**Dietary Macronutrient Composition Affects the Influence of Exogenous Prolactin-Releasing Peptide on Appetite Responses and Hypothalamic Gene Expression in Chickens**

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Abstract

**Background:** The interaction between the effects of exogenous neurotransmitters and dietary composition on appetite regulation in nonmammalian species is unclear.

**Objective:** The objective of this study was to determine the effects of exogenous prolactin-releasing peptide (PrRP) and dietary macronutrient composition on food intake regulation in broiler chicks.

**Methods:** Three isocaloric diets were formulated: high-carbohydrate (HC), high-fat (HF; 60% of ME from lard) and high-protein (HP) diets. In Expt. 1, 4-d-old Hubbard 3 Cobb-500 chicks fed 1 of the 3 diets since hatch were intracerebroventricularly injected with 0 (vehicle), 3, or 188 pmol PrRP (n = 10). Food intake was measured for 180 min. In Expt. 2, hypothalamic mRNA abundance of appetite-associated factors was measured in hypothalamus samples obtained 1 h postinjection of 0 or 188 pmol PrRP. In Expt. 3, chicks were given free access to all diets before and after intracerebroventricular injection and food intake was measured.

**Results:** Three and 188 pmol PrRP increased (P = 0.0008 and 0.04) HP diet intake, but only 188 pmol PrRP was efficacious at increasing HC (P = 0.0011) and HF (P = 0.01) consumption compared with the vehicle. There was a diet effect on mRNA abundance of all genes (P < 0.05), with greater expression in chicks fed the HF or HP than the HC diet. Whereas neuropeptide Y (NPY) mRNA was similar between vehicle- and PrRP-injected chicks that consumed HP or HF diets, expression was greater (P < 0.05) in PrRP- than vehicle-injected chicks that consumed the HC diet. When chicks had access to all diets, 188 pmol PrRP caused preferential (P < 0.0001) intake of the HP over the HC and HF diets.

**Conclusion:** The HP diet enhanced the sensitivity of chicks to the food intake–stimulating effects of PrRP, and PrRP in turn increased preference for the HP diet. Thus, dietary macronutrient composition influences PrRP-mediated food intake, and PrRP in turn affects nutrient intake and transcriptional regulation in chicks.

Keywords: prolactin-releasing peptide, macronutrient, food intake, chicken, hypothalamus

Introduction

Dietary macronutrient composition plays an important role in regulating appetite and body weight composition, and animals will select for specific nutrients in the diet. For instance, the preference of rats for protein was affected by the protein content of their previous meal, with a high-protein (HP) diet leading to selection of carbohydrates and vice versa (1). In general, HP diets reduce food intake (1, 2), whereas diets low or deficient in protein stimulate hyperphagic behavior (3–5). There are also reports on the effect of dietary macronutrient composition on food intake in chickens, with more research focused on the effect of different amounts of dietary protein than dietary fat and carbohydrate (6).
For example, HP diets were associated with decreased food intake in 15- to 27- (7) and 7-d-old broiler chickens (8).

The effect of dietary nutrients on food intake in mammals is influenced by exogenous appetite-related neuropeptides. Central administration of galanin stimulated the ingestion of fat and, to a lesser extent, carbohydrates, but not protein (9). In rodents, central injection of neuropeptide Y (NPY) increased carbohydrate (10–13) and fat (10, 14) intake, leading to obesity (15, 16). Macronutrient composition in turn influenced the effects of NPY on appetite. After 4 wk of consuming diets that differed in macronutrients, free-choice rats showed increased sensitivity in their food intake response of feed and fat but not sugar to exogenous NPY (17). We recently demonstrated that NPY also selectively influenced consumption of carbohydrate, fat, and protein in chicks, with central NPY injection increasing intake of an HP and high-carbohydrate (HC) but not high-fat (HF) diet under a free-choice scenario (18). We also showed that diet modulated the effects of NPY on food intake, with an HF diet enhancing NPY sensitivity (more robust increase in food intake and longer duration of response).

