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# A high fat diet enhances the sensitivity of chick adipose tissue to the effects of centrally injected neuropeptide Y on gene expression of adipogenesis-associated factors☆

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ABSTRACT

The purpose of this study was to determine how dietary macronutrient composition and exogenous neuropeptide Y (NPY) affect mRNA abundance of factors associated with lipid metabolism in chick adipose tissue. Chicks were fed one of three isocaloric (3000 kcal metabolizable energy (ME)/kg) diets after hatch: high carbohydrate (HC; control), high fat (HF; 30% of ME from soybean oil) or high protein (HP; 25% crude protein). On day 4 post-hatch, vehicle or 0.2 nmol of NPY was injected intracerebroventricularly and abdominal and subcutaneous fat depots collected 1 h later. In abdominal fat, mRNA abundance of peroxisome proliferator-activated receptor γ (PPARγ) and fatty acid binding protein 4 (FABP4) increased after NPY injection in HF diet-fed chicks. NPY injection decreased expression of PPARγ and sterol regulatory element-binding transcription factor 1 (SREBP1) in the subcutaneous fat of HC diet-fed chicks, whereas SREBP1 expression was increased in the subcutaneous fat of HF diet-fed chicks after NPY injection. An acutely increased central concentration of NPY in chicks affects adipose tissue physiology in a depot- and diet-dependent manner. The chick may serve as a model to understand the relationship between diet and the brain-fat axis' role in maintaining whole body energy homeostasis, as well as to understand metabolic distinctions among fat depots.

## 1. Introduction

Dietary macronutrient composition affects adipose tissue physiology in mammalian and avian species. Consumption of a high fat (HF) diet was associated with increased adipocyte volume in rats (Aslan et al., 2006). Effects on adipose tissue are depot-dependent in that visceral fat expands predominantly by hypertrophy while hyperplasia contributes more to subcutaneous fat development in mice fed a HF diet (Joe et al., 2009). Consumption of a high protein (HP) diet was associated with decreased gene expression of adipogenesis-associated transcription factors in the adipose tissue of 4 day-old chicks, and differences in adipocyte morphology and plasma non-esterified fatty acids (NEFAs) from chicks that consumed high carbohydrate (HC) or HF diets (Wang et al., 2017).

Adipose tissue development is also influenced by appetite-regulating peptides, such as neuropeptide Y (NPY), a 36 amino acid neurotransmitter that is involved in the regulation of energy balance. Obesity is associated with elevated hypothalamic NPY while induction of NPY expression in the hypothalamus results in obesity (la Fleur et al., 2010; Tiesjema et al., 2007; Wilding et al., 1993). NPY is a potent orexigenic factor that increases food intake in both mammalian (Clark et al., 1984) and avian species (Kuenzel et al., 1987), and the magnitude of response in chicks is affected by dietary macronutrient composition (McConn et al., 2017; Nelson et al., 2015). Besides its orexigenic effects, central NPY injection is associated with enhanced lipid storage in white adipose tissue (WAT) in rats (Billington et al., 1991), and our group demonstrated that NPY promotes adipogenesis during the early and later stages of chicken preadipocyte differentiation (Shipp et al., 2016; Zhang et al., 2015).

Thus, based on previous studies showing a role for NPY in regulating both appetite and adiposity in chickens and effects of dietary macronutrient composition on the appetite response to NPY and adipose tissue development in chicks, this study was designed to determine how dietary macronutrient composition and centrally-administered NPY affect gene expression of adipogenesis-associated factors in chicks.

