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RESEARCH ARTICLE

Dietary fat alters the response of hypothalamic neuropeptide Y to subsequent energy intake in broiler chickens

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ABSTRACT

Dietary fat affects appetite and appetite-related peptides in birds and mammals; however, the effect of dietary fat on appetite is still unclear in chickens faced with different energy statuses. Two experiments were conducted to investigate the effects of dietary fat on food intake and hypothalamic neuropeptides in chickens subjected to two feeding states or two diets. In Experiment 1, chickens were fed a high-fat (HF) or low-fat (LF) diet for 35 days, and then subjected to fed (HF-fed, LF-fed) or fasted (HF-fasted, LF-fasted) conditions for 24 h. In Experiment 2, chickens that were fed a HF or LF diet for 35 days were fasted for 24 h and then re-fed with HF (HF-RHF, LF-RHF) or LF (HF-RLF, LF-RLF) diet for 3 h. The results showed that chickens fed a HF diet for 35 days had increased body fat deposition despite decreasing food intake even when the diet was altered during the re-feeding period (P<0.05). LF diet (35 days) promoted agouti-related peptide (AgRP) expression compared with HF diet (P<0.05) under both fed and fasted conditions. LF-RHF chickens had lower neuropeptide Y (NPY) expression compared with LF-RLF chickens; conversely, HF-RHF chickens had higher NPY expression than HF-RLF chickens (P<0.05). These results demonstrate: (1) that HF diet decreases food intake even when the subsequent diet is altered; (2) the orexigenic effect of hypothalamic AgRP; and (3) that dietary fat alters the response of hypothalamic NPY to subsequent energy intake. These findings provide a novel view of the metabolic perturbations associated with long-term dietary fat over-ingestion in chickens.

KEY WORDS: Poultry, Appetite, Energy status, Hypothalamus, Energy balance

INTRODUCTION

The hypothalamus is a processing centre that integrates signals from the brain, peripheral circulation and gastrointestinal tract to regulate energy intake and expenditure. Two neuron populations in the arcuate nucleus (ARC) of the hypothalamus exert potent effects on food intake, energy balance and glucose homeostasis: agouti-related peptide/neuropeptide Y (AgRP/NPY) neurons are orexigenic (promote feeding and inhibit energy expenditure), while proopiomelanocortin (POMC) and corticotropin-releasing hormone (CRH) neurons promote anorexia (reduce food intake and increase catabolic processes) in the ARC of avian species and mammals (Boswell et al., 2002; Richards, 2003).

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Several studies have shown that these hypothalamic neuropeptides are sensitive to body energy status and critical to whole-body metabolic adjustment. NPY reduces energy expenditure by decreasing adipose tissue thermogenesis (Egawa et al., 1991; Patel et al., 2006; Chao et al., 2011; Zhang et al., 2014). The obesity of ob/ob mice is attenuated when NPY is knocked out (Erickson et al., 1996; Segal-Lieberman et al., 2003). NPY deficiency attenuates responses to a palatable high fat diet in mice (Hollopeter et al., 1998; Patel et al., 2006). Animals in which POMC neurons have been knocked out are obese and hyperphagic (Butler and Cone, 2002; Mencarelli et al., 2012; Diané et al., 2014; Raffan et al., 2016). Additionally, the release of these neuropeptides is closely associated with dietary fat level. Mice fed a higher fat diet had lower NPY and AgRP mRNA levels (Lin et al., 2000; Wang et al., 2002) and higher POMC mRNA levels (Lukaszewski et al., 2013). Therefore, we hypothesized that the response of hypothalamic neuropeptides to energy status could be changed by the dietary fat level.

In the present study, two experiments were conducted to evaluate the effect of dietary fat on the response of hypothalamic neuropeptides to different energy statuses in broiler chickens. The modern broiler chicken has a fast growth rate, large fat deposition rate and high insulin level, and therefore is an excellent model for a fat metabolism study (Sundick et al., 1996; Tsi et al., 2003). Two feeding states (fasted versus fed in Experiment 1) and two re-feeding diets with different energy levels (re-fed high fat diet versus re-fed low fat diet in Experiment 2) were employed to induce differential body energy statuses. Our results indicate that dietary fat alters the response of hypothalamic NPY to subsequent energy intake. NPY-based self-adjusting mechanisms can develop in lean chickens but do not readily occur in obesity induced by a high fat diet. These findings provide a novel view of the metabolic perturbations associated with long-term dietary fat over-ingestion in chickens.

MATERIALS AND METHODS

Ethics statement

All of the animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Shandong Agricultural University and performed in accordance with the 'Guidelines for Experimental Animals' of the Ministry of Science and Technology (Beijing, China). All efforts were made to minimize suffering.

