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Protein restriction does not affect body temperature pattern in female mice

Running Head: Protein nutrition and torpor in mice

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Abstract

Daily torpor is a physiological adaptation in mammals and birds characterized by a controlled reduction of metabolic rate and body temperature during the resting phase of circadian rhythms. In laboratory mice, daily torpor is induced by dietary caloric restriction. However, it is not known which nutrients are related to daily torpor expression. To determine whether dietary protein is a key factor in inducing daily torpor in mice, we fed mice a protein-restricted (PR) diet that included only one-quarter of the amount of protein but the same caloric level as a control (C) diet. We assigned six non-pregnant female ICR mice to each group and recorded their body weights and core body temperatures for 4 weeks. Body weights in the C group increased, but those in the PR group remained steady or decreased. Mice in both groups did not show daily torpor, but most mice in a food-restricted group (n = 6) supplied with 80% of the calories given to the C group exhibited decreased body weights and frequently displayed daily torpor. This suggests that protein restriction is not a trigger of daily torpor; torpid animals can conserve their internal energy, but torpor may not play a significant role in conserving internal protein. Thus, opportunistic daily torpor in mice may function in energy conservation rather than protein saving.

Key words: core body temperature, daily torpor, female mice, isocaloric, protein restriction

Introduction

Through long-standing multidisciplinary efforts by scientists, the nutrient requirements of laboratory animals have been precisely determined (e.g., AIN-93 described by the National Research Council [18]). These guidelines enable us to investigate the various influences of a lack of dietary nutrients on developmental, physiological, and behavioral traits. For example, a deficiency in dietary folic acid induces premature hearing loss [13], a deficiency in dietary zinc induces cutaneous disorders and/or idiopathic dysgeusia [10], and a deficiency in dietary thiamine (vitamin B) induces Wernicke–Korsakoff syndrome and related neurological disorders, which lead to delirium tremens, poor eyelid function, and ataxia [30]. On the other hand, there are still unresolved issues concerning the influence of a lack of dietary nutrients on several adaptive animal behaviors.

Daily torpor is a physiological adaptation in mammals and birds that is induced by energy-limited situations such as starvation or cold [23]. This adaptation is characterized by a controlled reduction of metabolic rate (MR) and body temperature (T_b) during the resting phase of the circadian rhythm [6]. As the surface area-to-volume ratio of small mammals is larger than that of large mammals, small mammals have greater energy requirements per body mass due to their greater heat loss [4]. Therefore, small mammals have a more pronounced tendency for torpor than large ones [23]. Some laboratory rodents also express daily torpor (e.g., the white-footed mouse (*Peromyscus leucopus*) [17] and the house mouse (*Mus musculus*) [5]).

Historically, many studies have suggested that daily torpor may play an important role in energy conservation by lowering MR and T_b. Additionally, studies have attempted to determine the novel functions of daily torpor applicable to the extension of life [8, 21, 29]. Daily torpor is also regarded as a confounding factor in some energy constraint experimental procedures [12, 19], because low MR and low T_b can modulate an animal's physiological

69 status, such as physical activity, blood properties, and cell mitotic activity [8, 24]. Therefore,
70 knowledge of the details of the mechanism and functions of daily torpor in laboratory animals
71 would be valuable.
72 Laboratory mice express daily torpor in response to fasting and dietary caloric restriction [5,
73 8, 15]. Additionally, obvious strain- and individual-related variations in torpor expression
74 have been observed [1, 21]. Furthermore, mice can regulate the depth of a torpid bout based
75 on the level of dietary or caloric supplementation [15, 25]. This suggests that mice have a
76 sensitive reaction to changes in energetic conditions. This area of research usually focuses on
77 integrated energy restriction events, although dietary energy sources are multifactorial,
78 containing fats, proteins, and carbohydrates. We examined whether a deficiency in specific
79 energy sources (i.e., fat, protein, or carbohydrate) corresponds to the expression of daily
80 torpor. If so, this might elucidate a novel function of daily torpor and/or a method for
81 regulating torpor expression.
82 Here, we focused on deficiency in dietary protein intake, because protein is one of
83 important nutrients in maintaining the energy balance of homeostasis and is a source of
84 several enzymes. Severe protein deficiency affects various endocrinological activities and in
85 some cases leads to death from malnutrition [18]. A deficiency in dietary protein intake can
86 be partially compensated for by the endogenous protein store (primarily in the liver) or
87 muscular degradation [18]. Recently, Mitchell et al. [15] reported that the C57BL/6 strain of
88 inbred male mice never expressed daily torpor following restriction of dietary protein intake
89 (dietary protein-restricted (PR) levels: 20%, 30%, and 40% compared to control diet), but
90 caloric-restricted groups (dietary caloric restriction levels: 20%, 30%, and 40% compared to
91 control diet) expressed daily torpor (see also Materials and Methods). This result appears to
92 indicate that caloric but not protein restriction is a principal trigger for the expression of daily
93 torpor by starvation. Although Mitchell et al. (2015) [15] used male mice in their protein

