The nonshivering thermogenesis of brown adipose tissue and fat mobilization of striped hamsters exposed to cycles of cold and warm temperatures

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Abstract
The adaptive adjustments in the capacity for metabolic thermogenesis are critical for the survival in many small mammals that are acclimated to cold winter conditions. In this study the striped hamsters (Cricetulus barabensis) were subjected to repeated cycles of cold (5°C) and warm (23°C) temperatures. Resting metabolic rate (RMR), nonshivering thermogenesis (NST) and energy intake, as well as the expression of uncoupling protein 1 (UCP1) of brown adipose tissue (BAT) and serum thyroid hormone levels were measured. Both RMR and NST were significantly increased in striped hamsters subjected to repeated cycles of short-term cold (5°C, 72 h) – warm (23°C, 4 days) temperatures compared to that of the hamsters consistently kept at 23°C. In these cycled hamsters, BAT UCP1 expression was significantly upregulated, whereas serum T3 and T4 concentration did not change significantly. Moreover, gross energy intake was considerably increased during both cold exposure and warm phases, whereas fat deposition was significantly decreased in these cycled hamsters compared to those consistently kept at 23°C. This indicates that small mammals may both increase energy intake and mobilize fat depots to cope with frequent cold exposure. Thyroid hormone may be not involved in the BAT UCP1-mediated thermogenesis and fat mobilization.

Keywords: Energy intake, fat deposit, striped hamsters, thermogenesis, thyroid hormone, uncoupling protein 1 (UCP1)

1. Introduction
In endothermic animals, ability to survive in cold environment requires proper physiological and behavioral strategy, and efficient thermoregulatory mechanisms (Jefimow et al. 2004a). The adaptive changes in the capacity for non-shivering thermogenesis (NST) are critical for the survival in many small mammals, such as rodents, when they are exposed to cold (Heldmaier et al. 1990; Jansky 1973; Haim & Izhaki 1993; Nespolo et al. 2001a; Woodley & Buffenstein 2002). The main advantage gained by using NST is the ability to generate heat over a short period of time to deal with acute cold exposure, therefore results in the widening of the thermal tolerance of a species without the need to maintain a high resting metabolic rate (RMR) (Heldmaier et al. 1990; Haim & Zisapel 1999). However, the animals that are chronically exposed to cold temperatures or acclimatized to a seasonal cold environment usually develop both high RMR and NST (Scantlebury et al. 2003; Song & Wang 2003; Wang et al. 2006; Jefimow 2007; Terrien et al. 2010). It has been proposed that RMR determines the metabolic scope and hence capacity for thermogenesis, and therefore RMR influences fitness by maximizing access to resources and winter survival (Bozinovic & Rosenmann 1989; Bozinovic 1992; Hinds et al. 1993; Woodley & Buffenstein 2002; Lovegrove 2003; Wang et al. 2006).
The whole body RMR can be estimated from the sum of weight of tissues and organs multiplied by their specific metabolic rate, suggesting that RMR would be determined by the weight of various organs and tissues of the body (Even et al. 2001). The NST is an adaptive form of thermogenesis that can be acutely induced by norepinephrine injection (i.e. an adrenergic thermogenesis). In mammals, the facultative NST is achieved mainly in brown adipose tissue (BAT), where the heat production occurred in the mitochondria as a consequence of a regulated uncoupling process mediated by uncoupling protein 1 (UCP1) (Nicholls 1976; Cannon & Nedergaard 2004). Animals usually increase food intake to meet the energy expenditure for increased RMR and NST (Cannon & Nedergaard 2004; Jefimow et al. 2004a, 2004b). It has been proposed that leptin signals reflecting nutritional state are sensed by the hypothalamus, which, in turn, modulates food intake and energy expenditure (Kennedy 1953; Friedman & Halaas 1998; Park & Ahima 2014). Leptin mediates its effects by binding to the long isoform of leptin receptor (ObRb) expressed in the hypothalamus (Park & Ahima 2014). Leptin promotes the gene expression and secretion of anorexigenic peptides of proopiomelanocortin (POMC) and cocaine-and-amphetamine-regulated-transcript (CART), but inhibit orexigenic peptides of neuropeptide Y (NPY) and agouti-related peptide (AgRP) expression in hypothalamus, thereby reducing food intake, increasing energy expenditure (Park & Ahima 2014; Xu et al. 2019). Leptin-mediated neuropeptides have been previously reported to regulate food intake and thermogenesis in small mammals acclimated to cold temperatures, whereas its role in the animals that were frequently exposed to cold condition remained uncertain (Lisboa et al. 2003).

