

Satiety induced by neuropeptide FF and gastrin in birds

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## ACADEMIC ABSTRACT

Mammalian and avian species differ in some appetite-related aspects including how and which neurotransmitters and hormones regulate appetite. The objective of this research was to determine how two satiety-inducing neuropeptides regulate feeding behavior in avian models. Neuropeptide FF (NPFF) was intracerebroventricularly (ICV) injected into Japanese quail and decreased food intake at a dose of 32 nmol. NPFF-injected quail had increased expression levels of hypothalamic melanocortin subtype 3 receptor and decreased expression levels of neuropeptide Y receptor subtype 1 mRNAs compared to vehicle-injected controls. In a second study, gastrin was ICV injected into broiler chicks and decreased food intake at a dose of 500 ng (0.12 nmol). There was increased c-Fos immunoreactivity in the lateral hypothalamus (LH), paraventricular nucleus (PVN), arcuate nucleus, nucleus of the solitary tract, and area postrema at 1 h post-injection. Although a variety of genes were measured in those activated nuclei, there were only differences in melanin-concentrating hormone mRNA in the LH and corticotropin-releasing factor (CRF) mRNA in the PVN, suggesting that CRF signaling was involved in the hypothalamic response to gastrin. However, co-injection of gastrin and astressin, a CRF receptor antagonist did not affect gastrin-induced suppression of food intake, implying that the CRF receptors may not be directly associated with gastrin-induced satiety. Identifying the molecular pathways that mediate the effects of anorexigenic neuropeptides in birds will lead to the development of novel treatment options for appetite-related diseases and increased understanding

of factors that affect production efficiency in commercial poultry and survival/resource allocation in wild birds.

# Anorexic effects of neuropeptides in Japanese quail and broiler chicks

Amanda Lynn Logan

## **PUBLIC ABSTRACT**

Compulsive eating behavior associated with obesity, and anorexia nervosa, are appetite-related disorders for which no effective pharmacological treatment options exist. From an agricultural perspective, understanding what drives eating behavior is important for informing management decisions that affect animal health and welfare. From an evolutionary point of view, understanding the neural mechanisms of appetite in different species is important because of the necessity of appetite for survival. This thesis research focused on elucidating the central mechanisms associated with the actions of two neuropeptides that inhibit food intake. In the first experiment, we explored the mechanisms underlying the appetite suppressive effects of neuropeptide FF (NPFF), a neuropeptide known for its morphine-modulating properties, and found that it decreased food intake in Japanese quail, a bird that is more representative of a bird in the wild than the chicken. NPFF affected the gene expression of several appetite-related factors in the hypothalamus, a region of the brain that regulates appetite, providing insights on the associated molecular pathways. In the next study, we examined the effect of gastrin, a digestive hormone known for its role in regulating gastric acid secretion, in broiler chicks. Broiler chickens are meat-type chickens genetically selected for growth and meat production. Gastrin significantly decreased food intake in broiler chicks, and this was associated with changes in the gene expression of melanin-concentrating hormone and corticotropin-releasing factor in the lateral hypothalamus and paraventricular nucleus of the hypothalamus, respectively. Overall, we were able to provide novel insights regarding the hypothalamic mechanisms that

regulate the inhibition of food consumption. These findings may lead to the development of a novel appetite suppressant or stimulant to adjust food consumption in individuals with eating disorders and chickens during specific stages of growth.

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## **Chapter 1: Literature Review**

### **Introduction**

As obesity becomes more prevalent, the research, medical, and general population continue to be concerned with appetite and food intake regulation. Energy homeostasis refers to the body having a normal balance of energy intake, storage, and expenditure in order to ensure that all physiological processes function to support optimal growth, health, and activity levels. However, a high percentage of the population is experiencing an increase in food intake and a decrease in energy expenditure, thus leading to weight gain and the current obesity epidemic. From an agricultural perspective, the broiler chicken industry is facing similar problems, with obesity causing secondary issues like leg and metabolic disorders and visually unappealing meat products for consumers. Elucidating the hypothalamic and brainstem mechanisms associated with appetite could lead to treatment options for those living with obesity and for those raising broiler chickens.

### **Energy Homeostasis**

In order to maintain energy homeostasis, the body needs to conserve or expend energy and consume food or stop eating depending on what the body requires in that moment. Following a depletion of energy, the homeostatic pathway increases the body's motivation to eat. When hunger arises, the extra energy that is stored in adipose tissue leads to release of signals from adipose, and the peripheral and central systems communicate with each other via hormones and neurotransmitters to regulate energy usage in order to maintain energy homeostasis [1]. This

signaling is essential to mammalian and avian species to keep this multiplex regulation of appetite under control.

The regulation of energy homeostasis requires two systems to work in unison: the peripheral and central nervous system. The peripheral system includes hormones that have a role in hunger and satiety and are released from a variety of tissues including the pancreas (insulin), adipose tissue (leptin), and gastrointestinal tract (ghrelin, cholecystokinin (CCK), glucagon-like peptide-1, and peptide YY) [2], while the central system includes numerous categories of neurotransmitters including, but not limited to, peptides and amino acids and their derivatives [3]. The hypothalamus, which is of paramount importance in the central nervous system, holds a dominant role in integrating signals from both of these regulatory systems.

### **Hedonic Food Intake**

Aside from homeostatic purposes, hedonic rewards have a strong influence on appetite and could be a cause of the growing obesity epidemic. This is an essential factor in causing obesity because a person or animal will want to keep eating if the food is stimulating their body's reward circuits and giving them a sense of euphoria. The major neurotransmitter involved in this category of eating is dopamine. The mesocorticolimbic (MCL) system, or reward circuit, consists of the ventral tegmental area (VTA), the medial prefrontal cortex, amygdala, hippocampus, and nucleus accumbens (NAc). Dopamine projects from the VTA to the other regions of the brain. The dopamine is released in response to cues in order to target the MCL [4]. The activation of the MCL system and the output of a feeling of euphoria is the cause of hedonic eating behaviors.

Palatable foods, or high-energy foods, are known to activate this reward system. There are several reasons that fats and carbohydrates are thought to be more palatable than other foods. Both can be quickly converted into energy. From a hunter-gatherer society perspective, it would be advantageous to eat a large amount when food was available because it provided them with energy [5] and allowed them to have a positive energy balance when food was unavailable [6]. In addition, sugary foods and animal fat were not readily available and were seen as valuable food items in the eyes of early humans and caused modern humans to evolve a motivation to seek out these foods [7]. In modern societies, this evolved mechanism can lead to obesity since these foods are readily available and cravings exist. The sweet taste of sugars plays a role in opioid activation which helps in analgesia [8]. Additionally, sugar was found to be addictive in male rats and the rats exhibited signs of opioid withdrawal when sugar withdrawal was implemented by naloxone injection or food deprivation [9]. These factors are a few of the reasons why the rate of obesity is increasing at such a high rate. In order to control these hedonic eating behaviors, changes in the diet and discovering the mechanisms associated with homeostatic regulation need to be closely reviewed so that proper solutions can be implemented.

This literature review will focus on a few neurotransmitters, including peptides and amino acids and their derivatives, that are essential in regulating homeostatic appetite in both mammalian species and avian species. Differences are present between mammals and birds and it is important to recognize the reasons for that in order to properly understand the role of the hypothalamus and brainstem in food intake. In this thesis research, the satiety-inducing mechanisms of neuropeptide FF (NPFF) in Japanese quail and gastrin in broiler chicks were investigated.

## **Mammalian Species**

### **a. Hypothalamic nuclei**

The hypothalamus contains many different nuclei that have a role in various physiological processes in the body. In order to achieve energy homeostasis, neurotransmitters, including peptides and amino acids and their derivatives from different areas of the brain, communicate to the rest of the body. There are at least five appetitive nuclei centers in the hypothalamus that form the basis of appetite regulation: ventromedial nucleus of the hypothalamus (VMH), the dorsomedial nucleus (DMN), the paraventricular nucleus (PVN), the lateral hypothalamus (LH), and the arcuate nucleus (ARC).

In the mid to late 20<sup>th</sup> century, multiple lesioning studies of hypothalamic nuclei were performed which led to the discovery of the roles these nuclei play in appetite regulation. Sometimes these lesions would result in a decrease in food intake, while other times they would cause an increase in food intake, depending on what part of the hypothalamus was lesioned [10]. In 1943, the VMH was bilaterally lesioned in rats which caused an increase in food intake [11]. In 1951, the lesioning of the LH in rats caused a decrease in food intake [10]. These classical lesion studies led to the “dual-center” hypothesis that the LH is the feeding center and the VMH is the satiety center. However, problems with this hypothesis began to arise when multiple studies showed that lesions to other areas also caused an increase in food intake [12] indicating that food intake regulation is much more complex. Since then, more research has been conducted in order to understand the specific signaling that occurs in each nucleus, some of which is discussed below.

The mammalian ARC, known as the infundibular nucleus in avian species, is at the base of the hypothalamus [13], located adjacent to the third ventricle in the hypothalamus. It is the site containing leptin receptors which when activated have potent effects on energy homeostasis [14]. Likewise, the VMH, LH, and brainstem contain leptin receptors for circulating leptin [15]. The ARC has at least two groups of neurons. One group contains neuropeptide Y (NPY) and agouti-related peptide (AGRP)-producing neurons. The other group contains pro-opiomelanocortin (POMC) and cocaine-and amphetamine regulated transcript (CART)-producing neurons. The AgRP and NPY population is orexigenic, while the CART and POMC population is anorexigenic [16].

The neurons in the ARC communicate with neurons in the PVN where thyrotropin-releasing hormone (TRH), corticotropin-releasing factor (CRF), and oxytocin are released. The neurons also communicate with the LH and the perifornical area where orexigenic melanin-concentrating hormone (MCH) and orexins are made. Signals from high levels of adiposity, when the body is in positive energy balance, stimulate the ARC to release anorexigenic peptides, while signals from low levels of adiposity, during times of fasting, would signal the release of the orexigenic peptides [2].

NPY and AgRP are co-expressed in neurons in the medial part of the ARC. The NPY/AGRP neurons cause the release of peptides that stimulate food intake by increasing NPY signaling and increasing the antagonistic effects of AgRP. NPY is one of the most abundant, orexigenic peptides which stimulates food intake and decreases energy expenditure. NPY that is released in the PVN, LH, and perifornical area (PFA) regions in the arcuate nucleus has an orexigenic effect [17]. Thus, an inhibition of the NPY system could be one way to reduce food intake; however, there are many systems are involved in food intake.

Melanocortins including  $\alpha$ -MSH are cleaved from POMC, are released in the PVN, and act to decrease food intake [17]. AgRP is a melanocortin receptor antagonist. Fasting increases the expression of AgRP, which in turn decreases the effects of melanocortin [18] by disrupting the melanocortin receptors (MCR) in order to stimulate food intake. Melanocortin subtype 3 receptors are expressed in some of the food intake-regulating hypothalamic nuclei: the ARC, LH, and VMH [19]. Melanocortin subtype 4 receptors (MC4R) are even more widespread. They are found in the ARC, DMN, LH, PVN, and VMH [20]. MC4R-deficient mice result in obesity, hyperphagia, hyperinsulinemia, and hyperglycemia [21]. During times of fasting or low levels of adiposity, orexigenic factors will stimulate AgRP and NPY neurons in the ARC in order to increase food intake to achieve energy homeostasis.

On the other hand, the other group of neurons works to inhibit food intake. POMC and CART neurons are co-expressed in the lateral part of the ARC. Fasting inhibits POMC and CART releasing neurons in the ARC so that POMC and CART are not released onto second order neurons, while leptin, released when the body has had enough food, activates anorexigenic POMC and CART neurons in the mammalian model [22]. A deficiency of POMC protein causes obesity and low levels of adrenocorticotrophic hormone (ACTH). POMC is the precursor for ACTH and MSH, which are anorexigenic, and beta-endorphin, which is orexigenic [20].

The LH, PVN, VMH and DMN contain second order neurons that respond to signals from the first order neurons in the ARC or directly from the signals themselves. The LH is known as a feeding center and thus destroying it will lead to a loss of regulation and the body will not perceive hunger. The LH contains melanin-concentrating hormone- and orexin-releasing neurons, which are orexigenic factors in mammals. The NPY/AgRP and  $\alpha$ -MSH-releasing neurons from the ARC project to the MCH- and orexin-releasing neurons in the LH, to stimulate

or inhibit food intake, respectively [23]. The PVN is next to the superior section of the third ventricle in the anterior hypothalamus located above the DMN, VMH, and ARC. The PVN is responsible for the majority of secretion of the hormones CRH and TRH, which are anorexigenic, and it also reacts to signals coming from the ARC [13]. The PVN has a role in both satiety and hunger. The VMH has a role in energy and glucose homeostasis and can sense circulating glucose, lactate, and leptin. As glucose and lactate levels rise in the blood, glucose-excited neurons located in the VMH increase their firing of action potentials, which is hypothesized to subsequently affect energy and glucose homeostasis [24]. The VMH also uses leptin signaling via its leptin receptors to regulate food intake [15]. The DMN has a role in both hunger and satiety. It contains many NPY, an orexigenic factor, and  $\alpha$ -MSH, an anorexigenic factor, terminals from the ARC which are important for regulating food intake [23]. After decades of research, the simple “dual-center” hypothesis has turned into a complex system of regulation by the hypothalamic nuclei associated with appetite.

### **b. Factors affecting food intake in the hypothalamus**

There are many factors affecting food intake in the hypothalamus. A few will be discussed here. As briefly discussed already, leptin receptors are expressed in the two groups of ARC neuron centers, NPY/AGRP and POMC/CART [16], the LH, VMH, and brainstem [15], which are essential for leptin signaling. Leptin is released by adipocytes to signal to the hypothalamus to reduce food intake. Therefore, leptin protein deficiency or a deficiency in leptin receptors causes obesity. Leptin was shown to inhibit NPY and POMC expression in mice [25]. Acting in opposition to leptin is ghrelin. Ghrelin is produced in the stomach [26] in X/A-like cells, secreted into blood vessels, and stimulates food intake since the ARC is the main active



location of ghrelin [27]. Ghrelin binds to growth hormone secretagogue receptors [26] which are found in the ARC and VMH of the hypothalamus, among other body tissues, [28] explaining its ability to effect food intake. Overall, when food restriction occurs, the levels of leptin fall and ghrelin rises activating NPY/AgRP neurons and inhibiting POMC/CART neurons to increase food intake.

### **c. Brainstem**

In addition to the hypothalamus, the brainstem plays an important role in appetite. The brainstem receives and sends signals from and to the hypothalamus. The PVN of the hypothalamus provides output to areas of the brainstem [29]. Three specific anatomies, the vagus nerve, the area postrema (AP), and the nucleus of the solitary tract (NTS), will be discussed. The vagus nerve serves as a link between the brain and the stomach [23]. Leptin not only activates the hypothalamic neurons but also the vagal pathway when leptin and CCK work together to regulate short-term satiety [30]. A knockdown of the leptin receptor in the NTS and the AP caused mice to experience an increase in food intake [31], showing the role of the leptin receptors in the brainstem to respond to circulating leptin [15]. However, in long term satiety, vagal afferents play a secondary role. The vagal afferent nerve fibers can communicate information regarding mechanical touch [32], tension, and muscle stretch and/or length [33] in the gastrointestinal tract.

Circulating amino acids also have the ability to control food intake. L-Arginine, L-lysine, and L-glutamic acid decrease food intake in rats when orally gavaged and injected intragastrically and intravenously. Upon administration of these amino acids, c-Fos immunohistochemistry revealed increased c-Fos-positive cells in both the AP and NTS [34]. The blood brain barrier

does not surround the AP and the NTS so these two nuclei receive vagal afferent signals, allowing them to respond to circulating nutrients, like amino acids. The anorexigenic effect seen in rats caused by arginine and glutamic acid require the area postrema neurons to be intact and the anorexigenic effect caused by lysine requires the vagus nerve to be intact [34], demonstrating the areas of the brainstem that respond to circulating amino acids. The brainstem can send and receive information to and from the hypothalamus, and can sense circulating nutrients and peptides in the blood, showing the brainstem as a link between the hypothalamus and the rest of the body.

#### **d. Conclusion**

Many factors play into appetite regulation. However, some neurotransmitters have different effects in avian species compared to their effects in mammalian species. In order to better understand appetite regulation and to properly research neuropeptides, the next part of the review will focus on parts of the central and peripheral appetite regulatory systems that are different between mammalian and avian species.

