Dietary Macronutrient Composition and Exogenous Neuropeptide Y Affect Feed Intake in Broiler Chicks

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Abstract

Understanding the central nervous system's role in appetite regulation is crucial to cure the obesity epidemic, which is more prevalent than any disease in the United States. Central appetite regulators, known as neuropeptides, are pivotal in understanding appetite regulation. Neuropeptide Y (NPY), a 36 amino acid peptide, plays a major role in regulating the hunger signals from the brain. In all vertebrates studied, it is a strong orexigenic neurotransmitter located throughout multiple nuclei of the hypothalamus. Peripheral hormones associated with hunger are able to activate NPY neurons in the arcuate nucleus, which leads to a cascade of events that activate orexigenic neurons throughout the hypothalamus. Although extensive research has gone into understanding the role of NPY in appetite regulation, the effects of macronutrient composition of diets on NPY function have not been elucidated in non-mammalian species.

This research investigates how food intake is affected by dietary macronutrient composition in broiler type chickens that are fed three varying macronutrient diets: high carbohydrate (22% CP, 3000kcal/kg) a broiler starter diet, high fat (60% ME from lard), high protein 30%CP). All diets were formulated to be isocaloric. When chicks are fed the high fat diet central NPY administration has a greater effect on feed intake compared to both the basal and high protein diet. Regardless of what diet the chick is fed from hatch, if they are switched to one of the other two diets post central administration of NPY the high fat diet stimulated feed intake

for the longest duration. Although, NPY had the strongest orexigenic effect on chicks fed the high fat diet, in a choice diet situation broiler chicks chose the high protein diet, independent of central NPY administration.

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Introduction

The central nervous system plays an integral role in the regulation of feeding behavior and overall energy homeostasis across various species [2]. Orexigenic (increase food intake) and anorexigenic (decrease food intake) signals are crucial in the regulation of appetite and many have been shown to be conserved throughout evolution. Homeostatic appetite control stems from the need to maintain a balanced energy supply within the body in order to support key physiological functions [3]. The steps involved in appetite regulation involve a series of signaling cascades that begin with a pre-prandial phase that includes a multitude of orexigenic signaling pathways, followed by the prandial state during which ingestion occurs, and finally the post-prandial state which is initiated when satiety signals are released to terminate feed intake [4].

The central nervous system communicates with various signaling pathways that perceive levels of nutrients and metabolites available in the body, and adjusts metabolism and energy expenditure to maintain physiological homeostasis. This homeostasis is key in regulating all physiological functions in order to avoid excess stress, energy expenditure, or surplus of energy. There are multiple inputs within the central nervous system that provide the regulatory components essential for appetite control [5]. Peripheral and central stimuli are responsible for appetite control systems in the following manner; neural events will trigger a cephalic phase that initiates a peripheral response, and subsequently translates into neurochemical brain activity which will trigger the behavior of eating [6]. Elucidating the relationship between central and peripheral signaling is crucial for gaining a greater understanding of complete appetite regulation and modulation of energy balance.

The connection between the hypothalamus as a central feeding control center of the brain and the various neuropeptides that influence food intake is a continuously growing field of research [7]. The influence of different neuropeptides on feed intake has been determined under basal diet conditions in the model organism, and more recently, varying macronutrient conditions to understand how protein, lipids, and carbohydrates influence intake.

It is well documented that obesity and its comorbidities occur when energy intake exceeds energy expenditure. Orexigenic neuropeptides increase food intake, and thus it is crucial to understand the link between obesity and orexigenic neuropeptide concentrations. These neuropeptides provide pharmacological targets for regulating hunger and treating obesity. Obesity is a nationwide epidemic, with more than two thirds of the US population classified as overweight or obese [8]. Anorexia, although not as prominent, currently afflicts about 8 million individuals and is also associated with other comorbidities [9]. These applications can also be extrapolated into the field of agriculture, on animals with a higher tendency to deposit excess carcass fat (ex. broilers and swine). One neuropeptide in particular, neuropeptide Y (NPY), has been researched extensively within mammalian models to reveal is orexigenic significance [10].

High fat diets have become popular as a result of increased availability of inexpensive meals through the fast food industry, whereas high protein diets have historically been used as a dieting strategy [11]. Determining how these types of diets affect NPY in chickens will enhance our understanding of how fat and protein influence appetite and feeding behavior from both an agricultural and biomedical standpoint.

To our knowledge, there are no reports of the effects of high fat and high protein diet consumption, on NPY synthesis and activity in an avian species. Neuropeptide Y activity in response to various feeding regimens differs among species, thus it is likely that macronutrient

composition will alter NPY regulation in a species-specific manner. The objective of this study is to investigate the effects that NPY and varying dietary macronutrient composition have on feed intake in HubbardxCobb-500 broiler chickens. Broilers were the chosen avian model because they are highly feed efficient on a basal diet that meets their requirements for metabolizable energy, macronutrients, and micronutrients. Therefore, variations in feed intake associated with NPY administration will likely be due to effects from nutritional variation.

The three dietary treatments are a relatively high fat diet (HF), a relatively high protein diet (HP), and a high carbohydrate diet (HC) that meets the nutrient requirements during the starter phase of the Cobb broiler chicken (Table 1). In the high-fat diet, 60% of the metabolizable energy is derived from fat in lard; while in the high-protein diet, crude protein is formulated to 30% balancing corn and soybean meal. The metabolizable energy is held constant across all three diets to ensure that increased or decreased food intake is not a result of differences in the energy density of the diet. The basal and high fat diet are isonitrogenous.

Literature Review

Appetite Regulation in Rodents

Appetite regulation has been studied extensively in rodents and has provided much of the information known about the central and peripheral signaling pathways associated with food intake. Within the rodent brain there have been many neuropeptides have been identified to have a role in appetite regulation [3].

Specific nuclei within the hypothalamus have been determined to be crucial in energy homeostasis. The arcuate nucleus (ARC), the paraventricular nucleus (PVN), the ventromedial nucleus (VMN), dorsomedial nucleus (DMN), and lateral hypothalamus (LH) affect ingestive

behavior in various ways. A mammalian model of appetite regulation was constructed by Schwartz et al. that diagrams the hypothalamic components of appetite regulation and the relationship between peripheral hormones and central neurotransmitters (figure 1)[1]. This communication between the brain and rest of the body is possible because the ARC lacks a blood brain barrier. In the case of an orexigenic hormone from the periphery acting on the ARC, NPY/AgRP neuronal activity within the ARC will increase and POMC/CART activity will decrease. This will create a signaling event to the PVN where NPY/AgRP expression will increase, not only increasing food intake in that manner, but by also decreasing α-MSH expression by blocking its receptor. These two mechanisms are what ultimately lead to increased food intake and obesity with prolonged activation. Inversely, if an anorexigenic hormone from the periphery signals to the ARC to decrease food intake, POMC/CART neuronal activity will increase, increasing α-MSH, and ultimately decreasing food intake. Prolonged anorexigenic signaling can lead to a state of anorexia.