Birds and husbandry

Male broiler chicks [Arbor Acres, *Gallus gallus domesticus* (Linnaeus 1758); 1 day old] were obtained from a local hatchery and were reared in an environmentally controlled room. In both Experiment 1 and Experiment 2, 128 chicks were reared in 16 floor pens each containing eight chicks (1 m^2 per pen). Each pen was equipped with a feeder and a nipple water line. The brooding temperature was maintained at 35°C (65% relative humidity) for the first 2 days, then decreased gradually to 21°C (45% relative

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List of symbols and abbreviations

AgRP	agouti-related peptide
ARC	arcuate nucleus
BMG	body mass gain
CRH	corticotropin-releasing hormone
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HF	high fat
INSR	insulin receptor
LF	low fat
NPY	neuropeptide Y
POMC	proopiomelanocortin
PVH	paraventricular nucleus
RHF	re-fed with HF diet
RLF	re-fed with LF diet
18S	18S ribosomal RNA

humidity) until day 28 and was thereafter maintained at 21°C through to the end of the experiment. The light regime was 23 h light:1 h dark. All of the birds had free access to food and water during the rearing period.

Experimental protocol and sample collection

Experiment 1

At 1 day old, 128 chicks with similar body mass were divided into two groups with eight replicates/pens per group and eight chickens per replicate. As shown in Fig. 1A, the chicks were randomly subjected to one of the following two treatments for 35 days: (1) high-fat (HF) diet (15.06 MJ kg^{-1}, 13.5% soy oil) or (2) low-fat (LF) diet (10.90 MJ kg^{-1}, 0% soy oil). The composition and nutrient levels of the experimental diets are shown in Table 1. Food intake and body mass of the eight chickens reared in one pen were weighed weekly using an electronic balance with an accuracy of 1 g (Senssun, Guangdong, China), and the daily average food intake, energy intake and body mass gain (BMG) of one chicken were calculated. At 36 days old, half of the experimental chickens in each diet treatment were sampled during the fasted state after 24 h of food withdrawal (HF-fasted, LF-fasted), and the other half were sampled in a fed state (HF-fed, LF-fed). At the end of the experiment, eight chickens with a similar body mass were randomly selected from each treatment for sampling. A blood sample was drawn from a wing vein using a heparinized syringe. Plasma was obtained following centrifugation at 400 ***g*** for 10 min at 4°C and stored at −20°C. Immediately after the blood samples were obtained, the chickens were killed by exsanguination. The abdominal, cervical and subcutaneous thigh fats were harvested and weighed using an electronic balance with an accuracy of 0.01 g (A&D, Tokyo, Japan). Hypothalamus samples were collected according to Yuan et al. (2009), snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction.

Experiment 2

At 1 day old, 128 chicks with similar body mass were divided into two groups with eight replicates/pens per group and eight chickens per replicate. As shown in Fig. 1B, the chicks were randomly fed the HF or the LF diet for 35 days. At 36 days old, all of the chickens were fasted for 24 h, then half of the experimental chickens in each group were randomly re-fed with HF diet for 3 h (HF-RHF, LF-RHF) or LF diet for 3 h (LF-RLF, HF-RLF). Food intake was recorded after re-feeding. At the end of the experiment, eight chickens with a similar body mass were randomly selected from each treatment for sampling. Plasma and hypothalamus samples were collected as above.

Measurement of plasma insulin levels

Plasma insulin was measured using a radioimmunoassay with guinea pig anti-porcine insulin serum (3 V, Weifang, China). A large cross-reaction has been observed between chicken insulin and the guinea pig anti-porcine sera (Simon et al., 1974). The insulin in

