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RESEARCH ARTICLE

Low-protein diet-induced hyperphagia and adiposity are modulated through interactions involving thermoregulation, motor activity, and protein quality in mice

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Blais A, Chaumontet C, Azzout-Marniche D, Piedcoq J, Fromentin G, Gaudichon C, Tomé D, Even PC. Low-protein diet-induced hyperphagia and adiposity are modulated through interactions involving thermoregulation, motor activity, and protein quality in mice. *Am J Physiol Endocrinol Metab* 314: E139–E151, 2018. First published November 14, 2017; doi:10.1152/ajpendo.00318.2017.— Low protein (LP)-containing diets can induce overeating in rodents and possibly in humans in an effort to meet protein requirement, but the effects on energy expenditure (EE) are unclear. The present study evaluated the changes induced by reducing dietary protein from 20% to 6%—using either soy protein or casein—on energy intake, body composition, and EE in mice housed at 22°C or at 30°C (thermal neutrality). LP feeding increased energy intake and adiposity, more in soy-fed than in casein-fed mice, but also increased EE, thus limiting fat accumulation. The increase in EE was due mainly to an increase in spontaneous motor activity related to EE and not to thermoregulation. However, the high cost of thermoregulation at 22°C and the subsequent heat exchanges between nonshivering thermogenesis, motor activity, and feeding induced large differences in adaptation between mice housed at 22°C and at 30°C.

body composition; diet-induced thermogenesis; energy balance; energy expenditure; energy intake; motor activity; low-protein diet; mouse; temperature; thermoregulation

INTRODUCTION

Protein and amino acid sufficiency in the body is tightly controlled and might modulate food motivation and food choice to prevent and/or counteract protein deficiency. The underlying mechanisms involve many signals, including amino acid signaling in the brain (23, 25, 38, 41, 42) and control of energy metabolism in peripheral tissues (particularly liver, muscle, and white and brown adipose tissue).

Among the different behaviors induced in subjects faced with a decrease in dietary protein concentration, increased food consumption has been proposed as a response to maintain protein intake and to meet protein requirements. Accordingly, different results showed that low-protein (LP) diets tended to induce overeating in rodents and possibly in humans (3, 10, 43, 44) in an effort to increase protein intake, which induces overconsumption of carbohydrates and fat, thus altering energy balance and inducing fat accumulation (protein leverage) (35). In addition, if such hyperphagia induced by LP diets is devoted to maintaining amino acid sufficiency in the body to support their metabolic functions, what would happen with a LP diet with a low content in one or several essential amino acids? These amino acids may be more rapidly limiting when the protein in the diet is reduced and animals would be more prone to increase energy intake and may possibly gain more adiposity than animals fed a low but well-balanced protein diet.

LP diets seem also to increase energy expenditure, at least in rodents (19, 30), as a result of either the passive cost of metabolizing an unbalanced diet or an active process through an increase in both adaptive diet-induced thermogenesis and heat dissipated in the brown adipose tissue (BAT) to dissipate part of the excess ingested energy (8, 29). In addition, since total energy intake is related to total energy requirements, more energy and, therefore, more protein are ingested when energy expenditure is increased by exercise or a cold environment, while protein requirements are not significantly increased (2, 21, 44). Accordingly, it has been reported that rats and mice submitted to regular exercise or to a low temperature could better adapt to a decrease in protein content of the diet (2), as the increased food consumption would be followed passively by an increase of protein intake subsequently oriented toward maintenance of lean tissues. This is the case particularly of the mouse whose thermal neutrality is at ~29°C. When they are kept in usual housing conditions of 20–22°C, their energy expenditure, and thus energy and protein intake, is about twice that measured at thermoneutrality (5, 11, 15, 33).

In this report, a first experiment compared behavioral and metabolic responses and the evolution of body composition (BC) of mice living in normal housing conditions (20–22°C) when subjected to a decrease in dietary protein content from 20% to 6% using a well-balanced animal protein (casein) or a plant protein (soy) diet in which methionine is the main limiting essential amino acid. To further investigate mouse capacity to adapt to such reduced protein diet, a second experiment was performed to compare mice housed at 22°C vs. 30°C (thermoneutrality).

MATERIALS AND METHODS

Pellet preparation. The food pellets were prepared in the laboratory from dry powders manufactured at the "Atelier de preparation des

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aliments" (Institut National de la Recherche Agronomique, Jouy en Josas, France). Food was mixed with water to obtain a paste and subsequently cut into pellets (~3 cm long, 1 cm large). The progressive loss of water was established to adjust the dry weight of the pellets, according to the time elapsed between the fabrication and the consumption of the pellet (between 3 and 8 days).

Animals and experimental design. The experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609 EEC) regarding the care and use of laboratory animals and was approved by the French Ethical Committee (Comethea 13-012). In *experiment 1* (E1) four groups of eight 7-wk-old female BALB/c mice (Harlan Laboratories) housed singly in Plexiglas cages bedded with wood shavings were used (Fig. 1). After 2 wk of adaptation to the laboratory conditions, the four groups were assigned one of the four diets described in Table 1. During the experiment, the mice were housed in a room with a reversed 12:12-h light-dark cycle (lights on at 2100), in which temperature was controlled at 22°C ± 1. Food intake and body weight (BW) were measured weekly throughout. During E1, energy expenditure and SMA under free living conditions were measured at the usual animal housing temperature, i.e., 22°C. Basal metabolic rate (BMR) and the thermogenic response to feeding (TEF) were measured at thermal neutrality (30°C), as we usually do to comply with the definition of BMR and to measure TEF free from errors due to meal-induced changes in thermoregulation (13, 14). These measurements led to conflicting results at 22°C and 30°C, which led us to suspect that the mechanisms of adaptation to LP diets could be room temperature-dependent. To verify this hypothesis, we performed a second experiment, in which we inverted the temperatures of E1, i.e., during which energy expenditure and spontaneous motor activity (SMA) under free living conditions were measured at thermal neutrality, i.e., 30°C, and RMR in fasted mice and TEF were measured at 22°C. We expected that direct comparison of the values measured at 22°C and 0°C in mice fed ad libitum and in mice that were food restricted and then refed would provide an explanation for the conflicting results of E1. In *experiment 2* (E2), four groups of five, 7-wk-old female BALB/c mice (Harlan Laboratories) were housed singly in the same conditions as in E1 during 8 wk, and then the temperature in the room was increased to 30°C during five more weeks. Energy intake and BW were measured weekly throughout, i.e., complementing the measurements done in E1.

Body composition and energy intake. BC was assessed by dual-energy X-ray absorptiometry (DEXA) using a Lunar PIXImus densitometer (DEXA-GE PIXImus). Fat mass (FM) gain and fat-free mass (FFM) gain were computed from DEXA measurements. In E1, BC was measured at the onset and at the end of the experimental period.

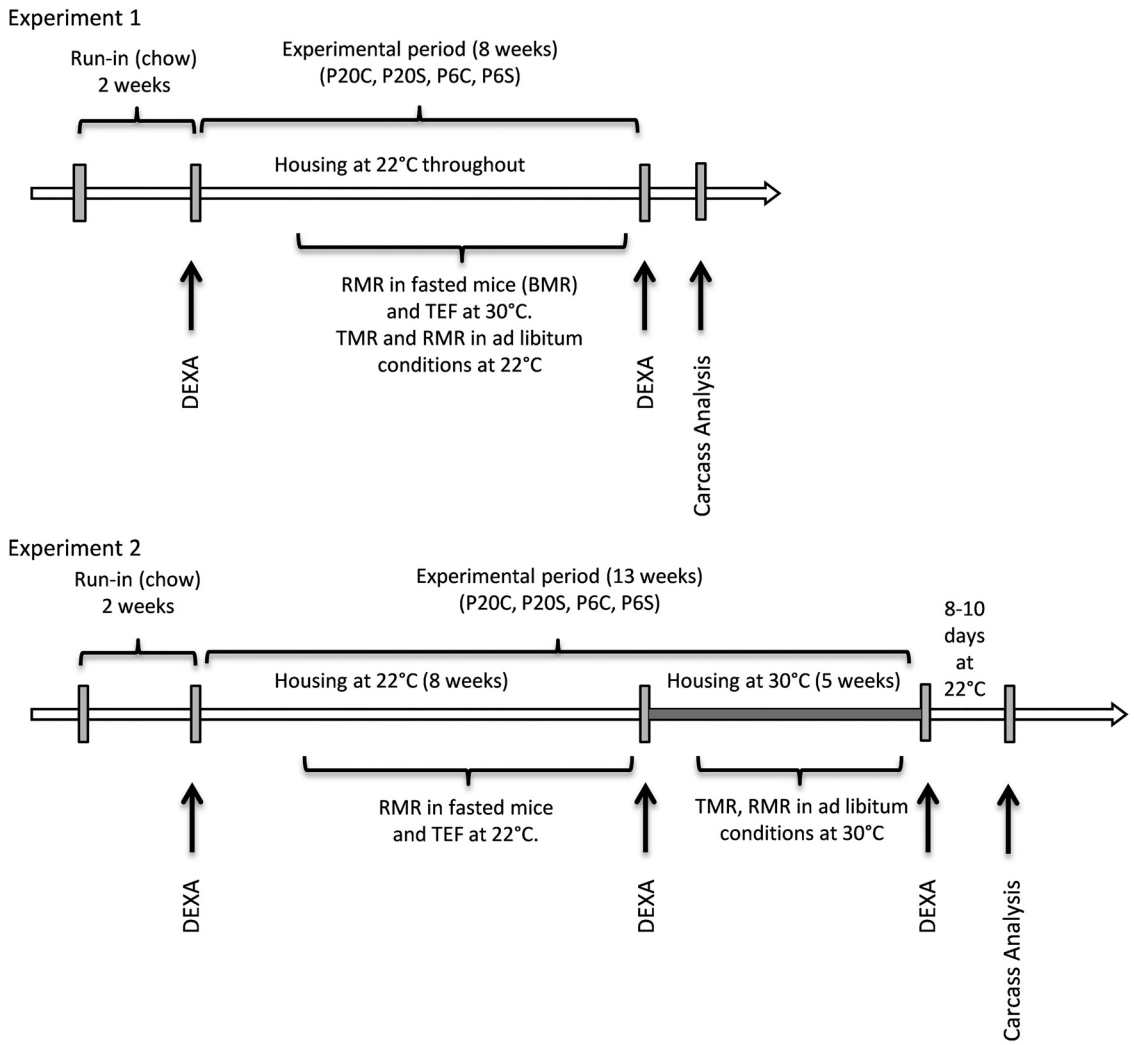


Fig. 1. Design of the experiments. In *experiment 1*, mice were housed 8 wk at 22°C and then killed for analysis of body composition by dissection of the main tissues and organs. In *experiment 2*, mice were housed 8 wk at 22°C and then 5 wk at 30°C. Mice were returned 8–10 days at 22°C before being euthanized for carcass analysis.

