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RESEARCH ARTICLE

Plasticity in gastrointestinal morphology and enzyme activity in lactating striped hamsters (*Cricetulus barabensis*)

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ABSTRACT

In small mammals, marked phenotypic plasticity of digestive physiology has been shown to make it easier for them to cope with energetically stressful periods, such as lactation. It has been proposed that the capacity of the gut to digest and absorb food is not the limiting factor to sustained energy intake (SusEI) during peak lactation. In this study, plasticity in energy intake and gastrointestinal morphology was examined in striped hamsters at different stages of reproduction and when raising litters of different sizes. Mechanisms associated with digestive enzymes and neuroendocrine hormones underpinning the plasticity were also examined. Females significantly increased energy intake, digestibility, digestive tract mass and the activity of stomach pepsin and small intestine maltase, sucrase and aminopeptidase in peak lactation compared with the non-productive and post-lactating periods. Further, females raising large litters significantly increased energy intake, digestibility, gastrointestinal mass and activity of digestive enzymes, and weaned heavier offspring compared with those nursing small and medium litters, indicating that the significant plasticity of digestive physiology increased reproductive performance. Agouti-related protein (AgRP) mRNA expression in the hypothalamus was up-regulated significantly in females raising large litters relative to those raising small litters. Serum leptin levels, and mRNA expression of hypothalamus neuropeptide Y (NPY) and the anorexigenic neuropeptides pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) did not differ among females raising small, medium and large litters. Leptin levels in lactation may only reflect a state of energy balance rather than being the prime driver of hyperphagia. Some hypothalamic neuropeptides, such as NPY, POMC and CART, may be involved in the limits to the SusEI during lactation.

KEY WORDS: Digestive enzymes, Lactation, Leptin, Litter size, Neuropeptide, Phenotypic plasticity, Sustained energy intake

INTRODUCTION

It has been demonstrated that in small mammals marked phenotypic plasticity in response to changes in ecological environment or physiological state can increase biological performance (Bozinovic et al., 1990; Hammond and Wunder, 1991, 1995; Naya et al., 2008a; Vézina and Williams, 2003). Moreover, these changes in organism traits due to changes in internal or external environmental conditions can occur over a short time scale and are reversible (Hammond et al., 1999, 2001; Karasov and Diamond, 1983; Karasov and Hume, 1997; Nagy and Negus, 1993; Piersma and Drent, 2003; Piersma and Lindström, 1997). For example, notable plasticity in digestive physiology has been shown in rodents in energetically stressful periods such as lactation, which is generally believed to determine an individual's reproductive strategy and thus influence the probability of reproductive success (del Valle et al., 2004; Hammond and Kristan, 2000; Koteja, 1996a; Naya et al., 2008a; Reilly et al., 2006).

Although digestive plasticity is used by females to meet the energetic demands of lactation, a limit to sustained energy intake (SusEI) or total energy expenditure exists during peak lactation (Hammond and Diamond, 1992; Hammond et al., 1994, 1996; Król and Speakman, 2003; Król et al., 2003; Rogowitz, 1998; Valencak et al., 2009, 2010; Weiner, 1992). During the search for the mechanism behind limitations on SusEI, studies focused on several hypotheses, one of which is the central limitation hypothesis (Koteja, 1996b; Simons et al., 2011; Speakman and Król, 2005, 2011). This hypothesis proposes that the limit may be imposed by the capacity of the gut to digest and absorb food (Koteja, 1996a,b). Previously, some studies have demonstrated that the dam can elevate her food intake dramatically beyond a previously supposed centrally imposed limit when exposed to the cold during late lactation (Hammond and Kristan, 2000; Johnson and Speakman, 2001; Rogowitz, 1998; Speakman and Król, 2005). This would indicate that the limit on energy intake was not imposed centrally (Speakman and Król, 2005). The marked plasticity in the size of the gut and associated organs may be a plausible refutation of this hypothesis; however, few studies have focused on the mechanisms associated with the digestive enzymes and neuroendocrine hormones underpinning this plasticity.

Digestive enzymes located on the apical membrane of enterocytes are one of the most important components of digestion in mammals (del Valle et al., 2004; Sabat et al., 1999). An adaptive regulation of digestive enzyme activity has been shown to meet the increased food intake in response to high energy demand conditions, such as cold exposure, low quality food, pregnancy and lactation (Bozinovic and Nespolo, 1997; Debray et al., 2003; Nespolo et al., 2002). During such conditions, fat depots are usually mobilized, resulting in decreased levels of leptin, an important endocrine factor secreted by adipocytes (Denis et al., 2003a,b). Leptin interacts in the brain with almost all neuropeptides known to be involved in the regulation of energy balance and especially food intake (Kalra et al., 1999; Wauters et al., 2000). It has been reported that leptin inhibits secretion of the orexigenic neuropeptides neuropeptide Y (NPY) and agouti-related protein (AgRP), which are both key downstream effectors of the leptin signal in the hypothalamus (Stephens et al., 1995; Speakman and Król, 2005). Decreased leptin levels during peak lactation would impair the control of NPY and AgRP secretion, and consequently females would be able to consume more food to meet the energy requirement of their offspring (Brogan et al., 2000; Denis et al., 2003a,b). Therefore, leptin and hypothalamic neuropeptides

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associated with food intake regulation may be involved in the digestive plasticity during lactation.

The striped hamster, *Cricetulus barabensis* (Pallas 1773), is a major rodent in northern China and is also distributed across Russia, Mongolia and Korea (Zhang and Wang, 1998; Zhang and Zhao, 2015). We have previously observed that food intake increased greatly in hamsters during lactation and when exposed to cold conditions (Zhao et al., 2010; Zhao, 2011), and that leptin may be involved in the energy balance regulation (Zhao et al., 2014a,b). Significant plasticity of gut size and digestive enzymes indicative of the activities of sucrase, maltase and aminopeptidase occurs in hamsters acclimated to different temperatures (Zhao et al., 2014b). Female hamsters reach sexual maturity at 3.0–3.5 months of age, and body mass of adult females averages 25.5 g; the length of pregnancy and lactation are 19–21 days and 17–19 days, respectively; natural litter size ranges from 3 to 8, and the mean litter size is 5.2. It has previously been demonstrated that SusEI during peak lactation is limited at 5× basal metabolic rate (BMR) (Zhao et al., 2010). This suggests that the lactating hamster is a suitable model for the study of the factors limiting SusEI. We firstly examined energy intake, morphology and digestive enzyme activity of the gastrointestinal tracts in non-lactating, peak-lactating and post-lactating hamsters, and then examined energy intake, digestive enzyme activity and hypothalamic neuropeptide mRNA expression in hamsters with a manually manipulated litter size. We hypothesized that reversible plasticity of the digestive system occurs over a short time scale in lactating hamsters to cope with marked variations of food intake, which would be even more significant under increased energy requirements, such as when raising large litters. Digestive enzymes and neuroendocrine mechanisms associated with leptin and hypothalamic peptides are proposed to be involved in the limits to SusEI during lactation.