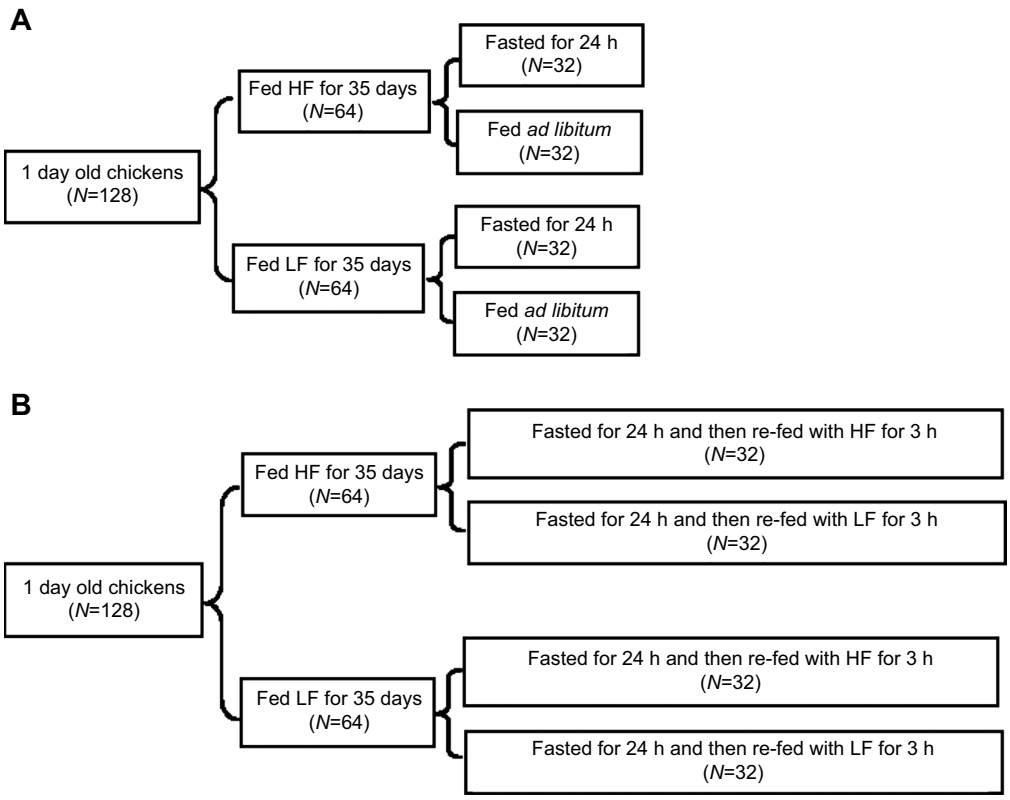

Fig. 1. Schematic diagram of the experimental design. Experiments were designed to assess the effect of dietary fat on chickens under different feeding conditions [A, Experiment 1; high fat (HF) and low fat (LF) diet] or re-feeding conditions (B, Experiment 2).

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Table 1. The composition and nutrient levels of the experimental diets (air dry basis)

	LF	HF
Ingredients (%)		
Maize	46.622	44.859
Soybean meal	30.676	36.984
Choline chloride	0.260	0.260
Dicalcium phosphate	1.424	1.643
Limestone	1.893	1.742
DL-Methionine	0.255	0.294
Lysine	0.271	0.246
NaCl	0.255	0.255
Soy oil	0	13.468
Bran	18.094	0
Vitamin premix*	0.050	0.050
Mineral premix‡	0.200	0.200
Calculated chemical composition		
Metabolizable energy (MJ kg^{-1})	10.900	15.060
Crude protein (%)	20.000	20.000
Met (%)	0.551	0.556
Lys (%)	1.064	1.073
Ca (%)	1.000	1.000
P (%)	0.450	0.450

HF, high fat; LF, low fat.
*Vitamin premix provided (per kg of diet): vitamin A, 8000 IU; vitamin D3, 3000 IU; vitamin E, 33 mg; vitamin K3, 2.3 mg; thiamine, 1.75 mg; riboflavin, 6.9 mg; niacin, 28.45 mg; pantothenic acid, 6.7 mg; biotin, 2.75 mg; folic acid, 0.6 mg; vitamin B12, 2.2 mg; choline (50%), 840 mg; cobalamin, 2.2 mg; and pyridoxine, 3.35 mg.
‡Mineral premix provided (per kg of diet): ferrous sulphate heptahydrate, 183.4 mg; heptahydrate zinc sulphate, 255 mg; monohydrous manganese sulphate, 276.8 mg; copper sulphate, 22 mg; calcium iodide, 2.2 mg; and pentahydrate sodium selenite, 0.6 mg.

this study is referred to as immunoreactive insulin. The sensitivity of the assay was 1 μIU ml^{-1}, and all of the samples were included in the same assay to avoid inter-assay variability. The intra-assay coefficient of variation was 1.99%.

RNA isolation and analysis

Total RNA extraction and real-time PCR were performed as described previously (Liu et al., 2014). The primer sequences are shown in Table 2. The PCR data were analysed with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The mRNA levels of target genes were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and 18S ribosomal RNA (18S) (ΔCt). On the basis of the Ct values, 18S and GAPDH mRNA expression were stable across the treatments in this study (P>0.1).

Statistical analysis

Data are presented as means±s.e.m. All of the data were subjected to one-way ANOVA to test the effect of energy status under pre-feeding HF or LF conditions using the Statistical Analysis Systems statistical software package (Version 8e, SAS Institute, Cary, NC, USA). Homogeneity of variances among the groups was confirmed using Bartlett's test (SAS Institute). When the main effect of the treatment was significant, the differences between the means were assessed by Duncan's multiple range analysis. The mean was considered significantly different at P<0.05.

RESULTS

Effects of dietary fat on production performance, fat deposition and plasma insulin

After 35 days of feeding, the food intake of the chickens fed the HF diet was significantly lower than that of the LF diet-fed chickens (P<0.05; Fig. 2A), but the energy intake and BMG showed the opposite result (P<0.05; Fig. 2B,C, Table 3). HF chickens had significantly enhanced body mass and fat deposition in the abdomen and cervical subcutaneous tissues (P<0.05, Fig. 3) than chickens fed the LF diet. Compared with the LF diet, the HF diet did not significantly affect insulin content in the plasma of either fasted or *ad libitum* fed chickens (P>0.05, Fig. 4A).