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Table 1. *Composition of the various diets*

	P20 Casein	P20 Soy	P6 Casein	P6 Soy
Casein or soy protein, g	183.0	180.0	51.0	51.0
Corn starch, g	585.4	588.0	699.1	699.1
Saccharose, g	94.3	94.8	112.7	112.7
Soy oil, g	40.0	40.0	40.0	40.0
Methionine, g	3.43	0.82	1.03	0.23
Minerals, g	35.0	35.0	35.0	35.0
Vitamins, g	10.0	10.0	10.0	10.0
Cellulose, g	50.0	50.0	50.0	50.0
Choline, g	2.3	2.3	2.3	2.3
Total, g	1,000	1,000	1,000	1,000
Energy, kJ/g	15.0	15.1	14.7	14.7
Carbohydrates, % energy	68.7	68.6	83.6	83.5
Fat, % energy	10.1	10.0	10.3	10.3
Protein, % energy	21.2	21.4	6.0	6.2

P6 Casein: 6% casein diet, P6 Soy: 6% soy diet, P20 Casein: 20% casein diet, P20 Soy: 20% soy diet. Note that the soy protein used in this study was cleared of phytoestrogens (Soy protein isolate; MP Biomedicals, cat. no. 205456).

In E2, BC was measured at the onset of the experiment, at the end of the 22°C period, and at the end of the 30°C period (Fig. 1). At the end of both experiments, mice were killed for a detailed analysis of BC by dissection and weighing of the main tissues and organs. Adiposity was expressed as a percentage of adipose tissue weight relative to BW. Pieces of liver, muscle, adipose tissue, and hypothalamus were dissected out rapidly and snap frozen for subsequent quantitative PCR analyses (data not shown). Energy intake was measured from changes in the calculated dry weight of the pellets. To correct energy intake for body size and composition, values were adjusted to 20 g of metabolically active mass (MAM), MAM being computed as FFM + 0.2 FM (4, 13). FM and FFM at the time of measurement were estimated by linear extrapolation between FM and FFM levels of the previous and subsequent DEXA measurements.

Spontaneous motor activity and total energy expenditure under free living conditions. Mice were housed individually in air-tight plastic cages placed on an activity platform equipped with force transducers that produced an electrical signal proportional to the intensity of the work generated by the mouse SMA (13). The SMA level was obtained by adjusting the force transducers signal to 20 g BW. For gas analysis, the cages (five simultaneously) were ventilated at 600 ml/min and connected, in turn, to the same gas analyzers. Oxygen consumption ($\dot{V}O_2$ in ml/min) and carbon dioxide production ($\dot{V}CO_2$ in ml/min) were measured in each cage during 100 s every 10 min (100 s for each cage plus 100 s in room air, to correct values for room O_2% and CO_2%). Total metabolic rate (TMR) was computed according to a modification of the Weir formula to obtain data in Watts [(16.3 $\dot{V}O_2$+ 4.57 $\dot{V}CO_2$)/60] (13). Measurements were performed over 2–3 days. *Day 1* in the metabolic cage was used for habituation, and *day 2* or the means of *days 2* and *3* were used for data analysis. $\dot{V}O_2$ and $\dot{V}CO_2$ recordings from the gas analyzers and SMA signal from the force transducers were synchronized a posteriori by adjusting a convolution of the SMA signal in order to get the best possible correlation between changes in TMR and intensity of SMA. The origin and slope of this correlation were used to compute resting metabolic rate (RMR) and metabolic cost of SMA (COST), respectively (11). For between-group comparisons, TMR and RMR were normalized to body size and composition (MAM), as described for caloric intake. In E1, temperature in the room was maintained at 22 ± 1°C, as in the usual living conditions. In E2, temperature in the room was maintained at 30 ± 1°C to reduce to ~0 the cost of thermal regulation. Temperature in the cages passively followed temperature in the room.

Basal/resting metabolic rate and thermogenic response to feeding. Mice were housed at 1800 in an air-tight plastic cage with water but without food. $\dot{V}O_2$, $\dot{V}CO_2$, and SMA (by means of highly sensitive piezo-resistive force transducers) were measured continuously, and data were stored at 2-s intervals. The next morning at 1000, a calibrated meal of 1 g of the usual diet (16 kJ) was given, and the data acquisition continued until 1700. In E1, temperature in the metabolic cage was regulated actively by means of a heating/cooling system at 30 ± 0.2°C, as usual for that kind of measurement (13). In E2, temperature in the metabolic cage was regulated at 22°C. Changes in $\dot{V}O_2$, $\dot{V}CO_2$, and SMA recorded at a 2-s interval were analyzed by a process of numerical filtering (13, 14) to get a precise calculation of COST, TMR, RMR, and by difference in metabolic rate of motor activity (MAct). TEF was computed in kilojoules as the energy expended by the increase in RMR over premeal RMR during 6 h after ingestion of the test meal and is presented as a percentage of the ingested energy. Mean value of premeal RMR measured during 2 h before meal delivery after the overnight fast was used to compute BMR (BMR↔post-absorptive RMR at thermoneutrality) when mice were housed at thermoneutrality (E1) or RMR in the fasting state when mice were housed at 22°C (E2). We performed also a detailed analysis of the short-term changes in TMR, MAct, COST, and RMR in response to activity bouts of 20–40 min that occurred spontaneously between 6 h after the onset of recording and meal delivery in the cage (i.e., in mice in the postabsorptive state) to perform a detailed analysis of the metabolic responses to SMA free from noise due to simultaneous feeding. TMR, RMR, and BMR were normalized to the body size and composition (MAM) of the mice.

Statistical analysis. Data are presented as means ± SE and as a boxplot (min, max, 1st and 3rd quartile, median). Blue boxes present data collected at 22°C, while red boxes present data collected at 30°C (Fig. 2). Statistical analysis was performed with R (R Core team, 2014, v.3.1.0, http://www.R-project.org). Two- or three-way ANOVA were used, taking protein source (casein vs. soy, "Diet" factor), protein level (20% vs. 6%, "Level" factor), and when required temperature (22°C vs. 30°C, "Temp" factor) as fixed factors. Repeated-factor ANOVA using the linear mixed-effects procedure were used when measurements were repeated in the same mouse (e.g., parameters measured at 22°C and 30°C in the same mouse, metabolic responses to bursts of SMA). Post hoc Tukey tests were performed using the least-square means procedure when main effects or interactions were significant. The level of significance was set at $P < 0.05$.

RESULTS

Energy intake, body weight, and body composition. In E1, energy intake was measured only at 22°C (Fig. 2) and showed

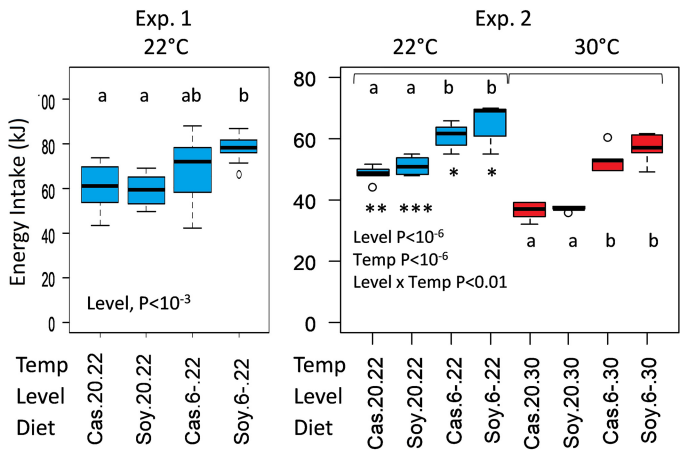


Fig. 2. Energy intake (kJ) at 22°C and 30°C measured in *experiments 1* (n = 8 par group) and *2* (n = 5 per group). [a,b,c]Post hoc Tukey tests at a given temperature. Groups that do not share the same letters are different at $P < 0.05$. *, **, ***, $P < 0.05$, 0.01, 0.001 or less, respectively, for differences in a group at 22°C vs. 30°C.

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a significant effect of the protein level (level effect), mice fed the 6% protein diets (P6) ate more than those fed the 20% protein diets (P20), but no diet effect was observed, indicating that casein-fed and soy-fed mice ate a similar amount when fed at similar protein levels. In E2, energy intake was first measured at 22°C, and then after the mice were acclimated to 30°C. Energy intake measured at 22°C was ~20% lower than that measured in E1, but as in E1, mice fed with the P6 diets ate more (level effect $P < 10^{-6}$) and casein- and soy-fed mice ate a similar amount when fed at similar protein levels (no diet effect). The same observation was made at 30°C, i.e., mice fed with the P6 diets ate more than those fed with the P20 diets, and when diets included the same protein level, no differences were observed between soy- or casein-fed mice. E2 also showed that energy intake was significantly lower at 30°C than at 22°C in P6-, as well as in P20-fed mice and in soy-fed, as well as in casein-fed, mice (level effect, $P < 10^{-6}$ and no diet effect). However, we also observed a significant Level × Temperature effect, indicating that the effect of temperature was not the same in P20- and P6-fed mice. Accordingly, post hoc Tukey tests showed that the energy intake measured at 30°C was significantly lower than at 22°C in the P20-fed mice ($0.001 < P < 0.01$) and also lower than in the P6-fed ones ($0.01 < P < 0.05$). Taken together, these results show that energy intake was reduced at 30°C vs. 22°C and always higher in P6- than in P20-fed mice. Also, the difference in energy intake between 22°C and 30°C was larger in P20- than in P6-fed mice.