It is by now an historical notion that thyroid hormones (THs) are unique in their ability to stimulate basal thermogenesis, that is BMR or RMR (Lannia et al. 2003). But it’s now known that THs are also involved in cold-induced BAT thermogenesis (Silva & Rabelo 1997). For example, hypothryoid rats do not survive cold well and fail to increase BAT recruitment (measured as UCP1 concentration and activation) in response to noradrenaline (Silva & Rabelo 1997; Lannia et al. 2003). In a variety of small mammals in the field, the seasonal enhancement of either RMR or NST in cold winter conditions are associated with an increased THs secretion and production (Freakle & Oppenheimer 1995; Lannia et al. 2003; Kim 2008; Silva 2011; Li et al. 2019). The absence of T3 blocks UCP1 synthesis, leading to hypothermia (Zaninovich 2001). However, BAT thermogenesis in hypothyroid rats did not change after acclimation to a chronic cold condition compared to that in normal controls (Zaninovich et al. 2002). These hypothyroid rats subjected to long-term cold exposure had normal body temperature, and had gained more weight than normal controls, suggesting that cold adaptation can be maintained in the absence of THs (Zaninovich et al. 2002).

To examine the roles of THs-mediated metabolic thermogenesis in burrowing mammals that are frequently subjected to cold exposure, we investigated energy budgets, metabolic thermogenesis, and serum THs concentrations in striped hamsters (Cricetulus barabensis) that were experimentally exposed to repeated cycles of cold and warm temperatures. The striped hamster is a common rodent in northern China, Russia, Mongolia and Korea (Zhang & Wang 1998; Song & Wang 2003). Striped hamsters show considerable seasonal fluctuations in their energy budget and metabolic thermogenesis. We previously documented a significant increase in the RMR and/or NST of striped hamsters under cold conditions when UCP1-based BAT thermogenesis was activated (Zhao et al. 2010a; Zhao 2011). Here, we presented the results of experiments designed to determine the effects of repeated exposure to alternating cold (5°C) and warm (23°C) temperature cycles on the energy intake, metabolic thermogenesis, UCP1 expression in BAT, body composition and serum THs concentrations, of striped hamsters. We hypothesized that the striped hamsters would increase NST after repeated exposure to these temperature cycles. Furthermore, we hypothesized that THs-mediated up-regulation of BAT UCP1 would contribute to this increase in metabolic thermogenesis, which was possibly different from these animals that were chronically acclimated to cold temperatures.

2. Results

2.1. Body mass

There was no significant difference in the body mass of the three groups before the start of the experiment (day 0, F2,25 = 0.21, P > 0.05, Figure 1(a)). The hamsters did not show significant changes in body mass throughout the experiment, during which body mass did not differ significantly among the three groups on any day (day 8, F2,25 = 0.16, P > 0.05; day 49, F2,25 = 0.19, P > 0.05).

2.2. Food intake

Food intake did not significantly differ among three groups before the start of the experiment (day 0, F2,25 = 1.95, P > 0.05, Figure 1(b)). The food intake of Cold-24 h and cold-72 h hamsters fluctuated
considerably throughout the experiment, increasing during the cold, and decreasing during the warm, phase of each cold and warm cycle. The food intake of Cold-24 h and Cold-72 h hamsters was significantly higher during the cold phase of each cycle than that of the Con group. For example, Cold-24 h and cold-72 h hamsters consumed 58.9% and 75.3% more food on the cold day during the first cycle than those in the 23°C group (day 8, \( F_{2,25} = 4.61, P < 0.01 \)).