During the re-feeding period, the chickens fed the HF diet for 35 days maintained a lower food intake than the chickens on the LF diet (P<0.05), even when they were re-fed a different diet after fasting (Fig. 2D); that is, the HF groups maintained a lower food intake than the LF groups, independent of the diet the chickens were re-fed. The plasma insulin level was not affected by the level of fat in the re-feeding diet (P>0.05, Fig. 4B).

Effects of dietary fat on mRNA expression of hypothalamic appetite-related genes in chickens with different energy statuses

In Experiment 1, 24 h fasting reduced the mRNA expression of NPY, AgRP, POMC, CRH and insulin receptor (INSR) compared with the fed condition (P<0.05; Figs 4C and 5A,C,E,G), independent of the diet the chickens ate before fasting. Under both fed and fasted conditions, HF diet-fed chickens had a lower AgRP mRNA level compared with the LF diet-fed chickens (P<0.05, Fig. 5C).

Table 2. Gene-specific primers used for the analysis of chicken gene expression

Gene	GenBank accession no.	Primer sequences (5′–3′)	Product size (bp)
AgRP	NM_001031457	F: GGAACCGCAGGCATTGTC R: GTAGCAGAAGGCGTTGAAGAA	163
CRH	NM_001123031	F: CTCCCTGGACCTGACTTTCC R: TGTTGCTGTGGGCTTGCT	86
GAPDH	NM_204305	F: ACATGGCATCCAAGGAGTGAG R: GGGGAGACAGAAGGGAACAGA	266
INSR	AF111857	F: CAAACGGTGACCAAGCCTCA R: CATCCTGCCCATCAAACTCCG	186
NPY	M87294	F: GAGGCACTACATCAACCTCATCAC R: TGTTTTCTGTGCTTTCCCTCAA	101
POMC	NM_001031098	F: CGCTACGGCGGCTTCA R: TCTTGTAGGCGCTTTTGACGAT	88
18S	AF173612	F: ATAACGAACGAGACTCTGGCA R: CGGACATCTAAGGGCATCACA	136

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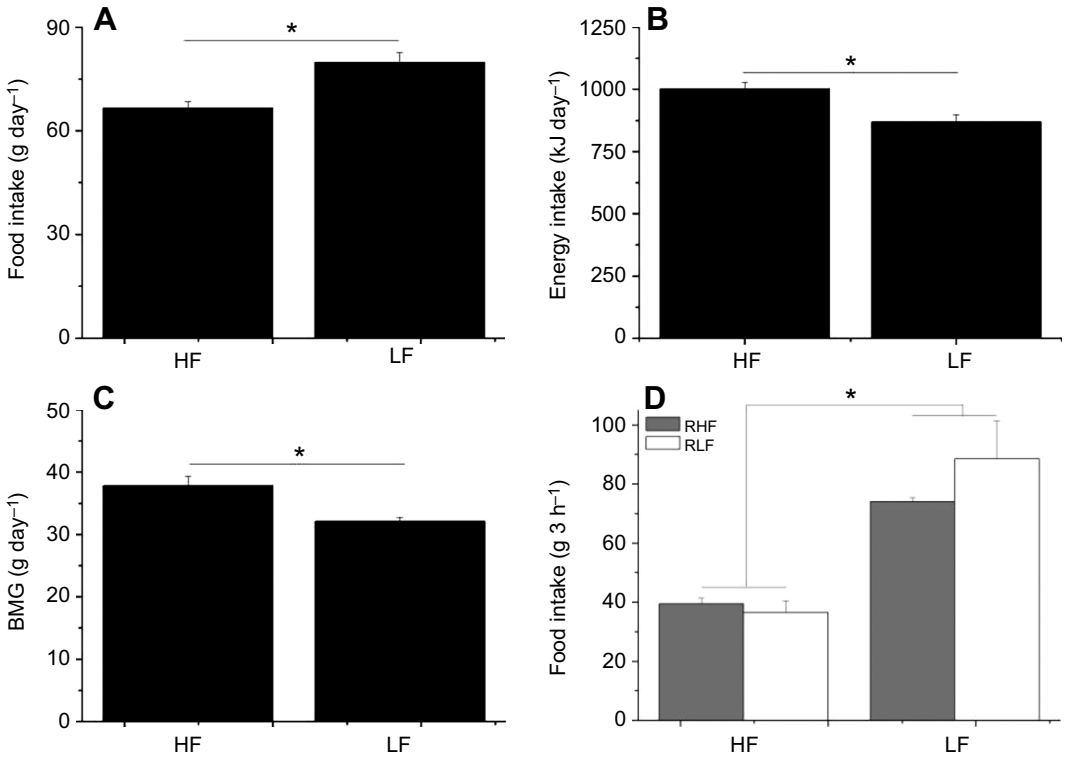

Fig. 2. Effects of the HF diet on food intake and body mass gain of chickens after 35 days of feeding and 3 h of re-feeding. (A–C) Food intake, energy intake and body mass gain (BMG) per bird, measured during 35 days of feeding. (D) Food intake per bird, measured after 3 h of re-feeding with the HF (RHF) or LF (RLF) diet. All of the data were subjected to one-way ANOVA. Values are means±s.e.m. (N=8 for A–C, N=4 for D). *Significant difference (P<0.05).