MATERIALS AND METHODS

Animals and experiment protocols

Striped hamsters were obtained from a colony that was maintained in the animal house of Wenzhou University. This colony was begun with animals that were initially trapped from farmland at the center of Hebei Province (115°13′E, 38°12′S), North China Plain. The hamsters were kept on a 12 h light:12 h dark photoperiod (lights on at 08:00 h) at an ambient temperature of 23+1°C. Food (standard rodent chow; Beijing KeAo Feed Co., Beijing, China) and water were provided *ad libitum*. Adult female hamsters (3.5–4 months old in the breeding colony), housed individually in clean plastic cages (29×18×16 cm), were used in this study. All experimental protocols and procedures were approved by the Animal Care and Use Committee of Wenzhou University.

Experiment 1 was designed to examine energy intake, morphology and digestive enzyme activity of the gastrointestinal tract in the non-lactating, peak- and post-lactating stages. Forty-eight virgin female hamsters were randomly assigned into one of three groups: non-lactation group (N=8), peak-lactation group (N=20) and post-lactation group (N=20). The females in the peak-lactation and post-lactation groups were paired with male hamsters for a week; if fighting occurred, the female and male were separated. Nine and 8 females, respectively, in the peak-lactation and post-lactation groups became pregnant and gave birth. The offspring were weaned on day 17 of lactation; the females in the peak-lactation group were killed following weaning, and those in post-lactation group were separated from their offspring and were maintained to day 17 after the weaning.

Experiment 2 was designed to examine energy intake, digestive enzyme activity of the gastrointestinal tract and hypothalamic neuropeptide mRNA expression in a second group of lactating hamsters in which the litter size was manipulated during early lactation to increase (to 8 pups) or reduce (to 1 pup) the number of pups. Female hamsters were paired with males according to the same methods as in experiment 1. However, on day 5 of lactation, females were randomly assigned to one of three groups: LS1 group (N=8), in which litter size (LS) was set to 1 pup; LS4 group (N=8), in which litter size was set to 4 pups; and LS8 group (N=9), in which litter size was set to 8 pups; females had to support this number of pups from day 5 till day 15 of lactation. Body mass, litter size, litter mass, energy intake and digestibility were measured on day 13 and 14 of lactation. Offspring in all three groups were weaned on day 15 of lactation.

Gross energy intake and digestibility

As described previously, gross energy intake and digestibility were measured at 2 day intervals. Briefly, food was provided quantitatively on day 13 of lactation, and uneaten food, food residues mixed with the bedding and feces were collected 48 h later. The spillage of food and feces were sorted and separated manually after they were dried at 60°C for 10 days to constant mass. The water content of the diet (%) was calculated from the decrease in mass of the diet. Gross energy content of the diet and feces was determined using a Parr 1281 oxygen bomb calorimeter (Parr Instruments, Moline, IL, USA). Gross energy intake, digestible energy intake and digestibility were calculated using the equation: gross energy intake (kJ day^{-1})=dry matter intake (g day^{-1})×energy content of food (kJ g^{-1}); digestive energy intake (kJ day^{-1})=gross energy intake−[dry mass of feces (g day^{-1})×energy content of feces (kJ g^{-1})]; digestibility (%)=digestive energy intake/gross energy intake×100% (Grodzinski and Wunder, 1975; Zheng et al., 2015).

Tissue sampling

In experiment 1, the lactating females and their non-lactating counterparts were killed on day 17 of lactation, and those in the post-lactation group were killed on day 17 after the weaning. All females in experiment 2 were killed on day 15 of lactation. Trunk blood was collected for later measurement of leptin levels. The hypothalamus was removed carefully and quickly, and stored in liquid nitrogen immediately. The stomach, small and large intestine and cecum were separated, and the contents were removed. They were weighed to ±1 mg (Sartorius, Germany) and then immediately preserved in liquid nitrogen for the enzyme assays.

Sample preparation and protein content

As described previously (Liu and Wang, 2007), the stomach and different segments of the digestive tract (duodenum, jejunum and ileum) were homogenized separately in 0.9% NaCl solution (1:10, w/v) using an electric glass homogenizer, during which temperature was controlled in an ice-water bath. The activity of several digestive enzymes was measured in whole-tissue homogenates rather than in mucosal samples to avoid underestimation of activity as previously reported (Brzęk et al., 2009; Liu and Wang, 2007; Martinez, 1990; Zhao et al., 2014b). The tissue protein content was determined by the Folin phenol method with bovine serum albumin as the standard (Lowry et al., 1951).

Activity of pepsin, maltase and sucrase

As described previously (Zhao et al., 2014b), we measured the activity of pepsin (EC 3.4.23.1), sucrase (EC 3.2.1.48) and maltase (EC 3.2.1.20) using commercial kits (Jiancheng Biotech Co. Ltd, Nanjing, China) according to the manufacturer's protocols. The inter- and intra-assay variations were, respectively, 5.6% and 2.5%

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for pepsin, 5.0% and 3.1% for sucrase and 5.1% and 3.1% for maltase.

Bovine albumin was used as the substrate for pepsin activity measurement. Briefly, 80 µl of centrifuged gastric juice (3500 ***g***, 10 min) was added to a mixture, including bovine albumin (0.5% w/v in 0.01 mol l^{-1} HCl, pH 2). A duplicate background control tube (gastric juice blank) included 0.01 mol l^{-1} HCl but excluded bovine albumin. The mixture was incubated for 20 min at 37°C, and the reaction was stopped by adding 10% trichloroacetic acid. The supernatant (3500 ***g***, 10 min) was mixed with 2.5 mol l^{-1} NaOH and Folin–Ciocalteu reagent. Absorbance was measured at 700 nm. Pepsin activity was calculated according to the kit instructions and expressed as U min^{-1}.

Sugar and maltose were used as the substrate for the sucrase and maltase measurements, respectively. Briefly, 10 µl of the homogenate was added to 20 µl of assay mix in each tube at 37°C for 20 min. The reaction was terminated by using a stop solution, followed by 1 ml detective reagent solution, and the absorbance was read at 505 nm. The activity of both enzymes was expressed in U min^{-1} (1 U was defined as 1 nmol sucrase or maltase hydrolyzed at 37°C by 1 mg tissue protein per minute; Zhao et al., 2014b).

Aminopeptidase activity

Aminopeptidase-N activity was measured using methods described previously (Brzęk et al., 2009; Liu and Wang, 2007; Maroux et al., 1973). Briefly, L-alanine *p*-nitroanilide was used as a substrate for the aminopeptidase-N assay. The reaction was started by addition of 10 µl of the homogenate to 1 ml of assay solution (2.04 mmol l^{-1} L-alanine *p*-nitroanilide in 0.2 mol l^{-1} phosphate buffer, NaH_2PO_4/Na_2HPO_4, pH 7.0). The solution was incubated at 37°C for 10 min, and then the reaction was stopped with 3 ml of ice-cold 2 mol l^{-1} acetic acid, and absorbance was read at 384 nm. Aminopeptidase activity was expressed as µmol min^{-1}. The inter- and intra-assay variations were 7.8% and 5.1%, respectively.