2.3. Gross energy intake (GEI) and digestibility

The GEI of Cold-24 h and Cold-72 h hamsters was higher by 75.8% and 77.8% during the phase of cold exposure than that of the hamsters kept at 23°C (\( F_{2,24} = 18.17, P < 0.01, \text{ Figure 2(a)} \)). The GEI of Cold-24 h hamsters did not differ significantly from that of 23°C hamsters during the warm phase, whereas that of Cold-72 h hamsters was significantly higher than that of 23°C hamsters (\( F_{2,24} = 17.47, P < 0.01 \)). Both Cold-24 h and Cold-72 h hamsters showed significantly higher GEF than 23°C hamsters during the phase of cold exposure, indicating that they produced significantly more feces (\( F_{2,24} = 12.70, P < 0.01, \text{ Figure 2(b)} \)). There was no significant difference in GEF between Cold-24 h and 23°C groups during the warm phase, but the Cold-72 h group produced significantly more feces than the other two groups (\( F_{2,24} = 4.55, P < 0.05 \)). Consistently, the Cold-24 h and Cold-72 h hamsters...
had significantly higher DEI during the cold phase ($F_{2,24} = 16.40$, $P < 0.01$, Figure 2(c)), and only Cold-72 h hamsters showed significantly higher DEI during the warm phase than the 23°C hamsters ($F_{2,24} = 17.74$, $P < 0.01$). There was no significant difference in digestibility among the three groups during either the cold ($F_{2,24} = 0.09$, $P > 0.05$) or warm phase of the temperature ($F_{2,24} = 2.59$, $P > 0.05$, Figure 2(d)).

### 2.4. RMR, NST and NSTr

The RMR of Cold-24 h hamsters did not significantly differ from that of the hamsters kept at 23°C, whereas that of Cold-72 h hamsters was significantly higher than that of Con hamsters (23.7%, $F_{2,24} = 5.81$, $P < 0.01$, Figure 3(a)). NST was significantly different among the three groups, and that of Cold-24 h and Cold-72 h hamsters had 12.2% and 17.4% higher than that of 23°C group ($F_{2,24} = 5.84$, $P < 0.01$, Figure 3(b)). Consistently, NSTr of Cold-24 h and Cold-72 h hamsters was significantly higher than that of 23°C group ($F_{2,24} = 3.66$, $P < 0.05$, Figure 3(c)).

### 2.5. Fat deposition

There was no significant difference in the carcass mass among the three groups ($F_{2,25} = 0.43$, $P > 0.05$, Figure 4(a)). Cold-24 h hamsters did not significantly differ in subcutaneous fat or abdominal fat mass from the 23°C group, whereas Cold-72 h hamsters had 21.1% and 55.4% less subcutaneous fat and abdominal fat, respectively, than the 23°C groups (subcutaneous fat, $F_{2,24} = 5.67$, $P < 0.05$, Figure 4(b); abdominal fat, $F_{2,24} = 17.54$, $P < 0.01$, Figure 4(c)). The mesenteric fat mass was not significantly different among the three groups ($F_{2,24} = 0.13$, $P > 0.05$, Figure 4(d)). There was no significant difference in the peritesticular fat or total body fat mass between the Cold-24 h and 23°C groups, but the cold-72 h group showed 32.3% and 32.5% less peritesticular fat and total body fat mass, respectively, than the 23°C groups (peritesticular fat, $F_{2,24} = 16.02$, $P < 0.01$, Figure 4(e); total fat mass, $F_{2,24} = 25.73$, $P < 0.01$, Figure 4(f)).

### 2.6. Organs mass

Liver mass was significantly different among the three groups, and that of Cold-24 h and Cold-72 h groups were higher by 19.4% and 18.3% than that of 23°C group (Table 1). Consistently, the Cold-24 h and Cold-72 h groups had significantly heavier heart and kidneys than 23°C group (Table 1). The stomach mass did not significantly differ among the three groups, whereas the mass of small and large, and caecum were significantly higher in either Cold-24 h or Cold-72 h group, than that of 23°C group (Table 1). The mass of total gastrointestinal tracts of the Cold-24 h
and Cold-72 h groups was higher by 12.6% and 18.0% than that of 23°C group (Table I).

There were significantly positive correlations between RMR and liver and lung mass (supplementary materials, Figure S1A and S1B). NST was positively correlated with the mass of liver, heart, and kidneys (Figure S1F, S1G and S1J). The gastrointestinal tracts were positively correlated with RMR (Figure S2). For example, significantly positive correlations were observed between RMR and small intestine (Figure S2B), and between NST and small, caecum, and total tracts (Figure S2G, S2I, and S2J), as well as between NSTr and total tracts (Figure S2O).