### **Avian Species**

#### **a. Differences**

Some of the neuropeptides and hormones that play a different role in food intake in avian species than in mammals include ghrelin, leptin, orexin, and prolactin-releasing peptide (PrRP). This may be due to differences in amino acid sequences, hypothalamic mechanisms, or other

physiological differences. Mammalian ghrelin inhibits food intake in neonatal chicks, but stimulates food intake in rats [35]. Avian ghrelin exhibits 54% amino acid identity to human and rat ghrelin [36], which may account for the differences observed. Like mammals, birds use ghrelin for growth hormone (GH) release. Unlike mammals, avian ghrelin has a greater effect on corticosterone release which could be used to further enhance the avian adrenal glands, the site of stress response [37]. In birds, NPY neurons do not respond to ghrelin, whereas in mammals the orexigenic effects of ghrelin is mediated by NPY. Instead, ghrelin activates corticotropin-releasing factor (CRF) to inhibit food intake in avian species [36].

The first avian leptin gene was discovered in zebra finches and is only 26%-29% identical to mammalian leptin [38]. The leptin gene was also discovered in rock doves. It only shares 47% similarity with human leptin [39]. In mice, leptin is mainly expressed in adipose tissue [40] and, when adiposity levels are high, signals to the brain to reduce food intake. In chickens; however, leptin is expressed in low levels in adipose tissue [41]. The avian leptin receptor is highly expressed in the pituitary, while the mammalian leptin receptor is highly expressed in the hypothalamus [41]. In mammals, leptin has an inhibitory effect on food intake; however, in chickens, when centrally injected, food intake was not affected [42], showing that leptin may serve a different role in avian species. As discussed in the mammalian food intake section of this literature review, leptin has a large role in regulation of mammalian food intake; however, this may not be true of avian species.

Central injections of both orexin-A and orexin-B stimulate food intake in rats [43]. However, chickens injected with orexin-A (sequence of human, rat, mouse, and bovine) and orexin-B (sequence of rat and mouse) decrease food intake. Human, rat, mouse, and bovine orexin-A share the same amino acid sequence. Rat and mouse orexin-B share the same sequence

[44]. In the rat, orexin-A and orexin-B are 46% identical in sequence [43]. But, as noticed with leptin, the avian orexin is not 100% identical to mammalian orexin. Chicken orexin A is approximately 85% similar to human, pig, and rat orexin A, while chicken orexin B is approximately 65% similar to human, pig, and rat orexin B [45]. In 12-week old female feed-restricted (25% of ad-libitum from hatch to 12-weeks) broilers, orexin mRNA was higher than in ad-libitum fed broilers, suggesting a role in appetite regulation [46], even if it does not directly decrease food intake.

Lastly, another neuropeptide that has the opposite effect in avian species is PrRP. Chicken PrRP-20 differs from rat PrRP-20 at only 3 amino acids. Aside from PrRP, PrRP2 was also discovered in chickens, but is not found in mammalian species and did not originate from the same gene as mammalian PrRP [47, 48]. In rats, PrRP is known to decrease food intake; however, when injected with rat PrRP-31, chickens increased food intake [49]. An ICV injection of PrRP into the PVN in rats did not have an effect on food intake; however, an injection into the DMN caused a decrease in food intake, suggesting that the DMN may be a site of action for mammalian PrRP. In addition, it was found that PrRP increased  $\alpha$ -MSH and neurotensin secretion, which are anorexigenic, and decreased CART release, which is orexigenic, suggesting multiple mediators of mammalian PrRP's effect on food intake [50]. Both ICV injection of rat PrRP and chicken PrRP2 into broiler chickens caused an increase in NPY mRNA expression in the diencephalon suggesting that NPY may mediate the orexigenic effects of PrRP and PrRP2 in broiler chicks when fed a high carbohydrate diet [47, 51]. The location of PrRP receptors may account for the differences seen in food intake. In both chickens and mammals, the PrRP receptors are similarly distributed in the central nervous system; however, they vary in location in the periphery. In chickens, PrRP receptor is located in the skeletal muscle, testis, ovary, lung,

and pituitary, while in mammals, PrRP receptor is located in the spinal cord, thyroid gland, lung, pancreas, kidney, gut, and testis. Further, in chickens the PrRP2 receptor is more widely distributed in the pituitary, heart, lung, liver, pancreas, kidney, gut, skeletal muscle testis, and ovary [47], suggesting that PrRP and PrRP2 may play different physiological roles in the chicken.

As noted above, differences between avian and mammalian species exist. Most peptides have a similar effect across the whole avian species, while some show differences in the effect on food intake or in hypothalamic mechanisms that mediate the effects of the peptides. For example, neuropeptide Y has been found to increase food intake in broilers, layers [52], quail [53], white-crowned sparrows [54], and ring doves [55]. However, even within the chicken species, some neurotransmitters can cause opposing effects on food intake. For example, AgRP, when ICV injected in broiler chicks, does not affect food intake 1, 2 or 4 h post-injection; however, when AgRP is ICV injected in layer chicks, food intake is increased at 1, 2, and 4 h post-injection [56]. Another example that occurs within the avian species is differing hypothalamic mechanisms of  $\alpha$ -MSH-induced satiety. In quail that were ICV injected with  $\alpha$ -MSH, there were increased c-Fos immunoreactive cells in the ARC, DMN, LH, and PVN [57]. In lines of chickens that have undergone selection for low body weight or high body weight, an ICV injection of  $\alpha$ -MSH caused increased c-Fos immunoreactive cells in the DMN, PVN, and VMH in both the low weight and high weight chickens compared to vehicle-injected, while the low-weights had a greater magnitude of increased c-Fos, showing that even between these two lines of chickens, signaling differs [58]. Lastly, in quail that were ICV-injected with  $\alpha$ -MSH, there was increased c-Fos immunoreactive cells in the PVN and VMH [59]. With varying nuclei being activated, it can be speculated that the hypothalamic mechanism of  $\alpha$ -MSH-induced satiety

varies between these birds. The differences within the chicken species may be because broiler chickens have undergone intensive genetic selection to increase efficiency and achieve faster growth rates and the low and high body weight lines have also been highly selected for, while Japanese quail are more wild-type birds.

## **Conclusion**

Figuring out how these processes work in mammals can lead to solutions for obesity and anorexia. While learning about the avian processes could have implications for humans, there are additional benefits in agriculture, like figuring out how to grow birds more efficiently by regulating their appetite. While the location and purpose of most neurons and neuropeptides are similar between avian and mammalian species, some differences like the effects of leptin and PrRP stand out. It is important to note that mammalian and avian forms of neuropeptides and hormones are not completely identical which could account for the differences in functions. Injecting a mammalian version of a neuropeptide into a bird could have different implications than if an avian version of the neuropeptide was injected into a bird or if an avian version was injected into a mammal. Further, avian species have many anatomical differences compared to humans which could lead to the varying effects of neurotransmitters. More research needs to be conducted in order to figure out the mechanisms of these peripheral and central signaling hormones and neuropeptides.

## **Chapter 2: Central neuropeptide FF injection is associated with anorexigenic effects in Japanese quail**

**Abstract:** Neuropeptide FF (NPFF), a neuropeptide known for pro- and anti- opioid like functions, including an anti-opioid effect on morphine-induced analgesia, decreases food intake in rats and chickens when centrally injected. In the current study, the Japanese quail was used as an avian model that has undergone less artificial selection than the broiler chicken. In Experiment 1, an ICV injection of 16 and 32 nmol NPFF decreased food intake during the first 30 min. In Experiment 2, at 1 h post-ICV injection of 32 nmol NPFF we collected the hypothalamus and measured the mRNA abundance of appetite-associated factors. NPFF-injected birds had an increased abundance of melanocortin subtype 3 receptor (MC3R) mRNA and a decreased abundance of neuropeptide Y receptor subtype 1 (NPYR1) mRNA, compared to the vehicle-injected quail. In Experiment 3, a comprehensive behavior analysis was conducted following an injection of 32 nmol NPFF. Quail injected with NPFF displayed a decrease in the the number of steps taken and the distance moved compared to vehicle-injected quail. In summary, NPFF caused a decrease in food intake and this may involve melanocortin and NPY signaling in the hypothalamus.

**Keywords:** Japanese quail, NPFF, appetite, hypothalamus

## 1. Introduction

Neuropeptide FF (NPFF) was first isolated from the bovine brain in 1985 and shown to have a role in morphine modulation in the rat [60]. NPFF has several functions including roles in pro- and anti-opioid-like effects on pain, food intake, memory, and blood pressure regulation [61]. Although NPFF does not bind directly to opioid receptors, NPFF is involved with pro- and anti-opioid-like effects. It was demonstrated in the broiler chicken that NPFF inhibits neuropeptide Y and B-endorphin, which are ligands of opioid receptors [62], which may account for its roles in both morphine modulation and food intake.

In the rat, NPFF is found in high concentrations in the posterior pituitary, spinal cord, hypothalamus, and pons-medulla [61]. NPFF is a member of the RFamide family of peptides, and this family is linked to G-protein-coupled receptors. RFamides have varying effects on appetite with pyroglutamylated RFamide peptide (QRFP) stimulating food intake in rodents and the others, NPFF, neuropeptide AF (NPAF), and prolactin-releasing peptide (PrRP), inhibiting food intake in rodents [63]. However, when mammalian PrRP is injected into chickens, it increased food intake [49].

In regards to appetite regulation, rats significantly decreased food intake following an intracerebroventricular (ICV) injection of NPFF, possibly due to NPFF's anti-opioid function [64]. In a separate group's study involving rats, an ICV injection of 1 nmol or 3 nmol NPFF caused a decrease in food intake that lasted 30 min post-injection and an increase in water intake that lasted 2 h post-injection, speculating that the reduction in food intake was a secondary effect to the potent increase in water intake. When 10 nmol were administered, food intake was decreased for 8 h post-injection and water intake was decreased for 8 h post-injection. The higher doses of NPFF caused barrel rolling effects in rats [60, 65].



To further understand the anorexigenic effects of NPPF, an avian model was used. The doses used in both the rat and chicken studies were similar; 0, 3, and 10 nmol for rats and 0, 4.16, 8.32, 16.16 nmol for chickens. NPPF decreased food intake in Cobb-500 broiler chickens; however, the significant increase in water intake was not observed nor were the barrel rolling behaviors, suggesting differences in the NPPF system between rats and chicks [66]. NPPF injection was associated with activation of the dorsomedial nucleus (DMN), nucleus dorsomedialis posterior thalami, paraventricular nucleus (PVN), and ventromedial hypothalamus (VMH) of the hypothalamus, which have roles in mediating satiety, but failed to activate the LH which is a hunger center, providing insights on pathways involved in decreasing food intake in broiler chickens [66].

In the present study, Japanese quail were ICV injected with NPPF in order to determine the hypothalamic mechanism of NPPF-mediated suppression of food intake in this avian model, which has not been as heavily selected for growth-related traits compared to the broiler chicken.

## **2. Materials and methods**

### **2.1 Animals**

Japanese quail were bred and hatched in our vivarium. The breeder flock was established with eggs donated by Dr. Mike Lacy at the University of Georgia. Upon removal from the hatcher, straight run chicks were group caged in a brooder for 4 days, then individually in galvanized wire cages (8 cm wide, 7 cm deep and 8 cm high) in a room at a constant temperature of  $35 \pm 1$  °C,  $50 \pm 5\%$  relative humidity, with a 14-h light/10-h dark period. At all times, unless otherwise noted, chicks had ad libitum access to tap water and a mash starter diet formulated to meet or exceed the recommended nutrient specifications for the starter phase of growth

(2,900 kcal ME/kg and 24% CP) [67]. The individual cages allowed visual and auditory contact with other chicks.

After the chicks were individually caged, they were handled twice daily to reduce the effects of stress on the day of data collection. The handling procedure consisted of the chick being removed from its cage and placed in a small Plexiglas box where it remained momentarily before being transferred to another Plexiglas box where it remained momentarily. The chick was removed from the second Plexiglas box and its head was inserted into a restraining device and held in this position for 5 s. The chick was then placed back into the Plexiglas box momentarily before having its head repositioned in the restraining device for another 5 s. Following removal from the restraining device for the second time the chick was placed back into its home cage. This procedure was conducted twice daily until the day prior to injection. The restraining device was a hardened block of clay that had been molded around the head of a 8 day-old quail chick cadaver and was designed such that the entire area of the frontal bone was not obstructed in order to facilitate free-hand intracerebroventricular (ICV) injection. The restraining device was designed with two air vents positioned to end at the nostrils. All experiments were conducted on day 7 post-hatch straight run quail. All 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**

On the day of the experiment (day 7 post-hatch), chicks were injected using a method adapted from Davis et al. [68] that does not appear to induce physiological stress [69]. The head of the chick was briefly inserted into the aforementioned restraining device. Injection coordinates

were 2 mm anterior to the coronal suture, .75 mm lateral from the sagittal suture, and 1.5 mm deep targeting the left lateral ventricle. Anatomical landmarks were determined visually and by palpation. Injection depth was controlled by placing a plastic tubing sheath over the needle. The needle remained at injection depth in the un-anesthetized chick for 5 s post-injection to reduce backflow. Chicks were assigned to treatments at random. Neuropeptide FF (American Peptide Company/Bachem, Sunnyvale, California) was dissolved in artificial cerebrospinal fluid [70] as a vehicle for a total injection volume of 5  $\mu$ l with 0.1% Evans Blue dye to facilitate injection site localization. After data collection, the chick was decapitated and its 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 at the time of decapitation following each experiment.

### **2.3. Experiment 1: food and water intake**

After a 6 h fast, chicks were randomly assigned to receive either 0 (vehicle only), 8, 16, or 32 nmol neuropeptide FF by ICV injection. After injection, chicks were returned to their individual home cages and given ad libitum access to both food and water. Food and water intake were monitored (0.01 g) every 30 min for 180 min post-injection. Water weight (g) was converted to volume (ml; 1 g = 1 ml). Data were analyzed using analysis of variance (ANOVA) within each time point, with the statistical model including the main effect of dose. When dose effects were significant, Tukey's method of multiple comparisons was used to separate the means within each time point. Statistical significance was set at  $P < 0.05$  for all experiments. Food and water intake results are shown on a cumulative and non-cumulative basis for each experiment.

## **2.4. Experiment 2: hypothalamic mRNA abundance of appetite-associated factors**

After a 6 h fast, chicks were randomly assigned to receive either vehicle or 32 nmol NPPF (based on results of Experiment 1) by ICV injection. Each chick was deeply anesthetized at 60 min post-injection with sodium pentobarbital via cardiopuncture, decapitated, and its brain removed. The whole upside-down brain was snap frozen in liquid nitrogen for 9 s. This duration freezes the outermost portion of the brain, providing firmness while leaving the center unfrozen to permit dissection without shattering. Cuts were made visually as per the following anatomy: perpendicular to the midline suture a cut was made at the septopallio-mesencephalic tract and at the third cranial nerves 1.8 mm parallel to the midline two cuts were made and finally the dorsal cut was made from the anterior commissure to 0.8 mm ventral to the posterior commissure [71]. This block (comprised primarily of the hypothalamus) was immediately stored in RNAlater (Qiagen).

## **2.5. Total RNA isolation, reverse transcription and real-time PCR**

The hypothalamus was homogenized using 5 mm stainless steel beads (Qiagen, Valencia, CA, USA), Tri reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and a Tissue Lyser II (Qiagen), and total RNA was extracted following the manufacturer's instructions (Molecular Research Center). The RNeasy Mini Kit (Qiagen) and RNase-free DNase I (Qiagen) were then used for total RNA purification. The integrity of total RNA samples was evaluated by agarose-formaldehyde gel electrophoresis and concentration and purity assessed by spectrophotometry at 260/280/230 nm with a Nanophotometer Pearl (IMPLEN, Westlake Village, CA, USA).

First-strand cDNA was synthesized in 20 µl reactions from 200 ng of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions. Reactions were performed under the following conditions: 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min. Primers for real-time PCR were designed with Primer Express 3.0 software (Applied Biosystems) (Table 1) and validated for amplification efficiency before use (95–105%). Real-time PCR was performed as we previously described [71].