Carcass mass and fat content

After the digestive tract was removed, the liver, heart, lungs, spleen and kidneys, as well as the reproductive tissues, were separated. The remaining carcass was weighed (to 1 mg) to determine wet mass, dried in an oven at 60°C to constant mass, and reweighed (to 1 mg) to determine dry mass. Total body fat was extracted from the dried carcass by ether extraction in a Soxhlet apparatus, and percentage fat content was calculated following the equation: fat content (%)=mass of extracted fat (g)/mass of dried carcass (g)×100% (Zhao et al., 2014a).

Serum leptin levels

The serum was obtained from the trunk blood 1.5 h following blood collection. Serum leptin levels were determined using radioimmunoassay with a commercial ^{125}I Multispecies Kit (Linco Research, St Charles, MO, USA), following the standard kit instructions. The inter- and intra-assay variations were 3.6% and 8.7%, respectively, and the lower and upper limits of the assay kit were 1 and 50 ng ml^{-1}. This kit was previously shown to be effective for striped hamsters (Zhao et al., 2014a).

Real-time reverse transcription-quantitative PCR analysis

Relative mRNA expression of several neuropeptides was quantified using real-time reverse transcription-quantitative PCR (RT-qPCR) analysis, as described previously (Zhao et al., 2014b). RNA isolation from the hypothalamus was carried out using a Trizol kit (Takara Bio, Dalian, China) according to the manufacturer's instructions. RNA concentration and purity were determined by A_{260} and A_{280} optical density measurements and A_{260}/A_{280} ratio was then calculated. cDNA was produced from 2 mg of total RNA samples using random primer oligo(dT)18 and avian myeloblastosis virus (AMV) reverse transcriptase (Takara Bio), according to the manufacturer's protocols; 2 µl of cDNA was used as a template in each PCR reaction using gene-specific primers: NPY forward, 5′-ACCCTCGCTCTGTCCCTG-3′, reverse, 5′-AATCAGTGTC-TCAGGGCTA-3′; AgRP forward, 5′-TGTTCCCAGAGTTCCC-AGGTC-3′, reverse, 5′-ATTGAAGAAGCGGCAGTAGCAC-3′; POMC forward 5′-GGTGGGCAAGAAGCGACG-3′, reverse 5′-C-TTGTCCTTGGGCGGGCT-3′; cocaine- and amphetamine-regulated transcript (CART), forward 5′-TACCTTTGCTGGGTGCCG-3′, reverse 5′-AAGTTCCTCGGGGACAGT-3′. The final reaction volume was 20 µl, including 10 µl of 2× SYBR Premix EX Tag TM (TAKARA), 0.4 µl of forward and reverse primer and 2 µl cDNA template. The reactions were performed using the Roche LightCycler480 real-time qPCR system (Forrentrasse CH-6343 Rotkreuz, Switzerland). The PCR conditions were 40 cycles of 5 s at 95°C, 30 s at 55°C and 30 s at 72°C, followed by thermal denaturation curves. All samples were quantified for relative quantity of gene expression with actin expression as an internal standard (actin forward 5′-AAAGACCTCTATGCCA-ACA-3′, reverse 5′-ACATCTGCTGGAAGGTGG-3′).

Statistics

Data are expressed as means±s.e.m. and were analyzed using SPSS 13.0 statistical software. All variables were tested for normality using the Kolmogorov–Smirnov test. For experiment 1, the differences in gross energy intake, digestive energy intake and digestibility, mass of digestive tract, digestive enzyme activity, body fat content, serum leptin levels and neuropeptide mRNA expression among the non-lactation , peak-lactation and post-lactation groups were examined using one-way ANOVA. Correlations between digestive enzyme activity and gross energy intake, digestive energy intake and digestibility were examined using Pearson's correlation analysis. For experiment 2, the effect of litter size on litter mass, mean pup mass and serum leptin levels, as well the other parameters mentioned above, were examined using one-way ANOVA. For both experiment 1 and experiment 2, one-way ANOVA was followed by Tukey *post hoc* comparisons. Correlations of gross energy intake and body mass, litter mass and digestibility, and of gross energy intake and litter mass were also analyzed using Pearson's correlation analysis. The tests were two-tailed, and the level of significance was set at $P<0.05$.

RESULTS

Experiment 1

Reproductive performance with natural litters, gross energy intake and digestibility

Litter size ranged from 3 to 8, and it averaged 5.0±0.4 at weaning. Litter mass averaged 52.9±4.7 g and mean pup body mass was 10.6±0.8 g at weaning. Hamsters in the peak-lactation group showed a significant increase in gross energy intake compared with those in the other two groups ($F_{2,21}=63.08$, $P<0.01$; Fig. 1A); values were 292.78% higher in the peak-lactation group than in the non-lactation group (*post hoc* $P<0.05$). Hamsters in the post-lactation group significantly reduced gross energy intake compared with those in the peak-lactation group (*post hoc* $P<0.05$), whereas the difference was not significant between the post-lactation and non-lactation groups (*post hoc* $P>0.05$). Consistent with gross energy intake, digestive energy intake significantly increased in the peak-lactation group, and it was 3.46-fold and 3.02-fold higher than

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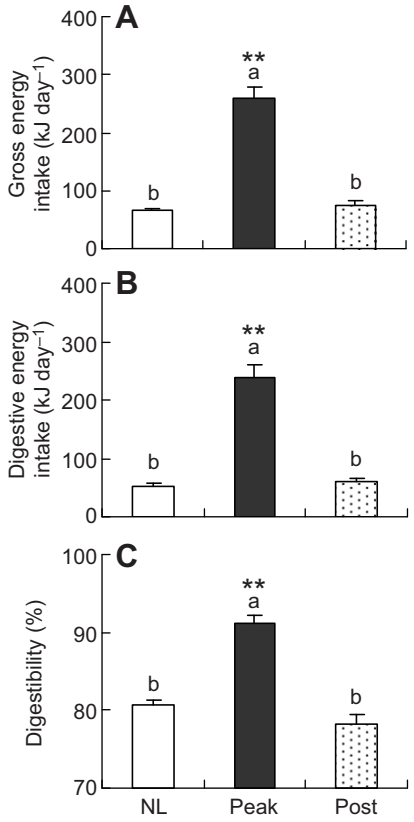

Fig. 1. Energy intake and digestibility in striped hamsters at different stages of lactation. (A) Gross energy intake, (B) digestive energy intake and (C) digestibility during non-lactation (NL), and peak- (Peak) and post-lactation (Post) periods. Data are means±s.e.m. Asterisks indicate a significant effect of lactation (**P<0.01). Different letters above the bars indicate a significant difference among the three groups (P<0.05).

in the non-lactation and post-lactation groups, respectively ($F_{2,21}$=56.66, P<0.01, *post hoc* P<0.05; Fig. 1B), whereas the difference between the non-lactation and post-lactation groups was not statistically significant (*post hoc* P>0.05). The hamsters in the peak-lactation group showed significantly higher digestibility than those in the other two groups ($F_{2,21}$=50.27, P<0.01, *post hoc* P<0.05; Fig. 1C). Digestibility in the post-lactation group was similar to that in the non-lactation group (*post hoc* P>0.05).