2.7. Serum T_3 and T_4, and leptin concentrations

Neither serum T_3 nor T_4 concentration differed significantly among the three groups (T_3, F_{2,25} = 1.37, P > 0.05, Figure 5(a); T_4, F_{2,25} = 1.17, P > 0.05,
Figure 4. Mass of carcass (a), subcutaneous fat (b), abdominal fat (c), mesenteric fat (d), peritesticular fat (e) and total body fat (f), in striped hamsters subjected to repeated cold (5°C) and warm (23°C) temperature cycles. 23°C animals maintained at room temperature (23°C) throughout the experiment. Cold-24 h and Cold-72 h, animals exposed to 6 cold and warm temperature cycles (24 h at 5°C followed by 6 days at 23°C, and 72 h at 5°C followed by 4 days at 23°C) from the 2nd to 7th week of the experiment. Data are means ± SD with individual data points; *P < 0.05, **P < 0.01.

Table I. Body composition of striped hamsters subjected to cold and warm temperature cycles.

| Organs          | 23°C          | Cold-24 h    | Cold-72 h    | P      |
|-----------------|---------------|--------------|--------------|--------|
| Liver (g)       | 0.425 ± 0.026 | 0.508 ± 0.025| 0.503 ± 0.030| **     |
| Heart (g)       | 0.184 ± 0.005 | 0.207 ± 0.008| 0.205 ± 0.007| **     |
| Lung (g)        | 0.192 ± 0.11  | 0.200 ± 0.005| 0.199 ± 0.008| NS     |
| Spleen (g)      | 0.026 ± 0.002 | 0.029 ± 0.003| 0.023 ± 0.002| NS     |
| Kidney (g)      | 0.327 ± 0.013 | 0.386 ± 0.015| 0.379 ± 0.022| **     |
| Stomach (g)     | 0.262 ± 0.017 | 0.286 ± 0.012| 0.264 ± 0.011| NS     |
| Small intestine (g) | 0.415 ± 0.013 | 0.456 ± 0.028| 0.548 ± 0.019| **     |
| Large intestine (g) | 0.141 ± 0.010 | 0.173 ± 0.004| 0.135 ± 0.008| **     |
| Caecum (g)      | 0.092 ± 0.006 | 0.111 ± 0.006| 0.127 ± 0.011| *      |
| Total tracts (g) | 0.910 ± 0.031 | 1.025 ± 0.030| 1.074 ± 0.025| **     |

23°C, animals maintained at room temperature (23°C) throughout the experiment. Cold-24 h and Cold-72 h, animals exposed to 6 cold and warm temperature cycles (24 h at 5°C followed by 6 days at 23°C, and 72 h at 5°C followed by 4 days at 23°C) from the 2nd to 7th week of the experiment. Data are means ± s.e.m.; NS, non-significance; *P < 0.05, **P < 0.01.

Figure 5(b)). The ratio of $T_3/T_4$ was also not statistically different among the three groups ($F_{2,25} = 0.06$, $P > 0.05$, Figure 5(c)). Serum leptin concentration did not differ significantly among the 23°C, 5°C-24 h and 5°C-72 h groups ($F_{2,21} = 0.43$, $P > 0.05$, Figure 5(d)). There were no significant correlations between metabolic thermogenesis (RMR, NST and NSTr) and serum $T_3$ or $T_4$ concentrations (8(a)-(f)).

2.8. Neuropeptides mRNA expression of hypothalamus

The ObRb mRNA expression of hypothalamus was not significantly different among the 23°C, 5°C-24 h and 5°C-72 h groups ($F_{2,21} = 2.81$, $P > 0.05$, Figure 6(a)). Neither the orexigenic peptide nor anorexigenic peptide were significantly different among the three groups (NPY, $F_{2,21} = 0.22$, $P > 0.05$, Figure 6(b); AgRP, $F_{2,21} = 1.36$, $P > 0.05$, Figure 6(c); CART, $F_{2,21} = 1.30$, $P > 0.05$, Figure 6(d); POMC, $F_{2,21} = 0.62$, $P > 0.05$, Figure 6(e)).

2.9. BAT UCP1 mRNA and protein expression

BAT UCP1 mRNA expression did not significantly differ among the three groups ($F_{2,21} = 2.15$, $P > 0.05$, Figure 6(f)). BAT UCP1 protein expression of Cold-24 h and Cold-72 h was increased by 29% and 34%, respectively, compared to that of 23°C group ($F_{2,25} = 8.36$, $P < 0.01$, Figure 7), whereas the difference between Cold-24 h and Cold-72 h was not
3. Discussion