restriction experimentation, it is important for experimental zoology to know sexual difference under the similar conditions. Even Sunagawa and Takahashi showed (2016) recently that male mice can enter daily torpor in certain conditions [27], it has been generally believed that male mice are less likely to enter daily torpor [28], mainly because of their high concentration of endogenous testosterone. Therefore, to determine the relationship between protein restriction and the expression of daily torpor, we need to consider the case in female mice. In this study, we subjected female laboratory mice to more severe restrictions in dietary protein intake than those of Mitchell et al. (2015) [15] and determined the T_b patterns, especially with regard to the expression of daily torpor.

Materials and methods

Animals and housing conditions

Females of the ICR strain of laboratory mice (n = 18, 9 weeks of age) were purchased from SLC Japan Bred. Co. Ltd. (Shizuoka, Japan). We selected this strain because they express daily torpor in response to starvation and have a high body mass, making it easier to implant a thermostat into the abdominal cavity [5, 20]. Mice were housed individually in plastic cages (W 225 × D 338 × H 140 mm, Crea Japan, Tokyo, Japan) with wood shavings as bedding, and allowed free access to a solid diet (Labo MR Stock, Nosan Corporation, Kanagawa, Japan) and tap water until the experimental period (see below). The room environment was strictly controlled as follows: room temperature throughout the experiment was maintained at 24°C, and the photoperiodic cycle was 12 h / 12 h (light / dark; light turned on at 08:00). All experimental procedures in this study were approved by the Animal Experiment Committee of the University of Miyazaki (Permission No. 2005–053–10).

Diets

The compositions of our experimental diets are described in Table 1. We prepared two types of powdered diet in accordance with AIN-93M, a common purified diet for laboratory mice [18, 20]. The control diet (C diet) maintained the exact same composition as AIN-93M, including 14.0% casein and 0.18% L-cystine as protein sources. The protein-restricted diet (PR diet) partially replaced casein and L-cystine with cornstarch (carbohydrate) and included 3.5% casein and 0.045% L-cystine. The PR diet had only a quarter of the protein amount relative to the C diet, while maintaining the same caloric level as the C diet with respect to gross energy (GE). Mitchell et al. (2015) [15] used four diets including 20%, 16%, 14%, and 12% protein in each diet, supplemented by increasing amounts of carbohydrate (see details in Mitchell et al. 2015 [15]). The PR diet used in this study had a more severe level of dietary protein restriction (about 3.5% casein as a protein source) than those used in Mitchell et al. (2015) [15]. Each diet was mixed about once monthly in 10-kg batches and stored at 4°C until feeding.

Core body temperature (T_b) measurement

To record core body temperature (T_b) and estimate the expression of daily torpor, we implanted a data logger (iButtons, DS1922L, Maxim Integrated, CA, USA) in the abdominal cavity of female mice under anesthesia (Sodium pentobarbital (54mg/kg): Somnopentyl, Kyoritsu Seiyaku Corporation, Tokyo, Japan). We allowed all mice at least 3 weeks of recovery under *ad libitum* feeding conditions and used their body mass as an indicator of recovery.

Data loggers were coated with a thin layer of a paraffin–Evaflex mixture (EV220, Du Pont–Mitsui Polychemical Co. Ltd., Tokyo, Japan) according to Masaki *et al.* (2005) [14] to avoid damage by serous fluid. These loggers were programmed to record temperature every

15 minutes at a 16-bit resolution (0.0625°C), which yielded 45 consecutive days of data. Logger weights were 3.56 ± 0.23g. This data logger is acceptable for implantation into the abdominal cavities of female mice weighing around 40 g (less than 10% of the body weight of female mice).

Experimental procedure

We transferred each mouse from its home cage to another cage lined with steel wire mesh. We supplied 10.0 g·day^{-1} of the C diet for 5 days to acclimate mice to a powdered diet, then gradually reduced the diet supply by 1.0 g every 3 days. When the diet supply reached 6.0 g·day^{-1}, mice consumed all the supplied diet. Hence, we continued this supplementation for 5 more days, but no loss of body weight was observed with this amount. Therefore, we determined the amount of the diet as 6.0 g·day^{-1}.

At the end of this estimation trial, we randomly assigned all mice to either the control group (C group, n = 6) or the protein-restricted group (PR group, n = 6). We also established a food-restricted group (FR group, n = 6), which was used to determine the reaction of the expression of daily torpor in the context of food restriction in the ICR strain of mice. The C and PR groups were supplied 6.0 g·day^{-1} of the C and PR diet for 4 weeks, respectively. The FR group was supplied 80% of the amount of the C diet compared to the C group (4.8 g·day^{-1}) for 4 weeks. We determined the expression of daily torpor in each mouse during this period. Body weights were measured every 2 days. If weight loss reached 15% of the initial body weight, we terminated the experiment for that mouse.