In Experiment 2, gene expression in response to re-feeding diet was compared between HF and LF groups. Among five appetite-related genes measured in the present study, *NPY* expression in response to re-feeding dietary fat was altered depending upon the feeding experience: in the HF group, re-feeding with the HF diet (RHF) increased *NPY* expression compared with re-feeding with the LF diet (RLF; P<0.05), while the LF group showed the opposite result, in which RHF decreased *NPY* expression compared with RLF (P<0.05; Fig. 5B). For *AgRP* (Fig. 5D), RHF decreased *AgRP* expression compared with RLF in the LF group (statistically significant, P<0.05) and showed a tendency to do so in the HF group (−51%, not statistically significant, P>0.05). The mRNA expression of POMC, CRH and INSR was not significantly affected by the level of fat in the re-feeding diet (P>0.05; Figs 4D and 5F,H).

DISCUSSION

In the present study, we investigated whether dietary fat influenced the response of hypothalamic appetite-related peptides to different energy statuses (fed versus fasted; RHF versus RLF). The results demonstrate that chickens fed a high fat diet show decreased food intake even when the subsequent diet type is changed. In addition, dietary fat alters the response of hypothalamic NPY to subsequent energy intake.

Table 3. Effect of HF diet on body mass of chickens over 35 days

BMG (g day^{-1})	HF	LF	P-value
Week 1	7.5±0.1	7.8±0.2	0.1993
Week 2	32.1±0.6	33.3±0.2	0.0876
Week 3	27.6±1.2	27.2±1.6	0.8376
Week 4	51.4±2.7^{a}	40.7±2.1^{b}	0.0199
Week 5	70.3±4.2^{a}	51.4±2.4^{b}	0.0077

Body mass was recorded weekly, and body mass gain (BMG) per bird was calculated week by week for 5 weeks (35 days). All of the data were subjected to one-way ANOVA. Values are means±s.e.m. (N=8). Different superscript letters indicate a significant difference (P<0.05).

HF diet decreases food intake even when the subsequent diet type is changed

Chickens fed for 35 days with the HF diet displayed decreased food intake, in line with our previous results demonstrating that chickens adjusted their food intake according to the dietary metabolizable energy concentration (Yuan et al., 2008). The HF effect on food intake is likely to be driven mainly by energy needs, because several reports have shown that taste or palatability was relatively less important (Blundell et al., 1993, 1996; Berridge, 1996). The viewpoint that a HF diet induces an increase in body mass has been widely verified in human and animal studies (West and York, 1998; Murtaugh et al., 2007) and was also shown in the present study. The increase in body mass resulted from a higher proportion of adipose tissue rather than skeletal muscle (West and York, 1998; Murtaugh et al., 2007). We observed that the adipose mass in the abdomen and cervical subcutaneous tissues relative to body mass was higher in HF diet-fed chickens than in LF diet-fed chickens, in line with previous work showing that high dietary energy promotes lipid deposition (Eits et al., 2002). The favourable effect of the HF diet on body mass and fat deposition can be ascribed to the higher energy ingestion, despite the lower food intake. In addition, fat accumulation is greater when more energy comes from dietary fat than from carbohydrates or proteins (Horton et al., 1995; Lean and James, 1988).

Chickens fed for 35 days with the HF diet maintained a lower food intake than those fed the LF diet, even when the diet was