An increase in thermogenic capability is important in many small mammals to cope with cold conditions in seasonal environments because it may allow an
increase in survival (Nespolo et al. 2001b). A considerable enhancement of metabolic thermogenesis, including RMR and NST, has been observed in a variety of animals acclimated to cold or winter like conditions, such as Mongolian gerbils (Meriones unguiculatus), desert spiny mice (Acomys russatus), naked mole-rats (Heterocephalus glaber), degu (Octodon degus), leaf-eared mice (Phyllotis darmani), common spiny mice (Acomys cahirinus), golden hamsters (Mesocricetus auratus) and plateau pikas (Ochotona curzoniae) (Kronfeld-Schor et al. 2000; Wang et al. 2000, 2003, 2006; Nespolo et al. 2001a; Woodley & Buffenstein 2002; Scantlebury et al. 2003; Jefimov et al. 2004b). In this study, we observed that both RMR and NST were significantly increased in striped hamsters exposed to repeated cycles of short-term cold (5°C, 72 h)- warm (23°C, 4 days) temperatures. This was consistent with the animals that were consistently exposed or acclimated to seasonal cold temperatures. Interestingly, here we found that NST were considerably increased, whereas RMR did not differ significantly, in the striped hamsters subjected to cycles of acute cold (5°C, 24 h)- warm (23°C, 6 days) temperatures. According to Degen (1997), lower RMR and high capacity for NST allow rodents a low metabolic heat production during activity and permit them to increase heat production quickly under cool-to-cold conditions (Nespolo et al. 2001a, 2001b). Thus, thermogenic capacity can change quickly through modifications of NST in response to environmental cues (Stearns 1989, 1992; Nespolo et al. 2001a, 2001b).

Consistent with the change in NST, in this study BAT UCP1 expression was significantly increased in the striped hamsters subjected to repeated cycles of cold and warm temperatures. It is well known that BAT UCP1 expression regulates the flux of protons through the ATP synthase, being increased by cold acclimation in many small mammals (Silva & Rabelo 1997; Nedergaard et al. 2001; Lannia et al. 2003; Wang et al. 2006; Zhang & Wang 2006). This indicates that the NST mediated by BAT UCP1 is possibly general physiological regulation in a diversity of mammals in response to cold environments (Nedergaard et al. 2001). It has been suggested that both BAT and muscle-based NST play important roles in temperature homeostasis, and the both thermogenesis processes are equally recruited during mild and severe cold adaptation (Bal et al. 2017a, 2017b). In addition, these two thermogenic processes functionally interplay in some situation, i.e. loss of heat production from one thermogenic pathway leads to increased recruitment of the other (Bal et al. 2017a, 2017b). In this study, the markers that are indicative of skeletal muscle thermogenesis

Figure 7. The protein expression of uncoupling protein 1 of brown adipose tissue (BAT UCP1) in striped hamsters subjected to repeated cold (5°C) and warm (23°C) temperature cycles. 23 °C, animals maintained at room temperature (23°C) throughout the experiment. Cold-24 h and Cold-72 h, animals exposed to 6 cold and warm temperature cycles (24 h at 5°C followed by 6 days at 23°C, and 72 h at 5°C followed by 4 days at 23°C) from the 2nd to 7th week of the experiment. Data are means ± s.e.m. ** P<0.01.
were unfortunately measured in these striped hamsters, thus we could not distinguish BAT-based NST from the muscle-based NST, suggesting that the both may contribute to the increased metabolic thermogenesis in striped hamsters exposed to repeated cycles of short-term cold-warm temperatures.

Thyroid hormones are active regulators of basal or resting metabolic rate, and energy expenditure by a number of mechanisms (Freake & Oppenheimer 1995; Silva 1995, 2001; Zaninovich et al. 2002), and it is also essential to initiate BAT thermogenesis, because triiodothyronine (T3) potentiates the action of norepinephrine on UCP1 gene transcription (Bianco et al. 1988; Silva 1988; Zaninovich et al. 2002). These findings suggest that thyroid hormones are required for increased thermogenic capacity to occur as an adaptation to long-term cold exposure, and this has been observed in many small rodents acclimated to cold winter conditions (Freake & Oppenheimer 1995; Silva & Rabelo 1997; Goglia et al. 2002; Lannia et al. 2003). Unexpectedly, we did not find significant changes in either serum T3 or T4 concentrations in the striped hamsters subjected to repeated cycles of cold and warm temperatures. Consistently, BAT-mediated thermogenesis, and UCP1 mRNA and protein expression was markedly increased in cold-exposed hypothyroid rats to levels, similar to those seen in cold-exposed normal rats (Rattus norvegicus) (Zaninovich et al. 2002). The findings of this study suggest that THs may be not involved in BAT thermogenesis of animals that were frequently subjected to cycles of cold-warm temperature. As described above, both BAT and muscle-based NST are recruited in response to cold adaptation, and a functional interplay between these two thermogenic processes (Bal et al. 2017a, 2017b). The findings of this study provide weak evidence for the THs-mediated BAT thermogenesis in response to cycles of cold-warm temperature, but may suggests alternate thermogenic mechanisms beyond BAT in these hamsters which needs careful consideration for proving the fact that despite increase in UCP1 expression.