Data handling and statistical analysis

We analyzed body weight data and T_b data throughout the experimental period. Changes in body weight from the start to the end of the experiment in each group were examined using

the paired t-test. Comparisons of terminal body weight data among groups were estimated by the Tukey–Kramer HSD test. We calculated three T_b parameters: daily mean T_b (Mean T_b), daily minimum T_b (Min. T_b), and daily maximum T_b (Max. T_b). These T_b parameters were also compared among groups using the Tukey–Kramer HSD test. The expression of daily torpor was defined as $T_b < 31°C$ [5] and compared among all groups.

Statistical analyses were performed using JMP 10 (JMP 10 Basic Analysis and Graphing, SAS Institute, 2012). A $P < 0.05$ was considered statistically significant, and results were expressed as means ± SD.

Results

Body weight and food consumption

Female mice consumed most of the supplied diets (C group: 5.76 ± 0.36g; PR group: 5.85 ± 0.18g; FR group: 4.79 ± 0.08g). Daily protein intake for each mouse was about 0.85 g for the C group, 0.21 g for the PR group, and 0.68 g for the FR group. These results suggest that the C and PR groups consumed the same amounts of calories, but protein intake was reduced by 75% in the PR group.

Despite the initial body weights being approximately the same (44.42 ± 1.68 g for the C group, 44.75 ± 1.51 g for the PR group, and 43.78 ± 2.17 g for the FR group), body weights after the experiment were 50.00 ± 2.61 g for the C group, 43.77 ± 2.78 g for the PR group, and 40.42 ± 3.20 g for the FR group. The C group had a significantly higher body weight than the PR and FR groups ($P < 0.05$) after the experimental period. The C group gradually became heavier, at 112.5% relative to the initial body weight ($P < 0.05$), but the weights were 97.8% ($P = 0.29$) and 92.3% ($P < 0.05$) for the PR and FR groups, respectively (Fig. 1).

194 ***Core T_b and torpor expression***

195 Five of six mice in the FR group exhibited torpor (T_b < 31°C [5]), but no mice did in the C
196 and PR groups (Table 2, Fig. 2). Additionally, we did not detect any significant differences in
197 T_b parameters (Mean T_b, Min. T_b, and Max. T_b) between the C and PR groups (P = 0.9728, P
198 = 0.8956, and P = 0.5478, respectively). The T_b parameters for the FR group were
199 significantly lower compared with those for the C and PR groups (P < 0.05). Female mice did
200 not exhibit torpor under PR conditions but did under energy-deficient conditions.

201 Daily T_b patterns in both the C and PR groups were similar, but the FR group was very
202 different in this regard; specifically, mice in the FR group had low T_b (below 35°C) starting
203 on the day of food restriction, and this ratio gradually increased throughout the experiment
204 (Fig. 2, Fig. S1–S3).

205

206

207 **Discussion**

208 We subjected female ICR mice to severe protein restriction (75% lower daily protein intake
209 compared to normal conditions; PR group); however, they did not exhibit any T_b reductions
210 during the resting phase. In contrast, in the food-restricted group (20% lower daily food
211 intake compared to the control group; FR group), five of six mice sporadically or frequently
212 expressed daily torpor (Fig. 2, Fig. S1–S3). Additionally, the T_b parameters (Mean, Min., and
213 Max. T_b) and daily T_b patterns indicated a similar pattern in the control (C group) and PR
214 groups (Table 2, Fig. 2, and Fig. S1–S3). Most mice consumed all their food, and none of the
215 mice in the C group decreased in body weight (Fig. 1), indicating that the amounts of calories
216 for the C and PR groups were sufficient, and they did not express daily torpor (Table 2).
217 Therefore, the results indicate that mice may preserve thermal homeostasis when caloric
218 intake is sufficient even if protein intake is insufficient.