Lesioning of individual nuclei in rodents confirmed that all are involved in the feeling of satiety. Stimulation of the PVN has been shown to play a role in both hunger and satiety signaling, while the LH is the only area in which lesioning inhibits food intake in the rodent [4-6].

The brain stem has also been identified as a region involved in the regulation of food intake and energy homeostasis. The relationship between gut and brain satiety signals start peripherally from the gastrointestinal tract and relay signals to the solitary tract nucleus (NTS) through the sensory vagus nerve. This connection was discovered when transection of the sensory vagal fiber affected intake by increasing meal size and duration. This result confirmed that vagal afferents are involved in the transmission of satiety signals from the periphery to the

brain [12]. Therefore, while the hypothalamus is crucial to energy homeostasis other areas of the brain cannot be dismissed for playing a role in appetite regulation.

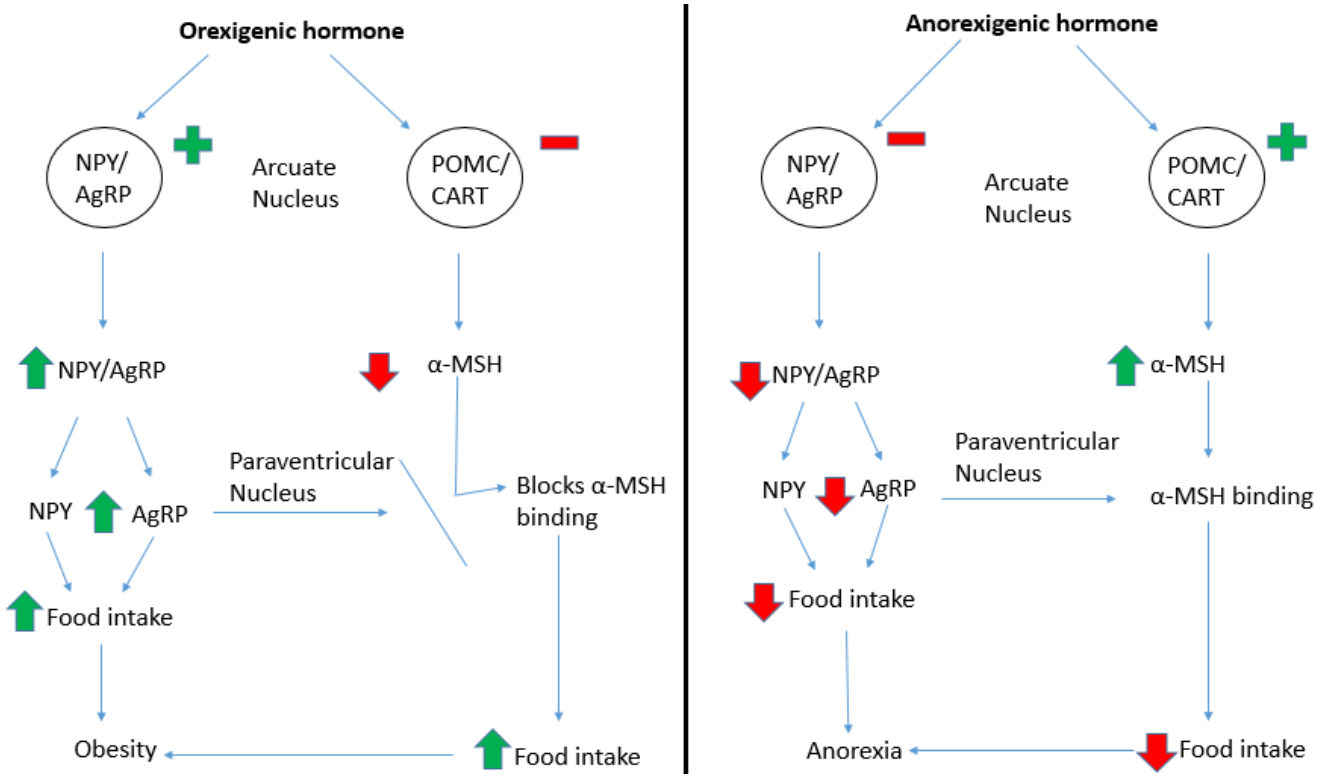

Figure 1. Model of appetite regulation in the hypothalamus (adapted from Schwartz et al.). NPY/AgRP and POMC neurons in the ARC play an essential role in the regulation of appetite. Shown are models of obesity and anorexia in detail to orexigenic and anorexigenic signaling, respectively [1].

Appetite Regulation in Aves

Appetite regulation is most widely understood in mammalian species; much is still unknown about the commonalities and differences that may exist between species. Various chicken breeds such as layers and broilers have been used to understand avian appetite regulation. The ARC and PVN have been identified as playing a significant role in controlling increased feeding behavior across various breeds of chickens [13]. Other neuroanatomical locations (eg. hypothalamic nuclei) and their role in appetite regulation have been recognized as having similar functions between chickens and rats.

Some of the appetite regulating peptides have been shown to not have the same effects on feed intake across species. Ghrelin, which is a known orexigenic hormone in mammals, has been shown to decrease feed intake in chicks [14]. Melanin-concentrating hormone, galanin, motilin, and orexins all increase food intake in rodent models, while they have no influence on food intake in chickens [15,16]. Little research has been published to further investigate why these peptides have opposite or null effects in chickens as compared to rodents.

A factor that may contribute to differences in avian: rodent appetite regulation is the vast difference in gastrointestinal tract structure and function. Rodents being digestion during chewing, where salivary amylase begins hydrolyzing carbohydrates. As a monogastric, with a single chambered stomach, swallowed food reaches the stomach via the esophagus where it is subject to chemical digestive processes, by where acids and enzymes continue to hydrolyze nutrients. This chyme travels through the small and large intestines where absorption of various nutrients and water occur, and finally feces are excreted through the rectum. Avians on the other hand lack teeth, which manipulates feed but does not allow for mastication. Food is first stored in the crop before moving to the proventriculus. The proventriculus is the glandular stomach where chemical digestion occurs with secretion of pepsin and HCl, analogous to the stomach in mammals. The gizzard is a thick muscular wall that participates in the mechanical digestion of feed, via muscular contractions. Digesta can move between the proventriculus and gizzard via peristaltic waves. The small and large intestines of aves are relativity similar in structure and function to that of their rodent counterpart except that chickens have paired ceca, which functions as a fermentation organ. Aves have a cloaca that is responsible for excreting feces and uric acid.