Digestive tract segment mass

There were significant differences in the mass of digestive tract segments between the three groups (stomach, $F_{2,21}$=9.69, P<0.01; small intestine, $F_{2,21}$=11.25, P<0.01; large intestine, $F_{2,21}$=8.92, P<0.01; and cecum, $F_{2,21}$=9.89, P<0.01; Table 1). The stomach, small and large intestine and cecum in the peak-lactation group were heavier by 30.4%, 66.9%, 42.8% and 36.7% than in the non-lactation group (Table 1). The mass of digestive tract segments in the post-lactation group was reduced relative to that of the peak-lactation group, and returned to the levels of the non-lactation group.

Pepsin activity

The three groups differed significantly in pepsin activity ($F_{2,21}$=32.21, P<0.01; Fig. 2A). The hamsters in the peak-lactation group showed 170.8% higher pepsin activity than those in the non-lactation group (*post hoc* P<0.05). Pepsin activity in the post-lactation group was significantly higher than that in the non-lactation group (*post hoc* P<0.05), but was lower by 39.2% than that in the peak-lactation group (*post hoc* P<0.05). There were strong positive correlations between pepsin activity and gross energy intake, digestive energy intake and digestibility (Table 2).

Table 1. Digestive tract mass in striped hamster

	Non-lactation	Peak-lactation	Post-lactation	F	P
Stomach (g)	0.307±0.019^{b}	0.401±0.019^{a}	0.319±0.010^{b}	9.69	**
SI (g)	0.529±0.034^{b}	0.883±0.056^{a}	0.707±0.068^{a,b}	11.25	**
LI (g)	0.206±0.013^{b}	0.294±0.020^{a}	0.240±0.012^{a,b}	8.92	**
Cecum (g)	0.188±0.012^{b}	0.257±0.008^{a}	0.224±0.013^{a,b}	9.89	**

SI, small intestine; LI, large intestine. Data are means±s.e.m., **P<0.01. Different letters in the same row indicate a significant difference among the three groups (P<0.05).

Maltase, sucrase and aminopeptidase activity

There were significant differences between the three groups in the activity of maltase, sucrase and aminopeptidase, which increased by 106.2%, 114.5% and 116.2% in the peak-lactation group compared with the non-lactation group (maltase, $F_{2,21}$=11.60, P<0.01, *post hoc* P<0.05; Fig. 2B; sucrase, $F_{2,21}$=13.68, P<0.01, *post hoc* P<0.05; Fig. 2C; aminopeptidase, $F_{2,21}$=23.27, P<0.01, *post hoc* P<0.05; Fig. 2D). The activities of the three digestive enzymes were all significantly decreased in the post-lactation group compared with those in the peak-lactation group (*post hoc* P<0.05). Strong positive relationships were observed between the activities of maltase, sucrase, aminopeptidase and pepsin and gross energy intake, digestive energy intake and digestibility (Table 2).

Carcass mass, body fat content and serum leptin levels

There was a significant effect of lactation on the mass of the wet carcass ($F_{2,21}$=4.23, P<0.05; Fig. 3A) and dry carcass ($F_{2,21}$=5.58, P<0.01; Fig. 3B). The dry carcass mass decreased by 14.9% in the peak-lactation group compared with the non-lactation group (*post hoc* P<0.05), and that of the post-lactation group returned to the levels of the non-lactation group (*post hoc* P>0.05). However, wet carcass mass was not significantly different among the three groups (*post hoc* P>0.05). Body fat content averaged 31.3±2.2% in the non-lactation group; it decreased to 23.5±1.1% in the peak-lactation

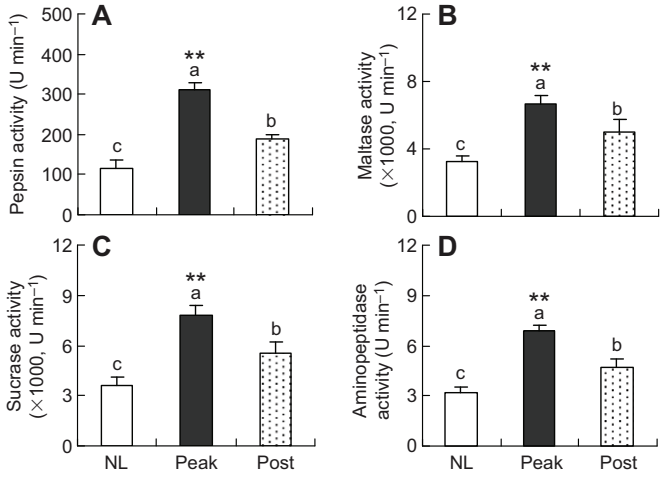

Fig. 2. Digestive enzyme activity of striped hamsters at different stages of lactation. (A) Stomach pepsin activity and (B–D) small intestine maltase (B), sucrase (C) and aminopeptidase (D) activity during non-lactation (NL), and peak- (Peak) and post-lactation (Post) periods. Data are means±s.e.m. Asterisks indicate a significant effect of lactation (**P<0.01). Different letters above the bars indicate a significant difference among the three groups (P<0.05).

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Table 2. Pearson's correlation coefficients for the relationship between the activity of pepsin, maltase, sucrase and aminopeptidase and gross energy intake, digestive energy intake and digestibility in the striped hamster

	Pepsin	Maltase	Sucrase	Aminopeptidase	GEI	DEI	Digestibility
Pepsin	1						
Maltase	0.70**	1					
Sucrase	0.73**	0.95**	1				
Aminopeptidase	0.76**	0.95**	0.94**	1			
GEI	0.80**	0.71**	0.71**	0.81**	1		
DEI	0.79**	0.70**	0.71**	0.80**	0.99**	1	
Digestibility	0.70**	0.65**	0.69**	0.76**	0.94**	0.94**	1

GEI, gross energy intake; DEI, digestive energy intake. **P<0.01.

group ($F_{2,21}$=4.81, P<0.05, *post hoc* P<0.05; Fig. 3C) but the non-lactation and post-lactation groups did not differ in fat content (*post hoc* P>0.05). Serum leptin levels in the peak-lactation group were lower by 28.4% and 38.3%, respectively, than in the non-lactation and post-lactation groups ($F_{2,21}$=8.44, P<0.01, *post hoc* P<0.05; Fig. 3D).

Experiment 2

Litter size and litter mass

Litter size and litter mass on day 13–14 of lactation were significantly different among the three groups (litter size, $F_{2,22}$=855.66, P<0.01; Fig. 4A; litter mass, $F_{2,22}$=249.09, P<0.01; Fig. 4B). The females in the LS1 group raised significantly lighter litters than those in the LS4 group (*post hoc* P<0.05). Litter size and litter mass in the LS8 group were higher by 7.1- and 6.5-fold than those in the LS1 group (*post hoc* P<0.05), and were higher by 1.9- and 1.7-fold than those in the LS4 group (*post hoc* P<0.05). Mean pup mass was not significantly affected by litter size during lactation ($F_{2,22}$=0.68, P>0.05; Fig. 4C), and pup mass in the LS4 group did not differ from that of the other two groups (*post hoc* P>0.05).