219 Dietary protein restriction clearly influenced body weight gain in female mice. They did
220 not gain any body weight throughout the experimental period (Fig. 1), implying that the mice
221 in the PR group may have had a zero energy balance. Generally, PR animals partially
222 compensate by using several amino acids for protein homeostasis and enhancing the
223 degradation of skeletal muscle and hepatic protein stores [7, 9, 11]. However, degradation of
224 internal proteins is an insufficient explanation of why female mice maintained their body
225 weight for so long under PR feeding (4 weeks in this experiment). We suspect that female
226 mice may uptake fecal protein, which is called "coprophagy" and which plays nutritionally
227 significant roles in providing microbial proteins to animals via feces. Coprophagy is closely
228 related to the cecum in terms of protein nutrition [26]. Ebino *et al.* (1993) [2] demonstrated
229 that laboratory mice also engage in coprophagy and that feces were a rich source of proteins
230 and other nutrients, such as vitamins. Therefore, it is possible that the mice we tested did not
231 express daily torpor following restriction of protein in their diet because they increased the
232 frequency of coprophagy. Torpor in the garden dormouse *Eliomys quercinus*, which does not
233 have a cecum, was induced by protein deficiency even though energy requirements were
234 amply satisfied [16], indicating that they may not ingest microbial proteins by coprophagy.
235 This would suggest that our results are not generally applicable to all mammalian species.
236 Therefore, we need to consider species differences, including feeding phenology and
237 morphological digestive capacities, to estimate the relationship between a deficiency of a
238 specific energy source and daily torpor.
239 Five of six mice in the FR group exhibited daily torpor (Table 2). Their daily torpor
240 patterns were frequent or sporadic, with significant variation among individuals. Additionally,
241 the FR group frequently had low T_b (below 35°C) during the restricted feeding period, but the
242 C and PR groups did not (Fig. 2, Fig. S1–S3). We initially focused on the expression of daily
243 torpor (T_b < 31°C); however, female mice showed a gradual adjustment to a

nutrition-restricted situation. This low T_b, but not daily torpor, appeared to be accompanied by a small reduction in metabolic rate and may have contributed to energy conservation as an alternative to daily torpor. Interestingly, the large Japanese field mouse (*Apodemus speciosus*) may be cognizant of the magnitude of a food cache and change torpor patterns [3]. Moreover, mice can regulate the depth of a bout depending on dietary restriction [25]. Hence, flexible expression of daily torpor and a minor reduction of T_b may be related to a psychological recognition of food quantity and body condition by the mouse itself. Therefore, more attention should be given to the influences of the feeding process and appetite on the expression of daily torpor and gradual changes in T_b.

In this study, we determined that dietary protein restriction failed to induce daily torpor in female laboratory mice. Although we did not identify a novel nutritional function for daily torpor, our research on daily torpor has only just begun. In our future research, we aim to determine the influence of deficiencies in other nutrients and feeding systems on the induction of daily torpor.

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Data Accessibility

All data used in the manuscript and the R codes can be downloaded via Dryad (http://datadryad.org/) with DOI:XXXXXXX.

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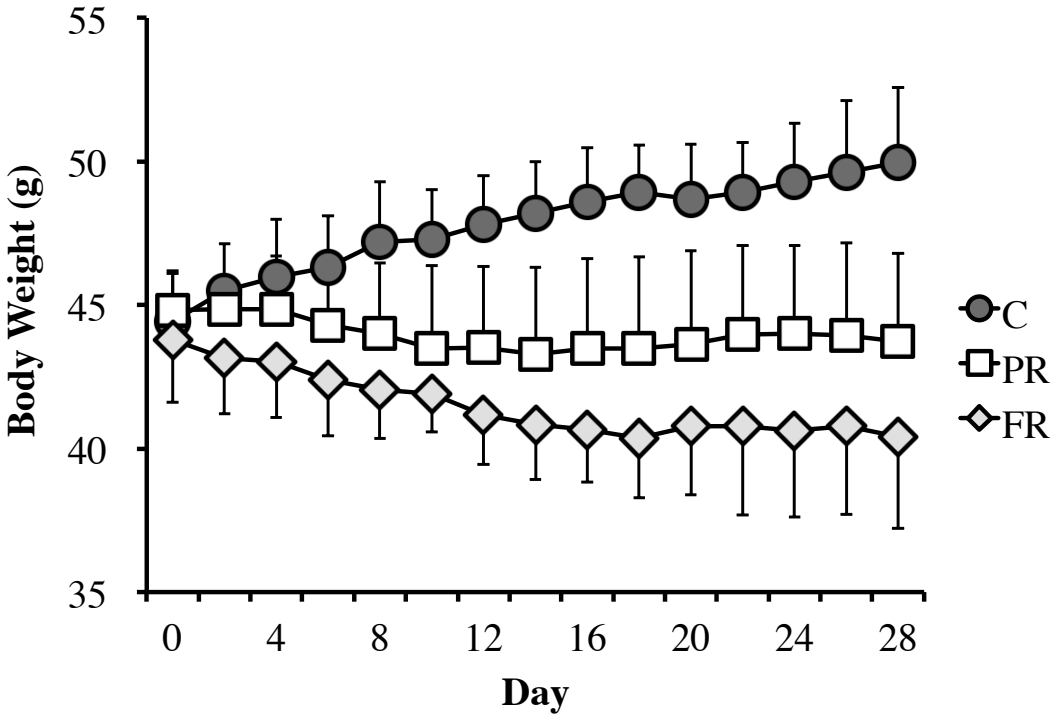

Fig. 1. Body weight changes in control (C), protein-restricted (PR), and food-restricted (FR) groups during the experiment.