The basal or resting metabolic rate is usually reported estimate of energy expenditure in endotherms (McNab 2019), and that of mammals is best explained by body mass, and is also interpreted as reflecting the size and metabolic intensity of energy-consuming tissues (Meerlo et al. 1997). In this study, we observed that RMR was positively correlated with the mass of liver and lung, and in particular, small intestine in striped hamsters subjected to cycles of cold and warm
temperatures. In addition, NST was significantly correlated with the mass of liver, heart, and kidneys, as well as, gastrointestinal tracts. The increased metabolic thermogenesis of the animals acclimated to cold conditions is usually compensated by the elevation in energy intake (Zhao 2011; Francis & Ebling 2015; Deem et al. 2020). Consistently, in the present study the striped hamsters showed considerably higher gross and digestive energy intake during the period of cold exposure. Interestingly, the hamsters that were subjected to 72 h cold and warm cycle also consumed significantly more food after they returned to the warm temperature than those kept at warm condition, which may be exploited to meet the energy requirement of increased RMR. Similarly, the Brandt’s voles (Lasiopodomys brandtii) increased energy intake during the cold acclimation, which was still higher than that in the control in the two weeks of rewarming (Zhang & Wang 2006). However, in this study, serum leptin concentration and the gene expression of ObRb in hypothalamus were not significantly different in the hamsters that were frequently subjected to cycles of cold-warm temperature compared to that of controls. Neither anorexigenic peptides nor orexigenic peptides gene expression was significantly changed flowing cycles of cold-warm temperature, suggesting that leptin pathway may be not involved in regulations of energy budgets of striped hamsters acclimated to cold-warm cycles. In addition to energy intake, we observed that the hamsters subjected to 72 h cold and warm cycle showed significantly less fat deposition than the control hamsters. This indicates that the fat depots are likely mobilized by the animals to cope with the next episode.

4. In conclusion

Both RMR and NST were significantly increased in striped hamsters subjected to repeated cycles of short-term cold (5°C, 72 h)- warm (23°C, 4 days) temperatures compared to that of the hamsters kept at 23°C. In these cycled hamsters, BAT UCP1 expression was significantly upregulated, whereas serum T3 and T4 did not change significantly. It suggests that BAT thermogenesis of animals that were frequently subjected to cycles of cold-warm temperature could not be mediated by thyroid hormone.

5. Materials and methods

5.1. Animals

The offspring of a colony at Wenzhou University that was founded with animals trapped in farmland in the center of Hebei province (115u139E, 38u129S) on the North China Plain in 2008. A randomized outbreeding protocol is used to maintain genetic diversity. Animals were housed individually in plastic cages (29 cm × 18 cm × 16 cm) with sawdust bedding, and kept at 23 ± 1°C under a 12 h:12 h (light: dark, lights on at 08:00 h) photoperiod. Food (standard rodent chow; produced by Beijing KeAo Feed Co., Beijing, China) and water were provided ad libitum.

Twenty-eight male striped hamsters (3–3.5 months of age) were randomly assigned into one of three groups: a control group (23°C, n = 9) which was kept at 23°C throughout this experiment, and two cold-warm cycle treatment groups (5°C-24 h, n = 10 and 5°C-72 h, n = 9) which were transferred into a cold room (5°C) for 24 h and 72 h, respectively, followed by six and four days of warm (23°C) temperature. The alternating cold-warm temperature cycle was repeated for 6 weeks. All three groups were fed ad libitum with the diet with a caloric value of 22.0 kJ/g (Research Diet, D12492, USA) for the duration of the experiment (60% fat, 20% carbohydrate and 20% protein).