Gross energy intake, digestive energy intake and digestibility

Gross energy intake was 109.2±7.7 kJ day^{-1} in the LS1 group, and it increased to 216.5±14.5 kJ day^{-1} and 386.5±12.5 kJ day^{-1} in the LS4 and LS8 groups, respectively ($F_{2,22}$=139.20, P<0.01; Fig. 4D). Gross energy intake in the LS4 and LS8 groups increased by 98.2% and 253.9% relative to that in the LS1 group (*post hoc* P<0.05). Similar changes were observed in digestive energy intake. Females raising large litters had higher digestive energy intake than those supporting small and medium litters ($F_{2,22}$=133.22, P<0.01, *post hoc* P<0.05; Fig. 4E). Digestibility was also affected by litter size, and females tended to increase digestibility with increases in litter size ($F_{2,22}$=33.65, P<0.01, *post hoc* P<0.05; Fig. 4F). No relationship was observed between gross energy intake and body mass (Fig. 5A), but significant correlations were found between gross energy intake and litter mass (Fig. 5B) and digestibility (Fig. 5C). Digestibility was significantly positively correlated with litter mass (Fig. 5D).

Mass and protein content of the stomach and small intestine

Litter size had a significant effect on the mass of the stomach ($F_{2,22}$=4.56, P<0.05; Fig. 6A) and small intestine ($F_{2,22}$=27.72, P<0.01; Fig. 6B). In the LS8 group, the stomach was heavier by 15.3% and 13.0% than in the LS1 and LS4 groups, respectively (*post hoc* P<0.05). The small intestine in the LS8 group was heavier

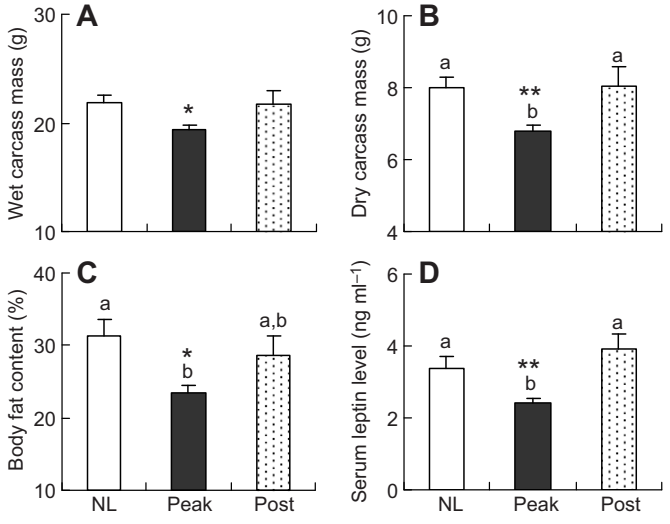

Fig. 3. Carcass mass, body fat content and leptin levels of striped hamsters at different stages of lactation. Wet (A) and dry (B) carcass mass, (C) body fat content and (D) serum leptin levels during non-lactation (NL), and peak- (Peak) and post-lactation (Post) periods. Data are means±s.e.m. Asterisks indicate a significant effect of lactation (*P<0.05, **P<0.01). Different letters above the bars indicate a significant difference among the three groups (P<0.05).

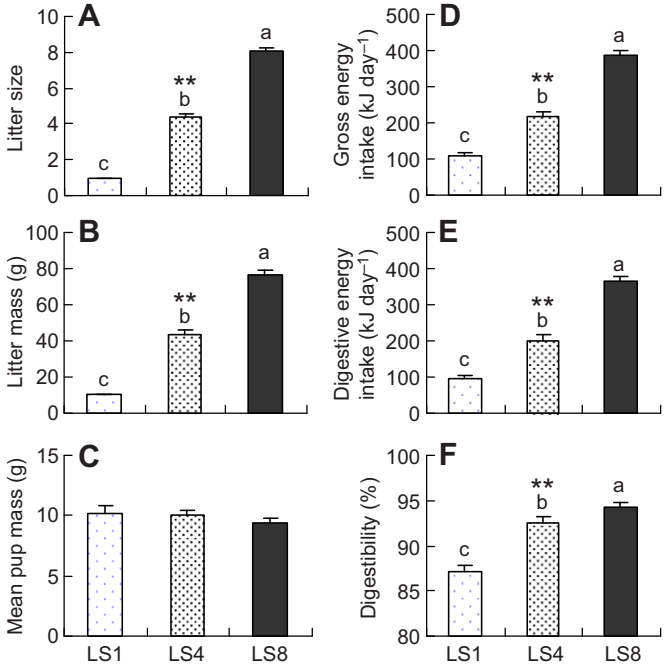

Fig. 4. Effect of litter size on mass, energy intake and digestibility on day 13–14 of lactation. (A) Litter size, (B) litter mass, (C) mean pup mass, (D) gross energy intake, (E) digestive energy intake and (F) digestibility in hamsters raising 1 (litter size, LS1), 4 (LS4) and 8 pups (LS8). Data are means±s.e.m. Asterisks indicate a significant effect of litter size (**P<0.01). Different letters above the bars indicate a significant difference among the three groups (P<0.05).

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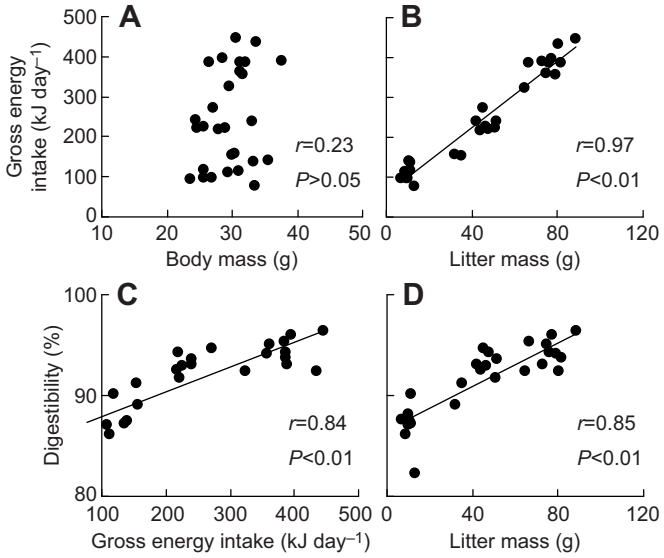

Fig. 5. Correlation between gross energy intake, mass and digestibility in striped hamsters. (A,B) Correlation between gross energy intake and body mass (A) and litter mass (B). (C,D) Correlation between digestibility and gross energy intake (C) and litter mass (D).