Body weights changed in the C (increased) and FR (decreased) groups, but the PR group maintained body weight over the experimental period. Error bars indicate mean ± SD.

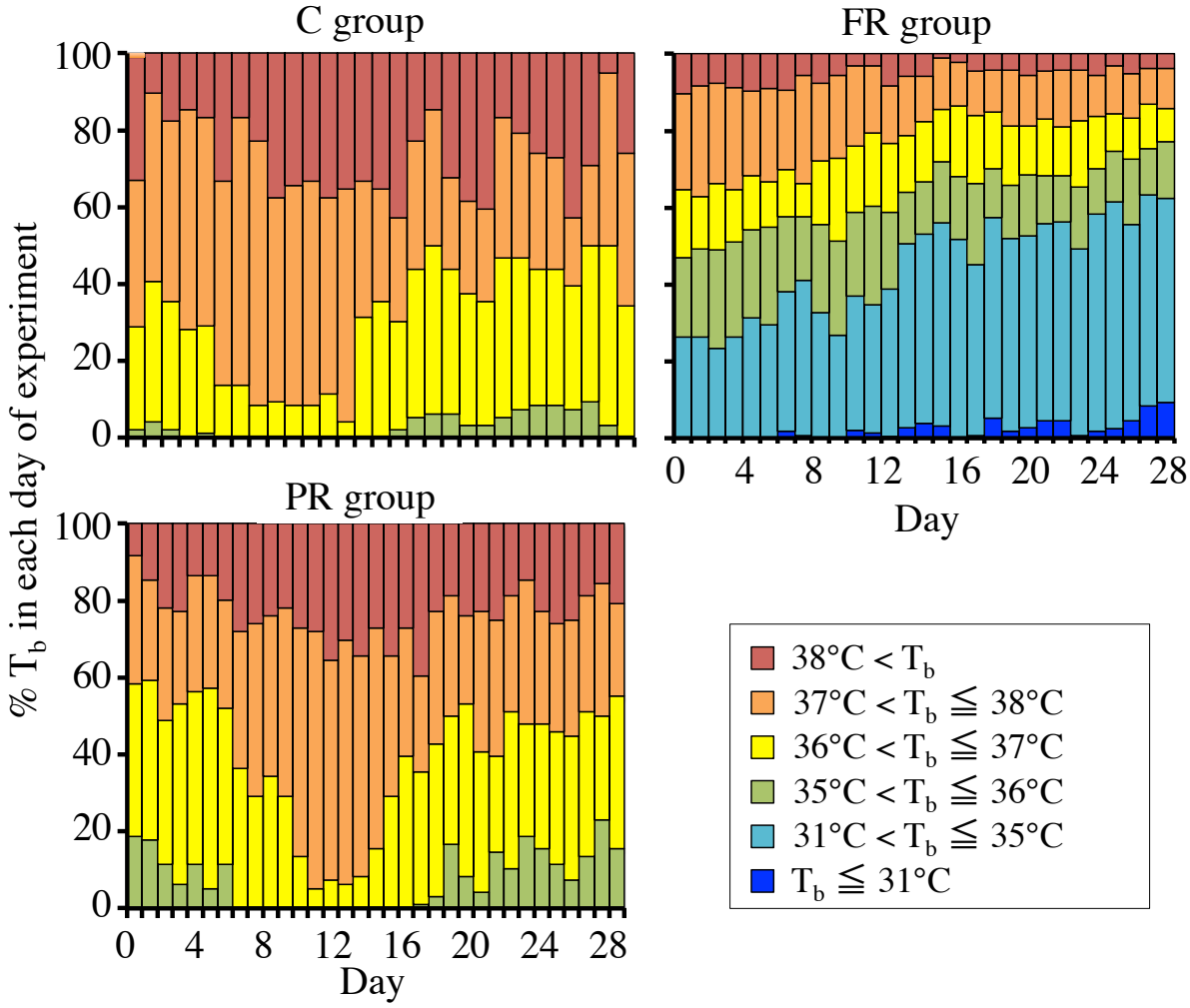

Fig. 2. Representative daily T_b patterns in control (C), protein-restricted (PR) and food-restricted (FR) groups during the experiment.

C and PR mice had similar daily T_b patterns. There were few measurements of T_b < 35°C in the C and PR groups, but the FR group frequently had a T_b < 35°C during this experiment. The vertical line shows the percentage of T_b in each day of experiment. C, PR, and FR labels indicate "group name" of the mice. Figs. S1–3 show individual daily T_b pattern data.

Table 1. Composition of the experimental diet.