Prolactin-releasing peptide (PrRP) is another potent orexigenic factor in chickens (19). Since it was first described as a hypothalamic prolactin releasing factor in cultured mammalian pituitary cells (20), many other physiologic functions have been ascribed to PrRP, including effects on energy metabolism, cardiovascular regulation, and sleep and pain mediation (21). Central injection of PrRP decreases food intake in rats, and hypothalamic PrRP mRNA is reduced in a negative energy balance state, such as during lactation and fasting in female rats (22). However, central administration of PrRP increases food intake in chickens, even at very low doses (19), although it is unknown whether its appetite-related functions are affected by dietary composition. Because there was an interaction between exogenous NPY and dietary macronutrient composition on food intake in chicks, and because PrRP is also an extremely potent orexigenic factor in chicks that was shown to be affected by nutrition status in rodents, the objective of this study was to investigate the effects of dietary macronutrient composition on the orexigenic effects of PrRP in broiler chicks.

**Methods**

**Chicks.** Hubbard × Cobb-500 day-of-hatch unsexed chicks (broiler-type chicks) were obtained from a local hatchery and caged individually in a room at a temperature of 30 ± 1°C and 50 ± 5% relative humidity. Chicks were handled daily to adapt to handling and to minimize stress during data collection, with ad libitum access to feed and water. Diets were formulated (Supplemental Table 1) and mixed at Augusta Cooperative Feed Mill. The HC diet was formulated to meet the minimum requirements defined for the starter phase of commercial broilers serving as a broiler starter industry standard starter diet (23). The HP diet was formulated to contain 30% crude protein and the HF diet to have 60% of the metabolizable energy derived from calories in refined lard, which is designed to be similar to a common rodent obesogenic diet (24). Diets were isocaloric and isonitrogenous (except for the HP diet) and formulated to meet minimum digestible amino acid requirements for commercial chicks (Supplemental 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 Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University.

**Intracerebroventricular injection procedure.** Chicks were injected with the use of an adapted method (25) that does not appear to induce physiologic stress (26), PrRP (RatPrRP; 3.994.0 molecular weight, American Peptide) was dissolved in avian artificial cerebrospinal fluid and injected at a total volume of 5 μL with 0.06% Evans blue dye to facilitate injection site localization. At the completion of data collection, chicks were deeply anesthetized with sodium phenobarbital, decapitated, and their brains dissected to determine accuracy of injection into the lateral ventricle. Chicks without dye present in the lateral ventricle were eliminated from the analysis. Sex was determined visually by dissection and gonadal inspection.

**Expt. 1.** In Expt. 1, chicks were randomly assigned to 1 of the 3 diets at day of hatch, with ad libitum access to food and water. On day 4 post-hatch, chicks were randomly assigned 1 of 3 intracerebroventricular PrRP doses: 0 (vehicle only), 3, or 188 pmol (n = 10 chicks per diet and intracerebroventricular treatment group). After intracerebroventricular injection, chicks were returned to their cages with continued ad libitum access to food and water. Food intake was quantified at 30 min intervals up to 180 min after injection. Food intake data were converted to a percentage of body weight by dividing food weight consumed by the chick’s body weight at injection time and multiplying by 100. All Expts. were replicated and the effect of replicate was not significant; thus, data were pooled. Data were analyzed with the use of 1-factor ANOVA within each diet and within each time point with the SAS 9.3 GLM procedure and the statistical model included the main effects of treatment. Sex was not significant in any Expt. and was removed from the model. Tukey’s method was used post hoc to separate the means. All data are presented as means ± SEs and differences considered significant at P < 0.05 for all Expts.