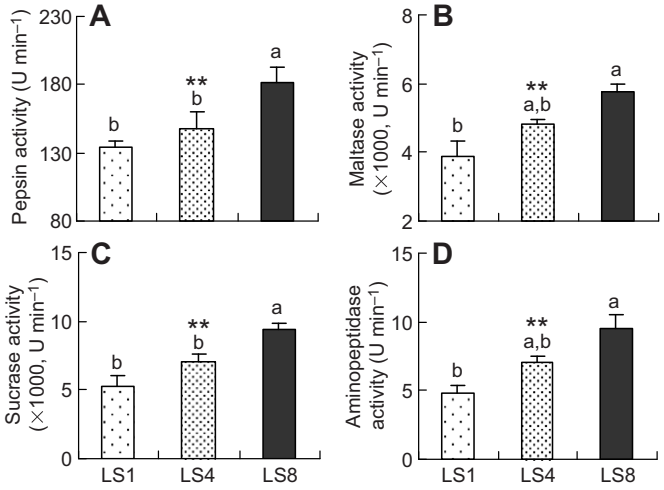

Fig. 7. Effect of litter size on digestive enzyme activity in the stomach and small intestine. (A) Stomach pepsin activity and (B–D) small intestine maltase (B), sucrase (C) and aminopeptidase (D) activity in striped hamsters raising 1 (LS1), 4 (LS4) and 8 pups (LS8). Data are means±s.e.m. Asterisks indicate a significant effect of litter size (**P<0.01). Different letters above the bars indicate a significant difference among the three groups (P<0.05).

by 54.6% and 32.5%, respectively, than in the LS1 and LS4 groups (*post hoc* P<0.05). The differences in the mass of the stomach and small intestine were not significant between the LS1 and LS4 groups (stomach, *post hoc* P>0.05; small intestine, *post hoc* P>0.05). The protein content of the stomach did not differ significantly between the three groups ($F_{2,22}$=1.49, P>0.05; Fig. 6C), whereas it did in the small intestine ($F_{2,22}$=18.60, P<0.01; Fig. 6D). The protein content of the small intestine in the LS8 group was higher by 57.8% and 33.4% than in the LS1 and 4 groups, respectively (*post hoc* P<0.05).

Digestive enzyme activity of the digestive tract

Pepsin activity was significantly affected by litter size, and it was higher by 35.2% and 22.5% in the LS8 group than in the LS1 and

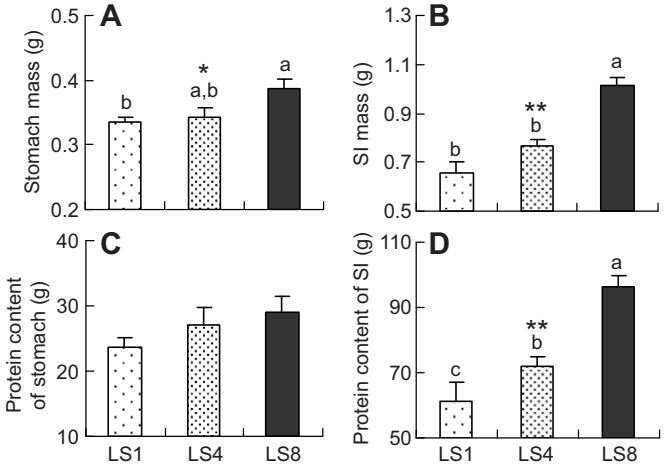

Fig. 6. Effect of litter size on mass and protein content of the digestive tract. Mass of stomach (A) and small intestine (SI, B) and protein content of stomach (C) and small intestine (D) in striped hamsters raising 1 (LS1), 4 (LS4) and 8 pups (LS8). Data are means±s.e.m. Asterisks indicate a significant effect of litter size (*P<0.05, **P<0.01). Different letters above the bars indicate a significant difference among the three groups (P<0.05).

LS4 groups ($F_{2,22}$=5.61, P<0.01, *post hoc* P<0.05; Fig. 7A). Maltase and sucrase activity were also affected by litter size (maltase, $F_{2,22}$=9.88, P<0.01; Fig. 7B; sucrase, $F_{2,22}$=12.91, P<0.01, Fig. 7C). The females in the LS8 group showed significantly higher maltase and sucrase activity than those in the LS1 group (*post hoc* P<0.05). There was a significant effect of litter size on aminopeptidase activity, and it increased by 98.3% in the LS8 group compared with the LS1 group ($F_{2,22}$=10.76, P<0.01, *post hoc* P<0.05; Fig. 7D).

Mass, protein content and digestive enzyme activity of the small intestine

Litter size had a significant effect on the mass of the duodenum ($F_{2,22}$=14.32, P<0.01) and jejunum ($F_{2,22}$=9.94, P<0.01), whereas no effect was observed on the ileum ($F_{2,22}$=1.85, P>0.05; Fig. 8A). The duodenum and jejunum in the LS8 group were significantly heavier than those in the LS1 and LS4 groups (*post hoc* P<0.05). Similarly, the protein content of the duodenum and jejunum was significantly higher in the LS8 group than in the LS1 and LS4 groups (duodenum, $F_{2,22}$=11.16, P<0.01; jejunum, $F_{2,22}$=8.27, P<0.01; Fig. 8B). Litter size had a significant effect on maltase activity in the duodenum ($F_{2,22}$=15.05, P<0.01), but not in the jejunum ($F_{2,22}$=0.43, P>0.05) or ileum ($F_{2,22}$=0.83, P>0.05; Fig. 8C). Sucrase activity differed significantly between the three groups in the duodenum ($F_{2,22}$=14.53, P<0.01) and jejunum ($F_{2,22}$=7.07, P<0.01), but not in the ileum ($F_{2,22}$=1.43, P>0.05; Fig. 8D). Sucrase activity in the LS8 group was higher by 113.4% than in the LS1 group in the duodenum (*post hoc* P<0.05) and was higher by 62.4% in the jejunum (*post hoc* P<0.05). Aminopeptidase activity in the LS8 group increased by 122.9% compared with that in the LS1 group in the duodenum ($F_{2,22}$=7.91, P<0.01, *post hoc* P<0.05), and increased by 119.5% in the jejunum ($F_{2,22}$=6.79, P<0.01, *post hoc* P<0.05; Fig. 8E). The three groups did not differ in aminopeptidase activity of the ileum ($F_{2,22}$=1.96, P>0.05).

Hypothalamic neuropeptide mRNA expression

Hypothalamic NPY mRNA expression in the LS4 and LS8 groups increased by 22.4% and 65.8%, respectively, compared with the

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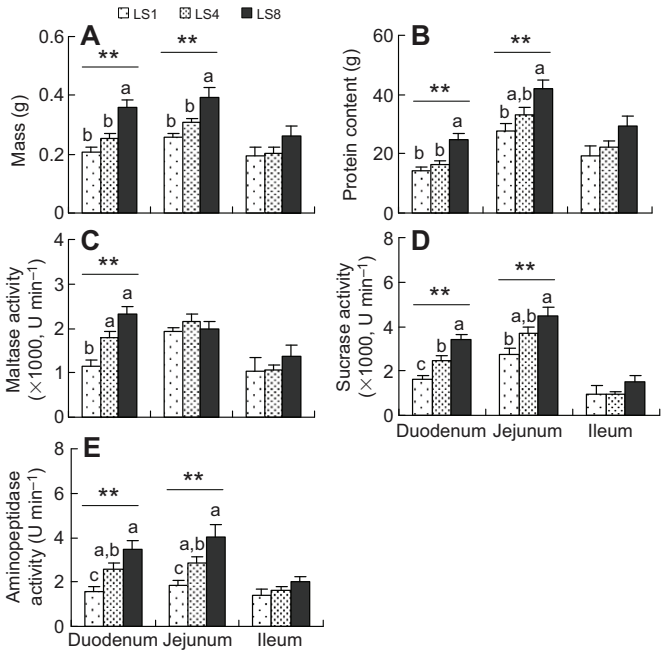

Fig. 8. Effect of litter size on mass, protein content and digestive enzyme activity of the small intestine. Duodenum, jejunum and ileum mass (A), protein content (B) and activity of maltase (C), sucrase (D) and aminopeptidase (E) in striped hamsters raising 1 (LS1), 4 (LS4) and 8 pups (LS8). Data are means±s.e.m. Asterisks indicate a significant effect of litter size (**P<0.01). Different letters above the bars indicate a significant difference among the three groups (P<0.05).