Ingredient	C diet	PR diet
	g/kg diet	
Cornstarch	465.692	572.042
Casein	140	35
Dextrinized cornstarch	155	155
Sucrose	100	100
Soybean oil	40	40
Fiber source (cellose)	50	50
Mineral mix	35	35
Vitamine mix	10	10
L-Cystine	1.8	0.45
Choline bitartrate	2.5	2.5
Tert-butylhydroquinone	0.008	0.008

We used two types of formula diet: the control (C) diet was AIN-93M [18, 20] and the protein-restricted (PR) diet replaced 75% of the protein sources (casein and L–cystine) with carbohydrate (cornstarch). These diets were isocaloric.

Table 2. Summary of Tb parameters in the C, PR, and FR groups.

T_b parameters	C group	PR group	FR group
Mean T_b(°C)	37.19±0.27	37.20±0.23	35.28±0.79*
Max. T_b(°C)	38.66±0.23	38.69±0.27	38.37±0.33*
Min. T_b(°C)	35.76±0.41	35.71±0.43	32.45±1.57*
No. of torpid mice	0 / 6	0 / 6	5 / 6

Note: * $P < 0.05$, Tukey–Kramer HSD test.

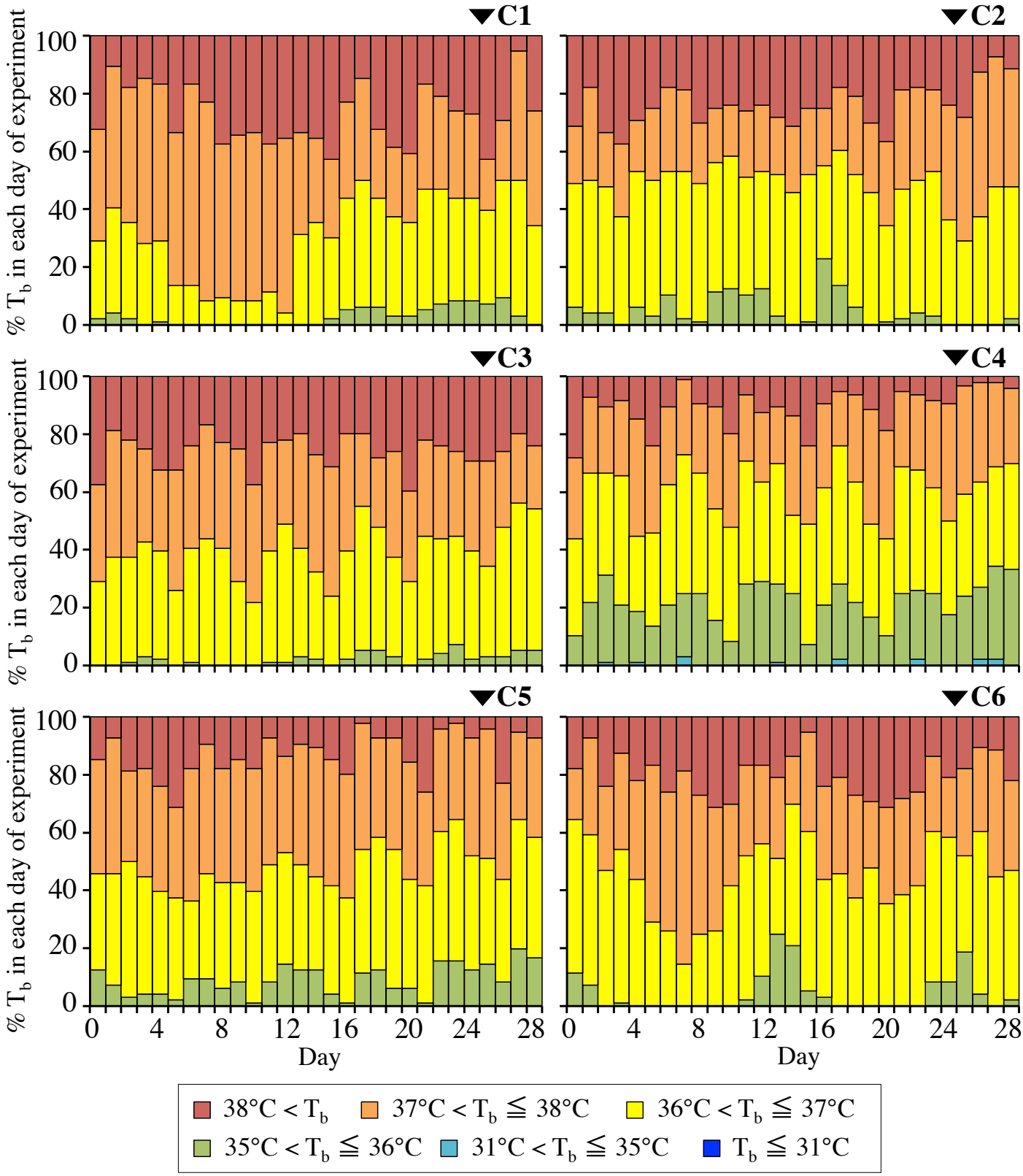

Fig. S1. Daily T_b patterns in each individual of mice during experiment in control group.