Initial BW, FFM, and FM (DEXA measurements) at the onset of the two experiments did not show any significant differences between groups, and thus, the data of the two studies were pooled for statistical analysis of the evolution of BC during the 22°C periods of both experiments. Final values for BW, FFM, fat mass, and adiposity did not reveal statistical differences between groups, but FFM gain was lower, and FM gain and adiposity gain were larger in the P6- than in the P20-fed mice (level effect, $P < 0.05$, Fig. 3). The differences were, however, rather limited, so that post hoc comparison did not reveal any between-group differences. In E2, the increase in room temperature during the last 5 wk of the experiment inhibited the gain in BW, FFM, FM, and adiposity in all groups. The P6 soy-fed mice were particularly sensitive because during the period at 30°C, all of the gains were significantly lower than zero (Fig. 4).

Carcass analysis was performed separately for the mice of *experiments 1* and *2* because of the differences in the duration of the experimental periods and the effects of the 30°C period on the evolution of BW and BC. In E1 (Table 2), we observed that protein level affected final BC more than "diet". In P6-fed mice, subcutaneous, visceral and total fat content were greater, and lean body mass (LBM, defined as BW − adipose tissue mass), carcass weight, and kidney weight were smaller than in P20-fed mice. Diet marginally affected fat content and adiposity ($0.03 < P < 0.07$). A Diet × Level interaction was found for subcutaneous fat, which indirectly affected the visceral to total fat ratio because the P20 soy group had less subcutaneous fat. Similarly, a Diet × Level interaction was found for kidney weights, pointing to the larger kidney weight in the P20 casein group. In E2 (Table 2), as reported above, mice were housed 5 wk at 30°C before euthanasia. This affected significantly final BC, and no differences were observed between groups in adipose tissue mass and adiposity. However, a significant, but less marked, effect was still observed in LBM and kidney weights. BAT and ovary weights showed specific changes with temperature. Ovary weights were not different in mice of E1 housed continuously at 22°C but were lower in the P6-fed mice of E2 that were housed at 30°C because of a specific decrease in weight in this group ($P < 0.01$). On the other hand, BAT weight was not different in mice housed at 22°C but was lower in casein-fed mice at 30°C, as it decreased specifically in this group ($P < 0.001$). These differences were observed despite the fact that the mice were returned to 22°C 8–10 days before BC analysis.

Components of energy expenditure in fasted and fed mice. Mean time course changes in RMR induced by ingestion of the test meals in all of the P6-fed mice and all the P20-fed mice are shown in Fig. 5*A*. This figure reveals several important features of the components of energy expenditure in mice and their dependency upon ambient temperature: *1*) premeal resting metabolic rate was two times higher at 22°C vs. 30°C and more stable at 30°C, *2*) the thermogenic response to feeding was the same in P6- and P20-fed mice at 30°C but was higher in P6- than in P20-fed mice at 22°C, *3*) at 22°C, postmeal RMR decreased below premeal RMR in P20- but not in P6-fed mice, suggesting that nonshivering thermogenesis (NST) decreased as a result of the heat released by TEF in P20- but not (or less) in P6-fed mice. Analysis of glucose and lipid oxidation before ingestion of the test meal (Fig. 5, *B* and *C*) showed that in all groups, the increase in RMR, thus NST, was fueled primarily by an increase in the rate of lipid oxidation. The TEF (Fig. 5*D*) was not different between groups at 30°C, but TEF was significantly reduced at 22°C vs. 30°C in the P20-fed mice. As shown in Fig. 5*A*, this apparent decreased TEF was due to a postmeal decrease in NST in the P20-fed mice.

In all groups, either fed and fasted, TMR and resting metabolic rate (RMR) were approximately two times larger at 22°C than at 30°C ($P < 0.001$), but we observed complex interactions between temperature, level, and diet. When mice were fasted and housed at 30°C, RMR that was equivalent to basal metabolic rate was quite identical in all groups at both temperatures, and we observed only a temperature effect (Fig. 5*E*). In contrast, when mice were fed, we observed a significant level effect for both RMR and TMR (Fig. 5, *F* and *G*), indicating that P6-fed mice had higher values than P20-fed mice. We observed also a "diet" effect because soy-fed mice generally had RMR and TMR values larger than casein-fed mice.

Spontaneous motor activity, metabolic cost of motor activity, and metabolic rate of motor activity in ad libitum-fed mice. To understand further the effects of diet and temperature on SMA, we performed an analysis of the distribution of SMA values measured in ad libitum conditions. Intensities of SMA values (units/20 g BW) ranged from 0 to 5. We classified them into four main categories, according to the usual profiles of the distributions: "resting" (intensities between 0 and <0.3), "fidgeting" (intensities between 0.3 and <0.7), "active" (intensities between 0.7 and <3), and "highly active" (intensities between 3 and 5). Figure 6*A* gives an example of such a distribution analysis performed in the P6 soy-fed mice housed at 22°C and 30°C. Comparisons between groups showed that time spent resting (Fig. 6*B*) was affected by temperature and level. There

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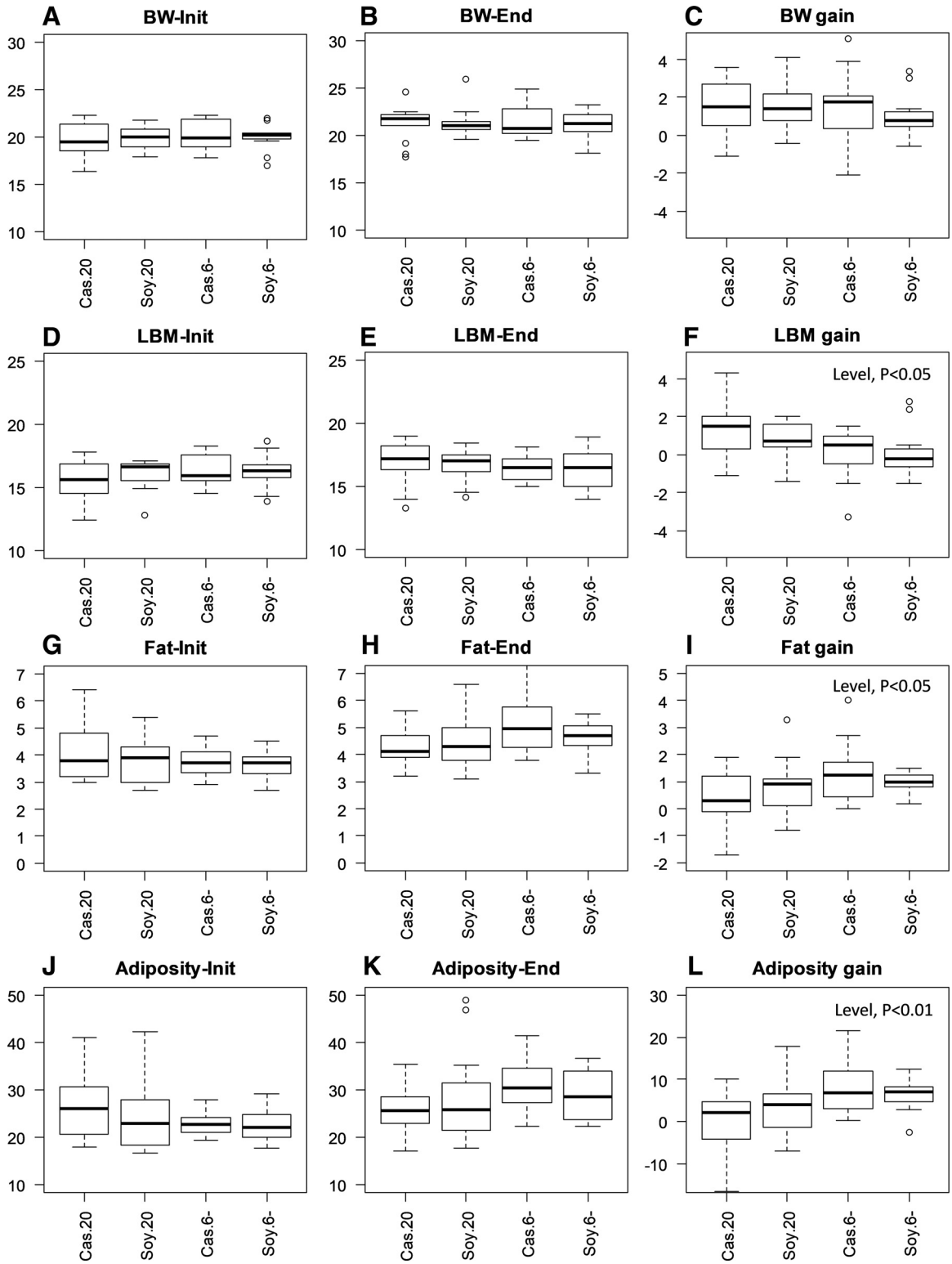


Fig. 3. Changes in body weight and body composition as measured by dual-energy X-ray absorptiometry (DEXA) in mice housed at 22°C (*experiment 1*). (Cas.20, Soy.20, Cas.6, $n = 8$; Soy.6, $n = 7$). LBM, lean body mass; BW, body weight.

was less effect observed at 22°C than at 30°C and less effect observed in P6- than in P20-fed mice. Time spent fidgeting (Fig. 6*C*) was affected only by level, being larger in P6- than in P20-fed mice. Time spent active (Fig. 6*D*) was affected by level, being higher in P6- than in P20-fed mice, and a significant Temperature × Level interaction was observed because the P6-fed mice were more active than the P20-fed mice at 22°C, but not at 30°C. Finally, time spent very active (not shown, but see Fig. 6*A*) was affected only by temperature, being larger at 22°C than at 30°C. Taken together, these results

