.445, p<0.001$; Figure 1B) and BMR ($F_{(4,48)}=2.929, p<0.05$; Figure 1D). No group differences in body mass or BMR were found prior to cold acclimation (Figure 1B & D). However, a significant increase in both body mass ($p<0.05$) and BMR ($p<0.01$) was apparent in the LTG after 1-week of...
acclimation to 15°C and these increases were sustained for the 4-week duration of the experiment (Figure 1B & D). A positive correlation was found between body mass and BMR ($r^2=0.610$, $p<0.001$; Figure 1E). The mean body temperature was about 42.3°C but no group differences were found in body temperature.

**Energy budget**

Four weeks of acclimation to 15°C significantly increased the GEI ($F_{(1,12)}=23.304$, $p<0.001$), FE ($F_{(1,12)}=12.328$, $p<0.01$), DEI ($F_{(1,12)}=17.560$, $p<0.01$), but not the digestive efficiency ($F_{(1,12)}=0.293$, $p=0.544$), of the LTG relative to the control. Significant group-by-time interactions were also found for GEI ($F_{(5,60)}=8.674$, $p<0.001$), FE ($F_{(5,60)}=8.923$, $p<0.001$), and DEI ($F_{(5,60)}=4.999$, $p<0.001$). No between-group differences were found in basal (day 0) levels of any of the above indices. However, just one day of acclimation to 15°C significantly increased the GEI ($p<0.01$) of the LTG relative to the control and this increase was sustained for the remaining 4 weeks of the experiment (Figure 2A). The LTG also had significantly higher FE than the control group on day 1 ($p<0.01$), week 1 ($p<0.05$), and week 3 ($p<0.01$) (Figure 2B), and significantly higher DEI on day 1 ($p<0.05$) and week 1 ($p<0.05$) (Figure 2C). A group-by-time interaction was also found for digestive efficiency ($F_{(5,60)}=2.890$, $p<0.05$). The LTG showed a significant, but transient, decrease in digestive efficiency on day 1 compared to the control group, ($p<0.05$), but this was not sustained over the entire 4 weeks of the experiment (Figure 2D).

**Organ mass**

The liver ($F_{(1,12)}=5.618$, $p<0.05$) and kidneys ($F_{(1,12)}=14.863$, $p<0.01$), but not the heart or pectoral muscle, of the LTG were significantly heavier than those of the control group (Figure 3A). Both partial and residual correlation analyses indicated significant, positive, partial correlations between BMR and dry liver mass, dry kidney mass, and dry pectoral muscle mass, but not between BMR and dry heart mass (Table 1).
Tissue state-4 respiration and COX activity

By the end of the experiment the LTG had significantly increased state-4 respiration in the liver \( t_{12}=2.718, p<0.05 \), kidney \( t_{12}=4.615, p<0.001 \), heart \( t_{12}=2.411, p<0.05 \), and pectoral muscle \( t_{12}=4.098, p<0.001 \), compared to the control group (Figure 3B). The LTG also displayed increased COX activity in the liver \( t_{12}=3.393, p<0.01 \), kidney \( t_{12}=3.525, p<0.01 \), and pectoral muscle \( t_{12}=2.689, p<0.05 \), but not in the heart (Figure 3C). Positive correlations were obtained between BMR and state-4 respiration, and between BMR and COX activity, in the kidney, heart, and pectoral muscle (Figure 4). However, BMR was only positively correlated with COX activity, and not with state-4 respiration, in the liver (Figure 4).

Discussion

Many small birds cope with cold stress in winter by adjusting their body mass (27, 32), internal organ mass (34, 43, 53), metabolic rate (17, 21), energy budget (45), and enzyme activity (38, 49, 50). The results of this study show that 4 weeks of cold acclimation caused significant changes in each of these variables in the Hwamei; an increase in body and organ mass, enhanced BMR, energy intake and cellular enzyme activity associated with bodily metabolic functions. Collectively, these data suggest that the Hwamei can improve its thermogenic capacity in response to a cold ambient environment, and provide further evidence to support the notion that small birds with small TNZs have high phenotypic plasticity with respect to thermogenic capacity (19, 22, 23).