This figure shows daily T_b fluctuation patterns of the control group animals for 28 days. The core T_b have monitored and recorded in every 12 min by implanted data logger. Almost all animals exhibited their T_b 35°C or higher for 28 days and none exhibited daily torpor (T_b < 31°C). Mice were fed 6.0 g/day of a control diet (Table 1) and C1–C6 indicate individuals in the control group. The vertical line shows the percentage of T_b in each day of experiment.

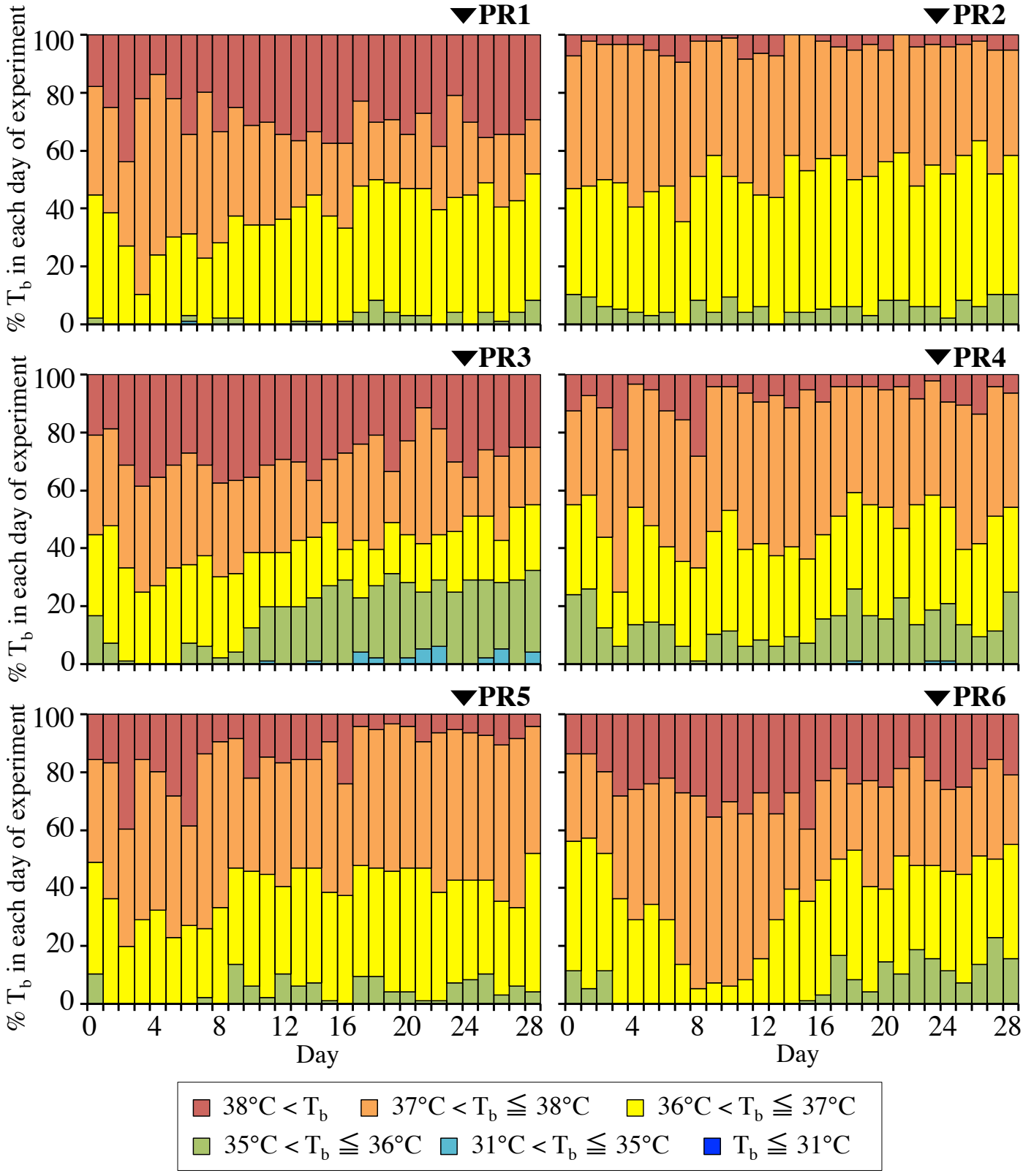

Fig. S2. Daily T_b patterns in each individual of mice during experiment in protein-restricted (PR) group.