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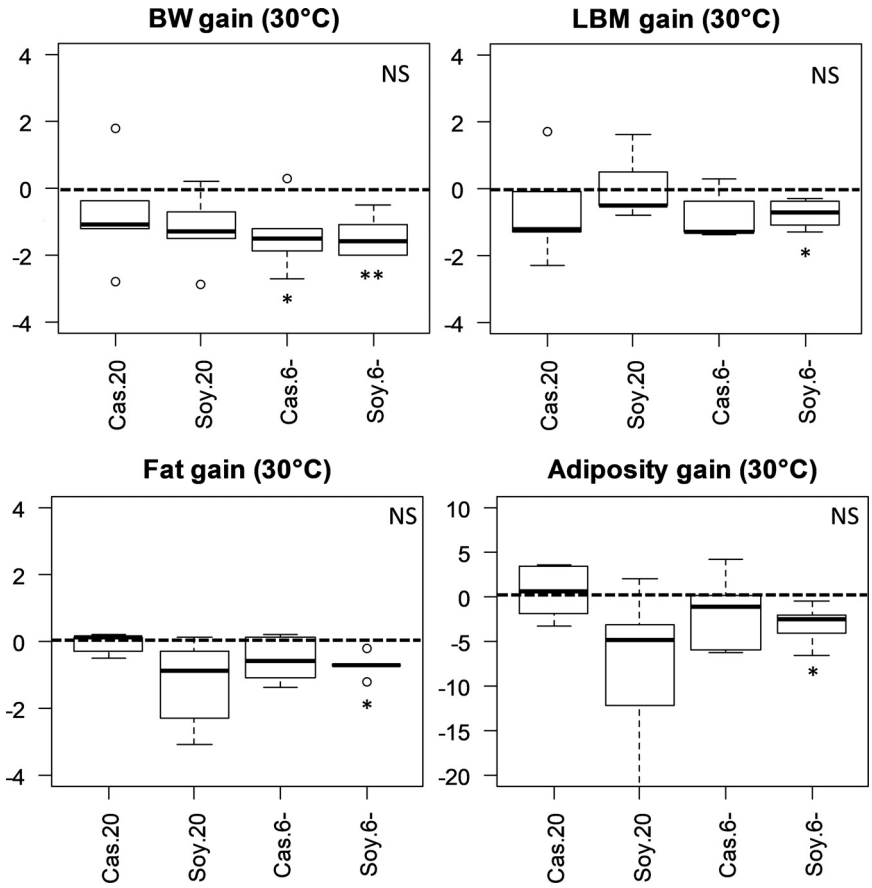


Fig. 4. Effect of increasing room temperature from 22°C to 30°C on the evolution of body composition (*experiment 2*). $n = 5$ per group. *Significant difference, $P < 0.05$. **Significant difference, $P < 0.01$.

show that temperature was the parameter most influencing SMA by reducing time spent resting and increasing time spent fidgeting, active, and very active. Level was also an important parameter since P6-fed mice housed at 22°C spent very little time resting, while they were globally fidgeting and active more than the P20-fed mice.

The mean SMA level during the recording periods (Fig. 6*E*) was affected only by temperature, being higher at 22°C than at 30°C and similar between groups at both temperatures. COST (Fig. 6*F*) was affected by temperature and level, being higher globally at 22°C than at 30°C and higher in P6 than in P20-fed mice, but only when mice were housed at 30°C. MAct (Fig. 6*G*) was also affected by temperature and level, and the differences were more contrasted than for COST, being significant between 22°C and 30°C and between P6- and P20-fed mice.

Analysis of the short-term changes between SMA, TMR, RMR, MAct, and COST in fasted mice. Periods of SMA lasting 20–40 min that occurred during the 10 h that preceded meal delivery in E1 devoted to measuring TEF (Fig. 5) have been used to analyze the short-term relation between activity, TMR, RMR, MAct, and COST in conditions free from artifacts due to the occurrence of feeding events. Figures 5, *I* and *II* show the mean changes observed in the P20 casein group. They show that at 22°C, the bursts of SMA were of much smaller amplitude, COST was much higher, and SMA induced an increase and then a decrease in RMR. Figure 7*III* defines the parameters that were compared between groups. No differences between groups were observed in the intensity of the activity bursts, TMR, MAct, and COST when mice were housed at 30°C (Fig. 7, *A–D*). In contrast, at 22°C, intensity of the activity bursts (Fig. 7*A*) was affected by temperature and diet, and a strong interaction between diet and temperature was observed as a result of a much lower intensity of the activity bursts observed in the casein-fed mice but not in the soy-fed mice at 22°C. In contrast, COST (Fig. 7*D*) was significantly increased at 22°C in the casein-fed mice, and larger in these mice than in the soy-fed mice. As a result of the opposite changes in activity bursts intensity and COST, the peak increases in TMR and MAct values were not different between groups (Fig. 7, *B* and *C*).

When mice were housed at 30°C, RMR remained fairly stable during activity bursts (Fig. 7, *I* and *E*). In contrast, in mice housed at 22°C, RMR exhibited an early increase that lasted ~5 min, and then a decrease that was maximal at the end of activity bursts (Fig. 7, *II* and *E*). These activity-induced changes in RMR were also marginally, but significantly, dependent on the protein level in the diet ($P < 0.05$). We observed also an interaction between level and temperature for the peak increase and peak decrease in RMR that were globally larger in P6-fed mice (Fig. 7, *E* and *F*). As a result of the postactivity decrease in RMR observed in all groups, the higher preactivity RMR at 22°C vs. 30°C (on the average 0.4 W vs. 0.25 W, Fig. 7*G*) was no longer observed at the end of the activity periods (~0.25 in all groups, Fig. 7*H*). This profile reflected the fact that during SMA, heat released by muscular contraction switched off NST.

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Table 2. *Body composition obtained by dissection and weighing of the main tissues and organs*

	P20 Casein	P20 Soy	P6 Casein	P6 Soy	ANOVA		
	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Diet	Level	Interaction
			Experiment 1: 8 Weeks at 22°C				
n	8	8	8	7			
BW	24.00 ± 0.4[a]	22.51 ± 0.24[b,c]	24.20 ± 0.5[b,c]	22.69 ± 0.43[a,c]		*	*
s.c. fat	0.725 ± 0.064[a]	0.407 ± 0.046[b]	0.885 ± 0.013[a]	0.835 ± 0.105[a]	*	**	*
Visc. fat	0.934 ± 0.1[a,b]	0.699 ± 0.061[a]	1.200 ± 0.004[b]	1.049 ± 0.135[a,b]		**	
Total fat	1.659 ± 0.146[a,b]	1.106 ± 0.095[a]	2.086 ± 0.017[b]	1.884 ± 0.214[b]	*	**	
Adiposity, %	6.87 ± 0.52[a,b]	4.90 ± 0.44[a]	8.63 ± 0.25[b]	8.33 ± 0.98[b]	*	***	
Visc. ratio, %	55.91 ± 2.31[a]	63.44 ± 2.37[a]	57.56 ± 0.26[a]	55.60 ± 2.66[a]			*
LBM	22.34 ± 0.29[a]	21.41 ± 0.27[a,b]	22.11 ± 0.52[b]	20.80 ± 0.48[b]		***	*
Carcass	7.86 ± 0.09[a]	7.43 ± 0.15[a,b]	7.71 ± 0.12[b]	6.94 ± 0.21[b]		***	
Kidneys	0.283 ± 0.006[a]	0.257 ± 0.009[a,c]	0.248 ± 0.013[b]	0.242 ± 0.009[b,c]		***	**
BAT	0.094 ± 0.012	0.089 ± 0.006	0.081 ± 0.01	0.094 ± 0.008			
Ovaries	0.21 ± 0.014	0.166 ± 0.012	0.178 ± 0.016	0.131 ± 0.019	**	*	
			Experiment 2: 8 Weeks at 22°C, Then 5 Weeks at 30°C				
n	5	5	5	5			
BW	20.62 ± 0.54	21.05 ± 0.86	20.34 ± 0.37	19.49 ± 0.44			
s.c. fat	1.12 ± 0.127	1.15 ± 0.109	1.032 ± 0.074	0.939 ± 0.1			
Visc. fat	1.201 ± 0.088	1.356 ± 0.195	1.47 ± 0.1	1.483 ± 0.085			
Total fat	2.321 ± 0.189	2.506 ± 0.297	2.502 ± 0.166	2.422 ± 0.168			
Adiposity, %	11.23 ± 0.8	11.77 ± 0.84	12.3 ± 0.77	12.4 ± 0.73			
Visc Ratio, %	52.11 ± 2.3[a]	53.59 ± 1.85[a]	58.74 ± 0.94[a,b]	61.56 ± 2.2[b]		**	
LBM	18.30 ± 0.47[a]	18.54 ± 0.57[a]	17.84 ± 0.36[a]	17.07 ± 0.36[a]		*	
Carcass	6.68 ± 0.22	6.85 ± 0.16	6.63 ± 0.13	6.33 ± 0.17			
Kidneys	0.21 ± 0.007[a,b]	0.220 ± 0.008[a]	0.192 ± 0.012[a,b]	0.170 ± 0.013[b]		***	
BAT	0.071 ± 0.006[a]	0.075 ± 0.007[a]	0.080 ± 0.005[a]	0.102 ± 0.011[a]		*	
Ovaries	0.148 ± 0.014[a]	0.160 ± 0.025[a]	0.08 ± 0.004[b]	0.077 ± 0.01[b]		***	

Unless specified, data are given in grams. BW, body weight; BAT brown adipose tissue; LBM, lean body mass; s.c., subcutaneous; visc. visceral. [a,b,c]Post hoc Tukey tests, numbers in a line that do not share the same symbols are different at $P < 0.05$. *$P < 0.05$ for global ANOVA. **$P < 0.01$ for global ANOVA. ***$P < 0.001$ for global ANOVA.