Effects of cold acclimation on BMR, body mass, and energy budget

Among the many environmental factors encountered by animals, low temperature is particularly stressful. It has been well documented that small mammals and birds can
alter several aspects of their physiology, morphology and behavior to cope with cold ambient temperatures (1, 6, 11, 17, 19, 27). BMR is a widely-accepted benchmark of metabolic expenditure for endotherms that is commonly used as a measure of the energetic cost of thermoregulation (18, 43). Our data show that cold-acclimated birds had a higher BMR than control birds, indicating higher heat output in the former than in the latter (18). Elevated BMR in response to cold, under either experimental or natural conditions, has been reported previously in other avian species (20, 43, 52). As BMR is directly related to the peak metabolic rate of thermogenesis during heightened cold tolerance (40), our data suggest that up-regulation of BMR may be an important component of improved cold tolerance in the Hwamei as it is in many other small birds (17, 28). Increased body mass may have contributed to the observed increase in BMR, as indicated by the positive correlation between these two variables. In addition, increased body mass enables animals to reduce heat loss by decreasing their surface to volume ratio (50).

Adjustments in energy intake and budget can compensate for the increased energy expenditure associated with thermogenesis in cold conditions (43). The significant increases in GEI and DEI observed in the LTG are consistent with the adaptive changes in energy intake and utilization in response to cold temperature documented in many other small birds (9, 28, 40, 45). Our repeated measurements of body mass, metabolic rate, and energy budget over the 4-week course of the experiment show the pattern of temporal change in these variables. For example, one interesting finding is the rapidity of the adjustments made by birds in response to cold acclimation. Significant increases in body mass and BMR were apparent in the LTG after just one week of acclimation. Moreover, significant increases in GEI, DEI and FE were apparent in the LTG after just 24 hours of acclimation. These data provide further evidence of the speed at which morphological, physiological, and behavioral responses to cold ambient temperature can occur (23, 34, 52). The ability to make such rapid physiological adjustments would
clearly be advantageous for small birds faced with sudden changes in ambient
temperature. It is noteworthy that a significant decrease in digestive efficiency in the
LTG was apparent after just 24 hours of acclimation to 15°C. This could suggest a
temporal mismatch between the sudden increase in GEI and initial digestive capacity.
However, digestion returned to normal after a week, suggesting that the digestive tract
required between 1-7 days to adapt to the increased food intake (28, 45).

Effects of cold acclimation on selected organs and their cellular metabolic activities

Cold acclimation has also been associated with changes in organ size and mass
(12, 43, 53). One idea is that energetically challenged birds may increase their food
intake, and at the same time, reorganize their internal organs to improve digestive and
thermal efficiency (40, 43). Elevation of BMR in the Hwamei is presumably related to
metabolic and/or morphological adjustments to meet the extra energy demands. Such
organs as the liver and kidneys may contribute significantly to BMR (3, 28). What are
the ecological implications of having larger liver and kidneys for cold-acclimated birds?
Williams and Tieleman (22, 35, 43) hypothesized that natural selection adjusted the size
of the internal organs to match energy requirements, and that body size independent
variation in BMR reflects the relative size of internal organs such as the liver, kidney and
heart, which are thought to have high mass-specific rates of oxygen consumption. Liver
is one of the largest and most metabolically active organs in endotherms, and it also has
the potential to contribute to the thermogenic response (42, 52). Mechanisms of heat
generation in liver include uncoupling of oxidative phosphorylation, futile cycling of
substrates and high mass-specific metabolic intensity (8, 17, 53). In the present study,
acclimation to 15°C for 4 weeks led to significant increases in the mass of the liver and
kidney, but not of the heart and pectoral muscles. Increases in liver and kidney mass are
associated with thermogenic capacity. In young swine, for example, the liver, kidneys,
and small intestine contribute up to 60% of the visceral, and 30% of total, heat production
Thus, the observed increase in liver and kidney mass in the Hwamei may be adaptive regulation of organ morphology to accommodate increased food intake and digestion (47), ultimately contributing to an altered metabolic rate.

It should be noted that, in birds, the pectoral muscle is considered to provide most of the heat required to cope with cold stress through shivering thermogenesis (16). Greater muscle mass allows higher heat production, thereby prolonging resistance to cold (40). Furthermore, birds living in cold environments have been reported to increase the size of the heart, which has been associated with increased cardiac output and oxygen transport (4, 43). However, we did not observe any significant changes in the dry mass of the heart and pectoral muscles in the Hwamei. The studies cited above may be evidence of species- and organ-specific changes in organ morphology in response to cold, however, we cannot exclude the possibility that these organs are involved in cold acclimation in the Hwamei (see below).