Neuropeptide Y

Neuropeptide Y (NPY) is a 36 amino acid peptide that was first isolated from the porcine brain in 1982, and is amply expressed within the central and peripheral nervous system [17]. NPY is a member of the pancreatic peptide family, and has been recognized in mammalian species to control many physiological mechanisms involved in sexual behavior, gonadotropin release, memory, cardiovascular and stress responses; however, it is best understood for its orexigenic effects [18]. NPY is one of the most potent endogenous orexigenic neurotransmitters in the body[19]. Its orexigenic effects were first confirmed in the rat, but have since been demonstrated to be similar across many species including fish, amphibian, avian, and other mammalian species including humans [20-23].

Due to its strong orexigenic effects, NPY has gained significant attention in the field of appetite regulation and body weight gain research [24]. Understanding the interactions between neuroanatomical regions within the brain has played an important role in determining the pathways involved in daily energy homeostasis. Research from the past decade has shown that NPY-ergic transmission is a crucial component because it leads to the final pathways involved in controlling energy homeostasis [2]. NPY is located in higher concentrations at distinct areas within the central nervous system: the locus coeruleus (LC) within the brainstem, and the ARC and PVN [3]. Within the brainstem, NPY is coproduced with norepinephrine, epinephrine, and galanin (GAL); which are also orexigenic signals. Co-release of these signals in the brainstem was shown to strongly stimulate target sites in the hypothalamus, although independently they also produce stimulatory effects [25]. The ARC of the hypothalamus contains NPY/AgRP neurons that can co-release both neurotransmitters or individually to stimulate feed intake. A co-release of these two neurotransmitters has been shown to magnify feed intake in comparison to

the release of only one, and NPY has a more potent effect on food intake than AgRP in rodents [26].

NPY Receptors

The NPY-ergic signaling pathways are mediated by several receptor subtypes, including Y1, Y2, Y4, Y5, Y6 and Y7 [27]. All of these receptors belong to the seven-transmembrane G protein-coupled receptors of the rhodopsin family, and although they all have a high affinity for NPY they have the greatest divergence between any other given receptor family [28]. All Y receptor subtypes bind endogenous NPY as well as peptide YY (PYY) with the exception of Y4r that only binds pancreatic peptide (PPs). Y1r and Y5r are considered to be the most important receptors involved in appetite regulation. In Y1r and Y5r knockout mice, food intake stimulated by central administration of NPY was significantly reduced when compared to wild type mice [28]. Y2r has been shown to be important in metabolism. Deletion of Y2r indicated an inhibitory role for Y2r in central regulation of body weight and food intake [29].

Methods for assessing NPY's effect on hypothalamic activity

NPY action is widespread throughout the central nervous system with known target sites in the hypothalamus. To verify these central locations more accurately, methods such as electrolytic lesioning, c-FOS activity assays, and exogenous NPY intracerebroventricular (ICV) injections have been performed across various species. These techniques revealed that NPY neurons innervate many other regions within the hypothalamus including the paraventricular nucleus (PVN), the dorsomedial nucleus (DMN),and the ventromedial nucleus (VMN) [13]. The c-FOS activity assay has been accepted as the gold standard for measuring neuronal activity, as c-FOS is a transcription factor that becomes expressed during an action potential [21].

Electrolytic lesioning of individual nuclei has also been performed to disrupt feeding which further supports the involvement of orexigenic signaling pathways [30]. Davis et.al. developed the intracerebroventricular (ICV) injection as a means to evaluate the effect of exogenous NPY on neuronal activation and feeding behavior [31].

In 1985, Clark et al. reported the results of a study where NPY was centrally administered to rats through ICV injection, which led to a physiological signal that dose-dependently stimulated feeding in satiated rats [32]. In 1987, Kuenzel et al. reported the results of an experiment where NPY was ICV injected into broiler chicks, which was associated with a dose-dependent increase in food intake during the first 60 minutes post injection [33].

NPY and Food Intake

After NPY's orexigenic effects were recognized, further studies were conducted to evaluate the effects of nutritional status and feeding state (fasting versus fed) on NPY concentration and activity. In a fasting state NPY mRNA expression is increased compared to the fed state. In a starvation state NPY mRNA expression decreases compared to the fed and fasted states, which is thought to be due to a regulatory mechanism that stops driving hunger signals when food is not available [34]. Blood glucose concentrations are reduced during the fasting state, which can affect NPY output because there are glucose-sensitive NPY neurons within the ARC. Exogenous insulin-induced hypoglycemia was associated with a greater feeding response in wild type mice as compared to their NPY-deficient counterparts [35]. A role for NPY in glucoprivic feeding was further supported by the finding that NPY mRNA content increased 2.4-fold in the hypothalamus of wild type mice at 7-hours post-insulin injection [36]. Zhou et al. investigated NPY in the PVN of broiler chicks, and its response to fasting and refeeding. In this experiment, 14 day old chicks were fasted for 48- or 72-hours and NPY content was measured in

the PVN and ARC after refeeding. NPY content was increased after 24 h of refeeding, an expected compensatory response after a period of nutrient deprivation [23].

Similar studies conducted with mice and goldfish have yielded conflicting results. Goldfish NPY levels returned to pre-fasting levels immediately after refeeding while mice NPY levels were more similar to chickens in response to fasting and refeeding [37]. This may suggest that although NPY plays a similar role in regulating appetite among species, NPY response to stressful feeding regimens could potentially differ across species [23]. This difference between the magnitudes of effect that NPY has on food intake does not only differ between species, but also among breeds of the same species. With respect to chickens, the broiler type chicken and the layer type chicken have relatively fast and slow growth rates, respectively. This difference in growth rate is present even though the proportion of time spent feeding is similar [38]. This was explained through measuring rate of consumption and actual food intake, which determined a significant increase in food intake in broilers, even when there is no significant difference between time spent feeding [39].

Endogenous NPY expression between these two stocks has also been investigated. Results show that there is no significant difference in NPY mRNA abundance between broiler and layer type chicks in the whole hypothalamus [40]. Further studies have detected NPY mRNA expression within specific nuclei associated with food intake in chickens. These results show that there is increased expression within the PVN and ARC of the layer type chicken compared to the broiler type chicken [41]. These results contradict NPY's strong orexigenic role, but may be explained by the observation that layer -type chicks exhibit an inability to habituate to stress, whereas broiler type chickens show a greater capacity to acclimate to novel

environments [42]. NPY has been shown to be co-release during stress-induced release of cortisol, which would account for the decreased food intake seen in layers.