DISCUSSION

This study investigated the effects of LP-containing diets, with either casein or soy protein as the protein source, in mice housed at ambient room temperature or at thermal neutrality. Energy intake was greater in P6-fed mice compared with P20, whether the protein was casein or soy and the temperature was 22°C or 30°C. In P6-fed mice, fat mass and adiposity were higher, marginally more in P6 soy- than in P6 casein-fed mice, and LBM was lower, but these differences were of limited amplitude between groups. Components of energy expenditure, RMR, COST, and MAct were in most cases larger in P6- than in P20-fed mice. Temperature had a strong influence on these parameters, and differences between P6- and P20-fed mice were not always similar at 22°C and 30°C. Few significant effects of the protein type were observed, except for COST measured at 22°C in fasted mice that was much higher in casein- versus soy-fed mice and for BAT weight that was lower in casein than in soy-fed mice when housed at 30°C.

The moderate 6% LP diet compared with normal 20% protein diet induced higher energy intake and adiposity, as well as lower lean tissue mass in the mice. Energy intake was measured in mice housed at the normal animal room temperature (22°C), but also in mice adapted to housing at thermal neutrality (30°C). At both temperatures, energy intake was higher in P6-fed mice compared with P20-fed mice, a result that agrees with previously reported data indicating that energy intake may be increased with moderately low dietary protein diets (3, 6, 10, 19, 24, 30, 34, 37, 39, 40). No differences in energy intake were noticed between P6 soy- and P6 casein-fed mice, suggesting that at this level of 6% protein, the lower level of several essential amino acids, particularly methionine in the soy diet, did not amplify the feeding response of the mice. This result does not allow us to conclude whether differences would have been visible at higher (10 or 15%) or lower (3%) protein levels. In all groups, energy intake was greater at 22°C than at 30°C, which reflected the increased cost of thermal regulation at 22°C (see below). In E1 with mice housed at 22°C, carcass analysis measured by dissection of the main tissues and organs showed that adipose tissue mass and adiposity were larger (~+45%) and LBM (−2%) smaller in P6 than in P20-fed mice, a result that agrees, in general, with most previous studies (10, 17, 29, 30), but not others (6, 9, 37). The increase in body fat and body adiposity induced by the LP diet was, however, of a lower magnitude than in response to high-fat or Western diets and whether such moderately low-protein diets can induce a real state of obesity remains questionable. The protein source (casein vs. soy) had some influence also on BC, but the effects were at the limit of significance ($0.04 < P < 0.05$), with a trend for casein-fed mice to be slightly fatter than soy-fed mice. No differences in body composition between P6 soy- and P6 casein-fed mice were observed, but fat mass and adiposity were significantly higher in the P6 soy- than in the P20 soy-fed groups, while the differences did not reach significance in the P6 casein vs. P20 casein groups. In addition, P6 soy-fed mice had smaller lean mass and carcass mass, suggesting that they were more challenged than the P6 casein-fed mice. This is in line with a previous experiment of this laboratory in which P6 soy- and P6 casein-fed mice were pair-fed to P20 soy mice (31) and where we observed that LBM and ovary weights were lower in P6 soy than in P6 casein-fed mice. Such a difference

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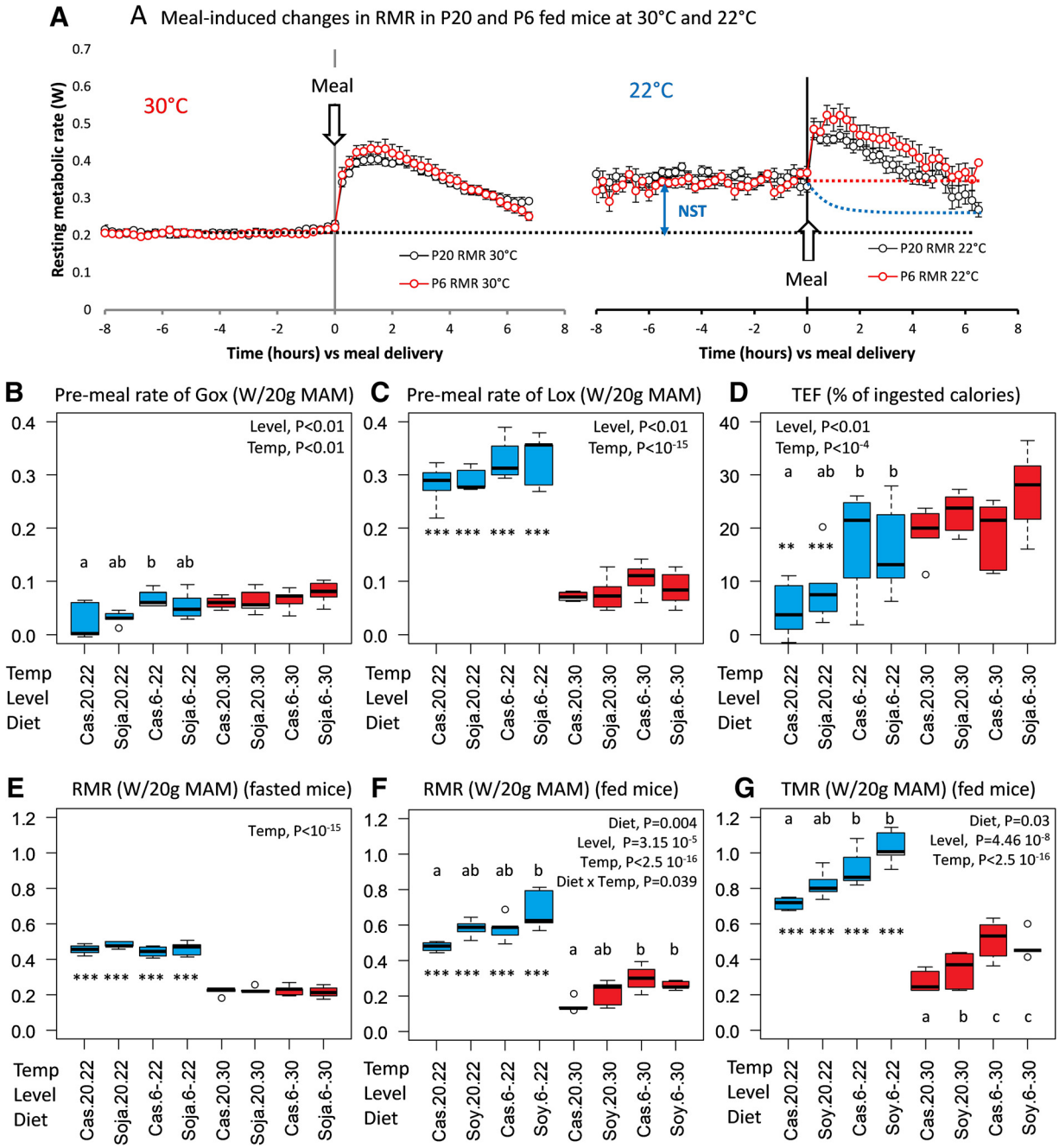


Fig. 5. Components of energy expenditure in fed and fasted mice. *A*: time course changes in resting metabolic rate (RMR) (means ± SE) measured from 8 h before to 6 h after ingestion of the test meals in the P20 (P20 Soy and P20 Casein pooled) and P6 (P6 Soy and P6 Casein pooled)-fed mice, at 30°C (*left*) and 22°C (*right*). Dashed blue line after meal ingestion at 22°C describes the estimated decrease in nonshivering thermogenesis (NST) intensity in the P20-fed mice, whereas the dashed red line represents the estimated stability of NST in P6-fed mice. Rates of glucose oxidation (Gox; *B*) and rates of lipid oxidation (Lox; *C*) measured during the 2 h before ingestion of the test meals. *D*: thermogenic response to feeding (TEF) computed as the cumulative increase in RMR measured during 6 h after ingestion of the test meals and expressed as a percentage of the ingested energy. *E*: RMR measured after an overnight fast during the 2 h before ingestion of the test meals in mice housed at 30°C. RMR (*F*) and total metabolic rate (TMR) (*G*) measured in mice fed ad libitum. [a,b,c]Post hoc Tukey tests at a given temperature: Groups that do not share the same letters are different at $P < 0.05$. *, **, *** $P < 0.05$, 0.01, 0.001 or less, respectively, for differences in a group between 22°C vs. 30°C. Number of mice per groups: 22°C: Cas.20, $n = 5$; Soya.20, $n = 5$; Cas.6, $n = 4$; and Soya.6, $n = 4$. 30°C: Cas.20, $n = 5$; Soya.20, $n = 5$; Cas.6, $n = 6$; and Soya.6, $n = 5$.

was reduced in the present experiments, probably because the mice were fed ad libitum and the P6 soy-fed mice were able to compensate partly for the lower quality of the soy protein by eating marginally more than P6 casein-fed mice.