Real-time PCR data were analyzed using the  $\Delta\Delta\text{CT}$  method with  $\beta$ -actin as the reference gene and the average of the vehicle-injected Japanese quail chicks as the calibrator sample [72]. Relative quantities calculated as  $2^{-\Delta\Delta\text{CT}}$  were used for statistical analysis. The statistical model included the main effect of treatment.

## **2.6. Experiment 3: behavior analysis**

In addition to the injection acclimation procedure, to acclimate chicks to the behavior recording arenas, chicks were placed in mock acrylic recording arenas for 1 h each day. On the day of the observation, chicks were fasted for 6 h then randomly assigned to receive either vehicle or 32 nmol NPFF by ICV injection. Following ICV injection, chicks were immediately placed in a 290 mm × 290 mm acrylic recording arena with food and water containers in diagonal corners. Chicks were simultaneously and automatically recorded from three angles for 30 min post-injection on DVD and data were analyzed in 5 min intervals using ANY-maze behavioral analysis software (Stoelting, Wood Dale, IL). At 30 min post-injection, food intake was measured. Locomotion (m traveled), the amount of time spent preening, in deep rest, standing, or sitting, and the number of steps, food pecks, jumps, defecations, exploratory pecks, escape

attempts, and water pecks were quantified. Food and water pecks were defined as pecks within the corresponding container, whereas any other pecks were counted as exploratory. Deep rest was defined as the eyes closed for greater than 3 s (and its timing starting 3 s after eye closure, ending when eyes reopened). Preening was defined as trimming or dressing down with the beak. Due to non-heterogeneous variance, behavior data were analyzed by the Mann–Whitney U test. Pecking efficiency at 30 min post-injection was calculated by dividing food consumed by number of food pecks for each chick. Pecking efficiency and food intake data were analyzed by ANOVA.

### **3. Results**

#### **3.1: Food and water intake**

Quail injected with 16 and 32 nmol NPPF decreased food intake in the first 30 min of the 180 min duration of the experiment (Fig. 1). There were no significant differences observed in water intake (Fig. 2).

#### **3.2: Whole hypothalamus mRNA abundance**

Quail injected with 32 nmol NPPF showed an increase in melanocortin subtype 3 receptor (MC3R) and a decrease in neuropeptide Y receptor subtype 1 (NPYR1) mRNAs relative to chicks injected with the vehicle (Fig. 3). There were no effects of NPPF injection on the mRNA abundance of any of the other genes measured.

### **3.3 Behavior analysis**

Quail injected with 32 nmol NPPF had alterations in count-type behaviors (Table 2). The chicks decreased their distance moved 5 min post-injection. Lastly, they decreased their number of steps 10, 25, and 30 min post-injection. The quail did not have alterations in timed-type behaviors (Table 3). In addition, food intake and feed peck efficiency were measured after the 30 min trial. Feed peck efficiency was not different between groups and food intake was decreased as we observed in Experiment 1.

## **4. Discussion**

In Experiment 1, the quail injected with 16 and 32 nmol of NPPF decreased food intake in the first 30 min. Unlike the previous study conducted in broiler chicks, the quail did not decrease food intake in response to 8.32 nmol NPPF, showing a dose threshold difference between the two species. In addition, the decrease in food intake lasted 90 min in broiler chicks with doses of 8.32 and 16.6 [66], whereas the decrease in quail food intake lasted 30 min with doses of 16 and 32, once again showing a possible difference in peptide potency between the two species. Similar to the broiler chicks, the quail did not have an increase in their water intake, unlike what was demonstrated in rats [65], suggesting that NPPF's anorexigenic effect is not associated with water intake in avian species.

In Experiment 2, the mRNA abundance of specific appetite-related genes in the whole hypothalamus was measured. Genes encoding factors that have a known role in regulating appetite in the hypothalamus were evaluated. There was greater MC3R and decreased NPYR1. In broiler chicks, it was concluded that inhibiting the ligands of both opioid receptor mu and

opioid receptor kappa, rather than the receptors themselves, mediates the anorexigenic effects of NPF [62] and in rats, it was also demonstrated that NPF does not bind directly to opioid receptors [73].  $\alpha$ -Melanocyte stimulating hormone ( $\alpha$ -MSH), an anorexigenic neuropeptide, binds to MC3R receptors in the second-order neurons of the hypothalamus like the lateral hypothalamus and ventromedial hypothalamus [74]. In addition, MC3R is found in POMC neurons in the arcuate nucleus, which are known for their role in inhibiting food intake [75]. MC3R may be partly mediating the effects of NPF on food intake, although data in this study represent mRNA abundance at a single time point after injection.

Neuropeptide Y (NPY), a potent orexigenic neuropeptide, binds to NPYR1 receptors to exert its effects. The use of an NPYR1 antagonist blocked NPY-induced feeding in mice, showing the importance of this receptor in appetite regulation [76]. NPF may induce its effects on food intake by downregulating NPYR1 in order to dampen the effects of NPY in quail.

In the present study, NPF-injected quail decreased distance moved and steps. In chickens, a central injection of NPF caused a decrease in feed pecks, jumps, standing time, and perching time, and an increase in exploratory pecks, sitting time, and sleeping time [66]. This suggests that NPF may cause behaviors that are competitive with food intake in both avian species.

In summary, the present study demonstrated that similar to the chicken and mammalian models, Japanese quail exhibit a decrease in food intake following a central injection of NPF. The NPF-induced satiety may be mediated through the upregulation of MC3R and the downregulation of NPYR1 signaling in the hypothalamus. Further research will need to be done to elucidate the exact hypothalamic nuclei that mediate NPF's appetite effects and to investigate the role of opioid receptors in NPF-injected quail.



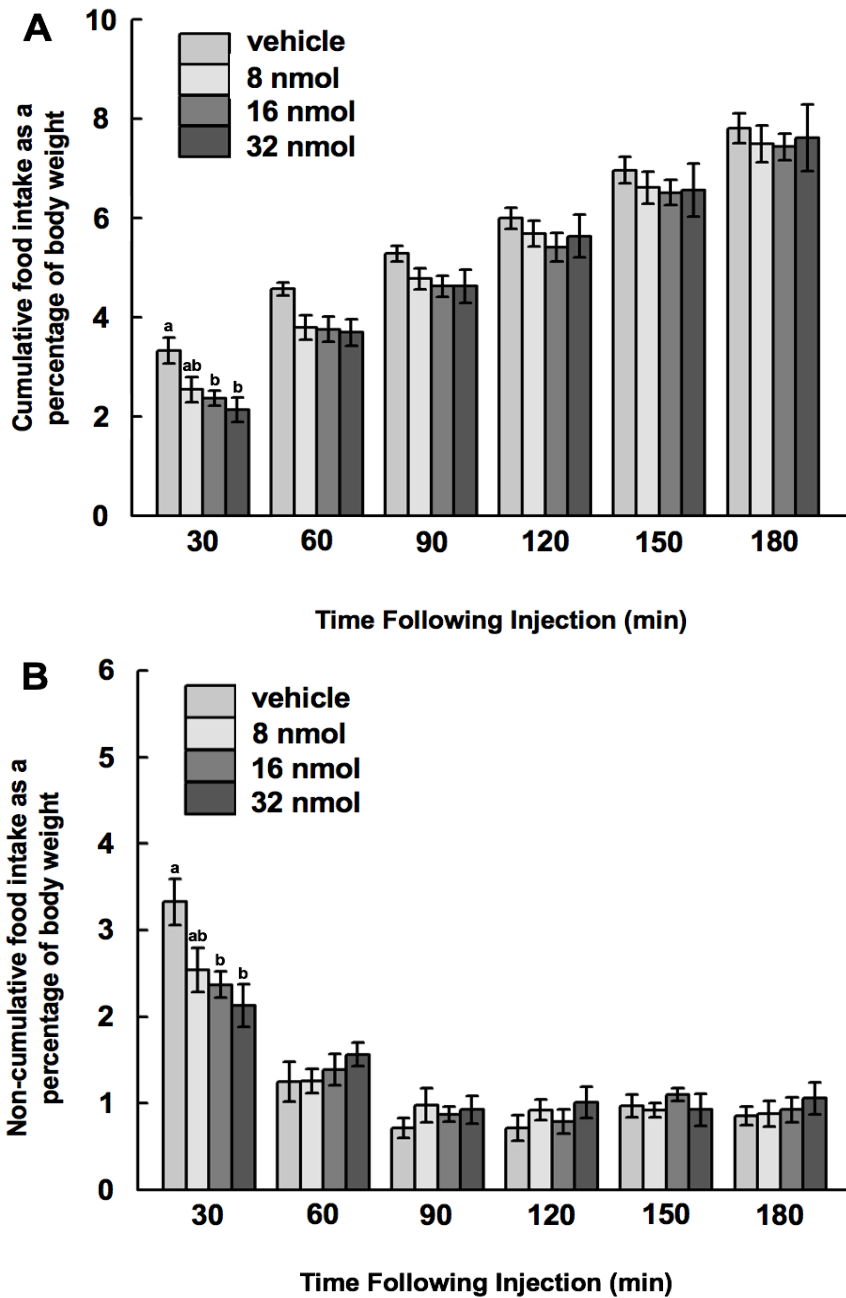


Figure 2-1. Food intake following intracerebroventricular injection of NPPF. Cumulative (A) and non-cumulative (B) food intake expressed as a percentage of body weight in Japanese quail. Values are means  $\pm$  standard error; bars with unique letters are different from each other within a time point ( $P < .05$ ). For this experiment, 10–12 chicks per treatment were available for the analysis.

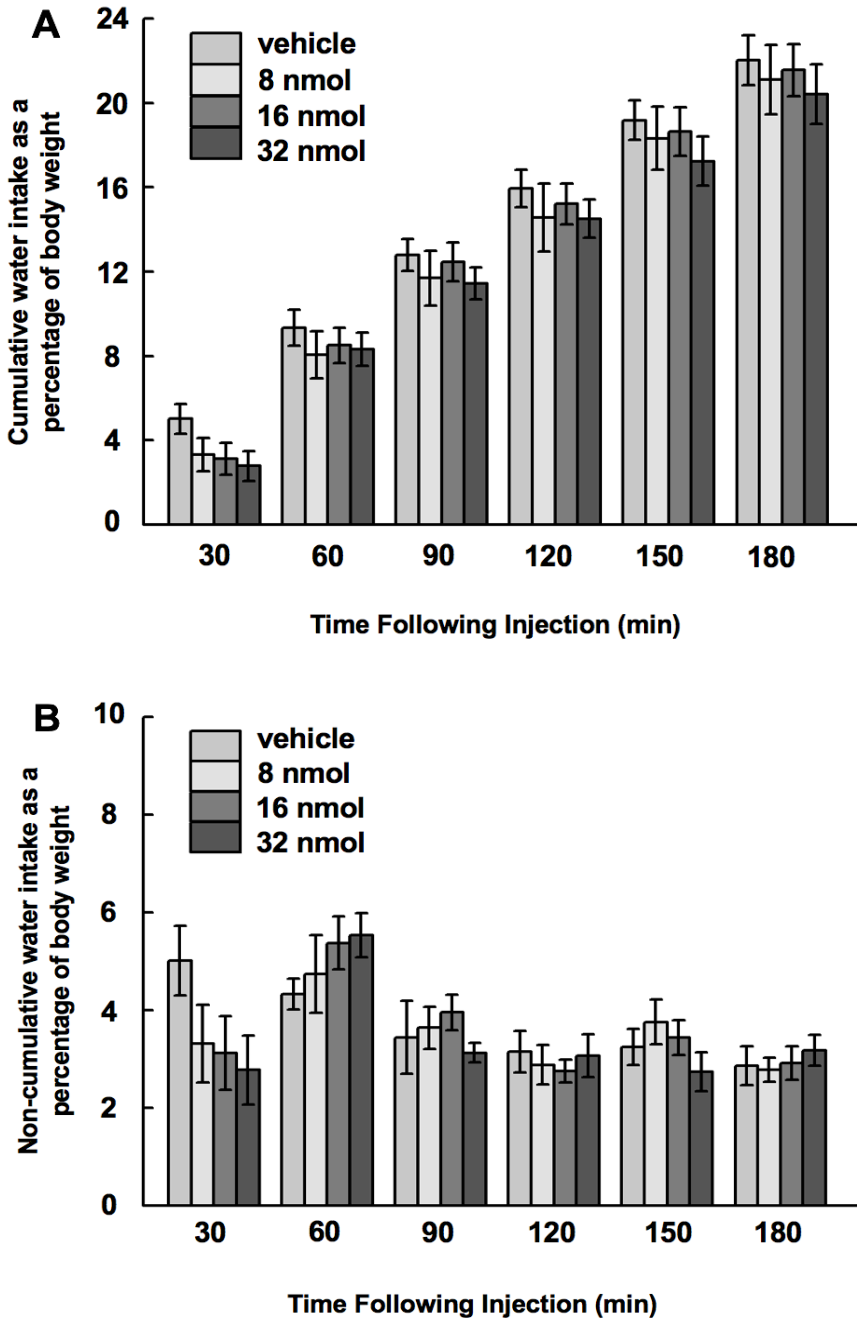


Figure 2-2. Water intake following intracerebroventricular injection of NPFf. Cumulative (A) and non-cumulative (B) water intake expressed as a percentage of body weight in Japanese quail. Values are means  $\pm$  standard error. For this experiment, 10–12 chicks per treatment were available for the analysis.

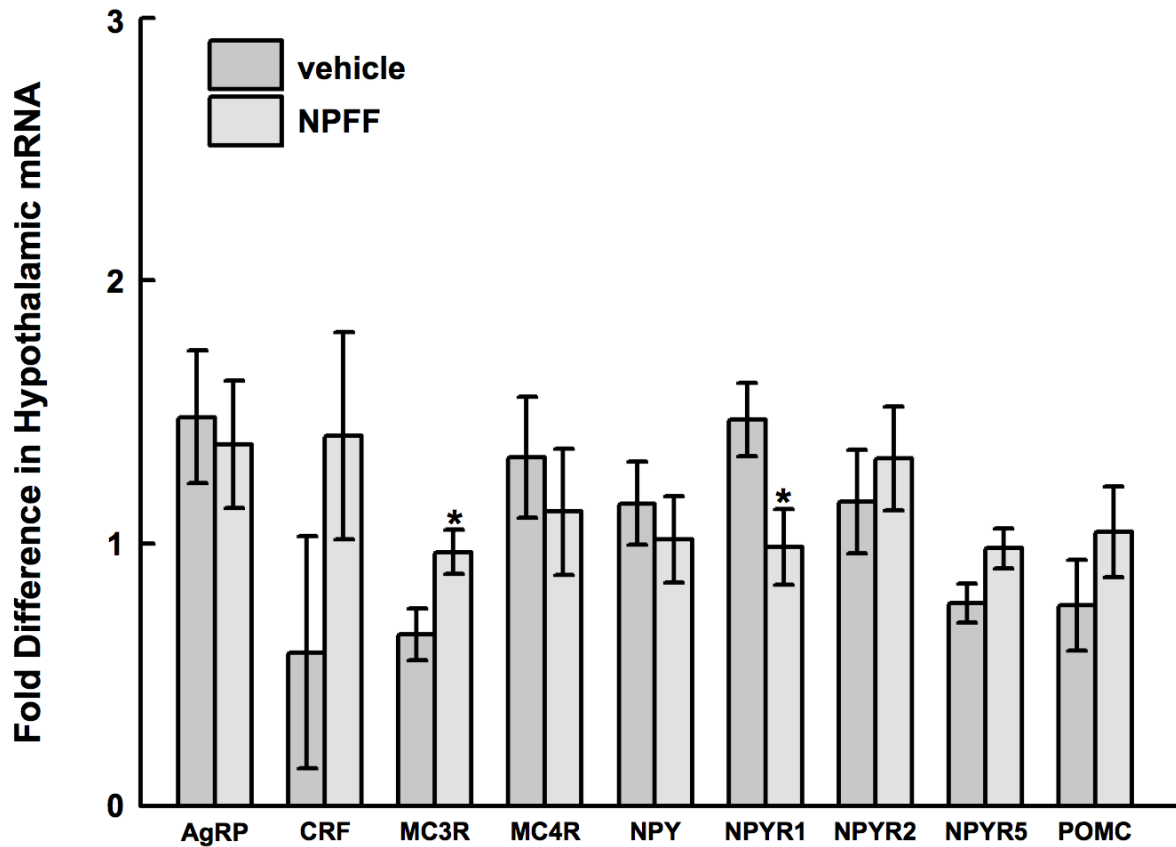


Figure 2-3. Fold difference in hypothalamic mRNA following intracerebroventricular injection of NPFF. Values are means  $\pm$  standard error; bars with \* are different from the vehicle-injected group within a gene ( $P < 0.05$ ). For this experiment, 7-12 chicks per treatment were available for the analysis. AgRP, agouti-related peptide; CRF, corticotropin-releasing factor; MC3R and MC4R, respectively, melanocortin receptors 3 and 4; NPY, neuropeptide Y; NPYR1, NPYR2, and NPYR5, respectively, neuropeptide Y receptors 1, 2, and 5; POMC, pro-opiomelanocortin.