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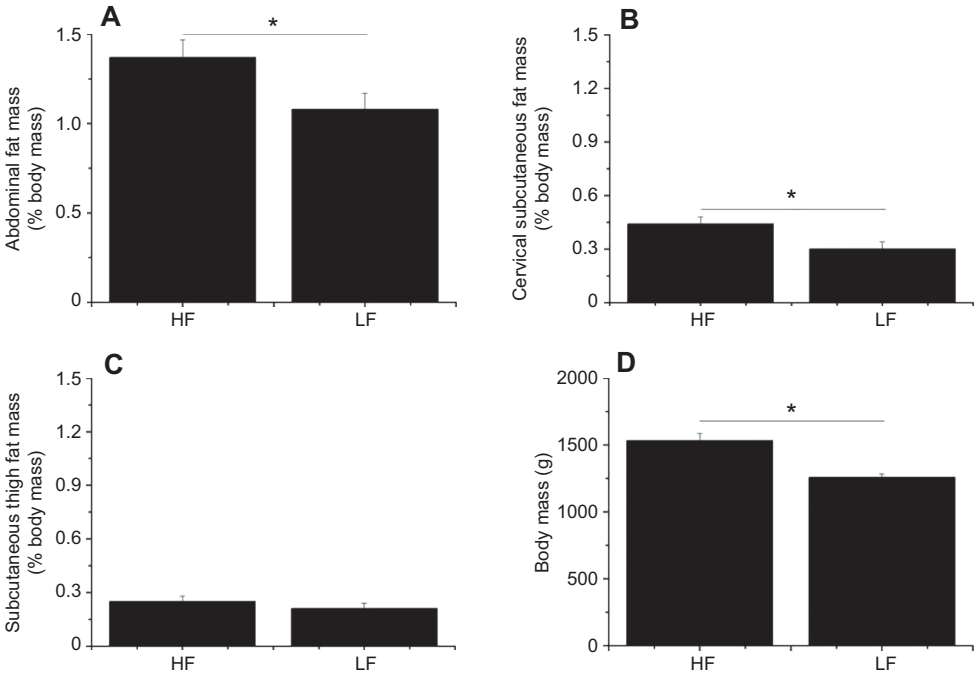

Fig. 3. Effects of the HF diet on fat deposition and body mass in chickens after 35 days. (A) Abdominal fat, (B) cervical subcutaneous fat, (C) subcutaneous thigh fat and (D) body mass. Body mass was measured at the end of the experiment and is given per bird. Fat mass is expressed as a percentage of body mass. All data were subjected to one-way ANOVA. Values are means±s.e.m. (*N*=8). *Significant difference (*P*<0.05).

altered during the re-feeding period. These results could be explained by the following. (1) Chickens have a short-term memory function regarding food intake; according to studies in humans (Higgs, 2002), a recent eating episode may be factored into decisions about how much to consume at the next meal. (2) Food intake is adjusted in response to changes in body fat content; the increased leptin activity following body fat deposition decreases food intake by increasing the hindbrain response to satiety signals (Schwartz et al., 2000). The positive energy balance induced by HF inhibits the rewarding properties of food while enhancing meal-induced satiety, thereby reducing food intake (Marx, 2003; Morton et al., 2014).

In mammals, circulating insulin signals the hypothalamus to effect long-term changes in energy balance by activating and/or inhibiting specific anabolic and catabolic pathways (Woods et al., 2006). Insulin receptors have been identified in the brains of chickens (Simon and Leroith, 1986), and intracerebroventricular insulin injection inhibited food consumption in layer hens (Shiraishi et al., 2011). Obrosova et al. (2007) found that a HF diet was accompanied by an increase in insulin; however, in the present