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**Table 1**
Ingredient and chemical composition of experimental diets.

| Ingredient (g/kg)[a] | High carbohydrate | High protein | High fat |
|---|---|---|---|
| Corn grain | 603.7 | 436.1 | 312.8 |
| Soybean meal | 339.9 | 488 | 369.4 |
| Soy hulls | 0 | 0 | 172.7 |
| Dicalcium phosphate | 15 | 14.2 | 15.6 |
| Limestone | 12.1 | 11.4 | 8.9 |
| Soybean oil | 11.3 | 36.5 | 102 |
| Vitamin/mineral premix[b] | 10 | 10 | 10 |
| Methionine 99% | 2.6 | 1.2 | 3.2 |
| L-Lysine HCl 78% | 1.9 | 0 | 1.6 |
| Sodium bicarbonate | 1.5 | 1.5 | 1.5 |
| L-Threonine | 0.9 | 0 | 1.2 |
| Coban 90[c] | 0.5 | 0.5 | 0.5 |
| Choline-Cl 60% | 0.5 | 0.5 | 0.5 |
| Phytase-Ronozyme-10000[d] | 0.1 | 0.1 | 0.1 |
| Kcal ME/kg | 3000 | 3000 | 3000 |
| Crude protein[e] | 22% | 25% | 22% |
| Crude fat[e] | 3.0% | 4.6% | 10.2% |

[a] Diets were formulated to meet or exceed minimum recommended specifications for Cobb-500 broilers during the starter phase (Cobb-Vantress).
[b] Guaranteed analysis (per kg of premix): Manganese, 25.6 g; selenium, 120 mg; zinc, 30 g; Vitamin A, 4409, 171.076 IU; Vitamin D3, 1410,934.744 ICU; 13,227.513 IU; d-biotin, 88.183 mg.
[c] Coban 90 (Elanco Animal Health) contains 90 g of Monensin sodium per pound of premix and is included in the diet as a coccidiostat.
[d] DSM Nutritional Products, Ltd.
[e] Analyzed at Experiment Station Chemical Laboratories at University of Missouri.

## 2. Materials and methods

### 2.1. Experimental animals

Cobb-500 broiler chicks were obtained from a commercial hatchery on the day of hatch, group caged and 1 day later were caged individually in a room at 30 ± 1 °C and 50 ± 5% relative humidity. Chicks were handled daily to adapt to handling and minimize stress, with ad libitum access to diet and tap water. From day of hatch, chicks were assigned to receive one of the three diets, which were formulated to be isocaloric (3000 kcal/kg) as shown in Table 1 (McConn et al., 2016, 2017). The HC diet was formulated to meet the minimum requirements defined for the starter phase of commercial broilers (Cobb-Vantress), the HP diet contained 25% crude protein, and the HF diet was formulated to have 30% of the metabolizable energy derived from soybean oil. Crude protein and fat values were experimentally verified for all diets (Table 1). Experimental procedures were performed according to the National Research Council Publication, Guide for Care and Use of Laboratory Animals and were approved by the Virginia Tech Institutional Animal Care and Use Committee.

### 2.2. Intracerebroventricular (ICV) injection procedure

At day 4 post-hatch, the chicks were assigned to treatment groups (three diet groups receiving injection of vehicle or 0.2 nmol NPY; 6 total groups with n = 10 chicks per group) using a randomized complete block design with body weight as the blocking factor. Chicks were injected using a method adapted from Davis et al. (1979) that does not appear to induce physiological stress (Davis et al., 1979; Furuse et al., 1999). The dose of chicken NPY was the same as that used in our previous study that examined the effects of diet and central NPY on dietary preference and hypothalamic gene expression (McConn et al., 2017). Chicken NPY (YPSKPDSPGEDAPAEDMARYYSALRHYINLITRQRY, AnaSpec, San Jose, CA, USA) was dissolved in artificial cerebrospinal fluid (Anderson and Heisley, 1972) as a vehicle for a total injection volume of 5 µL with 0.06% Evans Blue dye to facilitate injection site localization. After injection, chicks were returned to their home cages and food was withheld in order to eliminate effects of differential food intake on metabolic changes in the adipose tissue. After data collection, chicks were decapitated and the head sectioned along the frontal plane to determine the site of injection. Any chick without dye present in the lateral ventricle system was eliminated from analysis. Sex of chicks was determined visually by dissection. The number of chicks for each experiment are depicted in the figure legends.