Table 2-1. Primers used for real-time PCR for NPF<sup>1</sup>

Gene	Accession no.	Sequences (forward/reverse)
β-actin	XM_015876619.1	GTCCACCGCAAATGCTTCTAA/TGCGCATTATGGGTTTTGTT
AgRP	XM_015873898.1	GGTTCTTCAACGCCTTCTGCTA/TTCTTGCCACATGGGAAGGT
CRF	XM_015855730.1	TCAGCACCAGAGCCATCACA/GCTCTATAAAAATAAAGAGATGACATCAGA
MC3R	XM_015881722.1	GCCTCCCTTTATGTTACATGT/GCTGCGATGCGCTTCAC
MC4R	XM_015854466.1	CATCAGCTTGCTGGAGAACGT/GCGAATGGAGGTTCTTGTTCTT
NPY	XM_015853870.1	CATGCAGGGCACCATGAG/CAGCGACAGGGCGAAAGTC
NPYR1	XM_015861015.1	TAGCCATGTCCACCATGCA/GGGCTTGCCTGCTTTAGAGA
NPYR2	XM_015860997.1	TGCCTACATCCGCATTTGG/ATTCCCTGCCCCAGGACTA
NPYR5	XM_015861002.1	GGCTGGCTTTGTGGGAAA/CTGTCTTCTGCTTGCCTTTTGT
POMC	NM_001323229.1	GCCAGACCCCGCTGATG/CTTGTAGGCGCTTTTGATGAT

<sup>1</sup>Primers were designed with Primer Express, version 3.0 (Applied Biosystems). AgRP, agouti-related peptide; CRF, corticotropin-releasing factor; MC3R and MC4R, respectively, melanocortin receptors 3 and 4; NPY, neuropeptide Y; NPYR1, NPYR2, and NPYR5, respectively, neuropeptide Y receptors 1, 2, and 5; POMC, pro-opiomelanocortin.

Table 2-2. Count-type Behaviors for NPFF Injection Experiment<sup>1</sup>

Behavior	Treatment	Time post-injection (min)					
		5	10	15	20	25	30
Water pecks (n)	Control	0	0	0	0	1.1±0.9	3.5±2.0
	NPFF	0	0	0	0	0	0.4±0.4
Feeding pecks (n)	Control	32.9±8.9	73.3±16.2	123.3±19.4	164.1±20.6	189.5±22.8	221.8±25.8
	NPFF	25.2±7.4	64.9±13.1	97.9±16.0	136.0±20.2	166.2±26.2	198.8±30.1
Exploratory pecks (n)	Control	35.2±5.8	72.6±11.2	117.4±17.6	152.1±23.7	190.1±29.8	226.0±34.3
	NPFF	25.4±4.0	52.3±9.2	85.8±14.3	115.9±19.2	144.7±24.1	168.7±27.7
Jumps (n)	Control	0.5±0.1	1.2±0.4	1.2±0.4	1.4±0.4	1.6±0.5	1.8±0.5
	NPFF	0.3±0.2	1.0±0.3	1.4±0.3	1.8±0.4	2.0±0.5	2.3±0.5
Escape attempts (n)	Control	0	0	0	0	0	0
	NPFF	0	0	0	0	0	0
Defecations (n)	Control	0	0.1±0.1	0.2±0.1	0.2±0.1	0.3±0.1	0.5±0.1
	NPFF	0.1±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.3±0.1
Distance moved (m)	Control	5.0±0.6	10.9±1.2	17.3±1.9	23.2±2.6	29.6±3.2	36.4±3.8
	NPFF	3.7±0.5*	8.0±1.0	13.3±1.6	17.9±2.0	23.0±2.5	28.1±2.9
Steps (n)	Control	192.3±16.6	398.1±29.8	602.5±43.8	812.2±58.0	1049.4±66.0	1296.4±77.0
	NPFF	147.5±19.6	310.0±37.2*	497.1±53.5	671.9±67.2	856.2±83.2*	1038.7±96.3*

<sup>1</sup>Count-type behaviors recorded 5 to 30 min following ICV injection of 32 nmol NPFF to Japanese quail chicks (Experiment 3). Values are means ± standard error. \* denotes difference from vehicle within an observation time. For this experiment, 11-12 per treatment were available for the analysis.

Table 2-3. Timed-type Behaviors for NPFF Injection Experiment<sup>1</sup>

Behavior	Treatment	Time post-injection (min)					
		5	10	15	20	25	30
Standing	Control	297.4±0.7	597.0±0.8	895.9± 1.0	1191.4±2.8	1487.4±5.5	1782.4±6.4
	NPFF	288.2± 7.1	578.4±13.4	869.9±16.8	1164.4±17.9	1443.1±21.8	1711.4±28.3
Sitting	Control	0	0	0.1±0.1	0.1±0.1	0.2±0.1	0.3±0.2
	NPFF	0	0	0.1±0.1	0.1±0.1	0.1±0.1	0.2±0.1
Deep Rest	Control	0	0	0	0	0	0
	NPFF	0	0	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1
Preening	Control	0.2±0.1	0.5±0.2	1.3±0.7	3.7±1.8	4.5±2.0	4.5±2.0
	NPFF	0.3±0.2	0.9±0.6	3.8±2.0	6.4±3.0	10.5±4.4	12.9±4.9

<sup>1</sup>Timed-type behaviors were recorded 5 to 30 min following ICV injection of 32 nmol NPFF to Japanese quail chicks (Experiment 3). Values are the means ± standard error. \* denotes different from vehicle within an observation time. For this experiment, 14-15 chicks per treatment were available for the analysis.

### **Chapter 3: Hypothalamic mechanism of gastrin-induced satiety**

**Abstract:** Central injection of gastrin, a digestive hormone, decreases food intake in both mammalian and avian species. The physiological mechanism of gastrin-induced satiety in broiler chicks is unknown. Broiler chicks that were intracerebroventricularly (ICV) injected with 500 ng chicken gastrin decreased food intake for the first 120 min of the 180 min experiment. Chicks ICV injected with gastrin had increased c-Fos immunoreactivity in the lateral hypothalamus (LH), paraventricular nucleus (PVN) and arcuate nucleus of the hypothalamus, and area postrema and nucleus of the solitary tract of the brainstem. Injection of gastrin was associated with a decrease in melanin-concentrating hormone (MCH) mRNA in the LH and an increase in corticotropin-releasing factor (CRF) mRNA in the PVN, suggesting that gastrin is exerting effects on appetite via these pathways. However, co-injection of a CRF receptor antagonist, astressin, with gastrin did not affect gastrin-induced reductions in food intake. Thus, effects of gastrin on food intake in broiler chicks may not directly involve CRF receptor signaling, but are likely mediated via several key areas in the hypothalamus and brainstem.

**Keywords:** Chick, gastrin, appetite, CRF, hypothalamus

## 1. Introduction

Mammalian gastrin, a digestive hormone, is primarily expressed in gastrin cells (G cells) in the pyloric antrum [77]. In mammals, gastrin regulates gastric acid secretion and proliferation of cells in the gastric epithelium through cholecystokinin-2 (CCK-2) receptors [78]. Likewise, in chickens, gastrin stimulates gastric acid secretion [79] and binds to the CCK-CHR receptor which is similar in structure to CCK-2R, but it is unclear if the function is the same [80], which may be due to the structural differences between chicken and mammalian gastrin. The CCK-CHR receptor is present in the chicken brain and has binding affinity for gastrin [80, 81].

Chicken gastrin, isolated from the antrum, shares the same COOH-terminal containing  $\text{Typ-Met-Asp-PheNH}_2$  as mammalian gastrin and CCK. However, chicken gastrin more closely resembles the structure of mammalian CCK due to both having a sulfated tyrosine residue that is seven positions from the COOH-terminal. Although it has a similar structure to mammalian CCK, chicken gastrin has the same function as mammalian gastrin due to its ability to stimulate gastric acid secretion, but not pancreatic secretion [79].

The gastrin/CCK family members affect food intake when injected centrally into chickens [82]. Peripheral injection of gastrin-17 does not affect food intake in rats at a dose of 4 mg or 12 mg over a 30 min period [83]. However, a subcutaneous injection of 4 nmol gastrin produced a significant decrease in food intake in 7 day-old chicks for the duration of a 3 h experiment [84]. Both mice and chickens ate less when intracerebroventricularly (ICV) injected with gastrin [85, 86].

While it is evident that gastrin decreases food intake in chicks, the central mechanism is unknown. Specific hypothalamic and brainstem nuclei play a role in food intake. The lateral hypothalamus (LH), paraventricular nucleus (PVN), ventromedial hypothalamus (VMH),



dorsomedial nucleus (DMN), and the arcuate nucleus (ARC) of the hypothalamus are known for their roles in food intake. The area postrema (AP) and the nucleus of the solitary tract (NTS) of the brainstem are also known to help in the regulation of appetite. In mice, central injection of gastrin upregulated hypothalamic pro-opiomelanocortin (POMC) gene expression at 4 h post-injection, but the gene expression in individual nuclei was not measured [86]. Thus, the purpose of this study was to determine the hypothalamic and brainstem nuclei and corresponding appetite-associated mRNA that mediate gastrin's anorexigenic effect in the chicken.

## **2. Materials and Methods**

### **2.1 Animals**

Straight run Cobb-500 broiler chicks were obtained from breeders 30-40 weeks of age from a commercial hatchery on the morning of hatch. On day 1 post-hatch, they were individually caged in a room at  $30 \pm 2$  °C and  $50 \pm 5\%$  relative humidity with ad libitum access to mash diet (22% crude protein and 3000 kcal ME/kg) and tap water. All experiments were performed on day 4 post-hatch broiler chicks. The number of chicks used in each experiment are stated in the corresponding figures and tables. All experimental procedures were performed according to Virginia Tech's Institutional Animal Care and Use Committee.

### **2.2 Intracerebroventricular (ICV) injection procedure**

Chicks were injected (day 4 post-hatch) using a method adapted from [68] that does not appear to induce physiological stress [44]. The head of the chick was briefly inserted into a restraining device that left the cranium exposed and allowed for free-hand injection. Injection

coordinates were 3 mm anterior to the coronal suture, 1 mm lateral from the sagittal suture, and 2 mm deep targeting the left lateral ventricle. Anatomical landmarks were determined visually and by palpation. Injection depth was controlled by placing a plastic tubing sheath over the needle. The needle remained at injection depth in the un-anesthetized chick for 10 s post-injection to reduce backflow. Chicks were assigned to treatments at random. Chicken gastrin (4192.69 molecular weight, Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) was dissolved in artificial cerebrospinal fluid [87] as a vehicle for a total injection volume of 5  $\mu$ l with 0.1% Evans Blue dye to facilitate injection site localization. After data collection, the chick was decapitated and its 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 following the experiments.

### **2.3 Experiment 1: Effect on food and water intake**

After a 3 h fast with access to water, chicks were randomly assigned to receive either 0 (vehicle only), 125, 250, or 500 ng chicken gastrin by ICV injection. After injection, chicks were returned to their individual home cages and given *ad libitum* access to both food and water. Food and water intake were monitored (0.01 g) every 30 min for 180 min post-injection. Water weight (g) was converted to volume (ml; 1 g = 1 ml). Data were analyzed using analysis of variance (ANOVA) within each time point. When dose effects were significant, Tukey's method of multiple comparisons was used to separate the means within each time point. For all experiments, data are presented as means  $\pm$  SE and statistical significance set at  $P < 0.05$ .

## **2.4 Experiment 2: c-Fos immunohistochemistry in hypothalamic nuclei**

Chicks were randomly assigned to receive either vehicle or 500 ng gastrin (based on results of Experiment 1) by ICV injection. Chicks were fasted 3 h prior to injection, but given ad libitum access to water. Sixty min post-injection, as this is the time expected for the most robust c-Fos expression [88], chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture, then perfused via the carotid artery with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) containing 0.2% picric acid at pH 7.4. Brains were removed from skulls and post-fixed for 60 min in the same solution, after which they were blocked and placed through a series of graded sucrose incubations, consisting of 20 and 30% in 0.1 M PB, until they sank in each.

Several 60  $\mu\text{m}$  coronal sections that contained appetite-related nuclei based on anatomies described by Kuenzel [89] were collected in 0.02 M phosphate buffered saline (PBS) containing 0.1% sodium azide using a cryostat at  $-15\text{ }^{\circ}\text{C}$  [90]. The lateral hypothalamus (LH) was collected corresponding to 8.0 from interaural. The paraventricular nucleus (PVN) was collected corresponding to interaural 7.4 from interaural. The ventromedial hypothalamus (VMH) was collected corresponding to 6.8 from interaural. The dorsomedial nucleus (DMN) and the arcuate nucleus (ARC) were collected at 5.4 from interaural. Lastly, the nucleus of the solitary tract (NTS) and the area postrema (AP) were collected at 2.8 from the interaural. Sections were processed immediately after collection.

Procedures for the c-Fos immunohistochemistry assay were performed as we described and validated previously [91] using rabbit polyclonal anti-c-Fos at a dilution of 1:5,000 (Abcam, AB429) [91, 92]. Anatomy was confirmed and a digital micrograph captured for each section.

Overlays containing the respective nuclei boundaries were digitally merged with micrographs and the number of c-Fos immunoreactive cells within each respective nucleus counted by a technician blind to treatment. Data were analyzed by ANOVA and the model included the main effect of gastrin dose within a nucleus.

### **2.5 Experiment 3: mRNA abundance in hypothalamic nuclei**

After a 3 h fast with access to water, injections were performed in chicks that were randomly assigned to either vehicle or 500 ng gastrin (based on Experiment 1), and at 1 h post-injection, each chick was deeply anesthetized with sodium pentobarbital via cardiopuncture, then perfused via the carotid artery with 2.5 mL of RNA stabilizing buffer (16.7 mM sodium citrate, 13.3 mM EDTA, and 3.5 M ammonium sulfate; pH = 5.2). Within 30 min of perfusion, brains were sectioned in a cryostat at  $-10\text{ }^{\circ}\text{C}$  into 500  $\mu\text{m}$  thick coronal sections. Sections were collected from the rostral to caudal direction: the LH and PVN corresponding to 8.0 and 7.4 from interaural, respectively, and the DMN and ARC (also lateral to infundibular nucleus) corresponding to 5.4 from interaural based on published anatomy. The NTS and AP were collected from 2.8 from interaural [89] in a single biopsy and evaluated as a single nuclei due to the limitations of the 1 mm size of the punch extraction.

We selected to isolate nuclei for total RNA isolation that responded to treatment in Experiment 1 with increased c-Fos immunoreactivity. Nuclei biopsies were collected on a metal block housed on dry ice, using sterile disposable biopsy instruments (1 mm, Braintree Scientific Inc., Braintree, MA). The biopsies were immediately submerged in RNA lysis buffer with 1% beta-mercaptoethanol (Norgen Biotek, Ontario, Canada), vortexed, snap-frozen in liquid nitrogen, and stored at  $-80\text{ }^{\circ}\text{C}$ .

Total RNA was isolated from biopsies using the Total RNA Purification Micro kit (Norgen Biotek) and optional RNase-Free DNase-I kit (Norgen Biotek), following the manufacturer's instructions with some modifications. After thawing, the biopsies were vortexed vigorously for 30 s and incubated for 5 min at room temperature before the addition of 70% molecular-grade ethanol. Total RNA was eluted in nuclease-free water and assessed by spectrophotometry. RNA integrity was verified using Experion RNA StdSens Chips (Bio-Rad, Hercules, CA).