Effects of diet composition on appetite may also be species and breed specific. Throughout their growing phase, chickens can match their protein intake within a narrow range of their requirements in a choice situation where one diet is closer to the optimum protein composition [43]. To our knowledge effects of diet composition on NPY function have not been researched across species.

NPY and Dietary Nutrient Composition in Rodents

Dietary nutritional composition and palatability are important considerations when measuring feed intake. The gross feed intake of an animal is highly dependent on whether the nutritional composition of the feed meets the requirements for growth and maintenance of physiological functions. Dietary preferences have been observed to be influenced in part by genetic background but also on an individual basis in higher order species [44].

A negative correlation has been found between hypothalamic NPY expression and the carbohydrate-to-fat ratio in the diet [45]. Macronutrient preferences after central NPY injection in rats have shown that carbohydrate and protein are chosen over fat [46]. In a study conducted by Beck et al., when rats had the choice between a high carbohydrate (HC) or high fat (HF) diet, their choice was reflected in their PVN NPY content. An obesogenic diet, high in carbohydrates, decreased NPY in the ARC nucleus, although hyperphagia persisted until the end of the two month experiment. A further decrease in NPY was detected in the ARC and PVN when rats were fed a high fat diet. Even though their food intake was normophagic they were obese at the conclusion of the experiment [20]. Beck et al., suggested that these changes in NPY concentrations may be due to a regulatory mechanism that is crucial in preventing overeating and

fat deposition. Although both diets resulted in a decrease in NPY in the PVN, the concentration of NPY mRNA was lower after ingestion of high carbohydrates than it was after ingestion of the high fat diet. Feeding periods appear to play a critical role in dietary choice when rats were given a choice between HF and HC meals. During the beginning of the dark period (which for a nocturnal species like the rat would be the start of a feeding period) rats that had a higher NPY concentration showed a preferential consumption of carbohydrates [19].

Another study used Sprague-Dawley rats that were fed different diets consisting of a restricted protein, a restricted fat, or a restricted carbohydrate ration as well as paired feeding combining and restricting, or increasing different concentrations of the three macronutrients together. Restricted for this study was determined by decreasing intake of a specific macronutrient or energy by 50% compared to the control diet. The protein source was derived from casein, cornstarch as well as sucrose were the major sources of carbohydrate, and corn oil provided much of the saturated and unsaturated fatty acids. Rats on the restricted protein diet increased feed intake, while there was no effect on feed intake in the restricted fat or carbohydrate rations. In the paired feeding a high carbohydrate-restricted fat-normal protein ration as well as a high fat-restricted carbohydrate-normal protein ration was fed without an associated increase in NPY gene expression. The authors suggested this could be due to the normal protein rations in those diets fed, but future studies looking into NPY gene expression and protein intake would need to be conducted [47]. Although the diets in these two studies contained different nutritional compositions, in the Beck et al. study, the high carbohydrate diet had a lower protein percentage than the high fat diet. These results may explain the preferential carbohydrate intake and hyperphagia associated with carbohydrates in that study.

Food Choice in Chicks

There can be many arguments made for and against providing poultry with choices between feeds in a commercial setting. The most basic argument for giving them choice will be to provide them with opportunity to match their individual nutritional needs. From a production standpoint it is not always economically advantageous to give them such high quality feed and choice. A diet determined to meet the standard needs of a poultry stock is more beneficial for the poultry producer [44].

As compared to layers, broilers can better select between a high and low protein diet to meet their optimal concentration of protein for maximum growth [48]. It has been also shown that when both layers and broilers have the choice between diets varying in macronutrient content their visual cues between the diets play a larger role in determining immediate food choice rather than other sensory cues such as taste and smell [44]. After determining the food choice preferred by broilers Forbes et al. investigated the effect of endogenous corticosterone, the main glucocorticoid in chickens, on the choice between a high protein (381g CP/kg) and whole wheat diet (113g CP/kg). Corticosterone administered via intramuscularly demonstrated that chicks could detect metabolic changes caused by the corticosterone administration and try to restore their metabolic needs by modifying their diet choice [49]. Although there are several experiments supporting that poultry have the ability to detect and modify their nutritional requirements when given the choice in feed, there is still speculation as to if their accuracy is as good as their mammalian counterparts, rodents.

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NPYs Association with Obesity and Anorexia

Decreased or increased body conditions associated with feed restriction or over consumption have shown to influence NPY concentrations in short-term studies; but little is understood about NPY regulation during chronic excess food intake or food deprivation. Chronic excess food intake in most cases will lead to morbid obesity while chronic food deprivation can lead to a starvation state and in some cases, anorexia nervosa [57]. Understanding the physiological mechanisms underlying these extreme body conditions is of paramount importance to developing therapeutic strategies for eating disorders and obesity.

Leptin is an adipose-derived hormone that has been shown to regulate NPY and other orexigenic signals, although its existence and function in all avian models is still not completely known. Leptin activates POMC/CART neurons both directly through depolarization and also at the level of gene transcription. Although anorexia is poorly understood it is hypothesized that, NPY/AgRP neurons may be directly inhibited by anorexigenic factors, which prevents activation of stimulating feeding signals [58]. Ghrelin, a hormone predominately produced by the stomach, opposes the function of leptin and is a key regulator of NPY/AgRP neuron activation [59]. Although in chickens ghrelin signals satiety rather than hunger. It is known that leptin and ghrelin pathways are important contributors to body weight and maintenance, thus their signaling pathways are of major research interest, and thus is crucial to understand their functions. Dysegulation of any of the known appetite-regulatory pathways could affect food intake and provide opportunities to identify a pharmacological target for eating disorders.

From an agricultural standpoint, there are important implications for understanding appetite regulation in chickens. Excess energy intake in growing/finishing broilers is associated with deposition of energy as carcass fat, an undesirable trait. Broilers may also over-consume

and develop metabolic syndrome, which affects their reproductive efficiency and overall health. There are animal welfare issues associated with feed restriction in chickens; and there are currently no other strategies to modulate intake in chickens such that intake more closely matches post-absorptive requirements for protein synthesis.