The components of energy expenditure in fasted mice were determined from data acquired at 2-s intervals, which allowed a detailed and precise analysis of the effects of SMA on short-term changes in metabolic rate (12, 14). In contrast, in ad libitum-fed mice in which data were acquired at 10-min intervals, the analysis was coarser as they resulted from the origin (RMR) and slope (COST) of a single correlation between TMR and SMA established during the 24 h of recording (11). RMR

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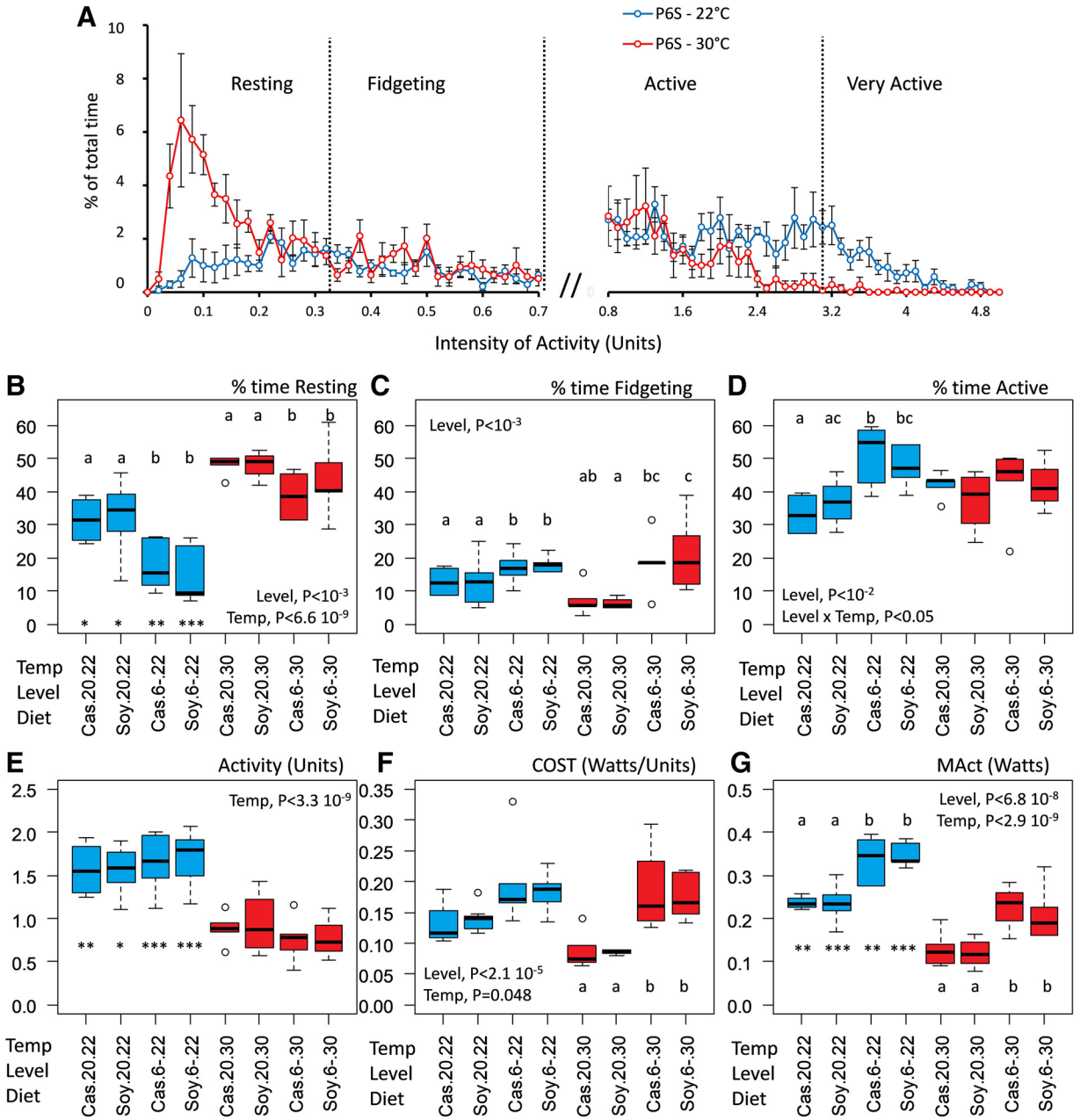


Fig. 6. Parameters of spontaneous activity measured in ad libitum-fed mice. Example of distribution of the intensities of the activity values at 22°C and 30°C in mice fed ad libitum the P6 soy diet. *B–D*: percentage of time spent resting, fidgeting and active in each group. Mean intensity of activity (*E*), cost of activity (*F*), and metabolism produced by activity (*G*). Legends are the same as in Fig. 5. Number of mice per group: 22°C: Cas.20, $n = 4$; Soya.20, $n = 7$; Cas.6, $n = 5$; Soya.6, $n = 5$. 30°C: Cas.20, $n = 5$; Soya.20, $n = 5$; Cas.6, $n = 5$; Soya.6, $n = 5$.

measured at 30°C in fasted mice reflected basal metabolic rate (BMR) and was extremely reproducible and similar in all groups with very small variability, showing that the cost of maintenance of basal cellular processes was affected neither by the protein level nor by the protein source. Therefore, the increase in total energy expenditure measured in P6-fed mice was not due to a BMR increase, and therefore, no adaptation occurred at this level (e.g., increased sympathetic activation of BAT or increased thyroid activity). When measured at 22°C, still in fasted mice, RMR was more than two times larger than BMR in all groups, confirming that in singly housed mice at the usual animal room temperature of 22°C, the cost of thermoregulation is at least as large as the cost of maintenance of the fundamental cellular processes (1, 5, 26). RMR at 22°C in fasted mice was also not different between P6 soy-, P6 casein-, P20 soy-, and P20 casein-fed mice, indicating that the cost of NST was not affected by the type and level of dietary protein. In contrast, when mice were fed ad libitum, there was a significant effect of the dietary protein level on RMR, with P6-fed mice having globally a higher RMR (in this situation RMR is the sum of BMR, NST, and TEF) than P20-fed mice. Theoretically, since BMR and RMR in fasted mice were not different between P6 and P20 mice, it could be concluded that the higher RMR observed in P6-fed mice would result neces-

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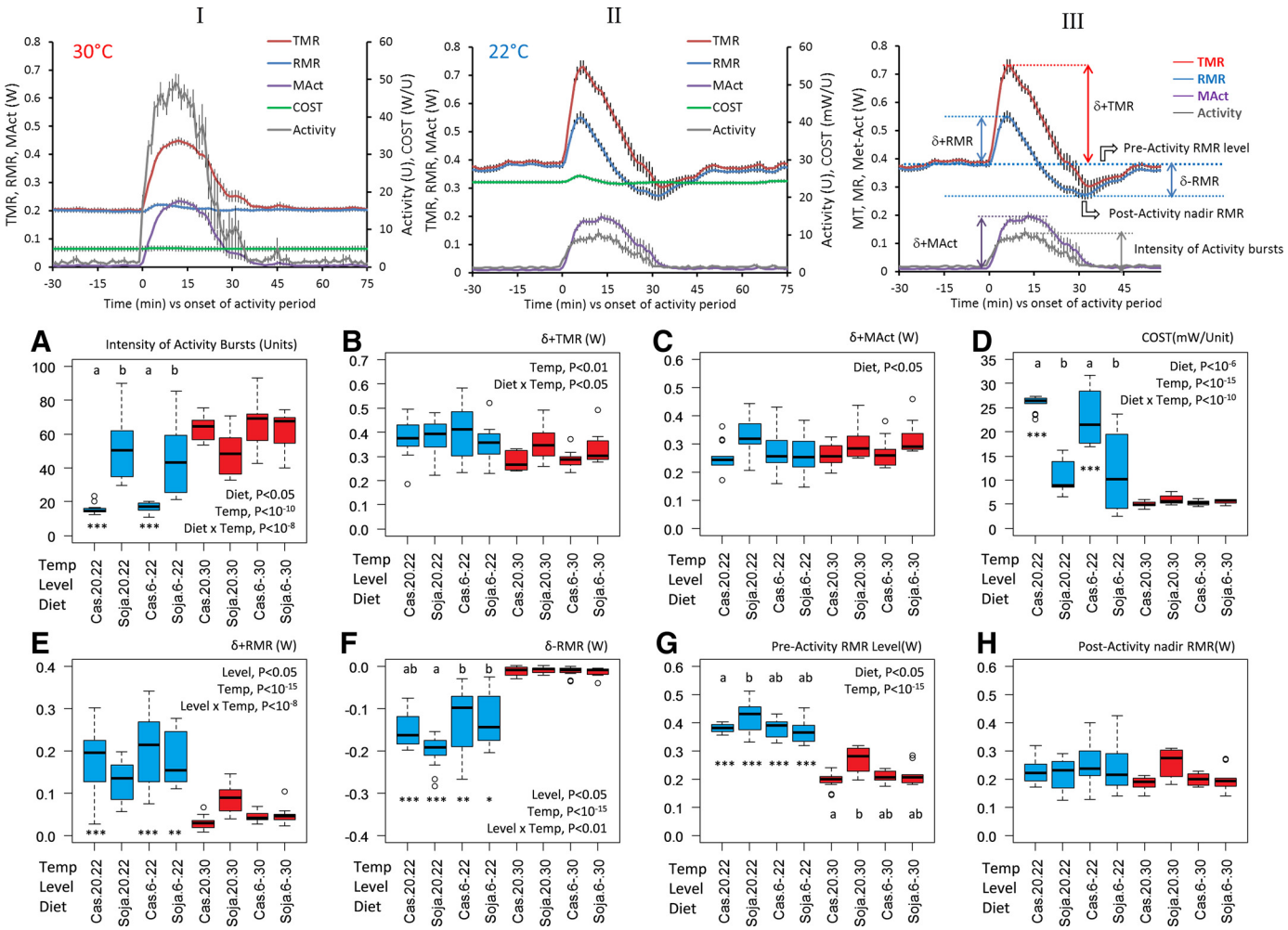


Fig. 7. *I* and *II*: time course changes presented at 1-min intervals induced on TMR, RMR, metabolic rate of motor activity (MAct), and COST by the occurrence of bursts of spontaneous activity in fasted P20 casein mice at 30°C (*experiment 1*, *I*) and 22°C (*experiment 2*, *II*). *III*: modification of II to describe the parameters characterizing the effects of activity of energy metabolism that were compared between groups and temperature in *A* to *H*. δ+TMR and δ−TMR peak increase and decrease in TMR, δ+RMR and δ−RMR peak increase and decrease in RMR, and δ+MAct peak increase in MAct. Postactivity nadir RMR is the lowest RMR value measured at the end of the activity period. Comparison between *I* and *II* shows the decrease in activity intensity, the increase in COST, and the decrease induced of RMR by the bout of activity, but the relative stability on the changes induced on TMR and MAct when P20 Casein mice were housed at 22°C vs. 30°C. Parts *A* to *H* describe statistical comparison of these parameters between groups and temperature. Legends are the same as in Fig. 5. Number of mice and activity periods (AP) per groups: 22°C: Cas.20 4 mice/12 AP; Soya.20 4 mice/16 AP; Cas.6 3 mice/9 AP; and Soya.6 3 mice/6 AP. 30°C: Cas.20 5 mice/13 AP; Soya.20 6 mice/10 AP; Cas.6 4 mice/11 AP; Soya.6 4 mice/8 AP.