Single-strand cDNA was synthesized from 100 ng total RNA in 20  $\mu$ L reactions with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) following the manufacturer's instructions. Reactions were performed under the following conditions: 25  $^{\circ}$ C for 10 min, 37  $^{\circ}$ C for 2 h, and 85  $^{\circ}$ C for 5 min. Primers for real time PCR were designed with Primer Express 3.0 (Applied Biosystems) (Table 1). Amplification efficiency was validated for all primer pairs before use (95–100% efficiency). Genes were selected to measure in each nuclei based on a report of the site-specific expression of hypothalamic neuropeptides and their receptors in different nuclei in mammals [93], and based on our previous hypothalamic gene expression studies [94]. Total RNA yield limited the total number of genes that could be measured in a single nucleus.

Real-time PCR reactions were performed in duplicate 10  $\mu$ L reactions that contained 5  $\mu$ L Fast SYBR Green Master Mix (Applied Biosystems), 0.5  $\mu$ L primers (0.25  $\mu$ L of 5  $\mu$ M forward primer and 0.25  $\mu$ L of 5  $\mu$ M reverse primer), 1.5  $\mu$ L nuclease-free water, and 3  $\mu$ L 5-fold diluted cDNA using a 7500 Fast Real-Time PCR System (Applied Biosystems). PCR was performed under the following conditions: 95  $^{\circ}$ C for 20 s and 40 cycles of 90  $^{\circ}$ C for 3 s plus 60  $^{\circ}$ C for 30 s. A dissociation step consisting of 95  $^{\circ}$ C for 15 s, 60  $^{\circ}$ C for 1 min, 95  $^{\circ}$ C for 15 s

and 60 °C for 15 s was performed at the end of each PCR reaction to ensure amplicon specificity. Factors were considered to be negligibly expressed when CT values were >35 and did not differ between cDNA generated from standard reactions and negative control reverse transcription reactions (assumed to represent genomic DNA amplification). All genes evaluated were included in the analysis due to these criteria being met.

Data were analyzed using the  $\Delta\Delta\text{CT}$  method, where  $\Delta\text{CT} = \text{CT target gene} - \text{CT actin}$ , and  $\Delta\Delta\text{CT} = \Delta\text{CT target sample} - \Delta\text{CT calibrator}$  [95]. Actin served as the reference gene and vehicle-treated chicks within a nuclei served as the calibrator sample. Relative quantities, calculated as  $2^{-\Delta\Delta\text{CT}}$ , were used for ANOVA with the model including the main effect of treatment, using JMP Pro 13 (SAS Ins.) and the Fit Model Platform. Significance was assigned at  $P < 0.05$ .

## **2.6 Experiment 4: Food intake following gastrin injection and a CRF receptor antagonist**

The experimental procedures were identical to those in Experiment 1 except that broiler-type chicks were randomly assigned to receive either vehicle only, 500 ng gastrin, 6 nmol astressin (3563.3 MW; American Peptide Co., Sunnyvale, CA, USA), or 500 ng gastrin + 6 nmol astressin by ICV injection after a 3 h fast with ad libitum access to water. The dose of astressin was based on previous experiments in broiler chicks [96].

## **2.7 Experiment 5: Behavior measurement**

Chicks were kept in individual cages with auditory but not visual contact with each other (to reduce isolation stress during the observational period), and were randomly assigned to

receive either vehicle or 500 ng chicken gastrin by ICV injection. Following 3 h of fasting with access to water, injections were performed and chicks were immediately placed in a 290 mm × 290 mm acrylic recording arena with food and water containers in diagonal corners. Chicks were simultaneously and automatically recorded from three angles for 30 min post-injection on DVD and data were analyzed in 5 min intervals using ANY-maze behavioral analysis software (Stoelting, Wood Dale, IL). At 30 min post-injection, food intake was measured. Locomotion (m traveled), the amount of time spent standing, sitting, preening, or in deep rest, and the number of jumps, steps, feeding and exploratory pecks, and escape attempts were quantified. Food pecks were defined as pecks within the food container, whereas any other pecks were counted as exploratory. Deep rest was defined as the eyes closed for greater than 3 s (and its timing starting 3 s after eye closure, ending when eyes reopened). Preening was defined as trimming or dressing down with the beak. Due to non-heterogeneous variance, behavior data were analyzed by the Mann–Whitney U test. Pecking efficiency at 30 min post-injection was calculated by dividing food consumed by number of food pecks for each chick. Pecking efficiency and food intake were analyzed by ANOVA. Significance was assigned at  $P < 0.05$ .

### **3. Results**

#### **3.1 Food and water intake**

Chicks injected with 500 ng gastrin decreased food intake relative to vehicle-injected chicks from 30 min to 120 min following injection on a cumulative basis. By 150 min post-injection, the effect subsided and there were no differences among treatments (Fig. 3-1A). On a non-cumulative basis, food intake was significantly decreased by 500 ng of gastrin relative to the

vehicle during the first 30 min (Fig. 1B). Water intake was not influenced by gastrin during the 180 min observation period on a cumulative basis (Fig. 3-2A), but was decreased at 120 min on a non-cumulative basis relative to that of chicks that were injected with the vehicle (Fig. 3-2B).

### **3.2 c-Fos immunoreactivity in hypothalamic and brainstem nuclei**

Chicks injected with 500 ng gastrin displayed changes in c-Fos expression in the hypothalamus and brainstem (Fig. 3-3 and 3-4). Those treated with gastrin had increased c-Fos immunoreactivity in the LH, PVN, ARC, AP, and NTS relative to chicks injected with the vehicle. There were no effects observed in the VMH and DMN (Fig. 3-3).

### **3.3 mRNA abundance in specific hypothalamic nuclei**

Chicks injected with 500 ng gastrin expressed less melanin-concentrating hormone (MCH) in the LH (Fig. 3-5A) and more corticotropin-releasing factor (CRF) mRNAs in the PVN relative to chicks injected with vehicle (Fig. 3-5B). There were no effects of gastrin injection on the mRNA abundance of any of the genes measured in the ARC (Fig. 3-5C) or AP/NTS (Fig. 3-5D).

### **3.4 Food intake following co-injection of gastrin and a CRF receptor antagonist**

During the first 30 min post-injection, chicks injected with 500 ng gastrin and 500 ng gastrin + 6 nmol astressin ate less than the vehicle-injected group, while the astressin-injected chicks were not different from the vehicle-injected chicks (Fig. 3-6A and 3-6B). On a cumulative basis, at 60 min, chicks injected with astressin consumed a similar amount of food as the vehicle-



injected chicks, but more than the gastrin and gastrin + astressin-injected chicks (Fig. 3-6A). At 60 min on a non-cumulative basis, there were no significant differences among groups (Fig. 3-6B). During the first 30 min, water intake was similar. However, on both a cumulative and non-cumulative basis, at 60 min, the gastrin + astressin-injected chicks drank less than the vehicle-injected chicks (Fig. 3-7C and 3-7D).

### **3.5 Behavior measurements**

Chicks injected with 500 ng gastrin displayed changes in timed-type behaviors (Table 2). The chicks injected with gastrin increased the amount of time spent standing at 5, 10, and 15 min following injection and decreased the amount of time spent sitting at 10 and 15 min following gastrin injection. Lastly, they decreased time spent in deep rest at 20 min following injection. There were no differences in count-type behaviors among groups (Table 3). In addition to the behavior analysis, food and water intake were measured after the 30 min period. Gastrin-injected chicks significantly decreased food intake, but did not affect water intake for the 30 min behavior experiment (data not shown).

## **4. Discussion**

We measured the effects of ICV-injected gastrin on food intake in fasted broiler chicks. A subcutaneous injection of two doses, 4 and 12 mg/kg gastrin-17, in both Zucker obese and lean rats did not affect food intake over a 30 min period [83]. In mice, an ICV injection of 1 ng of human gastrin-17 resulted in an almost 3-fold decrease in food intake during the 4 h period [86]. However, due to the constraint of the varying lengths of the study, a conclusion cannot be

reached on the true effect of gastrin on food intake in mammals. Further research will need to be done looking at additional time points.

A subcutaneous injection of 4 nmol/kg gastrin in 7 day-old chicks resulted in decreased food intake over a 3 h period [84]; however, only one time point was evaluated so it cannot be compared to the effect of a subcutaneous injection in the rat, especially since the rat study only included food intake data at 30 min post-injection. In 2 day-old chicks, both 275 and 550 ng chicken gastrin decreased food intake for 60 min post-injection, while the 550 ng dose continued to decrease food intake for 120 min. An additional experiment showed that the decrease of food intake associated with the 550 ng gastrin-injected birds started as early as 15 min post-injection [85]. In another study by the same group, 131 pmol chicken gastrin-36 decreased food intake at both 60 and 120 min following injection [82]. Consistent with these findings, we observed that 500 ng gastrin decreased food intake from 30 to 120 min post-injection over a 3 h period; however, the 250 ng dose did not affect food intake. It is important to note the threshold difference in dose between rodents and chicks. During ICV injection of gastrin, in rodents, a dose of 1 ng was used, while in chicks, a dose of 275 to 550 ng was needed to produce a response.

The c-Fos immunoreactivity was measured in Experiment 2 to identify the appetite-associated nuclei in the hypothalamus that were likely mediating the food intake effects of gastrin. Of the 7 brain nuclei evaluated, c-Fos immunoreactivity was increased in the LH, PVN, ARC, NTS, and AP of gastrin-treated chicks. An intravenous injection of 20 µg gastrin-17 in Wistar rats increased c-Fos expression in the LH and NTS-DMX (dorsal motor nucleus of the vagus nerve), but did not increase the c-Fos expression in the PVN [97]. Further, an intraperitoneal injection of 195 µmol/kg of pentagastrin in Sprague-Dawley rats increased the

number of c-Fos immunoreactive cells in both the AP and the NTS [98]. Both of these studies are similar to our findings, even though both involved peripheral injections.

The hypothalamus is a major center for appetite regulation. The ARC contains first order neurons. These neurons release neuropeptide Y and agouti-related peptide, which increase food intake, and release pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript, which decrease food intake. POMC is a precursor protein which can be cleaved into peptides, some of which can reduce food intake, while another can actually increase food intake. When a signal of adiposity (e.g., leptin from the circulation) communicates with the ARC, the anorexigenic first order neurons will signal via projections to second order neurons located in nuclei such as the PVN. In the PVN, neuropeptides such as CRF are released that induce satiety. On the other hand, when hunger-inducing signals are communicated to the ARC, the orexigenic first order neurons will signal via projections to second order neurons located in regions such as the LH to secrete hunger-inducing neuropeptides [2]. The hypothalamus and brainstem also communicate to regulate appetite. For example, the PVN provides output to the brainstem [29]. Additionally, the brainstem receives vagal afferents from the periphery and a knockdown of the leptin receptor in both the AP and NTS caused an increase in food intake in mice [31]. Projections from the NTS innervate with nuclei in the hypothalamus, including the PVN, DMN, ARC, and LH [29].

In the present study, an increase in c-Fos immunoreactivity in the ARC indicates an increase in the activity of the first-order neurons associated with satiety. An increase in c-Fos immunoreactivity in the PVN suggests that there might be increased anorexigenic signaling via projections from the ARC. We also observed an increase in c-Fos immunoreactive cells in the LH of the hypothalamus and AP and NTS of the brainstem. Thus, gastrin-induced satiety may

involve pathways associated with activation of hypothalamic nuclei leading to activation of brainstem nuclei. For instance, the PVN projects to the caudal brainstem and serration of this projection caused hyperphagia and obesity in adult female rats [99]. The brainstem projects to the ARC to communicate appetite regulatory signals [100].

In mice, an ICV injection with gastrin increased hypothalamic POMC gene expression [84]. POMC production is associated with an increase in anorexigenic signals [2]. In order to more precisely define the molecular mechanisms associated with gastrin-induced reductions in food intake, we measured mRNA abundance of appetite-associated factors in the nuclei that were activated in response to gastrin injection. A decrease in MCH mRNA was observed in the LH, while an increase in CRF mRNA was observed in the PVN. No changes in gene expression were found in the ARC or AP/NTS, which was surprising although we measured a relatively small subset of genes and may have missed factors that were associated with gastrin's effects, or missed the time at which maximal changes in gene expression occurred.

Satiety signals are projected to the PVN from the ARC, which causes the PVN to release anorexigenic neuropeptides, including CRF [2]. CRF-immunoreactive perikarya was detected in numerous nuclei of the chicken hypothalamus, including the PVN [101]. CRF injection decreased food intake in fed broilers for 3 h post-ICV injection, and decreased food intake at 150 and 180 min post-ICV injection in fasted broilers [102]. The activation of the PVN coupled to the upregulation of CRF mRNA in the PVN thus suggest that the satiety effects of central gastrin are partly mediated through increased CRF signaling in the PVN.

Astressin is a CRF receptor antagonist that binds to CRF receptors with high affinity. [103]. In addition to CRF binding to CRF receptors, urocortin binds with higher affinity than CRF to CRF receptor 2 [104]. Therefore, astressin will block the CRF receptors, and if we see a

change in response it may be due to blocking CRF, urocortin, or other endogenous ligands. To evaluate if gastrin-induced reductions in food intake were being mediated by CRF receptors in the PVN, we co-injected gastrin and astressin. The dual injection did not affect gastrin-induced reductions in food intake, suggesting that CRF receptors might not be directly involved in gastrin-induced satiety in chicks.

The behavior analysis was conducted to determine if the effect of gastrin injection was behaviorally specific to food intake. In 2 day-old chicks, ICV gastrin decreased drinking frequency at 60 min following injection and increased locomotor activity at 60 and 120 min following injection [85]. In the present study, gastrin-injected 4 day-old chicks spent less time sitting and in deep rest, and more time standing during the 30 min trial. However, these significant differences are only present in a few of the time points. Although the dose used in chicks is substantially increased compared to the dose used in rodents, it does not seem that gastrin is producing overdose- or malaise-related behavior, and seems likely that the effects of gastrin are specific to food intake.

In summary, ICV injection of gastrin was associated with a decrease in food intake, activation of the LH, PVN, ARC, AP, and NTS, an increase in CRF expression in the PVN, and a decrease in MCH expression in the LH in broiler chicks. Thus, gastrin's effects on food intake likely involve pathways in these key appetite-related nuclei. However, because dual-injection of gastrin and astressin did not affect gastrin-induced inhibition of food intake, it is unlikely that CRF receptor signaling is directly involved in gastrin-induced satiety. Future studies should explore the contribution of more molecular pathways in the hypothalamic and brainstem regions that were activated in response to gastrin injection.

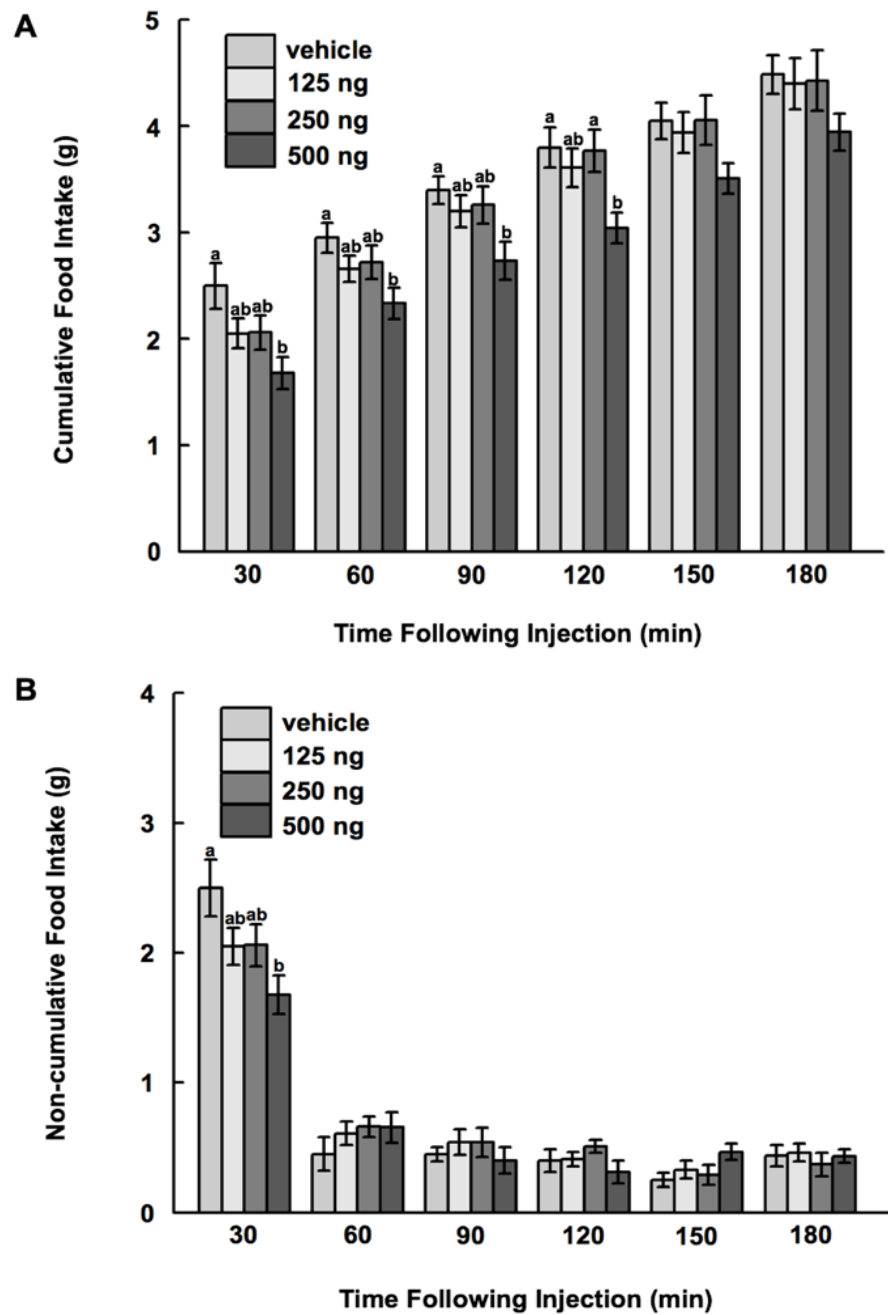


Figure 3-1. Food intake following intracerebroventricular injection of gastrin. Cumulative (A) and non-cumulative (B) food intake in 4 day-old broiler chicks. Values are means  $\pm$  standard error; bars with unique letters are different from each other within a time point ( $P < 0.05$ ). For this experiment, 8-10 chicks per treatment were available for the analysis.