Advances in animal models through artificially selecting specific traits to better mirror the physiological state present in people with eating disorders has contributed greatly to this field of research. As previously mentioned, a polygenic model of hypo-and hyperphagia has been produced in the White Plymouth Rock chicken through more than 50 years of divergent selection for body weight at 8 weeks of age [60]. Eating disorders in humans are typically associated with genetic polymorphisms, therefore this chicken population is a promising genetic model in uncovering many of the central mechanisms involved in anorexia and obesity. Although there are a number of monogenic and polygenic rodent models of anorexia and obesity, the body weight chicken lines are the only model containing both anorexic and obese individuals from the same founder population as a result of divergent selection for low or high body weight, respectively. When 5-day old low weight line (LWS) chicks received central injection of NPY, their cumulative feed intake as a percentage of body weight did not increase significantly, whereas the high weight line (HWS) chicks increased their cumulative food intake dose-dependently. Interestingly, c-FOS activation was similar among appetite-associated nuclei although the feeding behaviors were quite different [60].

Effects of dietary macronutrient composition on exogenous neuropeptide Y's orexigenic effect in chicks

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Abstract: In mammalian models it is well documented that the potent orexigenic factor, neuropeptide Y (NPY) causes preferential intake of high carbohydrate and fat diets, and that diet composition influences levels of NPY in the hypothalamus. However, to our knowledge information on this is limited in non-mammalian species. The purpose of this study was to determine the effect of dietary macronutrient composition on NPY's orexigenic effect in chicks. Three isocaloric diets were formulated:1) high carbohydrate 22% crude protein (CP), 3,000 kcal metabolizable energy (ME)/kg starter diet, 2) high fat with 60% ME derived from lard and 3) high protein containing 30% CP. In Experiment 1, chicks were fed the three diets and at 4 days post hatch received an intracerebroventricular injection of NPY. Chicks that consumed the high carbohydrate and protein diets had a non-dose dependent similar magnitude of increased food intake after NPY injection (136% and 143% greater than vehicle-treated chicks, respectively), but those on the high fat diet had a dose dependent food intake increase, 139% and 197% greater than vehicle-treated chicks for 0.2 and 2.0 nmol NPY, respectively. In Experiment 2, when chicks were given free access to all three diets, injection of 0.2 nmol NPY caused preferential increase in intake of only the high protein diet whereas 2.0 nmol NPY caused preferential increases in intake of the high carbohydrate and protein diets. Neither dose of NPY affected high fat diet intake, an effect opposite that of mammals. In Experiment 3, chicks were raised on one of the three diets and then switched to the others at the time of NPY

injection. When chicks were raised on the high fat and protein diets and then switched to the other diets, stimulation of cumulative food intake occurred for the same duration, 180 min following injection of NPY. However, when chicks were raised on the high carbohydrate and then switched to high fat, NPY injection caused a sustaining increase in cumulative food intake that lasted the entire observation period of 360 min. These results demonstrate that effects of dietary macronutrient composition on exogenous NPY-mediated food intake stimulation are different as compared to mammals. In chicks, exogenous NPY does not cause preferential fat ingestion, although a high fat diet enhances NPY's magnitude of increased food intake.

Key words: neuropeptide Y, hypothalamus, macronutrient, feed intake, chick

Diet composition has been extensively studied for its effect on food intake and body weight and compositional regulation (Mickelsen et al., 1955, Sclafani, 1987, Lucas et al., 1989). For example, in rodents, protein ingestion causes reduced food intake beyond its energy content (Li and Anderson, 1982), and rodents fed a diet deficient in a nutrient will alter food intake to compensate (Anderson, 1979, Anderson and Li, 1987). One factor that plays a significant role in innate food intake stimulation is neuropeptide Y (NPY), one of the most abundant neuropeptides in the central nervous system. NPY is abundantly expressed in the hypothalamus (a key region in regulation of food intake) and of particular orexigenic importance are NPY-ergic arcuate nucleus projections to the paraventricular nucleus (Chronwall et al., 1985). In rodents, exogenous injection of NPY causes increased carbohydrate (Stanley et al., 1989, Welch et al., 1994) and fat (Stanley et al., 1989, Chavez et al., 1998) intake, which can result in obesity (Chronwall et al., 1985, Giraudo et al., 1994).

Dietary macronutrient composition also affects food intake in chickens. There was reduced food intake when chickens were fed an isoenergetic high-protein (30% crude protein) diet or isonitrogenous diet with an imbalance of amino acids (Swennen et al., 2007). Less is known about the effects of dietary fat on food intake regulation in chickens and to our knowledge reported effects during the early post-hatch stage of growth are sparse. The majority of studies conducted to evaluate dietary macronutrient composition effects on growth in chickens used diets that were not isocaloric, confounding the effects of protein or fat content with energy density of the diet (Swennen et al., 2007). Moreover, the relationship between dietary macronutrient composition and NPY is not well understood in non-mammalian vertebrates. It was therefore our objective to measure food intake responses after exogenous NPY injection in chicks as a function of dietary macronutrient composition in isoenergetic diets. This model may

provide novel insight into mechanisms of how NPY-mediated food intake is influenced by dietary factors.

Hubbard X Cobb 500 day of hatch chicks (a common commercial broiler type chick) were obtained from a local hatchery and caged individually in a room with 30 ± 2 ˚ C and 50 ± 5% relative humidity. Chicks were handled daily to adapt to handling and minimize stress during data collection, with ad libitum access to diet and tap water. Diets were formulated as shown in Table 1 and mixed at Augusta Cooperative Feed Mill (Staunton, Virginia, USA). The high carbohydrate diet was formulated to meet the minimum requirements defined for the starter phase of commercial broilers (http://www.cobb-vantress.com) and serves as a broiler industry standard starter diet. The high protein diet was formulated to contain 30% crude protein and the high fat 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 (Beck et al., 1994) to allow for extrapolation of our results to mammalian models. All diets were isocaloric and isonitrogenous and formulated to meet minimum digestible amino acid requirements for commercial chicks.

All experiments were conducted at 4 days post hatch with a free hand intracerebroventricular (ICV) injection method as described previously (Davis et al., 1979, Newmyer et al., 2013). Chicken NPY (YPSKPDSPGEDAPAEDMARYYSALRHYINLITRQRY, AnaSpec, San Jose, CA, USA) was custom synthesized and dissolved in avian artificial cerebrospinal fluid and injected at a total volume of 5µL with 0.06% Evans blue dye to facilitate injection site localization. At the completion of data collection, chicks were euthanized and their brains dissected to determine

accuracy of injection into the lateral ventricle. Any chick without dye present in the lateral ventricle was eliminated from the analysis. Sex was determined visually by dissection.