**Expt. 2.** In Expt. 2, chicks were randomly assigned to 1 of the 3 diets at day of hatch, with ad libitum access to food and water. On day 4 post hatch, chicks were randomly assigned to receive vehicle or 188 pmol PrRP via intracerebroventricular injection. After injection, food was withheld to prevent effects associated with food consumption (n = 10 chicks per diet and treatment group). Sixty minutes after injection, chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture and decapitated, brains were removed and hypothalamus was isolated as we described (27). Total RNA isolation, reverse transcription, and real-time PCR were performed as described (27). Briefly, the hypothalamus was collected in RNA later (Qiagen) and homogenized with the use of 5 mm stainless steel beads and 1 mL Isol- RNA Lysis reagent (5-PRIME) for 2 × 2 min at 20 Hz with a Tissue Lyser II (Qiagen). After the step involving addition to 70% ethanol, total RNA was isolated with an RNaseasy Mini Kit (Qiagen), including the optional on-column RNase-free DNase I step (Qiagen). Single-strand cDNA was synthesized with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primers for real-time PCR are listed (Supplemental Table 2) and amplification efficiency was validated for all primer pairs before use (95–100% efficiency). Real-time PCR reactions were performed in duplicate with Fast SYBR Green (Applied Biosystems) and a 7500 Fast instrument (Applied Biosystems). Data were analyzed with the ΔΔCt method, where β-actin served as the reference gene and the average of the vehicle-treated chicks fed the HC diet served as the calibrator for all samples. Relative quantity values (2−ΔΔCt) were used for statistical analysis with the SAS 9.3 GLM procedure, where the model included the effects of treatment and diet and the interaction between them. Tukey’s test was used for pairwise comparisons.

**Expt. 3.** In Expt. 3, procedures were the same as in Expt. 1, except that each chick had access to all of the 3 diets before and after intracerebroventricular PrRP injection (n = 10 chicks per intracerebroventricular treatment group). The position of the 3 diets was randomized at day of hatch and maintained until the end of the Expt. Data were analyzed with the use of 2-factor ANOVA within treatment with the SAS 9.3 GLM procedure, and the statistical model included time and diet and their interaction. The diet × time interaction was significant and thus secondary ANOVAs were conducted within each time point. Sex was not significant in any Expt. and was removed from the model. Tukey’s method was used post hoc to separate the means.

**Results**

**Expt. 1.** At 4 post-hatch, body weight was greater in the HC group (70.1 g) than in the HP group (52.8 g; P < 0.001); it was intermediate and not different from the other 2 in the HP group.
Abundance of neuropeptide Y receptor subtype 5 (NPYR5) and melanocortin receptor 4 (MC4R) was greater (P < 0.0001) in chicks fed the HF diet than in chicks fed either of the other 2 diets. Expression of melanocortin receptor 3 (MC3R) and melanocortin receptor 4 (MC4R) was greater (P < 0.0002) in chicks fed the HP and HF diets than in those fed the HC diet. Abundance of neuropeptide Y receptor subtype 5 (NPYR5) mRNA was greatest (P < 0.0001) in the HP-fed group, intermediate in the HF-fed chicks, and lowest in the HC group. There was greater mRNA abundance of PrRP (P < 0.001), corticotropin-releasing factor (CRF) (P = 0.0069), and neuropeptide Y receptor subtype 1 (NPYR1) (P < 0.0001) in chicks fed the HP diet than in chicks fed the HC and HF diets. Expression of oxytocin (OXT) (P = 0.0004) and orexin (ORX) (P = 0.01) was greater in chicks fed the HF diet than in those fed the HC diet.

There was a main effect of PrRP treatment on mRNA abundance of NPYR2 (P = 0.01), which was decreased after PrRP administration. There was an interaction of diet and treatment (P = 0.04) on mRNA abundance of NPY (Expt. 2), where PrRP treatment increased NPY mRNA abundance in chicks that consumed the HC diet, but not the HF or HP diets at 1 h post-injection.

Expt. 3. In general, irrespective of treatment, chicks selected the HC and HP diets over the HF diet (Figure 3). The administration of 3 pmol PrRP did not change the preference for the HC and HP diets; however, chicks that received 188 pmol PrRP consumed more of the HP diet than either the HC or HF diet (P < 0.0001), although for 60 min postinjection there was no significant difference between consumption of HC and HF diets. At 180 min postinjection in vehicle-treated chicks, the percentage of HC, HF, and HP diet consumption as a fraction of total diet consumed was 38%, 9%, and 53%, respectively; the percentage for chicks treated with 188 pmol PrRP was 29%, 9%, and 62, respectively.