sarily from a facultative increase in TEF, i.e., from stimulation of diet-induced thermogenesis (DIT). Accordingly, Fig. 5*F* suggests that at 22°C, TEF is larger in P6- than in P20-fed mice. However, factoring in the effects of several parameters causes us to refrain from this interpretation. First, P6-fed mice had a greater energy intake than P20-fed mice, which may explain part of the larger RMR measured when mice are fed. In addition, Fig. 5*F* clearly shows that it is not TEF that is increased in P6-fed mice housed at 22°C vs. 30°C, but rather that TEF is decreased in P20-fed mice housed at 22°C vs. 30°C. According to Fig. 5*A*, our interpretation of the data is that NST was reduced by the heat released in relation to TEF more in the P20- than in the P6-fed mice. This resulted in a larger RMR decrease in P20- than in P6-fed mice and, subsequently, to an apparent decrease in TEF in the P20-fed mice. Therefore, we consider that the differences in RMR observed at 22°C between P6- and P20-fed mice were not due to a stimulation of DIT in P6-fed mice but rather to an inhibition of NST that occurred only (or was larger) in P20- than in P6-fed mice (see Ref. 11 for more details).

DIT may burn part of the excess energy ingested in various circumstances and reduce fat deposition (7, 18, 36), and some (29, 30, 37, 44), although not all (see review by Ref. 20), studies have suggested that BAT activity is increased in LP-fed rats and may be responsible for a larger DIT that could participate in the reduced fat deposition. However, key regulators of thermogenesis have been reported to be lower in mice fed LP diets despite an increased BAT size (19). In the present study, BAT size was larger at 22°C in casein-fed, but not in soy-fed mice, and these changes did not correlate with the intensity of TEF. When measured at thermal neutrality, i.e., in conditions where NST is absent, TEF was similar in P6- and P20-fed mice, while BAT size was significantly larger in P6- than in P20-fed mice. In contrast, in P20 casein-fed mice, TEF

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was apparently lower, while BAT size was larger at 22°C than at 30°C. Therefore, it appears that there is a dissociation between BAT size/capacities for NST and DIT. Similarly, no difference was observed in meal-induced increase in energy expenditure between lean and obese Zucker rats fed a high- or a low-protein diet, despite very large differences in BAT UCP levels (35). Taken together, these observations suggest that the thermogenic capacities of the P6- and P20-fed mice were similar, that the true thermogenic response to feeding measured at thermal neutrality was the same despite large differences in BAT size, but that when TEF was measured at usual room temperature, heat exchanges between NST and TEF were larger in P20- than in P6-fed mice, giving a false-positive result that TEF is larger in P6-fed mice, and, thus, that DIT is increased. In fact, if the estimated decrease in RMR that occurs in parallel to TEF in P20-fed mice is taken into account, TEF was similar at 30°C and 22°C in P20-fed mice (11). This observation can explain why many previous experiments performed at room temperature reported that LP diets increased DIT.

Importantly, the present study indicates that extra heat dissipated by the P6-fed mice came primarily from changes in spontaneous activity energy expenditure. Indeed, energy expended with SMA was significantly larger in P6-fed mice, in particular, at 22°C. In agreement with a previous experiment (37), the extra heat dissipated with SMA in P6-fed mice was not due to differences in the overall activity level but because COST was larger in P6- than in P20-fed mice, this difference being particularly striking at 30°C between P6 soy- and P6 casein-fed mice and between P20 soy- and P20 casein-fed mice, respectively. Taken together, these results demonstrate that the metabolic cost of SMA can be changed to a large extent by complex interactions between diet and temperature and that, as suggested by Fig. 7*II*, muscle warming at lower temperatures may be involved. Increasing the cost of SMA increases the heat released in muscles and in the body by muscular contraction and, therefore, can help to warm the body when temperatures are low. This mechanism can be used also to dissipate energy to adjust energy balance, as it was possibly the case in the P6 soy- and P6 casein-fed mice housed at thermal neutrality. Differences in the metabolic cost of SMA may reflect a large variability in coupling efficiency in muscles that was reported to range from 50% to nearly 100%, depending on the experimental conditions (22, 27, 28). It could depend also on the intensity of various thermogenic ATP cycles, such as that observed in bumble bees or endothermic fish, and/or on differences in UCP2 and UCP3 gene expression [see review by Harper et al. (16)]. However, in the present experiments, analysis of gene expression in muscles (data not shown) did not reveal any differences in UCP2 and UCP3 gene expression as a simple explanation for differences in the metabolic cost of SMA, as we observed previously, for example, in COUP-TFII KO mice (32). Other than shivering, futile cycles of ATP synthesis and hydrolysis have not been described in mammals, but the results of these experiments suggest strongly that differences in TMR between groups were related to differences in the metabolic cost of SMA, as this cost was similar in fasted mice at 30°C and at 22°C when RMR was also similar but was also larger in P6 mice fed ad libitum when RMR and TMR were also larger. Therefore, differences in the evolution of the metabolic cost of SMA as a function of ambient temperature in P6- and P20-fed mice can play a significant role in the adaptation to low-protein feeding.

Differences in the SMA pattern may also be involved. Mean daily SMA in ad libitum-fed mice was approximately two times greater at 22°C than at 30°C but did not reveal differences between groups at 22°C nor at 30°C. Analysis of the distribution of the four main states of SMA (resting, fidgeting, active, and very active) revealed striking differences related to temperature and dietary protein level. Time spent resting was decreased at 22°C and more so in P6- than in P20-fed mice. This strong decrease reflected probably that the mice felt uncomfortable and/or needed to remain active to generate heat and maintain body temperature. In this context, the fact that the P6-fed mice reduced the time spent resting more than the P20-fed mice suggests that they relied less on NST and more on activity to generate heat. Shivering in P6-fed mice can be excluded since shivering increases the intensity of the activity signal based on force transducers and would have generated a peak of frequency at a specific intensity in shivering mice, which was not the case. Therefore, when housed at 22°C, P6-fed mice generated more heat than P20-fed mice by being more active and by having a higher activity cost rather than by stimulating NST in BAT. This induced an overall increase in TMR that prevented the development of significant obesity despite the increased energy intake.

In conclusion, this study reports that in mice, energy intake and adiposity are increased in response to low-protein feeding. This observation is in line with the protein leverage hypothesis. However analysis of the other side of the energy balance equation indicated a significant increase in energy expenditure related to the energy expended with SMA. Consequently, in LP-fed mice, the energy balance was only slightly more positive than in mice fed a normal protein diet, and in the time framework of this study, we did not observe the establishment of a real state of obesity. The other main observations of these experiments are the dramatic differences observed in the components of energy expenditure and SMA as a function of temperature, dietary protein level, protein quality and fed versus fasted state. The heat exchanges between NST, SMA, and feeding may explain many discrepancies between previous experiments (temperature in the animal facilities, single or group housing, fed or fasted …). The increase in total energy expenditure in P6 mice fed ad libitum, revealed several differences at 22°C and 30°C but was sustained mainly by a higher energy expenditure due to SMA resulting from a higher metabolic cost of SMA and more time spent active or fidgeting. In contrast, in fasted mice, there were no differences in the intensity and cost of SMA when mice were housed at 30°C or at 22°C. Differences in the intensity and metabolic cost of SMA were observed between casein- and soy-fed mice primarily due to diet, with a larger metabolic cost but lower intensity of SMA in casein- versus soy-fed mice. These results reveal that the high level of energy expended for thermoregulation in mice housed at normal animal room temperatures affects the mechanisms of adaptation to a LP diet as a result of interactions involving thermoregulation, motor activity, and protein quality. In this context, if the mouse is used as a model of human physiology, the cost of thermal regulation should be more carefully considered. In this study, the long-term evolution of energy intake and body adiposity were not measured in mice housed at thermal neutrality from the onset of the study, but the evolution of body composition during E2 (Fig. 4) and

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the final differences in body composition measured at the end of E1 and E2 (Table 2) suggest that the consequences of LP diets on body composition are influenced by the ambient temperature. Studies performed at thermoneutrality should be considered in the mouse and/or rat model to better appreciate how low-protein diets may affect energy balance in humans.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.B. and P.C.E. conceived and designed research; A.B., J.P., and P.C.E. performed experiments; A.B., G.F., C.G., D.T., and P.C.E. edited and revised manuscript; A.B., C.C., D.A.-M., J.P., G.F., C.G., D.T., and P.C.E. approved final version of manuscript; C.C., D.A.-M., J.P., and P.C.E. analyzed data; C.C., D.A.-M., and P.C.E. interpreted results of experiments; D.T. and P.C.E. drafted manuscript; P.C.E. prepared figures.