This figure shows daily T_b fluctuation patterns of the protein-restricted group animals for 28 days. The core T_b have monitored and recorded in every 12 min by implanted data logger. Almost all animals exhibited their T_b 35°C or higher for 28 days and none exhibited daily torpor (T_b < 31°C). Mice were fed 6.0 g/day of a protein-restricted diet (Table 1) and PR1–PR6 indicate individuals in the protein-restricted group. The vertical line shows the percentage of T_b in each day of experiment.

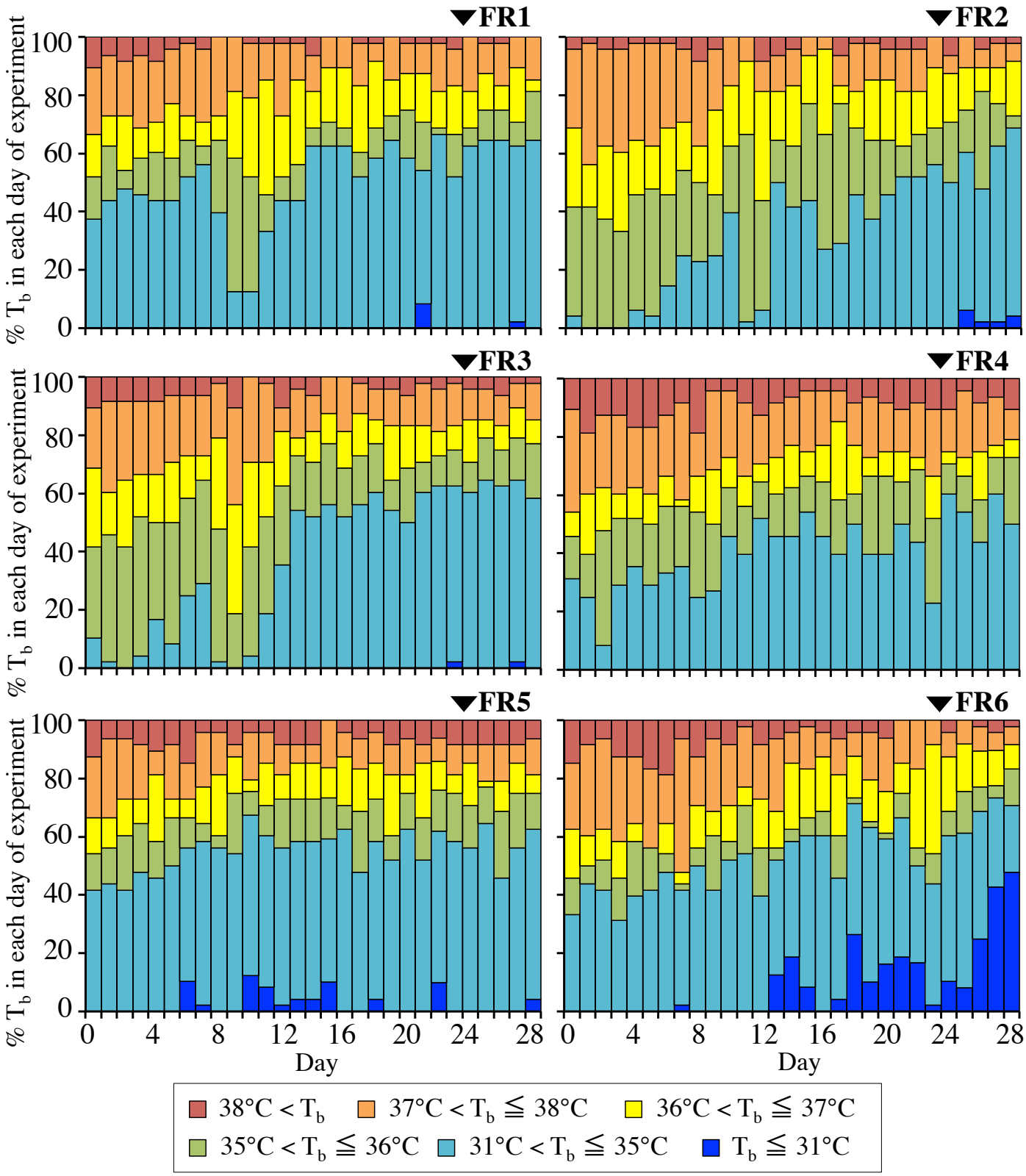

Fig. S3. Daily T_b patterns in each individual of mice during experiment in food-restricted (FR) group.

This figure shows daily T_b fluctuation patterns of the food-restricted group animals for 28 days. The core T_b have monitored and recorded in every 12 min by implanted data logger. Almost all animals showed low T_b (below 35°C) on the day of food restriction, and the ratio was gradually increased over the duration of the experiment, and exhibited daily torpor (T_b < 31°C). Mice were fed 4.8 g/day of a control diet (Table 1) and FR1–FR6 indicate individuals in the food-restricted group. The vertical line shows the percentage of T_b in each day of experiment.