Discussion

Consistent with previous reports (19, 28), intracerebroventricular PrRP increased food intake in chicks. Little is known about how PrRP affects macronutrient selection (22), particularly in chicks, in which it has the opposite effect on food intake than with mammals. In a study that used the same diet formulations as reported for the present study, intracerebroventricular NPY dose-dependently increased food intake in chicks fed the HF diet, with the highest magnitude of increase in chicks treated with 2 nmol NPY at 180 min after injection (18). In general, the HF diet increased sensitivity to exogenous NPY, and NPY in turn increased selection of the HC and HP diets, but not the HF diet (18). In the present study, chicks that were fed the HF diet and received 188 pmol PrRP also had the highest magnitude of food intake. Unlike in the NPY study, however, the dose-dependent response in food intake only occurred in chicks fed the HP diet, which implies that dietary macronutrient composition also affects the response to specific exogenous neuropeptides. That NPY and PrRP are both potent orexigenic in chicks but are affected differently by diet composition implies that their mechanism of action is different and/or that dietary macronutrient composition affects distinct neurotransmitter signaling pathways in the brain.

Unlike the previous study with NPY that focused solely on measuring food intake responses in chicks (18), the present study also included an Expt. designed to investigate the hypothalamic molecular mechanism mediating the differential response to PrRP in chicks that consumed different diets. For Expt. 2, a single dose of PrRP was used that corresponded to the greatest effect on food intake in Expt. 1 in order to maximize the potential for capturing differences in gene expression. Overall, most of the appetite-associated genes tested (except for PrRP, OXT, ORX, and CRF) were expressed less in the hypothalamus of chicks fed the HC diet.
diet than those fed the other 2 diets. That all genes were affected suggests that most changes in gene expression were generalized responses to the different diets. There was increased hypothalamic NPY and AgRP mRNA in the hypothalamus of chicks that ate the HF diet compared with chicks fed the HC and HP diets, contradictory to a rodent study in which rats fed an HC diet had increased hypothalamic NPY expression compared with rats that consumed an HF diet (29). Moreover, PrRP injection was associated with increased NPY mRNA abundance in chicks that were fed the HC diet. NPY is one of the most potent orexigenic factors in chicks and AgRP increases food intake in rats (30, 31). Although central AgRP injection did not increase food intake in broiler chickens, it is still important in the regulation of appetite, because the anorexigenic effect of α-melanocyte-stimulating hormone in chicks is attenuated by intracerebroventricular AgRP (32).

Chicks fed the HP diet had a lower threshold response in food intake to exogenous PrRP. This increased sensitivity to PrRP could be related to the increased expression of PrRP, NPYR1, NPYR5, and MC4R mRNA in chicks fed the HP diet compared with chicks fed the HF and HC diets. NPYR2 and NPYR5 may be associated with the regulation of food intake in chickens; however, the fact that activation of NPYR2 by NPY (13–36) only increased food intake at 30 min postinjection may indicate that the role of NPYR2 in food intake is weaker than that of NPYR5 (33).

The effect of intracerebroventricular PrRP on NPY mRNA abundance in chicks that consumed the HC diet is consistent with a previous report (that used the same HC diet) (37). According to rodent studies, there are NPY-expressing neurons in the paraventricular nucleus (PVN) and the expression of NPY is c-Fos dependent (34–36, 38). This may indicate that the orexigenic effect of PrRP in chicks may be associated with upregulated NPY mRNA of PVN origin. Thus, the effects of PrRP on food intake may involve transcriptional regulation of appetite-associated factors in the hypothalamus, although results should be interpreted with caution without accompanying peptide abundance data for specific hypothalamic nuclei at additional time points.