REFERENCES

1. **Abreu-Vieira G, Xiao C, Gavrilova O, Reitman ML.** Integration of body temperature into the analysis of energy expenditure in the mouse. *Mol Metab* 4: 461–470, 2015. doi:10.1016/j.molmet.2015.03.001.
2. **Andik I, Donhoffer S, Farkas M, Schmidt P.** Ambient temperature and survival on a protein-deficient diet. *Br J Nutr* 17: 257–261, 1963. doi:10.1079/BJN19630028.
3. **Apolzan JW, Carnell NS, Mattes RD, Campbell WW.** Inadequate dietary protein increases hunger and desire to eat in younger and older men. *J Nutr* 137: 1478–1482, 2007.
4. **Arch JR, Hislop D, Wang SJ, Speakman JR.** Some mathematical and technical issues in the measurement and interpretation of open-circuit indirect calorimetry in small animals. *Int J Obes* 30: 1322–1331, 2006. doi:10.1038/sj.ijo.0803280.
5. **Cannon B, Nedergaard J.** Nonshivering thermogenesis and its adequate measurement in metabolic studies. *J Exp Biol* 214: 242–253, 2011. doi:10.1242/jeb.050989.
6. **Chalvon-Demersay T, Even PC, Tomé D, Chaumontet C, Piedcoq J, Gaudichon C, Azzout-Marniche D.** Low-protein diet induces, whereas high-protein diet reduces hepatic FGF21 production in mice, but glucose and not amino acids up-regulate FGF21 in cultured hepatocytes. *J Nutr Biochem* 36: 60–67, 2016. doi:10.1016/j.jnutbio.2016.07.002.
7. **Danforth E Jr.** Dietary-induced thermogenesis: control of energy expenditure. *Life Sci* 28: 1821–1827, 1981. doi:10.1016/0024-3205(81)90353-2.
8. **de França SA, dos Santos MP, Przygodda F, Garófalo MA, Kettelhut IC, Magalhães DA, Bezerra KS, Colodel EM, Flouris AD, Andrade CM, Kawashita NH.** A low-protein, high-carbohydrate diet stimulates thermogenesis in the brown adipose tissue of rats via ATF-2. *Lipids* 51: 303–310, 2016. doi:10.1007/s11745-016-4119-z.
9. **Donald P, Pitts CC, Pohl SL.** Body weight and composition in laboratory rats: effects of diets with high or low protein concentrations. *Science* 211: 185–186, 1981. doi:10.1126/science.7444462.
10. **Du F, Higginbotham DA, White BD.** Food intake, energy balance and serum leptin concentrations in rats fed low-protein diets. *J Nutr* 130: 514–521, 2000.
11. **Even PC, Blais A.** Increased cost of motor activity and heat transfer between non-shivering thermogenesis, motor activity, and thermic effect of feeding in mice housed at room temperature—implications in preclinical studies. *Front Nutr* 3: 43, 2016. doi:10.3389/fnut.2016.00043.
12. **Even PC, Mokhtarian A, Pele A.** Practical aspects of indirect calorimetry in laboratory animals. *Neurosci Biobehav Rev* 18: 435–447, 1994. doi:10.1016/0149-7634(94)90056-6.
13. **Even PC, Nadkarni NA.** Indirect calorimetry in laboratory mice and rats: principles, practical considerations, interpretation and perspectives. *Am J Physiol Regul Integr Comp Physiol* 303: R459–R476, 2012. doi:10.1152/ajpregu.00137.2012.
14. **Even PC, Perrier E, Aucouturier JL, Nicolaïdis S.** Utilisation of the method of Kalman filtering for performing the on-line computation of background metabolism in the free-moving, free-feeding rat. *Physiol Behav* 49: 177–187, 1991. doi:10.1016/0031-9384(91)90252-J.
15. **Feldmann HM, Golozoubova V, Cannon B, Nedergaard J.** UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell Metab* 9: 203–209, 2009. doi:10.1016/j.cmet.2008.12.014.
16. **Harper ME, Green K, Brand MD.** The efficiency of cellular energy transduction and its implications for obesity. *Annu Rev Nutr* 28: 13–33, 2008. doi:10.1146/annurev.nutr.28.061807.155357.
17. **Hartsook EW, Hershberger TV, Nee JC.** Effects of dietary protein content and ratio of fat to carbohydrate calories on energy metabolism and body composition of growing rats. *J Nutr* 103: 167–178, 1973.
18. **Himms-Hagen J.** Thermogenesis in brown adipose tissue as an energy buffer. Implications for obesity. *N Engl J Med* 311: 1549–1558, 1984. doi:10.1056/NEJM198412133112407.
19. **Huang X, Hancock DP, Gosby AK, McMahon AC, Solon SM, Le Couteur DG, Conigrave AD, Raubenheimer D, Simpson SJ.** Effects of dietary protein to carbohydrate balance on energy intake, fat storage, and heat production in mice. *Obesity (Silver Spring)* 21: 85–92, 2013. doi:10.1002/oby.20007.
20. **Kozak LP.** Brown fat and the myth of diet-induced thermogenesis. *Cell Metab* 11: 263–267, 2010. doi:10.1016/j.cmet.2010.03.009.
21. **Maloney SK.** Low protein feeding and energy transduction in rats. *Temperature (Austin)* 1: 97–98, 2014. doi:10.4161/temp.28661.
22. **Marcinek DJ, Schenkman KA, Ciesielski WA, Conley KE.** Mitochondrial coupling in vivo in mouse skeletal muscle. *Am J Physiol Cell Physiol* 286: C457–C463, 2004. doi:10.1152/ajpcell.00237.2003.
23. **Morrison CD, Reed SD, Henagan TM.** Homeostatic regulation of protein intake: in search of a mechanism. *Am J Physiol Regul Integr Comp Physiol* 302: R917–R928, 2012. doi:10.1152/ajpregu.00609.2011.
24. **Peng YS, Meliza LL, Vavich MG, Kemmerer AR.** Changes in food intake and nitrogen metabolism of rats while adapting to a low or high protein diet. *J Nutr* 104: 1008–1017, 1974.
25. **Peters JC, Harper AE.** Influence of dietary protein level on protein self-selection and plasma and brain amino acid concentrations. *Physiol Behav* 33: 783–790, 1984. doi:10.1016/0031-9384(84)90048-9.
26. **Ravussin Y, LeDuc CA, Watanabe K, Leibel RL.** Effects of ambient temperature on adaptive thermogenesis during maintenance of reduced body weight in mice. *Am J Physiol Regul Integr Comp Physiol* 303: R438–R448, 2012. doi:10.1152/ajpregu.00092.2012.
27. **Rolfe DF, Newman JM, Buckingham JA, Clark MG, Brand MD.** Contribution of mitochondrial proton leak to respiration rate in working skeletal muscle and liver and to SMR. *Am J Physiol Cell Physiol* 276: C692–C699, 1999. doi:10.1152/ajpcell.1999.276.3.C692.
28. **Rolfe DFS, Brand MD.** Contribution of mitochondrial proton leak to skeletal muscle respiration and to standard metabolic rate. *Am J Physiol Cell Physiol* 271: C1380–C1389, 1996. doi:10.1152/ajpcell.1996.271.4.C1380.
29. **Rothwell NJ, Stock MJ.** Influence of carbohydrate and fat intake on diet-induced thermogenesis and brown fat activity in rats fed low-protein diets. *J Nutr* 117: 1721–1726, 1987.
30. **Rothwell NJ, Stock MJ, Tyzbir RS.** Mechanisms of thermogenesis induced by low-protein diets. *Metabolism* 32: 257–261, 1983. doi:10.1016/0026-0495(83)90190-7.
31. **Rouy E, Vico L, Laroche N, Benoit V, Rousseau B, Blachier F, Tomé D, Blais A.** Protein quality affects bone status during moderate protein restriction in growing mice. *Bone* 59: 7–13, 2014. doi:10.1016/j.bone.2013.10.013.
32. **Sabra-Makke L, Maritan M, Planchais J, Boutant M, Pégorier JP, Even PC, Vasseur-Cognet M, Bossard P.** Hypothalamic ventromedial COUP-TFII protects against hypoglycemia-associated autonomic failure. *Proc Natl Acad Sci USA* 110: 4333–4338, 2013. doi:10.1073/pnas.1219262110.
33. **Selman C, Korhonen TK, Bünger L, Hill WG, Speakman JR.** Thermoregulatory responses of two mouse *Mus musculus* strains selectively bred for high and low food intake. *J Comp Physiol B* 171: 661–668, 2001. doi:10.1007/s003600100217.
34. **Sørensen A, Mayntz D, Raubenheimer D, Simpson SJ.** Protein-leverage in mice: the geometry of macronutrient balancing and consequences for fat

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deposition. *Obesity (Silver Spring)* 16: 566–571, 2008. doi:10.1038/oby.2007.58.
35. **Specter SE, Hamilton JS, Stern JS, Horwitz BA.** Chronic protein restriction does not alter energetic efficiency or brown adipose tissue thermogenic capacity in genetically obese (*fa/fa*) Zucker rats. *J Nutr* 125: 2183–2193, 1995.
36. **Stock MJ.** Thermogenesis and energy balance. *Int J Obes Relat Metab Disord* 16, *Suppl* 2: S13–S16, 1992.
37. **Swick RW, Gribskov CL.** The effect of dietary protein levels on diet-induced thermogenesis in the rat. *J Nutr* 113: 2289–2294, 1983.
38. **Vander Tuig JG, Beneke WM.** Low-protein diet blocks development of hyperphagia and obesity in rats with hypothalamic knife cuts. *J Nutr* 126: 1713–1721, 1996.
39. **Webster AJ.** Energy partitioning, tissue growth and appetite control. *Proc Nutr Soc* 52: 69–76, 1993. doi:10.1079/PNS19930038.
40. **White BD, Du F, Higginbotham DA.** Low dietary protein is associated with an increase in food intake and a decrease in the in vitro release of radiolabeled glutamate and GABA from the lateral hypothalamus. *Nutr Neurosci* 6: 361–367, 2003. doi:10.1080/10284150310001640365.
41. **White BD, He B, Dean RG, Martin RJ.** Low-protein diets increase neuropeptide Y gene expression in the basomedial hypothalamus of rats. *J Nutr* 124: 1152–1160, 1994.
42. **White BD, Porter MH, Martin RJ.** Effects of age on the feeding response to moderately low dietary protein in rats. *Physiol Behav* 68: 673–681, 2000. doi:10.1016/S0031-9384(99)00229-2.
43. **White BD, Porter MH, Martin RJ.** Protein selection, food intake, and body composition in response to the amount of dietary protein. *Physiol Behav* 69: 383–389, 2000. doi:10.1016/S0031-9384(99)00232-2.
44. **Zhao XQ, Jørgensen H, Gabert VM, Eggum BO.** Energy metabolism and protein balance in growing rats housed in 18°C or 28°C environments and fed different levels of dietary protein. *J Nutr* 126: 2036–2043, 1996.



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