In Experiment 1, chicks were randomly assigned to one of the three diets at day of hatch, with ad libitum access to food and water. On day 4 post hatch, chicks were randomly assigned one of three ICV NPY doses: 0 (vehicle only), 0.2 or 2.0 nmol, which were administered between 05:00 and 07:00. After ICV injection, chicks were returned to their individual cages and had ad lib access to both diet and water. Food intake was quantified up to 180 minutes following injection (Figure 1). Food intake data were converted to a percentage of body weight by taking food weight consumed dividing by the chick's body weight at injection time and multiplying by 100. All experiments were replicated and the effect of replicate was not significant, thus data were pooled. Data were analyzed using ANOVA within time point using the GLM procedure of SAS 9.3 (SAS Institute, Cary, NC) and the statistical model included the main effects of treatment and diet and their interaction. The diet by NPY dose was significant and thus secondary ANOVAs were conducted within each diet. Sex was not significant in any experiment and was removed from the model. Tukey's method was used post hoc to separate the means. All data are presented as means ± standard error and differences considered significant at $P < 0.05$ for all experiments.

Firstly, vehicle treated chicks on all 3 diets consumed a similar amount of food on a percentage of body weight basis. Those chicks that received 0.2 or 2.0 nmol NPY on both the high carbohydrate and high protein diets increased their food intake, but the magnitude of this increase did not differ between NPY doses. At 180 minutes following injection, 2.0 nmol NPY-treated chicks on the high carbohydrate and fat diet consumed 139% and 143% the amount of vehicle-treated chicks, respectively. However, chicks on the high fat diet had a dose-dependent

increase in food intake after 120 min following injection. Prior to this time, both doses of NPY caused a similar magnitude of food intake stimulation.

In Experiment 2 procedures were the same as in Experiment 1, except that each chick had access to each of the 3 diets prior to and after ICV NPY injection. Vehicle-treated chicks had a preference for the high carbohydrate and proteins diets, with very little high fat diet consumed (Figure 2). The chicks that received 0.2 nmol NPY increased their food intake, but only by consuming more of the high protein diet. Those that were injected with 2.0 nmol NPY also increased their food intake, but at a level higher than those injected with 0.2 nmol, by preferentially consuming more of both the high carbohydrate and protein diets.

In Experiment 3 procedures were the same as in Experiment 1, except that chicks were raised on one of the 3 diets and then switched to another at the time of 0.2 nmol NPY injection and food intake was recorded up to 360 min following injection (Figure 3). Chicks that were raised on the high carbohydrate diet (Figure 3 panel A) and remained on the high carbohydrate diet after injection increased food intake up to 180 min following injection, a result consistent with Experiment 1. Vehicle-treated chicks that were switched to the high fat diet consumed very little of that diet for the first 2 hours and by 180 min consumed less than half as much as vehicle-treated chicks that remained on high carbohydrate or were switched to the high protein diet. The chicks that were switched to the high fat diet and were injected with NPY increased food intake, an effect that was significant at all observation times. Chicks that were switched to the high protein diet and received NPY injection increased food intake, but only up to 180 min following injection. Chicks that were raised on the high fat diet and then switched to the high carbohydrate diet and received NPY injection increased food intake at a greater magnitude (Figure 3, panel B) than chicks that were raised and remained on the high carbohydrate diet (Figure 3, panel A), and

this effect was not significant after 180 min. The same trend was true for chicks switched to the high protein diet. However, chicks that were raised on the high fat diet and remained on it after NPY injection increased food intake at all observation times, an effect similar to Experiment 1. Lastly, Figure 3, panel C depicts chicks that were raised on the high protein diet. Those that remained on the high protein diet after NPY injection increased food intake up to 180 min following injection, consistent with Experiment 1, and this was also the trend for those switched to the high protein diet. Vehicle-treated chicks switched to the high fat diet exhibited a similar food intake response as those raised on the high carbohydrate diet and then switched to the high fat diet: very little food intake just after the switch. However, unlike in the group raised on high carbohydrate diet, chicks switched to high fat and NPY injected only increased food intake up to 180 min following injection.

In rodent models, it is well documented that dietary macronutrient composition affects NPY concentrations (Beck et al., 1992a, Wilding et al., 1992, Giraudo et al., 1994, Wang et al., 1999, Widdowson et al., 1999). Rats consuming a high carbohydrate diet had decreased NPY in the parvocellular part of the paraventricular nucleus as compared to rats consuming a high fat diet (Beck et al., 1990). In rats, the paraventricular nucleus is thought to the be the primary site of NPY-induced food intake stimulation (Leibowitz et al., 1988) and in chicks we have demonstrated that ICV NPY injection induces increased c-Fos immunoreactivity in this nucleus (Newmyer et al., 2013). Therefore, enhancement of NPY action at the paraventricular nucleus of chicks consuming the high fat diet may be responsible for the increased food intake response to NPY injection (Figure 1). Rats fed a high fat diet had less NPY in the lateral hypothalamic area than rats on normal chow (Beck et al., 1990) and we also demonstrated that the lateral hypothalamic area has increased c-Fos immunoreactivity after ICV NPY in chicks (Newmyer et

al., 2013). Therefore, in our study the diets may have caused alterations in NPY signaling or NPY receptors in the hypothalamus, particularly at the paraventricular nucleus or lateral hypothalamic area which is responsible for the differential appetite-associated effects after exogenous NPY injection. In rats, high carbohydrate and fat diets did not affect the level of NPY expression in other hypothalamic nuclei related to appetite (Beck et al., 1990).

Vehicle treated chicks had a preference for the high carbohydrate and protein diets, with very little high fat diet consumed (Figure 2). In rodents, NPY causes a potent preferential enhancement of carbohydrate intake (Stanley et al., 1985, Beck et al., 1992b) and to a lesser extent fat intake (Beck et al., 1992b). In general, most of the research on the relationship between dietary macronutrient quantity and food intake in chickens was conducted with diets that were low in fat, protein or carbohydrate relative to the control diet (Swennen et al., 2007). Based on those studies, dietary protein had the greatest effects on food intake, with lesser effects from altering fat and carbohydrate levels in the diet (Swennen et al., 2007). An advantage of a precocial species in this research is that experimental diets can be fed from hatch, allowing for an understanding of how diet affects the physiology of the animal independent of previous exposure to other nutrition. Further studies will focus on elucidating the molecular mechanisms underlying these effects of diet and NPY on appetite regulation in the hypothalamus of the chick.