### 2.3. Total RNA extraction, reverse transcription, and real time PCR

At 1 h post-injection, chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture and decapitated. Adipose tissue depots, including abdominal and subcutaneous, were collected and processed for total RNA isolation as described (Wang et al., 2017). Briefly, samples were homogenized in 1 mL Isol RNA Lysis reagent (5-Prime, Gaithersburg, MD, USA) using 5 mm stainless steel beads (Qiagen, Valencia, CA, USA) and a Tissue Lyser II (Qiagen). Total RNA was separated following the manufacturer's instructions (5-Prime) and following the step of addition to 70% molecular-grade ethanol, samples were transferred to spin columns and further purified using the RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized from 200 ng total RNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Primers for real time PCR were designed with Primer Express 3.0 (Applied Biosystems) and validated for amplification efficiency before use (Table 2). Real time PCR was performed in duplicate in 10 µL reactions that contained 5 µL Fast SYBR Green Master Mix (Applied Biosystems), 0.25 µL of 5 µM forward primer, 0.25 µL of 5 µM reverse primer, 1.5 µL of nuclease free water and 3 µL of 10-fold diluted cDNA using a 7500 Fast Real-Time PCR System (Applied Biosystems). A dissociation step was performed at the end of each PCR reaction to ensure amplicon specificity.

### 2.4. Statistical analysis

The real time PCR data were analyzed using the ΔΔCT method, where $\Delta CT = CT_{target\ gene} - CT_{actin}$, and $\Delta\Delta CT = \Delta CT_{target\ sample} - \Delta CT_{calibrator}$ (Schmittgen and Livak, 2008). The average of subcutaneous fat from chicks fed the HC diet that also received the vehicle treatment was used as the calibrator sample. The fold difference (relative quantity; RQ) was calculated as $2^{-\Delta\Delta CT}$. Analysis of variance was performed with RQ values using the Fit Model platform of JMP Pro11 (SAS Institute, Cary, NC). The statistical model included the main effects of diet, NPY treatment and the interaction between them, within an adipose tissue depot. Sex was excluded as initial tests revealed that no effect involving sex was significant. Significant dietary effects were separated using Tukey's test and significant interactions separated with the Slice function of JMP and effects sliced within dietary group for each gene. When Slices were significant, a secondary ANOVA was performed with the model including the main effect of treatment within the diet and Tukey's test was used to separate the means for significant treatment effects. Significance was assigned at $P < 0.05$.

Data were transformed (Kirk, 1982) as follows to reduce heterogeneity of variance, where x is the relative quantity value: 1/x: neuropeptide Y (NPY) (both depots), NPY receptor 2 (NPYR2) (both depots), CCAAT/enhancer-binding protein beta (C/EBPβ) (both depots), thioredoxin-dependent peroxidase 2 (TPX2) (abdominal), adipose triglyceride lipase (ATGL) (abdominal), comparative gene identification-58 (CGI58) (abdominal), GATA Binding Protein 2 (GATA2) (abdominal), Ki67 (subcutaneous), lipoprotein lipase (LPL) (subcutaneous), 1-acylglycerol-3-phosphate O-acyltransferase 9 (AGPAT9) (subcutaneous), and fatty acid synthase (FASN) (subcutaneous); Square root (x) + square root (x + 1): LPL (abdominal), acyl-CoA dehydrogenase, long chain (ACADL) (abdominal), and monoglyceride lipase (MGLL) (subcutaneous); Ln(x + 1): perilipin 1 (PLN1) (both depots), MGLL (abdominal), ACADL (subcutaneous), CGI58 (subcutaneous), and GATA2 (subcutaneous); Log10(x): AGPAT9 (abdominal) and ATGL (subcutaneous); Log10(x + 1): CCAAT/enhancer-binding protein alpha (C/EBPα) (both depots), sterol regulatory element-binding transcription