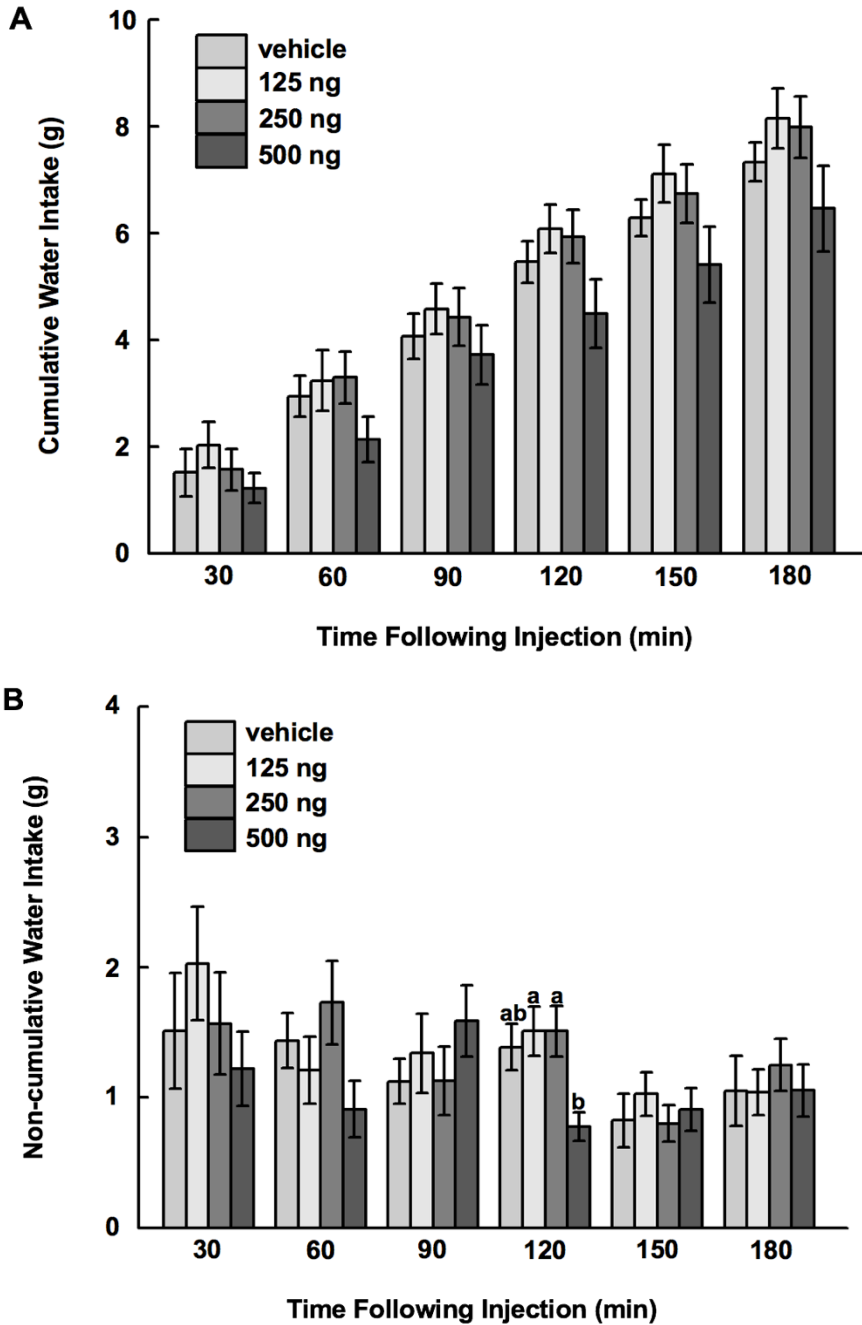


Figure 3-2. Water intake following intracerebroventricular injection of gastrin. Cumulative (A) and non-cumulative (B) water intake in 4 day-old broiler chicks. Values are means  $\pm$  standard error; bars with unique letters are different from each other within a time point ( $P < 0.05$ ). For this experiment, 8-10 chicks per treatment were available for the analysis.

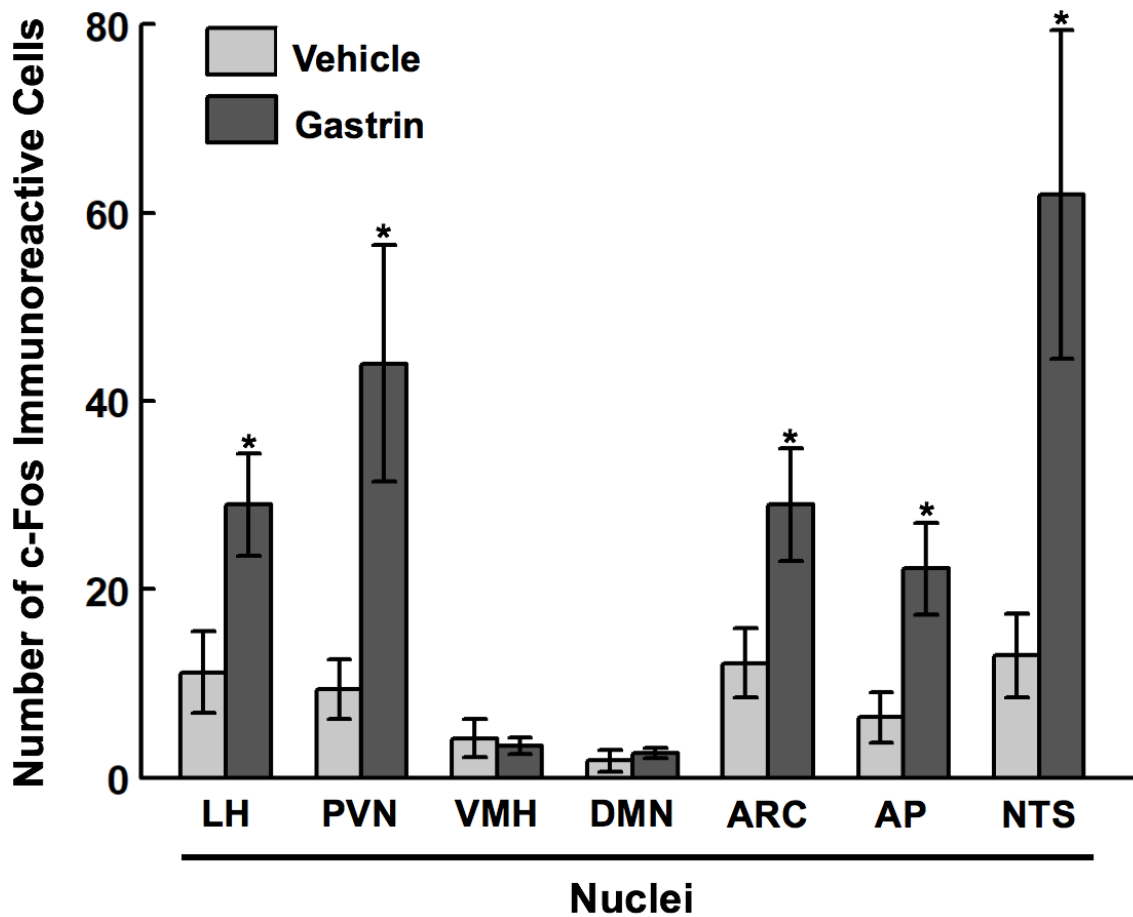
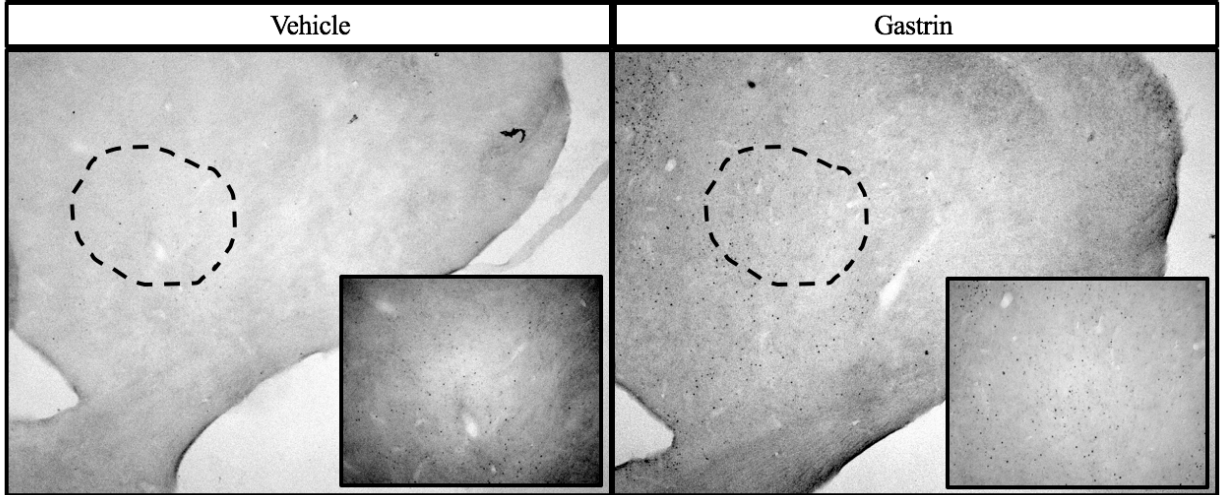


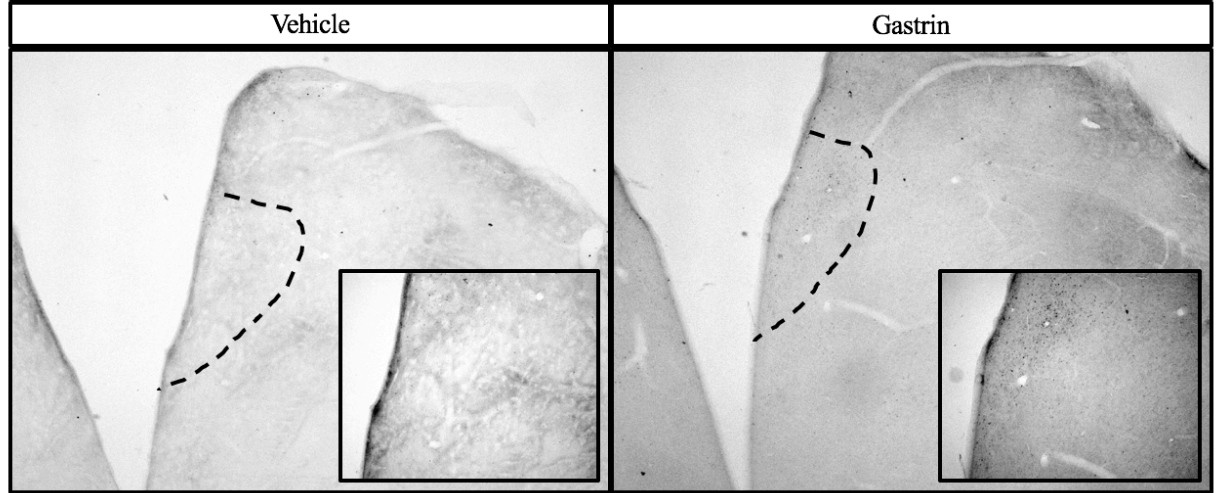
Figure 3-3. Number of c-Fos immunoreactive cells following intracerebroventricular injection of gastrin in 4 day-old broiler chicks at 60 min post-injection in the paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), lateral hypothalamus (LH), dorsomedial nucleus (DMN), arcuate nucleus of the hypothalamus (ARC), and area postrema (AP) and nucleus of the solitary tract (NTS) of the brainstem. Values are means  $\pm$  standard error; bars with \*are different from the vehicle-injected group within a nucleus ( $P < 0.05$ ).



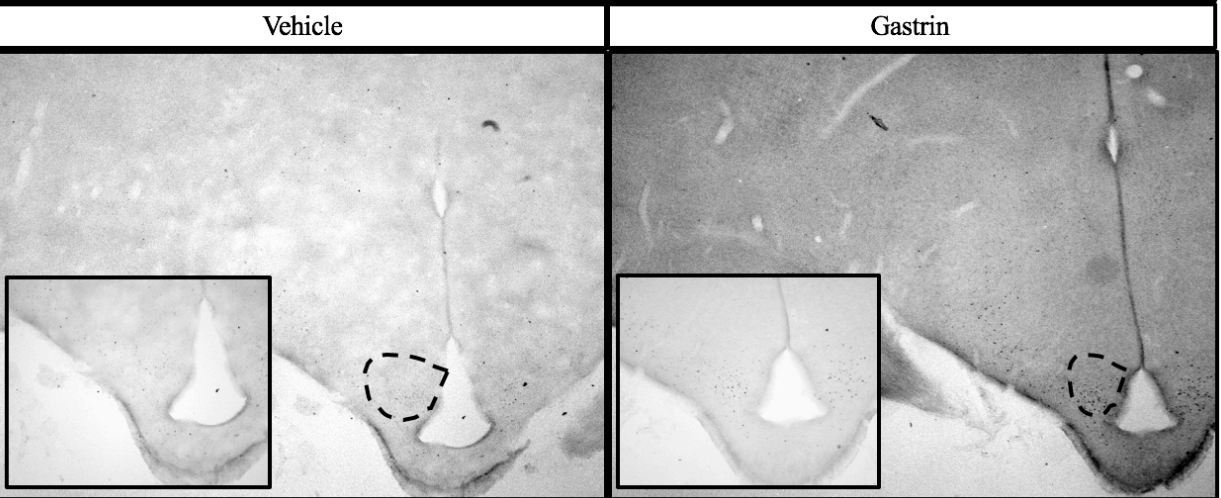
**A. Lateral Hypothalamus**



**B. Paraventricular Nucleus**



**C. Arcuate Nucleus**



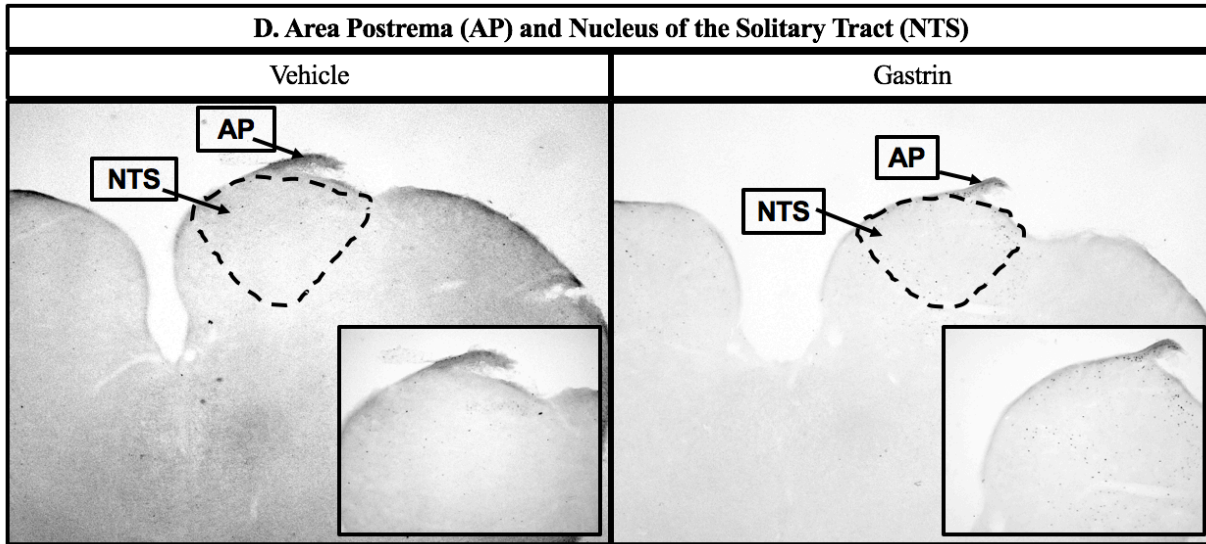


Figure 3-4. Representative photomicrographs of c-Fos immunohistochemical staining following intracerebroventricular injection of gastrin in the LH (A), PVN (B), ARC (C), and AP/NTS (D). Photomicrographs taken at greater magnification are located in the bottom corner of each image. Treatment is indicated above each photomicrograph. For this experiment, 5 chicks per treatment were available for the analysis.