In our study, birds acclimated to 15°C displayed higher levels of state-4 respiration in all 4 organs measured, compared to those kept at 35°C. In addition, cold-acclimated birds showed higher levels of COX in the liver, kidneys, and pectoral muscles than the control group. These results are consistent with those of previous studies. For example, winter-acclimatized Chinese bulbuls (Pycnonotus sinensis) showed increased levels of state-4 respiration and COX in the liver, suggesting that high aerobic capacity in liver could play an important role in the thermogenesis of cold-exposed animals (42, 52). Likewise, cold acclimation of ducklings increased the oxidative capacity of the pectoral muscles by 195%, and the liver by 47%, which could collectively make a significant contribution to non-shivering thermogenesis (2). High levels of state-4 respiration and COX activity are related to elevated BMR (52), a finding that is supported by the significant correlations between state-4 respiration, COX and BMR in this study. These results are also consistent with those obtained on other avian species, including the Eurasian tree sparrow (50) and Chinese bulbuls (52, 53). It would be interesting to
examine, in further studies, whether increased state-4 respiration and COX activity in the
Hwamei are also due to their responses to chronic shivering.

Our data seem to indicate the existence of two non-exclusive, organ-specific, adaptive strategies in the Hwamei. With respect to the liver and kidneys, there was an increase in both total tissue mass and enzymatic activity. However, with respect to the pectoral muscles and heart, there was only an increase in enzymatic activity with no accompanying change in mass. The observed increase in enzymatic activity in these organs may provide the cellular basis by which heightened activities of respiratory enzymes can support a higher metabolism during cold acclimation. It should be noted that enzymatic activity in the heart and kidneys in response to cold has been rarely studied in small birds. Our data suggest that changes in enzymatic activity in these organs may potentially contribute to increased metabolic activity and thermogenesis in small birds under cold conditions.

Perspectives and Significance

Our study demonstrate that Hwameis display a general, elevated, whole-body response to cold temperature, including increased body and organ mass, enhanced BMR and energy intake, and increased enzymatic activity in specific organs. These morphological, behavioral and physiological changes occurred rapidly in response to cold exposure. Such high phenotypic flexibility may be important to enable the Hwamei to survive periodic, sudden, cold stress (25). Further study are required to measure maximum metabolic rate (MMR), which is positively related to cold tolerance in birds to unravel the mystery of the narrow geographical distribution of Hwameis (36). In addition, more solid and convincing evidence on molecular biology will help us better understand the complex mediation process during the cold acclimation (30).

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Figure legends:

Figure 1. Comparisons of group differences in body mass and the basal metabolic rate (A & C) as well as the time courses of such group differences over the 4 weeks experimentation (B & D) between Hwameis (*Garrulax canorus*) that had been acclimated to either 35˚C (control group), or to 15˚C (low temperature group). The body mass and basal metabolic rate are also significantly correlated with each other (E). Data are shown as mean ±SEM, *: *p* < 0.05, **: *p* < 0.01. BMR = basal metabolic rate.

Figure 2. Comparison of gross energy intake (A), feces energy (B), digestible energy intake (C), and digestive efficiency (D) between Hwameis (*Garrulax canorus*) that had been acclimated to either 35˚C (control group), or to 15˚C (low temperature group), for 4 weeks. Arrow indicates the beginning of the acclimation. Data are shown as mean ±SEM, *: *p* < 0.05, **: *p* < 0.01. GEI = gross energy intake, FE = feces energy, DEI = digestible energy intake.

Figure 3. Differences in dry mass (A), state-4 respiration (B), and cytochrome C oxidase (C) in the liver, kidneys heart, and pectoral muscle of Hwameis (*Garrulax canorus*) that had been acclimated to either 35˚C (control group), or to 15˚C (low temperature group), for 4 weeks. Data are shown as mean ±SEM, *: *p* < 0.05, **: *p* < 0.01.

Figure 4. Correlations between basal metabolic rate (BMR), state-4 respiration, and cytochrome C oxidase in the liver, kidneys heart, and pectoral muscle of Hwameis (*Garrulax canorus*) acclimated to either 35˚C (control group), or to 15˚C (low temperature group), for 4 weeks.
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Table 1. Partial, and residual, correlations (after controlling for tarsus length) between basal metabolic rate (BMR) and the dry mass of different organs in the Hwameis (Garrulax canorus).
Table 1. Partial correlation and residual correlation for basal metabolic rate versus dry organ mass in Hwamei (controlling for tarsus length)

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