In sum, we have demonstrated that chicks on high carbohydrate, fat, and protein diets have differential food intake response after exogenous NPY injection. NPY dose-dependently causes preferential ingestion of a particular diet: low doses causes increased high protein diet consumption whereas higher doses cause increase ingestion of both high protein and high carbohydrate diet intake. This is an effect which is not consistent with rodents and warrants

further study with attention to molecular aspects of the NPY system at the hypothalamic level.

Table 1. Ingredient and chemical composition of experimental diets

Ingredient (% as-fed)[1]	Basal	High protein	High fat
Ground corn	58.80	34.64	2.16
Soybean meal	36.12	57.48	42.48
Soybean hulls	0.00	0.00	27.71
Lard	0.00	0.00	24.00
Soybean oil	1.2	4.80	0.00
Methionine 99%	0.28	0.04	0.35
Threonine	0.10	0.00	0.07
L-Lysine 78%	0.09	0.00	0.00
Dicalcium Phosphate	1.54	1.41	1.62
Calcium carbonate	1.15	1.07	1.01
Sodium bicarbonate	0.15	0.18	0.02
SALT920831	0.37	0.36	0.37
Coban 90[2]	0.05	0.05	0.05
Phytase-RONOZYME[3]	0.05	0.05	0.05
Vitamin and mineral premix[4]	0.10	0.10	0.10
Choline Chloride-60%	0.00	0.00	0.01
Kcal ME/kg	3,000	3,000	3,050
Crude protein	22%	30%	22%
Crude Fat	3.7%	6.7%	25%
Crude Fiber	2.5%	2.6%	12.4%

[1]Diets were formulated to meet or exceed minimum recommended specifications for Cobb-500 broilers during the starter phase (Cobb-Vantress)

[2]Coban 90 (Elanco Animal Health) contains 90 grams of Monensin sodium per pound of premix and is included in the diet as a coccidiostat

[3]DSM Nutritional Products, Ltd.

[4]Guaranteed analysis (per kg of premix): Manganese, 25.6 g; selenium, 120 mg; zinc, 30 g; Vitamin A, 4,409,171.076 IU; Vitamin D_3, 1,410,934.744 ICU; 13,227.513 IU; d-biotin, 88.183 mg

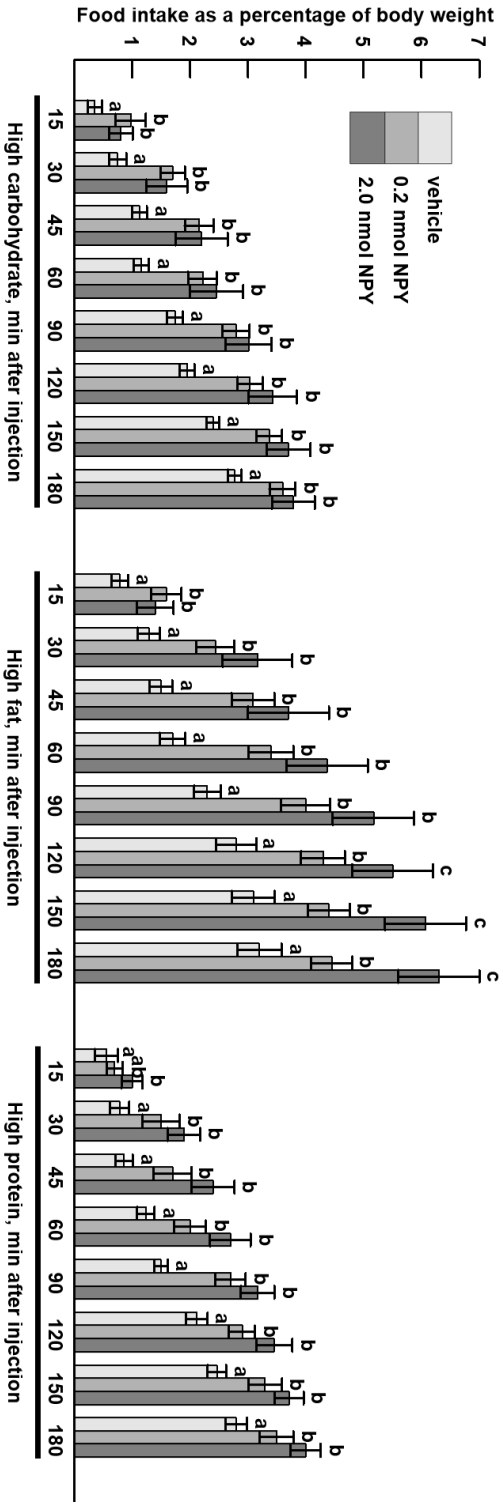

Figure 1. Cumulative food intake expressed as a percentage of body weight of NPY-injected chicks on either high carbohydrate, high fat or high protein diet. n = 17 to 20 chicks per NPY dose per diet, bars with different superscripts are significantly different from one another within a time within a diet.

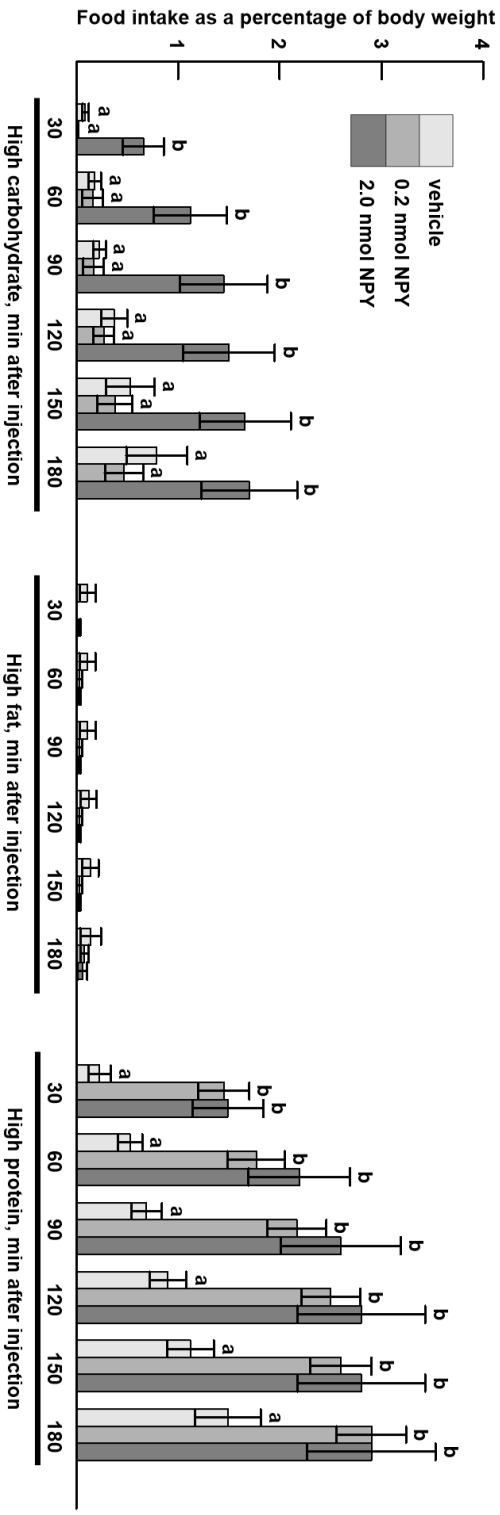

Figure 2. Cumulative food intake expressed as a percentage of body weight of NPY-injected chicks given free access to high carbohydrate, high fat and high protein diets simultaneously. n = 16 to 18 chicks per NPY dose per diet, bars with different superscripts are significantly different from one another within a time within a diet.