5.2. Body mass and food intake

At the beginning of the experiment, animals were weighed on a daily basis to the nearest 0.1 g to establish their baseline body mass in the first week. Food intake was also measured daily during these baseline measurements. Food intake was calculated from the difference between the food provided and the uneaten food on the next day, minus food residues mixed with bedding material (Xu et al. 2019).

5.3. Energy intake and digestibility

Gross energy intake (GEI) and digestibility were measured during the 6th cold-warm temperature cycle at 5°C and 23°C, respectively. As described by Deng et al. (2020), a known quantity of food was provided and any uneaten food and orts in bedding material were collected 24 h later. Food and feces were separated manually after drying to a constant mass at 60°C. The gross energy content of feces was determined with an IKA C2000 oxygen bomb calorimeter (IKA, Germany). GEI, digestive energy intake (DEI), gross energy of feces (GEF) and digestibility were calculated as follows (Grodzinski & Wunder 1975; Yu et al. 2020),

\[ \text{GEI}(\text{kJ/d}) = \text{food intake (g/d)} \times \text{dry matter content of food (\%)} \times \text{gross energy content of food(\text{kJ/g})}. \]
GEF (kJ/d) = dry feces mass (g/d) 
\times gross energy content of feces (kJ/g);

DEI(kJ/d) = GEI − GEF;

Digestibility (%) = DEI/GEI × 100%.

5.4. Resting metabolic rate (RMR) and non-shivering thermogenesis (NST)

RMR was estimated from the rate of oxygen consumption, which was measured with an open-flow respirometry system (TSE systems, PhenoMaster/LabMaster, Germany) at the end of the 6th cold-warm cycle. As described by Wen et al. (2018a, 2018b), air was pumped through a cylindrical, sealed, Perspex chamber (35 cm in length and 8 cm in diameter) at a rate of 1000 mL/min at a temperature of 30 ± 0.5°C, which is within the thermal neutral zone of the striped hamster (Zhao et al. 2010b). Gases leaving the chamber were dried and directed through the oxygen analyzer at a flow rate of 380 mL/min. Data were collected every 10s by a computer connected via an analogue-to-digital converter, and analyzed using standard software (TSE). RMR was measured for 3 h, and was calculated from the consecutive minimum rate of oxygen consumption over 10 min, corrected to standard temperature and air pressure conditions (STAP), and expressed as mL O₂/h per animal (Zhao et al. 2020).

The NST was estimated from the maximum rate of oxygen consumption, induced by the subcutaneous injection of a dose of norepinephrine proportional to the body weight of each animal (Heldmaier 1971; Zhao et al. 2010a). Dosages were calculated with the formula: NE (mg/kg) = 6.6·Mb–0.458 (g) (Heldmaier 1971). NST was measured for 60 min at 25 ± 1°C using the same method as RMR. The consecutive maximum rate of oxygen consumption over 10 min were used to calculate NST, which was corrected to SATP conditions and expressed as mL O₂/h. The regulated NST (NSTr) was calculated from the difference between NST and RMR (NSTr = NST-RMR).

5.5. Body composition

Animals were euthanized by decapitation the day after NST measurements. Scapular BAT and hypothalamus were removed and weighed carefully and quickly, frozen in liquid nitrogen and stored at −80°C until required for analysis. Subcutaneous fat, perirenal fat, mesenteric fat and abdominal fat (but not periovarian fat), were collected and weighed (to ±1 mg). Total body fat was the sum of the above fat deposits. Liver, heart, lung, spleen, and kidneys were collected and weighed separately (to ±1 mg). In addition, stomach, small and large intestine, and caecum were removed separately and weight without the contents (to ±1 mg).

5.6. Real-time RT–qPCR analysis

The mRNA expression of agouti-related protein (AgRP), cocaine- and amphetamine-regulated transcript (CART), neuropeptide Y (NPY), and pro-opiomelanocortin (POMC) of hypothalamus, and BAT UCP₁, were measured using real-time RT–qPCR analysis. Total RNA of hypothalamus was extracted using TRIZol Reagent (TAKARA, Dalian,