This study also revealed that, when given a choice of diets, chicks selected the HC and HP diets over the HF diet and that PrRP enhanced the preference for the HP diet. When rats were offered similar diets, the HP diet was the least consumed, with HC or HF diets being the most preferred (13). To explain differences across species is beyond the scope of our study, but may involve differences in age- and species-specific physiology; source, quantity, and balance of nutrients; duration of the feeding trial and timing and type (e.g., meal vs. continuous access) of feeding; and interaction of other nutrients in affecting physiology. According to studies conducted with rodents, HP diets tend to be more satiating than HC and HF diets (2), but in our study, the HF was the least consumed in a choice environment. Consumption of the HF diet also induced the greatest changes in gene expression of appetite-related factors in the hypothalamus, with most genes evaluated being more highly expressed in the HF group. In other studies, adult rodents consumed different diets before switching to the experimental diets. In the present study, chicks were fed experimental diets immediately after hatch, which is advantageous because it allows us to understand how the physiology of the animal is affected by diet without the influence of previous nutrition. These findings may have relevance for treating eating disorders because they demonstrate that the appetite-associated

### TABLE 1

Expt. 2: Hypothalamic mRNA abundance of appetite-associated neurotransmitters and receptors in 4-d-old broiler chicks fed different diets and treated with central injection of PrRP

<table>
<thead>
<tr>
<th>Effects</th>
<th>AgRP</th>
<th>PrRP</th>
<th>OXT</th>
<th>NPY</th>
<th>ORX</th>
<th>CRF</th>
<th>NPYR1</th>
<th>NPYR2</th>
<th>NPYR5</th>
<th>MC3R</th>
<th>MC4R</th>
</tr>
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<tbody>
<tr>
<td>Diet</td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>HC</td>
<td>1.10 ± 0.09</td>
<td>1.05 ± 0.05</td>
<td>0.95 ± 0.04</td>
<td>1.06 ± 0.03</td>
<td>0.96 ± 0.04</td>
<td>1.01 ± 0.07</td>
<td>0.99 ± 0.04</td>
<td>0.98 ± 0.05</td>
<td>0.97 ± 0.04</td>
<td>0.96 ± 0.03</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>HF</td>
<td>2.88 ± 0.15</td>
<td>0.96 ± 0.05</td>
<td>1.27 ± 0.05</td>
<td>1.42 ± 0.04</td>
<td>1.13 ± 0.05</td>
<td>1.12 ± 0.08</td>
<td>1.17 ± 0.04</td>
<td>2.24 ± 0.06</td>
<td>1.57 ± 0.05</td>
<td>1.10 ± 0.03</td>
<td>1.19 ± 0.03</td>
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<tr>
<td>HP</td>
<td>1.62 ± 0.13</td>
<td>1.43 ± 0.08</td>
<td>1.11 ± 0.06</td>
<td>1.23 ± 0.03</td>
<td>1.05 ± 0.03</td>
<td>1.53 ± 0.12</td>
<td>1.69 ± 0.09</td>
<td>1.36 ± 0.05</td>
<td>2.13 ± 0.09</td>
<td>1.17 ± 0.05</td>
<td>1.40 ± 0.06</td>
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<td>0.0006</td>
<td>&lt;0.0001</td>
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<td>Dose</td>
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<tr>
<td>0</td>
<td>1.85 ± 0.20</td>
<td>1.13 ± 0.06</td>
<td>1.09 ± 0.04</td>
<td>1.23 ± 0.05</td>
<td>1.06 ± 0.04</td>
<td>1.25 ± 0.08</td>
<td>1.31 ± 0.07</td>
<td>1.60 ± 0.12</td>
<td>1.58 ± 0.10</td>
<td>1.11 ± 0.03</td>
<td>1.17 ± 0.04</td>
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<td>188</td>
<td>1.80 ± 0.15</td>
<td>1.16 ± 0.06</td>
<td>1.13 ± 0.06</td>
<td>1.24 ± 0.03</td>
<td>1.03 ± 0.03</td>
<td>1.19 ± 0.09</td>
<td>1.25 ± 0.07</td>
<td>1.44 ± 0.10</td>
<td>1.54 ± 0.11</td>
<td>1.04 ± 0.04</td>
<td>1.21 ± 0.05</td>
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<td>P</td>
<td>0.72</td>
<td>0.65</td>
<td>0.55</td>
<td>0.82</td>
<td>0.51</td>
<td>0.61</td>
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<td>0.01</td>
<td>0.65</td>
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<tr>
<td>Interaction²</td>
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<td>0.95</td>
<td>0.80</td>
<td>0.04</td>
<td>0.13</td>
<td>0.79</td>
<td>0.97</td>
<td>0.50</td>
<td>0.60</td>
<td>0.39</td>
<td>0.33</td>
</tr>
</tbody>
</table>