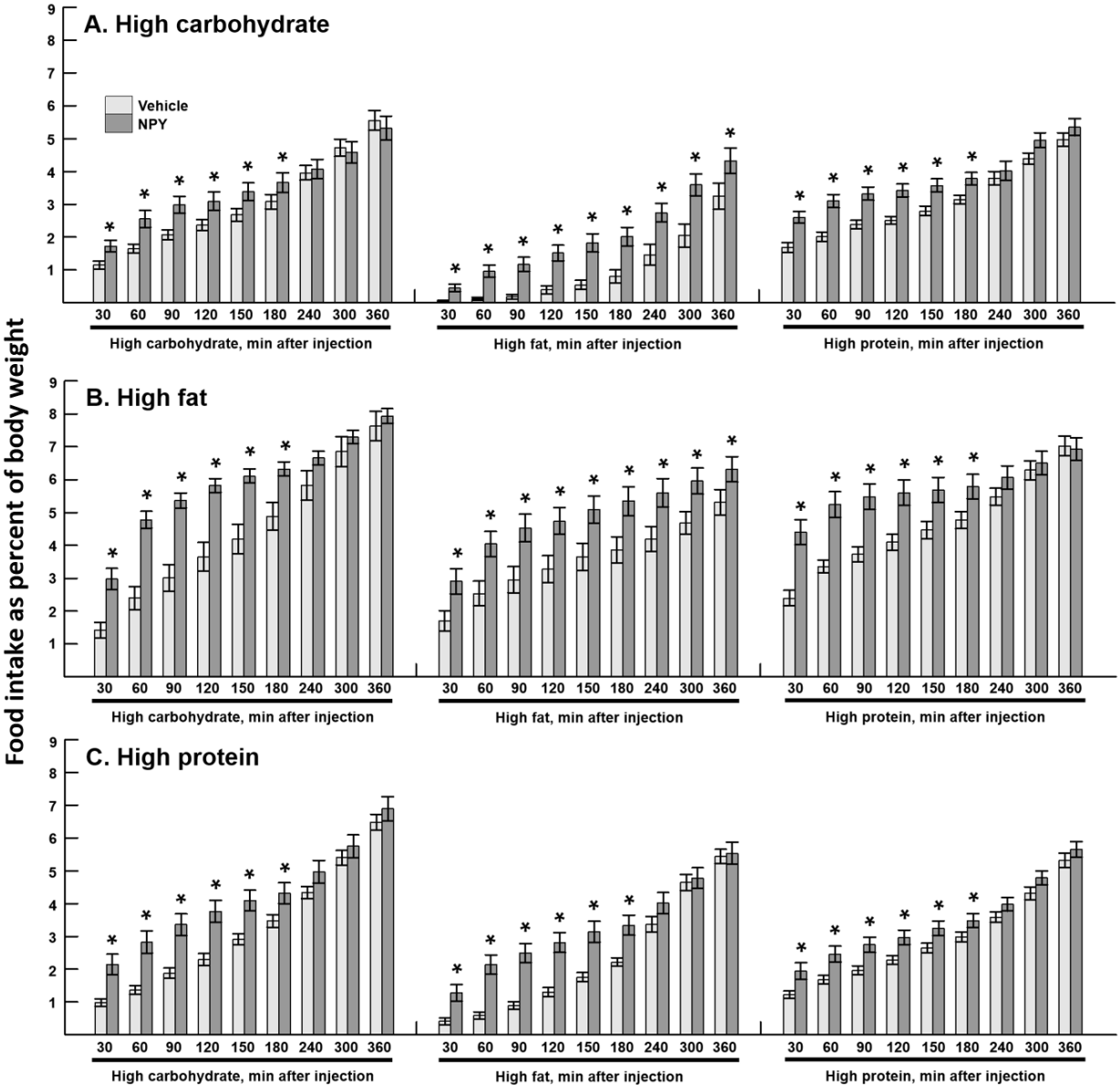

Figure 3. Cumulative food intake expressed as a percentage of body weight of NPY-injected chicks raised on either A) high carbohydrate, B) high fat or C) high protein diets and then switched to another diet at NPY injection. n = 15 to 20 chicks per NPY dose per diet, bars with an asterisk re significantly different from vehicle within a time within a diet.

Epilogue

Summary of Thesis

In conclusion, this research beings to shed light on the complex mechanisms involved in central appetite regulation and the nutrient sensing capabilities involved when broiler chickens are fed varying macronutrient rich diets. Results from this study suggest that diets high in saturated fats increase food intake when paired with icv injected NPY. High protein diets may trigger increased expression of anorexigenic neuropeptides which shortens the effect that exogenous NPY has on food intake. Understanding how macronutrients fluctuate food intake from a practical level will not only aid in agriculture to formulate a better ideal diet for production animals, but it may also help to better develop research strategies to understand obesity.

Future work

To further investigate how these particular diets influence food intake it would be beneficial to determine mRNA expression of various appetite associated factors during all food intake experiments. Although mRNA is an important molecular component to understand their function in food intake, protein content would also be very important to determine how much of that mRNA becomes actively expressed for the purpose of driving food intake. The diets formulated for this study were of extreme macronutrient concentrations that would not be seen in agriculture or normal diet. It would be interesting to see the threshold of macronutrient content that could be used to yield similar results found in this study. In addition to varying macronutrient concentrations it would also be informative to see how unsaturated fats influence endogenous NPY content and their response to exogenous NPY. These experiments were performed on 4 day old chicks to determine how diet effects early development. The next step

would be to determine these effects of mature chickens. The end result of this research would be to determine the complete mechanisms driving these food intake results.

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