| Gene              | Primers (5’ to 3’)          | Size (bp) |
|-------------------|-----------------------------|-----------|
| Actin (forward)   | AAGACCTCTATGCCAACA          | 196       |
| Actin (reverse)   | ACATCTGCTGAAAGTG          |           |
| AgRP (forward)    | TGTTCCCAGAGTTCCAGGTC       | 227       |
| AgRP (reverse)    | ATGGAAGAACCAGCAGCAGC       |           |
| CART (forward)    | TACCTTTTGTGCGGGTCCG        | 260       |
| CART (reverse)    | AGTCTTGCTGGGAGACGT         |           |
| NPY (forward)     | ACCCTGCCTCCTGCTCGG         | 186       |
| NPY (reverse)     | ATACGTCAGTCAGGCTA          |           |
| ObRb (forward)    | CAGTTGTCGATAAGCCTG         | 200       |
| ObRb (reverse)    | TTGCAATATTAACTGAGGT        |           |
| POMC (forward)    | GGTGGCGGAAGAACGAG          | 205       |
| POMC (reverse)    | CTGGTCTTGGGCGGGGCT         |           |
| UCP₁ (forward)    | GGACCACTACACCACCTGGGAAAA   | 330       |
| UCP₁ (reverse)    | GCTTCTGTGGGGTCTAT          |           |

AgRP, agouti-related protein; CART, cocaine- and amphetamine-regulated transcript; NPY, neuropeptide Y; ObRb, long isoform of leptin receptor; POMC, pro-opiomelanocortin; UCP₁, uncoupling protein 1.
As described previously (Xu et al. 2019), cDNA was synthesized in a final reaction volume of 50 μL with random primmer oligo (dT)18 and AMV Reverse Transcriptase (TAKARA). 2 μL cDNA samples were taken for the subsequent PCR reaction using gene-specific primers (Table II). The final reaction volume of 20 μL contained 10 μL of 2 × SYBR Premix EX Tag TM (TAKARA), 0.4 μL of forward prime and reverse primer (final concentration 0.2 μM per primer), 2 μL cDNA template and 7.2 μL DEPC H2O. qPCR was performed using Roche Light Cycler480II real-time qPCR system. Actin was used as an internal standard. Samples were quantified for relative quantity of gene expression (Zhao et al. 2014; Xu et al. 2019).

5.7. Western blot analysis of UCP1

BAT was lysed in RIPA buffer (0.5% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl [pH 7.5]) supplemented with phosphatase inhibitor cocktails. Protein extracts were diluted in 5× sample buffer (50 mM Tris at pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.1% bromophenol blue) separated on a discontinuous SDS-polyacrylamide gel (12% running gel and 5% stacking gel) and then transferred onto a PVDF membrane (millipore, IPVH00010). The blotting membranes were blocked with 5% (wt/vol) milk powder and incubated overnight at 4°C with the primary antibody β-actin (Servicebio GB12001) and UCP1 (proteintech 23673-1-AP). Secondary antibody (anti-rabbit IgG HRP conjugate; 1:3,000; Servicebio GB23303) was then added, and Super Signal Western blot Enhancer (Thermo Scientific) was used to visualize protein bands. Blots were analyzed with Bio-Rad Quantity One and normalized to β-actin.

5.8. Serum tri-iodothyronine (T3), thyroxine (T4), and leptin concentration

The trunk blood collected from each animal, and serum was separated from each blood sample and stored at −80°C until required for analysis. Serum T3 and T4 levels were determined by radioimmunoassay using125 IRIA kits (Beijing North Institute of Biological Technology, Beijing, China); the intra- and inter-assay coefficients of variation were 2.4% and 8.8% for T3, and 4.3% and 7.6% for T4. Serum leptin concentrations were determined using a commercial leptin (hamster) ELISA kit (Fine Biotech Co., Ltd., Wuhan, China). This kit is validated for striped hamsters according to the standard curve. The lower limit of the assay kit is 7.8 pg/mL, and the intra- and inter-assay coefficients of variation are 8% and 10%.

5.9. Statistical analysis

Data were analyzed using SPSS statistical software (version 20.0) after all variables were first confirmed to be normally distributed by a Kolmogorov–Smirnov test. The statistical significance of differences in body mass, food intake, RMR, NST, energy intake, digestibility, body composition, BAT UCP1 mRNA and protein expression, neuropeptides mRNA expression of hypothalamus, and serum T3, T4 and leptin among the 23°C, 5°C-24 h and 5°C-72 h groups were assessed using one-way ANOVA or ANCOVA, with body mass as a covariate. We also run a model of correlation analysis and linear regression analysis between RMR, NST and body composition, and T3 and T4 concentration. Data are presented as means ± s.e.m, or individual data points with means ± SD. All tests are two-tailed and P-values < 0.05 were considered statistically significant.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Supplementary material

Supplemental data for this article can be accessed here.

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