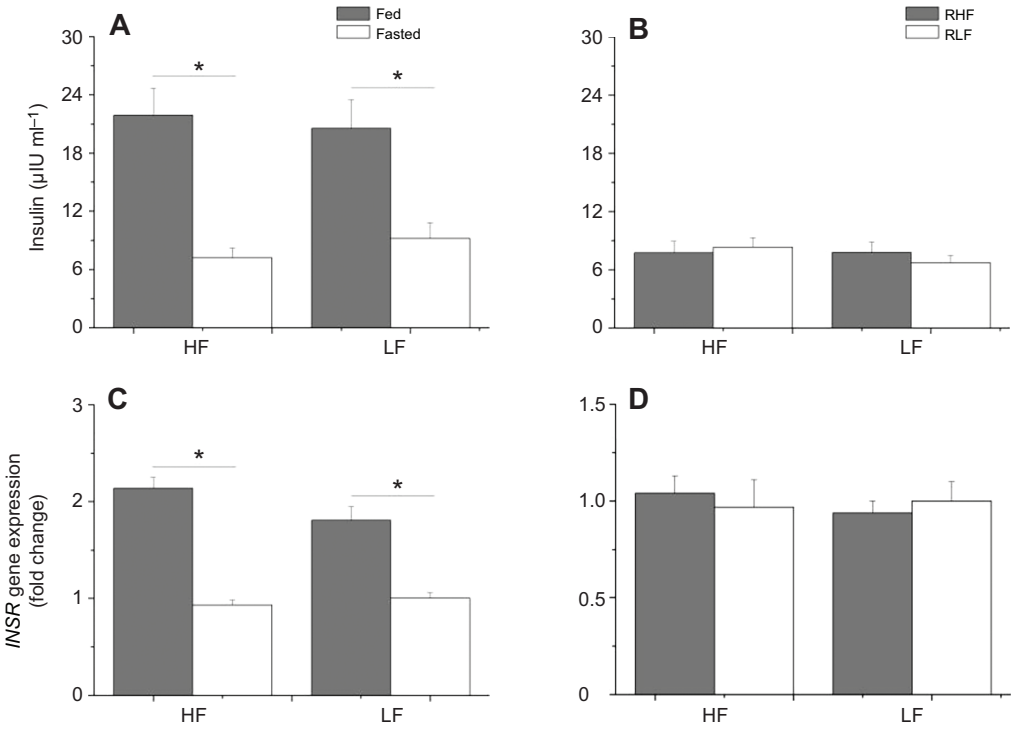

Fig. 4. Effects of the HF diet on plasma insulin levels and hypothalamic insulin receptor (INSR) mRNA expression in chickens under different feeding and re-feeding conditions. (A,C) Insulin (A) and INSR expression (C; fold increase of LF-fasted levels) in the fed and fasted state. (B,D) Insulin (B) and INSR expression (D; fold increase of LF-RLF levels) following re-feeding with the HF diet (RHF) or LF diet (LFR). Values are means±s.e.m. (*N*=8). *Significant difference (*P*<0.05).

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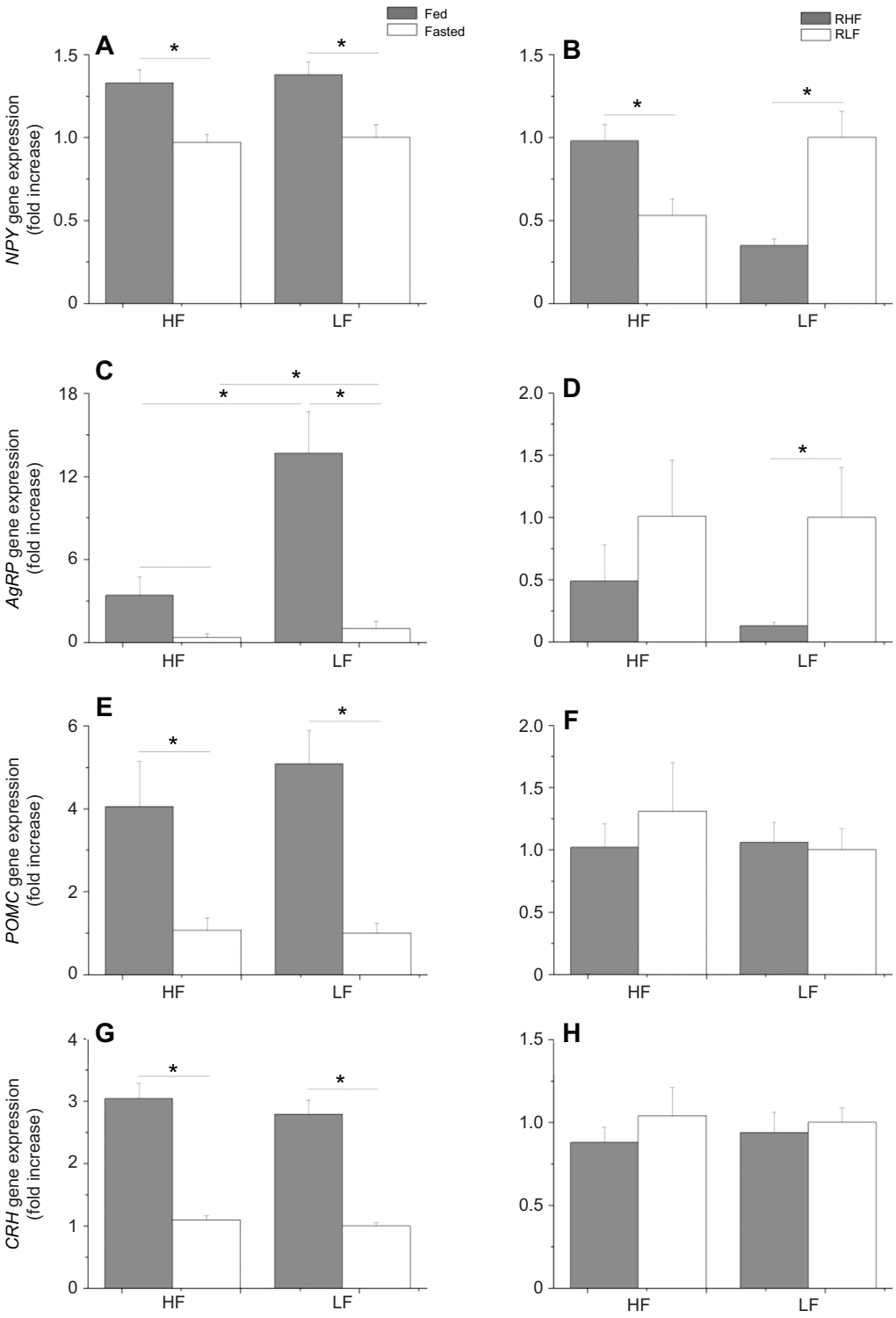