1 Values are least squares means ± SEMs, n = 7–10 for main effects of diet, PrRP treatment, and P values for the main effects and the 2-way interaction of diet and treatment. Labeled means in a column within an effect without a common letter differ, P < 0.05 (Tukey’s test). AgRP, agouti-related peptide; CRF, corticotropin-releasing factor; HC, high-carbohydrate; HF, high-fat; HP, high-protein; MC3R, melanocortin receptor 3; MC4R, melanocortin receptor 4; NPY, neuropeptide Y; NPYR1, neuropeptide Y receptor subtype 1; NPYR2, neuropeptide Y receptor subtype 2; NPYR5, neuropeptide Y receptor subtype 5; ORX, orexin; OXT, oxytocin; PrRP, prolactin-releasing peptide.  
2 Hypothalamic mRNA abundance.  
3 P value for the 2-way interaction of diet and PrRP dose on mRNA abundance.

### FIGURE 2

Expt. 2: Expression of hypothalamic NPY mRNA in chicks fed the HC, HF, or HP diet that received either vehicle or 188 pmol PrRP. There was an interaction between diet and PrRP treatment (P = 0.04). Values are means ± SEMs, n = 7–10. *Different from vehicle within diet, P < 0.05. HC, high-carbohydrate; HF, high-fat; HP, high-protein; NPY, neuropeptide Y; PrRP, prolactin-releasing peptide.
effects of neurotransmitters in the brain can be influenced by nutritional history and also that the macronutrient composition of the present diet in turn affects neurotransmitter signaling to mediate changes in appetite.

In conclusion, chicks fed the HP diet had a lower threshold response in food intake to intracerebroventricular PrRP than those fed the 2 other diets. Most of the genes evaluated in this study were more highly expressed in the hypothalamus of chicks that consumed the HF and HP diets than in those consuming the HC diet. Injection of PrRP increased mRNA abundance of NPY in the hypothalamus of chicks that consumed the HC diet, but not the HP or HF diet. Chicks that were injected with PrRP also selected the HP diet over the HC and HF diets when provided free-choice access to all 3 diets. Results demonstrate that dietary macronutrient composition influences appetite regulation, perhaps via transcriptional regulation of appetite-associated factors in the hypothalamus, and diet also affects PrRP-mediated food intake in chicks, whereas PrRP in turn affects nutrient intake. In exploring strategies to affect appetite in individuals with eating disorders, these results may have implications for highlighting the importance of understanding the effect of background nutrition on neurotransmitter-mediated responses in the brain.

Acknowledgments
GW, ERG, and MAC designed the research; GW and MAC conducted the research; GW analyzed the data; GW, TT, ERG, and MAC wrote the paper; and MAC had primary responsibility for the final content. All authors read and approved the final manuscript.

References

FIGURE 3 Expt. 3: Cumulative food intake expressed as a percentage of body weight of PrRP-injected chicks provided free access to HC, HF, and HP diets simultaneously at 30, 60, and 90 min (A) and 120, 150, and 180 min (B) postinjection. Bars represent means ± SEMs, n = 18 chicks per PrRP dose per diet. There was an effect from diet within each treatment group at each time point (P < 0.05). Bars with different superscript letters are significantly different from one another within a treatment and within a time (P < 0.05; Tukey’s test). HC, high-carbohydrate; HF, high-fat; HP, high-protein; PrRP, prolactin-releasing peptide.