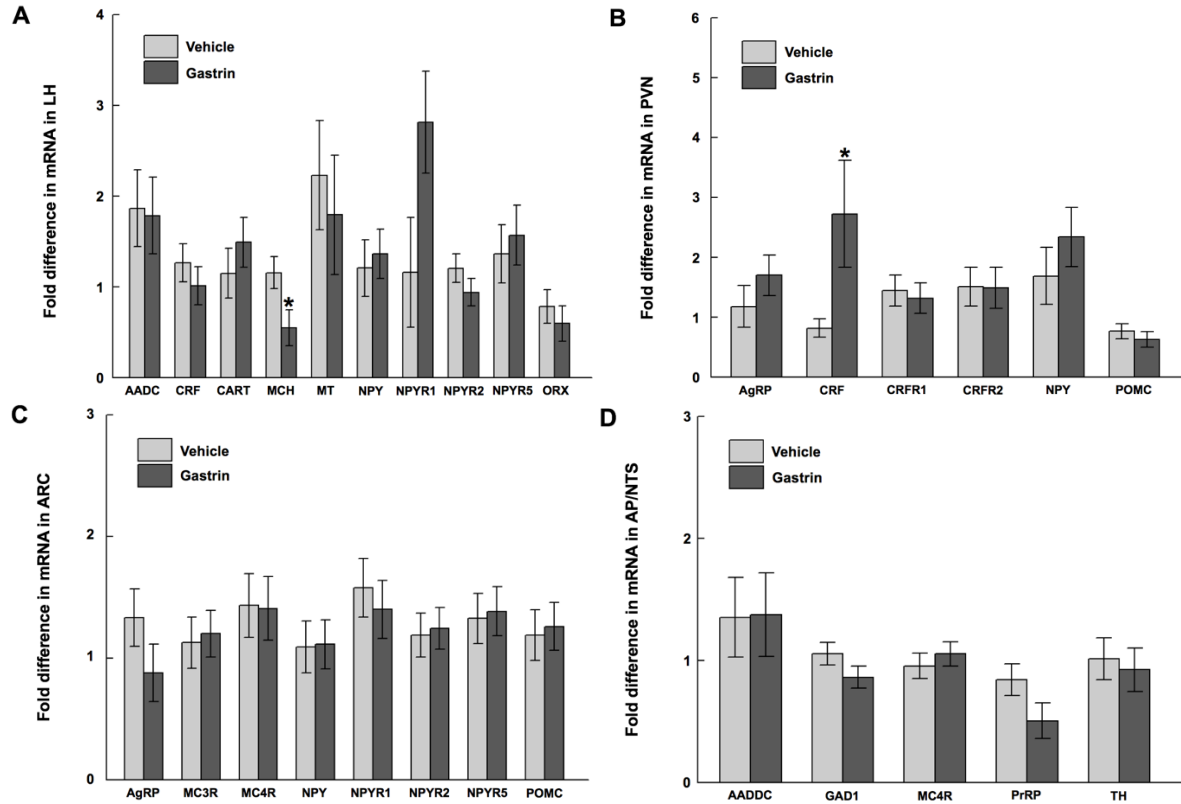


Figure 3-5. mRNA abundance in specific nuclei following intracerebroventricular injection of gastrin in 4 day-old broiler chicks at 60 min post-injection in the lateral hypothalamus (LH) (A), paraventricular nucleus (PVN) (B), arcuate nucleus (ARC) (C), and the combined biopsy of the area postrema (AP) and nucleus of the solitary tract (NTS) (D). Values are means  $\pm$  standard error; bars with \*are different from the vehicle-injected group within a gene ( $P < 0.05$ ). For this experiment, 6-18 chicks per treatment were available for the analysis. AADC, aromatic L-amino acid decarboxylase; AgRP, agouti-related peptide; CART, cocaine and amphetamine-regulated transcript; CRF, corticotropin-releasing factor; CRFR1 and CRFR2, respectively, corticotropin-releasing factor receptor subtypes 1 and 2; GAD1, glutamate decarboxylase isoform 1; MC3R and MC4R, respectively, melanocortin receptors 3 and 4; MCH, melanin-concentrating hormone; MT, mesotocin; NPY, neuropeptide Y; NPYR1, NPYR2, and NPYR5, respectively, neuropeptide Y receptors 1, 2, and 5; ORX, orexin; POMC, pro-opiomelanocortin; PrRP, prolactin-releasing peptide; TH, tyrosine hydroxylase.

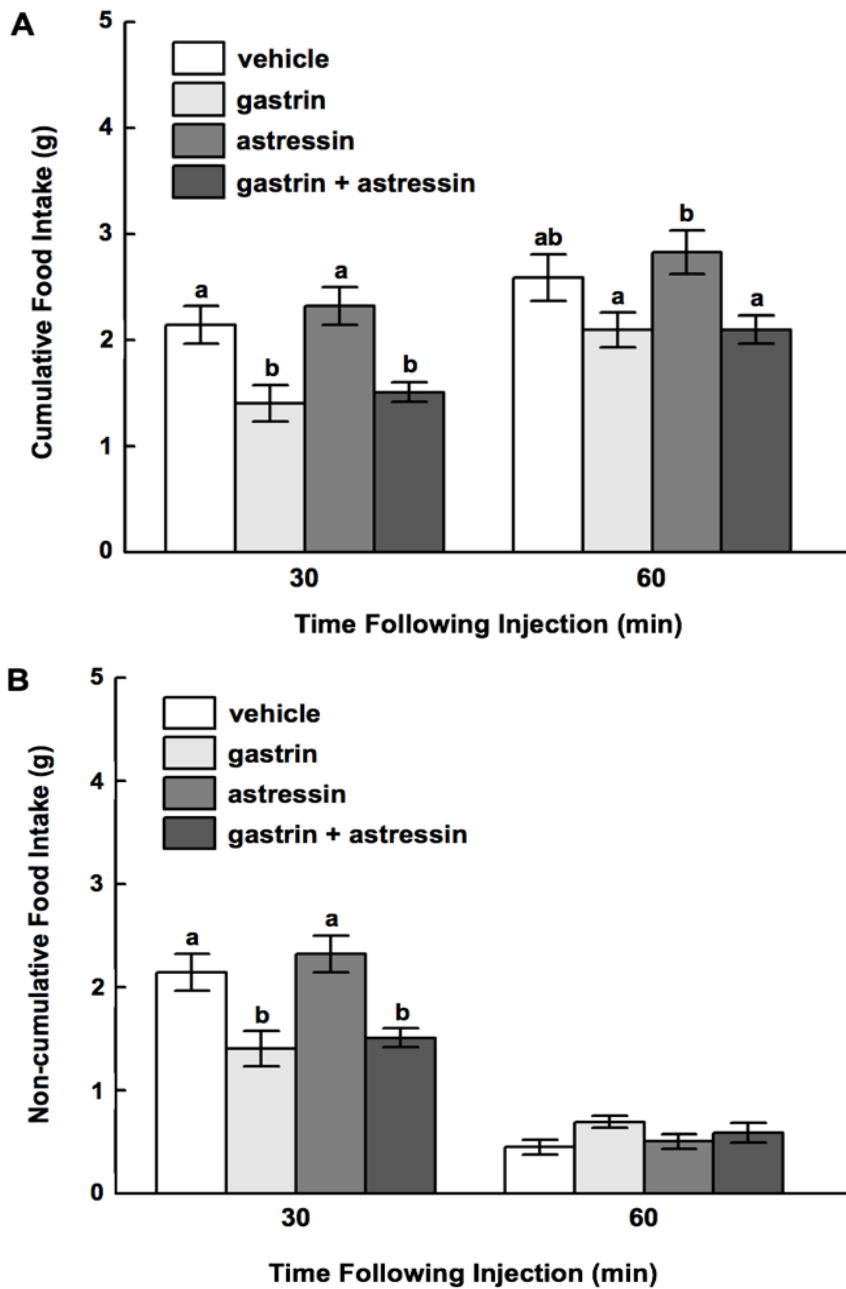


Figure 3-6. Food intake following intracerebroventricular injection of 500 ng gastrin, 6 nmol aestressin, or 500 ng gastrin + 6 nmol aestressin. Cumulative (A) and non-cumulative (B) food intake in 4 day-old broiler chicks. Values are means  $\pm$  standard error; bars with unique letters are different from each other within a time point ( $P < 0.05$ ). For this experiment, 8-10 chicks per treatment were available for the analysis.

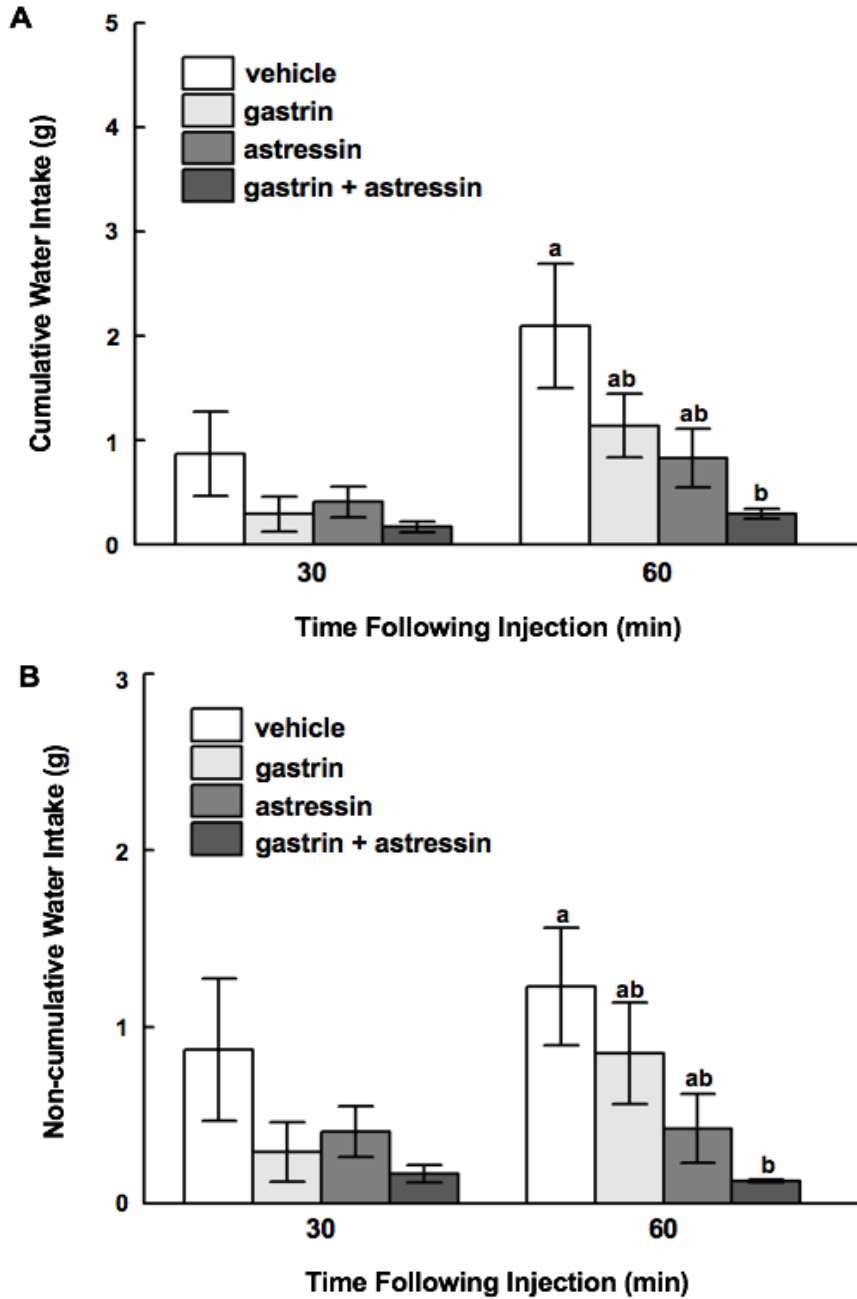


Figure 3-7. Water intake following intracerebroventricular injection of 500 ng gastrin, 6 nmol astressin, or 500 ng gastrin + 6 nmol astressin. Cumulative (A) and non-cumulative (B) water intake in 4 day-old broiler chicks. Values are means  $\pm$  standard error; bars with unique letters are different from each other within a time point ( $P < 0.05$ ). For this experiment, 8-10 chicks per treatment were available for the analysis.

Table 3-1. Primers used for real-time PCR for the gastrin experiment<sup>1</sup>

Gene	Accession no.	Sequences (forward/reverse)
β-actin	NM_205518.1	GTCCACCGCAAATGCTTCTAA/TGCGCATTTATGGGTTTTGTT
AADC	XM_004935144.2	TGGAATCCACCCACGTCAA/TCGGTCGCCAGCTGTGA
AgRP	NM_001031457.1	GGTTCTTCAACGCCTTCTGCTA/TTCTTGCCACATGGGAAGGT
CART	XM_003643097.3	GCTGGAGAAGCTGAAGAGCAA/GGCACCTGCCCGAACTT
CRF	NM_001123031.1	TCAGCACCAGAGCCATCACA/GCTCTATAAAAAATAAAGAGGTGACATCAGA
CRFR1	NM_204321.1	CTGCTGTCCTTGCTGGGAAT/ATCCTCCCCCGGATTGAC
CRFR2	NM_204454.1	GGATCAAATACAACACCACAAAAAAT/GGCCCATGTCCCATTGC
GAD	NM_204913.1	CCTGAAGATATGCGGGTTCTT/CACGATCCGGCCTTTGTCT
MC3R	XM_004947236.1	GCCTCCCTTTACGTTACATGT/GCTGCGATGCGCTTAC
MC4R	NM_001031514.1	CCTCGGGAGGCTGCTATGA/GATGCCAGAGTCACAAACACTT
MCH	NM_001195795.1	GTGGGCAGAAAGCAACTACCTT/TCAGTGTGAGCTGGAAAAGCA
MT	XM_004936280.2	TGGCTCTCTCCTCAGCTTGTTAT/GGCACGGCAGCTTACC
NPY	NM_205473.1	CATGCAGGGCACCATGAG/CAGCGACAAGGCGAAAGTC
NPYR1	NM_001031535.1	TAGCCATGTCCACCATGCA/GGGCTTGCCTGCTTTAGAGA
NPYR2	NM_001031128.1	TGCCTACACCCGCATATGG/GTTCCCTGCCAGGACTA
NPYR5	NM_001031130.1	GGCTGGCTTTGTGGGAAA/TTGTCTTCTGCTTGCCTTTGT
ORX	NM_204185.2	CCAGGAGCACGCTGAGAAG/CCCATCTCAGTAAAAGCTCTTTGC
POMC	NM_001031098.1	GCCAGACCCCGCTGATG/CTTGTAGGCGCTTTTGACGAT
PrRP	NM_001082419.1	GAGCGCTCCATGGAAATCAG/ATGCCACGGCCGGTGTAC
TH	NM_204805.2	CAGGACATTGGGCTTGCAT/TGTTGCCAGTTTCTCAATTTCTTC

<sup>1</sup>Primers were designed with Primer Express, version 3.0 (Applied Biosystems).