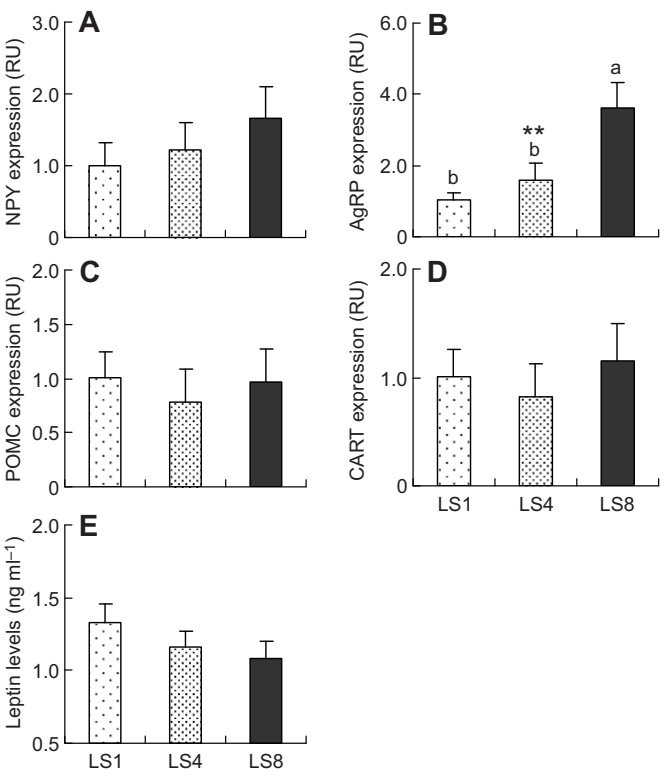

Fig. 9. Effect of litter size on mRNA expression of hypothalamic neuropeptides. (A) Hypothalamus neuropeptide Y (NPY), (B) agouti-related protein (AgRP), (C) pro-opiomelanocortin (POMC) and (D) cocaine- and amphetamine-regulated transcript (CART) mRNA levels in hamsters raising 1 (LS1), 4 (LS4) and 8 pups (LS8). Data are means±s.e.m. RU, relative units. Asterisks indicate a significant effect of litter size (**P<0.01). Different letters above the bars indicate a significant difference among the three groups (P<0.05).

LS1 group, but the effect of litter size was not statistically significant ($F_{2,22}$=0.76, P>0.05; Fig. 9A). The hypothalamus AgRP mRNA expression was significantly affected by litter size, being up-regulated with increasing litter size ($F_{2,22}$=7.15, P<0.01; Fig. 9B). AgRP expression in females raising 8 pups increased by 262.1% and 126.7% compared with that in females raising 1 and 4 pups, respectively (*post hoc* P<0.05). POMC and CART mRNA expression was not significantly affected by litter size (POMC, $F_{2,22}$=0.19, P>0.05; Fig. 9C; CART, $F_{2,22}$=0.32, P>0.05; Fig. 9D).

Leptin levels

Serum leptin levels tended to decrease with increases in litter size. Females raising 8 pups showed 18.5% lower leptin levels than those supporting 1 pup, but the difference among the three groups was not statistically significant ($F_{2,22}$=1.18, P>0.05; Fig. 9E).

DISCUSSION

Lactation is the most energy-demanding state in the lifetime of most female mammals, which in small mammals is met primarily by increasing energy intake (Hammond and Kristan, 2000; Johnson and Speakman, 2001; Król and Speakman, 2003; Rogowitz, 1998; Speakman and Król, 2005; Valencak et al., 2009, 2010; Weiner, 1992). In the present study, energy intake increased by 292.8% in lactating hamsters compared with the non-reproductive group. Energy intake dropped in hamsters after lactation, showing marked plasticity at different stages of reproduction. Consistently, energy intake during peak lactation increased by 3.3-fold in MF1 mice (Johnson et al., 2001), 153.0% in Brandt's voles (*Lasiopodomys brandtii*) (Zhang et al., 2008), 168.3% in Swiss mice (Zhao and Cao, 2009) and 68% in European hares (*Lepus europaeus*) (Valencak et al., 2009) in comparison with non-reproductive controls. Females not only consumed more food but also increased assimilation efficiency (digestibility) to meet the high energy requirement during lactation (Valencak et al., 2009). Here, we observed a significant elevation of digestibility in lactating hamsters (91.2%) compared with that in non-reproductive (80.7%) and post-lactating animals (78.2%). Conversely, digestibility averaged 83.7% in late-lactating MF1 mice, a significant decrease relative to non-reproductive controls (86.1%) (Speakman and McQueenie, 1996). This suggests that the strategies used by females to meet the energetic demands of lactation differ across rodent taxa, during which the plasticity of the gut morphology and digestive enzymes may be involved.

The striped hamster in this study showed significant plasticity of the digestive tract at different stages of reproduction. The stomach, small and large intestine and cecum in lactating hamsters were larger in size than in the non-reproductive and post-lactating animals. Such marked plasticity has also been observed in many other animals (Campbell and Fell, 1964; Derting and Austin, 1998; Hammond et al., 1996; Koteja, 1996a; Reilly et al., 2006; Simons et al., 2011; Speakman and McQueenie, 1996). Moreover, significant plasticity was observed in the digestive enzymes among the non-reproductive, lactating and post-lactating hamsters. Pepsin activity of the stomach, and the activity of maltase, sucrase and aminopeptidase in the small intestine were all significantly increased during peak lactation, probably resulting in the increased energy-processing capacity (Fig. 2). This suggests that animals can use phenotypic plasticity of the gut morphology and digestive enzyme activity to cope with the increased food intake in response to high energy demand conditions

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(Bozinovic and Nespolo, 1997; Debray et al., 2003; Nespolo et al., 2002). This may explain why the SusEI is not constrained centrally by the mother's capacity for food (energy) assimilation.

In order to examine the phenotypic plasticity of the gastrointestinal tract in response to conditions of higher energy demand, we manipulated litter size. This is because the mothers raising larger litters have more energy requirements for raising their offspring than those supporting small litter sizes (Hammond et al., 1996; Johnson et al., 2001; Liu et al., 2003; Rogowitz, 1998; Scantlebury et al., 2001; Zhang et al., 2008; Zhao et al., 2010, 2013). We observed that the female hamsters supporting large litters raised significantly heavier litters than those supporting smaller litters, indicating that the mass of individual pups was not affected by litter size. During peak lactation, gross energy intake in females raising 8 pups (386.5±12.5 kJ day^{-1}) increased considerably beyond that observed in females supporting a natural litter size (259.33±20.43 kJ day^{-1}). Digestibility also increased significantly in the mothers with 8 pups, indicating that female hamsters could increase energy intake and energy assimilation efficiency further to cope with the increased energy requirements of additional pups. This was inconsistent with a previous study performed on the same strain of hamster (Zhao et al., 2010), where female hamsters cannibalized some of the pups when given additional pups to raise, and finally weaned similar-sized litters to those supporting natural litter sizes. It is unknown why different results were obtained from the two studies. One plausible reason is that females and pups were measured daily following parturition in that study, which may have caused the cannibalization of addition pups (Zhao et al., 2010). In the present study, no measurement was carried out following litter size manipulation. This may suggest that wild hamsters are more sensitive to disturbance during the early lactation period than laboratory mice and rats, rodents in which infanticide does not occur.