Fig. 5. Effects of the HF diet on the mRNA expression of hypothalamic appetite-related genes in chickens under different feeding and re-feeding conditions. (A,C,E,G) Neuropeptide Y (*NPY*, A), agouti-related peptide (*AgRP*, C), proopiomelanocortin (*POMC*, E) and corticotropin-releasing hormone (*CRH*, G) gene expression (fold increase of LF-fasted levels) in the fed and fasted state. (B,D,F,H) *NPY* (B), *AgRP* (D), *POMC* (F) and *CRH* (H) gene expression (fold increase of LF-RLF levels) following re-feeding with the HF diet (RHF) or LF diet (LFR). Data were subjected to one-way ANOVA. Values are means±s.e.m. (*N*=8). *Significant difference (*P*<0.05).

study, the plasma insulin and hypothalamic INSR mRNA levels were not affected by dietary fat. These results are inconsistent with the previous report that HF induces an increase in insulin concentration compared with a control diet in mice (Obrosova et al., 2007) and rats (Sinitskaya et al., 2007). Our results suggest that insulin secretion is not sensitive to dietary fat. Accordingly, Shiraishi et al. (2011) found that intracerebroventricular insulin treatment did not affect food consumption in chicks under fasted and *ad libitum* conditions. The role of insulin as an afferent signal for energy stores in poultry remains to be determined.

Involvement of AgRP in the HF-induced decrease in food intake

Multiple neuronal populations distributed throughout the brain influence the decision to seek and consume food. AgRP neurons are thought to positively regulate feeding behaviour. AgRP increased food intake when injected into the brain (Hahn et al., 1998). Previous studies have shown that mice on a high saturated fat diet for 1, 4 and 7 weeks had decreased ARC AgRP mRNA levels (Wang et al., 2002). Similarly, our study showed that the HF diet-fed chickens had a lower AgRP mRNA level than the LF

diet-fed chickens under both fed and fasted conditions. Hypothalamic AgRP neuron activation might be associated with the higher food intake in LF diet-fed chickens, confirming the previous finding on the orexigenic effect of hypothalamic AgRP. In contrast to AgRP, we found that NPY, POMC and CRH were not affected by 35 days of HF diet in the present study. These results are inconsistent with studies in mammals which showed that NPY, POMC and CRH levels were affected by a HF diet (Guan et al., 1998; Chavez et al., 1998; Lin et al., 2000; Lukaszewski et al., 2013), implying a genetic bluntness in the response to dietary fat in avian species.

Dietary fat alters the response of hypothalamic NPY to subsequent energy intake

Hypothalamic NPY can control energy expenditure and contributes to overall energy homeostasis. Administration of NPY to the paraventricular nucleus (PVH) increases the activity of PVH neurons by presynaptically inhibiting local γ-amino butyric acid release (Cowley et al., 1999) and decreases brown adipose tissue thermogenesis (Egawa et al., 1991). NPY knockout mice have increased energy expenditure (increased O_2 consumption), increased uncoupling protein-1 (UCP-1) in brown adipose tissue and increased cold-evoked thermogenesis in white adipose tissue and are less susceptible to diet-induced obesity (Patel et al., 2006; Chao et al., 2011).

In the present study, chickens fed the HF diet for 35 days to induce obesity (fat) were compared with chickens fed the LF diet for 35 days (lean). During the re-feeding period (Experiment 2), lean chickens in the RHF group had lower NPY expression than chickens in the RLF group, while fat chickens showed the opposite result. Given that NPY decreases body energy expenditure, NPY inhibition by energy input (RHF) in lean chickens demonstrates an increase in energy utilization, implying a self-adjusting response to dietary fat to maintain whole-body energy stores. The findings in fat chickens, however, suggest that NPY-based self-adjusting mechanisms do not readily occur in fat chickens, and the impaired response of NPY to the energy intake in these chickens leads to a persistent dysregulation of energy balance. On the whole, the NPY response to dietary fat can vary among lean and fat individuals, and depends upon whole-body energy reserves and feeding experience.

Nevertheless, the disparity between lean and fat individuals in response to different re-feeding diets (Experiment 2) was not apparent for the different feeding statuses (Experiment 1) – all of the neuropeptides tested were lower in chickens under fasted compared with fed conditions independent of the diet that was previously consumed. These results are in accordance with previous studies of POMC (Brady et al., 1990) and CRH (Fekete et al., 2000), but inconsistent with results on NPY/AgRP (Morton and Schwartz, 2001; Flier, 2004). These conflicts might be attributed to fasting duration and species variation.

In summary, a HF diet results in decreased food intake even when the subsequent diet is altered. Additionally, hypothalamic AgRP inhibition might be associated with the lower food intake in HF diet-fed chickens. Finally, dietary fat alters the response of hypothalamic NPY to subsequent energy intake.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

H.L. conceived and designed the experiments, X.J.W. performed the experiments, S.H.X. and L.L. collected and analyzed the data, X.J.W. interpreted the data and wrote the article, H.C.J. and Z.G.S. revised the article. All authors read and approved the final manuscript.

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