AgRP, agouti-related peptide; CART, cocaine and amphetamine-regulated transcript; CRF, corticotropin-releasing factor; CRFR1 and CRFR2, respectively, corticotropin-releasing factor receptor subtypes 1 and 2; DDC, dopamine decarboxylase; GAD, glutamate decarboxylase; MC3R and MC4R, respectively, melanocortin receptors 3 and 4; MCH, melanin-concentrating hormone; MT, mesotocin; NPY, neuropeptide Y; NPYR1, NPYR2, and NPYR5, respectively, neuropeptide Y receptors 1, 2, and 5; ORX, orexin; POMC, pro-opiomelanocortin; PrRP, prolactin-releasing peptide; TH, tyrosine hydroxylase.

Table 3-2. Count-type behaviors for the gastrin injection experiment<sup>1</sup>

Behavior (n)	Treatment	Time post-injection (min)					
		5	10	15	20	25	30
Feed pecks	Vehicle	151.73±45.61	396.47±95.63	560.67±139.18	659.40±138.08	750.73±138.59	848.40±132.07
	Gastrin	273.57±62.78	577.64±107.58	767.57±143.34	914.43±162.37	1037.57±175.81	1122.71±189.24
Water pecks	Vehicle	0	0	0	0	0	0
	Gastrin	0.07±0.07	0.07±0.07	0.07±0.07	0.07±0.07	0.07±0.07	0.07±0.07
Exploratory pecks	Vehicle	7.60±2.13	15.73±5.04	29.00±7.82	43.93±12.90	58.93±18.28	72.73±25.49
	Gastrin	9.07±4.84	22.57±6.97	33.21±9.09	37.21±9.63	48.50±12.85	59.79±19.38
Jumps	Vehicle	0.13±0.09	0.13±0.09	0.33±0.21	0.33±0.21	0.47±0.22	0.53±0.24
	Gastrin	0.36±0.20	0.71±0.44	0.79±0.43	0.79±0.43	0.86±0.43	0.86±0.43
Defecations	Vehicle	0.13±0.09	0.33±0.13	0.40±0.13	0.67±0.16	0.87±0.13	0.93±0.12
	Gastrin	0.21±0.11	0.43±0.17	0.57±0.17	0.64±0.17	0.64±0.17	0.71±0.19
Steps	Vehicle	50.73±7.76	130.67±27.81	205.00±49.48	279.6±68.26	347.13±83.75	387.07±97.95
	Gastrin	76.29±15.18	166.29±32.73	256.21±44.51	343.00±53.38	409.79±97.95	459.71±71.97
Escape attempts	Vehicle	0.20±0.20	0.20±0.20	0.20±0.20	0.47±0.34	0.47±0.34	0.47±0.34
	Gastrin	0.57±0.50	1.21±0.93	2.21±1.70	2.79±2.07	3.07±2.23	3.07±2.23
Distance moved (m)	Vehicle	1.95±0.61	3.76±1.02	5.67±1.60	7.64±2.13	9.43±2.62	10.37±2.83
	Gastrin	2.26±0.82	4.37±1.35	6.14±1.69	8.08±1.98	9.50±2.19	10.62±2.44

<sup>1</sup>Count-type behaviors recorded 5 to 30 min following ICV injection of 500 ng gastrin into broiler chicks (Experiment 4). Values are means ± standard error. \* denotes difference from vehicle within an observation time. For this experiment, 14-15 chicks per treatment were available for the analysis.

Table 3-3. Timed-type behaviors for the gastrin injection experiment<sup>1</sup>

Behavior (s)	Treatment	Time post-injection (min)					
		5	10	15	20	25	30
Standing	Vehicle	276.31±9.87	562.71±13.54	841.71±21.82	1102.81±32.69	1286.35±67.64	1436.71±96.53
	Gastrin	296.24±2.75*	594.27±3.63*	883.46±10.42*	1167.30±12.26	1358.84±42.12	1560.78±62.06
Sitting	Vehicle	7.19±5.66	17.09±7.65	35.18±13.40	69.69±27.02	111.65±33.92	185.72±52.89
	Gastrin	2.47±2.18	4.19±2.85*	13.18±8.53*	37.00±22.71	70.00±33.74	107.84±39.20
Deep rest	Vehicle	12.30±8.67	19.85±12.28	23.57±12.37	73.65±26.07	125.70±42.16	206.99±61.72
	Gastrin	0	0	0.56±0.44	15.92±8.16*	57.31±23.12	131.66±50.06
Preening	Vehicle	0.12±0.08	0.15±0.08	0.23±0.11	0.23±0.11	0.23±0.11	0.54±0.29
	Gastrin	0.08±0.08	0.17±0.12	0.24±0.13	0.44±0.17	1.04±0.70	1.20±0.73

<sup>1</sup>Timed-type behaviors were recorded 5 to 30 min following ICV injection of 500 ng gastrin into broiler chicks (Experiment 4). Values are the means ± standard error. \* denotes difference from vehicle within an observation time. For this experiment, 14-15 chicks per treatment were available for the analysis.



## Chapter 4: Epilogue and Future Direction

Overall, this research was able to provide novel insights into the satiety-inducing effects of both NPFF and gastrin in Japanese quail and broiler chickens, respectively. In the first study, changes in NPYR1 and MC3R mRNA expression were detected in NPFF-injected Japanese quail. However, in broiler chicks, the satiety-inducing effect of NPFF was found to be mediated by inhibiting two orexigenic ligands, NPY and  $\beta$ -endorphin (activators of opioid receptor types mu and kappa) [62]. Likewise, in rats, it was concluded that NPFF did not directly bind to opioid receptors [73]. Therefore, future studies should investigate the involvement of opioid receptors and their ligands in appetite-regulatory mechanisms in Japanese quail.

Moreover, individual hypothalamic and brainstem nuclei should be extracted and mRNA levels of appetite-associated genes in those specific nuclei measured, similar to what was done in Experiments 2 and 3 for the gastrin study. There were two attempts to measure c-Fos immunoreactive cells in hypothalamic nuclei; however, in the first trial the results (a decrease in the LH) were very different from the results found in the broiler study and in the second trial there were no significant differences. Thus, it should be repeated again or concluded that the decrease in food intake by NPFF is not associated with activation of the hypothalamic nuclei, which is what is observed in rats injected with NPFF [65]. After following up with these experiments, there will be a better understanding of the mechanism of NPFF-induced satiety. It is possible that there are other regions of the hypothalamus or brain that are involved in mediating its effects, and future studies could lead to the elucidation of those appetite-regulatory regions.

In the second study utilizing gastrin, expression of CRF mRNA in the PVN was increased in gastrin-injected chicks, showing that CRF may mediate the effects of gastrin-

induced satiety. However, when a CRF receptor antagonist was injected, food intake did not increase, demonstrating that it is likely not CRF directly inducing the satiety effects. Further, MCH mRNA decreased in the LH. MCH is an orexigenic neuropeptide; however, it was shown to have no effect on food intake in broiler chicks when rat MCH was injected. The authors speculated that the amino acid sequence of chicken and rat MCH may differ, thus leading to the rat MCH not acting as an orexigenic factor [105]. The effect of chicken MCH in chicks need to be further evaluated to determine if it is an amino acid sequence difference or if MCH truly does not have an effect in chicks. If MCH causes orexigenic effects in the chick, then a decrease in MCH mRNA in the LH could help explain decreased food intake. For now, it is difficult to draw a conclusion about the difference in expression of MCH mRNA.

The ARC, AP, and NTS were also activated in gastrin-injected broiler chicks; however, none of the appetite-associated genes that were analyzed by real-time PCR differed in expression level in these specific nuclei. This experiment should be repeated, looking at a wider array of genes, especially in the AP and NTS, since our lab has not examined the brainstem nuclei in great depth despite that the brainstem is known to communicate reciprocally with the hypothalamus to regulate appetite.

It is important to note that the molecular experiments that I conducted focused mainly on measuring abundance of mRNA, which does not always provide an accurate depiction of the protein levels and activity. To gain more understanding, the measurement of protein levels and function of the protein (at more time points) would provide a more comprehensive picture of how a particular neuropeptide is influencing the physiology and neural networks in the hypothalamus and brainstem to regulate appetite.

Overall, I reported in this thesis novel findings regarding the role of satiety factors in appetite regulation in birds. Understanding how these neuropeptides signal within the hypothalamus or brainstem could assist other research groups that are searching for satiety-inducing drugs (or alternatively an appetite stimulant) or feed additives in order to combat diet-related or agricultural-related issues, respectively.

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## Appendices

### Appendix A. c-Fos Immunohistochemistry Protocol

#### Brain fixation, harvest and preparation

Typical brain harvest time is 1 hour following treatment

- A. Anesthetize chick via cardiopuncture with sodium pentobarbital
- B. Decapitate immediately by cutting as close to the 9<sup>th</sup> cervical vertebra as possible (long neck)
- C. In the hood, perfuse brain with normal saline for 2 minutes using a Fisher peristaltic pump set to speed 1 on the “low” setting
- D. Perfuse brain with 4% paraformaldehyde for 6 min with the same peristaltic pump settings
- E. Immediately after paraformaldehyde perfusion flush lines with normal saline
- F. Place head in a cup with identifying number
- G. Remove brain from skull as soon as practicable
- H. Post fix in 4% paraformaldehyde for at least 30 min
- I. Transfer to 20% sucrose in PB until it sinks (about 10 hours at RT)
- J. Transfer to 30% sucrose in PB (about 24 hours at RT)  
(Procedure may be stalled at sucrose steps by placing in 4°C)
- K. After it has sunk in 30% sucrose in PB it is ready for cryosectioning
- L. Orient brain per Kuenzel (2008) and block brain frontally immediately behind the arcuate nucleus such that the optic lobe is cut down the middle

- M. Collect 60 micron frontal sections at -10°C to -15°C chamber and specimen temperature
  - N. Place sections in 0.02 M PBS until further processing
- (Procedure may be stalled at this point in Storage Buffer at 4°C)

### **Day “One”**

- A. Rinse 3 x 10 min in 0.02 M PBS
  - B. Sodium borohydride (NaBH<sub>4</sub>) treatment (breaks double C=C bonds to improve antibody penetration)
    - i. Incubate for 15 minutes

\*Many bubbles may form, stir occasionally to ensure all sections transfer
  - C. Rinse 2 x 10 min in 0.02 M PBS
  - D. Incubate sections in blocking solution for 1 hour at RT on a shaker
  - E. Rinse sections 2 x 10 min in wash buffer at RT
  - F. Incubate sections in nonspecific binding solution for 30 min
  - G. Rinse sections 3 x 10 min in wash buffer at RT
  - H. Incubate sections with primary antibody at 4°C on shaker
- \*For assay control, do not add primary antibody
- \*For chick c-Fos assay use anti c-Fos 1:5000 (1:5000 → 5 μL in 25 mL) and incubate 72-96 hours;
- \*AB429 is in a clear tube with blue cap in the freezer

## Day "TWO"

- A. Rinse sections 3 x 10 min in wash buffer at RT
- B. Incubate sections with biotinylated goat anti-rabbit antibody (Vector) diluted 1:200 (10 drops in 100 mL) in 1% NGS / 0.02 M PBS for 2 hours at RT on a shaker (one drop of antibody is 50  $\mu$ L)
- C. Prepare horseradish peroxidase avidin-biotin complex (Vectastain Elite ABC Kit)
- D. Rinse sections 3 x 10 min in 0.02 M PBS (not wash buffer)  
(Procedure may be stalled at this step by placing in 4°C)
- E. Incubate with ABC solution prepared earlier for 1 hour at RT on a shaker
- F. Prepare DAB solution (Need to be used within 30- 60 min of preparation time)
  - \* Glucose oxidase (in blue box in same freezer as DAB)
  - \* No staining will occur at this point because there is no peroxide present.
  - \* Glucose will allow glucose oxidase to generate peroxide and the reaction will begin
  - a. Staining typically develops for Ni-DAB within 5-10 minutes (maybe longer?).  
You can view the sections under a dissecting microscope as they stain and see the reactions develop in real-time. When the reactions look strong, you can inactivate the DAB by transferring the sections to a well of PBS.
- G. Rinse sections 3 x 10 min in 0.05 M Tris-HCl at RT
- H. Add (~3 mL to the small wells) 3,3'-Diaminobenzidine (DAB; Sigma-Aldrich; Cat. No. 91-95-2) to well, add 3  $\mu$ L Glucose Oxidase Type X (MP; Cat. No. 195196), add sections, add 15  $\mu$ L 10% beta-D glucose (Acros; Ca. No. 50-99-7)
- I. Rinse sections 1 x 10 min in 0.05 M Tris-HCl at RT

J. Rinse sections 2 x 10 min in 0.02 M PBS at RT

(Procedure may be stalled at this step by placing in 4°C)

**“Day THREE”**

A. Mount sections on gelatin coated slides and let them dry

B. Dehydrate slides

i) 5 min 70% EtOH

ii) 3 min 70% EtOH

iii) 3 min 90% EtOH

iv) 3 min 100% EtOH

v) 3 min HistoClear

vi) 3 min HistoClear

C. Coverslip while still wet with HistoClear using Vectamount

## Appendix B. ANY-maze Behavior Analysis Instructions

1. Open the AnyMaze program
2. Click New experiment
3. Enter your experiment title
4. Enter name of peptide under 'Treatment' and the under Treatments on the right, enter the TOTAL number of animals in your experiment (do not separate by doses)
5. Go to 'Protocol'
6. Select 'Video Sources', click +, 'New Video Source', and name the video source anything you want
7. Select 'Apparatus', click +, 'New Apparatus', and name the apparatus anything you want
8. Click and drag the orange ruler at the bottom of the screen on the right and place it along a distance you have measured on the behavior apparatus. You may need to resize the orange line by clicking and dragging the ends
9. Enter the distance the orange line represents (290 mm for 31 Curie arenas)
10. Use the shapes above the video to outline the ENTIRE apparatus you will be using. Do not only outline the floor of the apparatus
11. Select 'Keys', click +, 'New Key', enter a behavior you want to analyze by pressing a key and the key you want to use for that behavior
12. Repeat for each behavior you want to analyze by individual key presses (i.e. step, jump peck, etc). Uncheck 'count this behavior as activity'
13. Leave the way you'd like the key to work as 'Simple'
14. Repeat for timed behaviors (i.e. sleep, sit, stand, preen, etc) with the exception of how you'd like it to work. For timed behaviors select 'Radio' as the way you'd like it to work

15. Select 'Test Settings and Options', and 'Animal colour'. Choose the color setting that corresponds to how your animals are colored compared to the floor color
16. Select 'Tracking the animal's head' and check 'yes'
17. Select 'Automatic starting of tests' and select 'no – I'll start the tests manually'
18. Select 'What to display while testing'. 'Mark the animal's centre point' and 'shade the entire area of the animal' should be checked. Check 'List any active keys' and select 'show these indicators in the top right corner of the image'
19. Select 'Advanced tracking options' and check 'the apparatus is inside an enclosure'
20. Go to "stages," click "first stage," enter "1800" for test duration (in seconds) if you want to analyze 30 min per bird.
21. Select 'Results, reports, and data'. Select 'Data page' and check the animal information you would like to appear in the data spreadsheet. Click 'Apparatus Measure's and select the ones you would also like to appear in your spreadsheet
22. Go to 'Data' at the top of the screen and click 'Select data to show'. On the right, select the ones you would like to appear in your data table. Be sure 'Segment of Test' is also selected
23. Under 'Actions' on the left of the screen, click 'Set segment length for analysis across time'. Under 'Settings', enter the segment length for analysis, for example if you want your results for every minute, enter 60 and if you want the data broken down by 5 minute intervals, enter 300
24. Go to "Tests" at the top of the screen and press the green arrow when ready to begin your analysis
25. During the analysis, keys that were set up for timed behavior only have to be pressed once when that behavior starts. When the animal switches to a different timed behavior (i.e. stands and



then sits down), press the key for the new timed behavior and it will stop timing the first one and start timing the new behavior.