The digestive enzymes, one of the most important components of digestion, have been reported to be regulated plastically to meet high energy demand conditions (Bozinovic and Nespolo, 1997; Debray et al., 2003; del Valle et al., 2004; Nespolo et al., 2002; Sabat et al., 1999). In this study, the female hamsters raising larger litters showed an increase in the mass, protein concentration and digestive enzyme activity of the stomach and small intestine above those observed in the females raising a natural litter size, suggesting that the mothers had a higher capacity to meet increased energy intake. Consistent with this, maltase and aminopeptidase-N activity did not change with lactation in degus (*Octodon degus*) (Naya et al., 2008b). However, marked plasticity of several central organs was observed in degus, which possibly contributed to the increased food assimilation during lactation (Naya et al., 2008b). It has been observed that maltase and aminopeptidase-N had greater activity values in the middle portion of the small intestine (Naya et al., 2008b). In the present study, significant plasticity of maltase, sucrase and aminopeptidase activity was observed in the middle section of the small intestine (jejunum), and also in the first section of the small intestine (duodenum). These results may reflect a different capacity of the digestive system across species, which determines the strategies used by females to meet their energetic demands (Bozinovic and Nespolo, 1997; Bozinovic et al., 2010; Debray et al., 2003; del Valle et al., 2004; Naya et al., 2008b; Nespolo et al., 2002; Sabat et al., 1999; Zhao et al., 2010). The remarkable plasticity of digestive enzyme activity in the stomach and in particular in the jejunum and duodenum likely drove the digestive mechanisms underpinning the plasticity of the gut morphology, providing strong refutation of the central limitation hypothesis.

Several other hypotheses regarding the factors limiting SusEI during peak lactation have been proposed (Hammond and Diamond, 1992, 1994; Hammond et al., 1994, 1996; Koteja, 1996b; Król and Speakman, 2003; Król et al., 2003; Rogowitz, 1998; Simons et al., 2011; Speakman and Król, 2005, 2011; Valencak et al., 2009, 2010; Weiner, 1992). The saturated neural control hypothesis explains that hypothalamic control of energy homeostasis may be involved in the limits to SusEI during lactation (Speakman and Król, 2005). It has been proposed that food intake is stimulated by a number of peripheral signals that act with several pathways in the brain to promote feeding behavior. However, it may reach a point of maximal stimulation during the second half of the lactation period because receptors become saturated (Speakman and Król, 2005). Leptin has been reported to be a peripheral signal involved in the hypothalamic control of energy intake during lactation (Kalra et al., 1999; Pickavance et al., 1996, 1999; Speakman and Król, 2005; Stephens et al., 1995; Wauters et al., 2000). In the present study, serum leptin tended to decrease with increasing litter size in lactating striped hamsters, but the change was not statistically significant. So far, the reported changes in leptin levels in lactating animals have been inconsistent. Some studies found that leptin levels were significantly reduced during peak lactation (Brogan et al., 1999, 2000; Cui et al., 2011; Denis et al., 2003a; Herrera et al., 2000; Kunz et al., 1999; Pickavance et al., 1998; Speakman and Król, 2005; Vernon et al., 2002; Zhang and Wang, 2007), but others suggested that leptin levels increased in lactating animals (Mistry and Romsos, 2002; Mukherjea et al., 1999). López-Soriano et al. (1999) demonstrated that leptin levels were unchanged during lactation. The diversity of leptin levels in lactation suggests that it only reflects a state of energy balance rather than being the prime driver of hyperphagia (Vernon et al., 2002).

The neuroendocrine basis of hyperphagia in lactation is suggested to be triggered by hypothalamic neuropeptides, such as the orexigenic neuropeptides NPY and AgRP, and the anorexigenic neuropeptides POMC and CART (Brogan et al., 2000; Chen et al., 2004; Crowley et al., 2004; Malabu et al., 1994; Li et al., 1999; Pickavance et al., 1999; Smith, 1993; Sorensen et al., 2002; Suzuki et al., 2014). In the present study, AgRP mRNA expression was significantly up-regulated in striped hamsters raising larger litter sizes, whereas NPY, POMC and CART mRNA expression did not differ between the hamsters raising different litter sizes. This is partly consistent with the observation that in lactating rats, both NPY and AgRP are greatly elevated (Chen et al., 2004; Crowley et al., 2004; Li et al., 1999; Malabu et al., 1994; Pickavance et al., 1999; Smith, 1993; Suzuki et al., 2014), but the increase in AgRP mRNA expression during lactation is especially marked (Suzuki et al., 2014). POMC and/or CART mRNA expression significantly decreased during lactation in comparison with the non-lactation period (Brogan et al., 2000; Smith, 1993; Sorensen et al., 2002; Suzuki et al., 2014). The findings of this study suggest that, in striped hamsters, hyperphagia during lactation may be caused by an increase in AgRP expression in the hypothalamus, rather than by NPY, POMC and CART. The absence of adaptive changes in NPY, POMC and CART mRNA expression seems to be weak evidence for the saturated neural control hypothesis, but it indicates that some hypothalamic neuropeptides, such as NPY, POMC and CART, may be involved in the limitation to the SusEI during lactation.

Conclusions

Female striped hamsters showed significant plasticity of gross energy intake, digestibility, gut morphology and digestive enzyme activity among the non-reproductive, peak-lactation and post-lactation periods. Our findings suggest that females not only increased the size of the gut but also increased digestive enzyme

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activity to meet the increased food intake during the high energy demand period of lactation. Further, energy intake, digestibility, gut mass and digestive enzyme activity significantly increased in the females raising additional pups above that observed in the females supporting natural litters, indicating that the significant plasticity of digestive physiology increased reproductive performance. These findings provide strong refutation of the central limitation hypothesis. Serum leptin levels and hypothalamus NPY, POMC and CART mRNA expression did not differ among females raising different litter sizes. Thus, leptin levels in lactation might only reflect a state of energy balance rather than being the prime driver of hyperphagia. Some hypothalamic neuropeptides, such as NPY, POMC and CART, may be involved in the limitation to SusEI during lactation, but this does not provide strong support for the saturated neural control hypothesis.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Z.-J.Z. conceived and designed the experiments. J.-Y.Z., X.-Y.Z., J.W. and S.T. performed the experiments. J.-Y.Z., X.-Y.Z. and Z.-J.Z. analyzed the data. J.-Y.Z. and Z.-J.Z. wrote the paper.

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