Effects of exogenous and endogenous factors on appetite regulation in broiler chicks and Japanese quail

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

Master of Science

In

Animal and Poultry Sciences

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May 2021

Blacksburg, VA

Key words: chicks, Japanese quail, ferulic acid, oxyntomodulin, arcuate nucleus

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Abstract (Academic)

Understanding how appetite is regulated, via exogenous or endogenous factors, is essential to animal agriculture in order to maximize production capabilities, as well as in human medicine to generate ways to treat conditions such as eating disorders or obesity. The aim of this thesis was to evaluate the effects of ferulic acid (FA), an exogenous factor found within plant cells, and oxyntomodulin (OXM), an endogenous hormone generated in the gastrointestinal tract, on food intake in avian models, as well as elucidate the hypothalamic mechanisms responsible. In broiler chicks (*Gallus gallus*), FA administered peripherally (IP) resulted in a transient yet potent reduction of food intake. A behavior analysis revealed that FA-treated chicks defecated fewer times than control birds. Within the arcuate nucleus (ARC) there was an increase in c-Fos immunoreactivity, indicating neuronal activation, in FA-treated chicks. Within the hypothalamus, there was a decrease in mRNA abundance of galanin, ghrelin, melanocortin receptor 3, and pro-opiomelanocortin (POMC), however within the ARC there was a decrease in POMC and an increase in c-Fos mRNA after FA treatment. OXM, a proglucagon-derived peptide produced in the gastrointestinal tract, administered intracerebroventricularly (ICV) or IP in Japanese quail (*Coturnix japonica*), resulted in a decrease in food intake for 3 hours post-injection. There was an increase in c-Fos immunoreactivity within the ARC as well as the dorsomedial nucleus (DMN) in quail ICV injected with OXM. In conclusion, these novel data provide insights on the similarities and differences between factors that can affect appetite regulation via anorexigenic effects.

Abstract (Public)

Exogenous and endogenous factors affect appetite regulation. Exogenous factors originate in feed components, additives, and other environmental factors that can affect bodily functions but are derived from an external source. Endogenous factors are made within the body, such as hormones and neurotransmitters, usually in response to a stimulus, and serve to communicate signals both locally and distantly in the body. Ferulic acid (FA), a natural exogenous factor originating within plant cells, is found in commonly consumed plant-based foods. When administered peripherally into broiler chicks (meat-type birds), FA caused a direct and potent, yet quickly diminishing, decrease in food intake via activation of cells within the hypothalamus, the region of the brain that is responsible for appetite regulation. Oxyntomodulin (OXM), an endogenous peptide hormone generated within the gastrointestinal tract in response to the digestion of nutrients, is known to decrease food intake in humans, rodents, and the broiler chick. However, its effects in Japanese quail, a model closer to a "wild-type" bird, are unknown. Quail injected peripherally (outside the brain) or intracerebroventricularly (ICV; into lateral ventricle of brain) with OXM showed a reduction in food intake that was more persistent than FA's effects with the effects also mediated via activation within the hypothalamus, although through slightly different molecular mechanisms. Understanding different factors that can regulate appetite in animals is necessary for agricultural applications to maximize production and improve health and welfare, as well as in humans to elucidate methods to treat appetite-related conditions, such as eating disorders and obesity.

Acknowledgements

Dr. Gilbert: I cannot convey how grateful I am to you for accepting me as one of your students and for being my advisor. You had a large part in inspiring me to further my own education and have been so supportive through the entire process. You serve as an incredible role model, as someone so accomplished, thoughtful, and dedicated. I value every piece of advice, suggestion, and nudge in the right direction you have given me through our time together. I can't thank you enough for what I consider taking a chance on me and providing me with an immense amount of encouragement and teaching me so much.

Dr. Cline: I am so grateful you gave me the opportunity to join your lab as an undergraduate, not to mention a purely average student, with the original purpose to help others with their work. You were the first to introduce me to the world of research, something I thoroughly enjoy and hope to be involved with for as long as I can. You routinely demonstrate a commitment and passion for what you do, whether it is in your research or teaching with such enthusiasm and strive to make experiment days or classes enjoyable for anyone involved. I have learned so much from you and I greatly appreciate all of your insights you have provided through my years here.

Dr. Campbell: I would like to thank you for being a member of my committee. Even only having the opportunity to have as many conversations as we have had, I am a so grateful to have received such thoughtful advice from you. Your words of encouragement and calming advice have allowed me to gain more confidence and assurance in myself and have helped me realize even those who have taught and given presentations for years, can still be nervous beforehand.

Mark and Chang: Both of you have been integral to my successes during my time in research through two separate degrees. I can count on either of you for any form of help I may need at any time of the day, whether it is for experiments, helping me understand something, or late-night math sessions. Not only have you both been so helpful, but you have been some of my closest friends for the past few years, and I am so appreciative of that.

Patricia Williams: I am so beyond grateful to have met you. Believe it or not, I enjoyed all the mopping of hallways, vacuuming of walls, and clearing out of broken freezers. But most of all I enjoyed all our conversations throughout the day. It is so nice having someone so understanding and supportive.

Friends and family: I thank all my friends and family for baring with me and supporting me through this eventful year, as well during my years of undergraduate research. I would especially like to say thank you to Grace for providing me with so much support, advice, and so many fun times and memories. Even from a distance, I always felt supported and surrounded by people who rooted for me.

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Chapter 1: Introduction

Appetite regulation is an essential concept to understand because of its application to many research areas in animal agriculture as well as human health. In animal production, a goal is to maximize efficiency and production of the animals used for various food products. Having high feed efficiency is cost effective, as it uses the least amount of feed possible to grow the animals to a certain body weight and composition standard. In the commercial poultry industry, broiler meat chickens have been genetically selected for factors such as body weight, breast size, and feed conversion ratio to lead to the highest financial return once they are processed and sold. Between the years 1957 and 2005, as a result of selection, broiler growth has increased by over 400%, breast size increased by 80%, and feed conversion ratio has reduced by 50% [1]. As a result of this intense selection, broilers are known to eat nearly continuously, however this is not ideal for the parent breeder lines of the broiler birds. Breeder lines are often feed restricted to prevent continuous feed consumption and development of obesity. Breeders are kept at an optimum weight that maximizes fertilization and egg quality, which decrease as the weight of the bird increases. If breeder lines are fed ad libitum, both sexes of birds display reduced fertility, heat stress, lameness, and heart failure [2]. So, alternative methods are sought to halt the desire of the bird to continuously feed without the need to restrict feed.

The concept of understanding appetite regulation is also applicable to humans. Obesity is a disease in which the amount of energy consumed in food outweighs the amount of energy expelled, resulting in hypertrophy and/or hyperplasia of fat cells and storage of energy as fat in adipose tissue and other tissues such as the liver. As a result, obesity can

lead to other diseases such as diabetes, heart disease, gallbladder disease, osteoarthritis, forms of cancer, and more [3]. The prevalence of overweight and obese young adults within the U.S. was estimated to be 68% in 2007-2008 [4]. While the over consumption of calories is a common cause of obesity, other factors such as genetic makeup, medication consumed, economic, psychological, and more can be responsible for excess weight gain [5], one of which is endocrine related. For example, a common cause for exacerbation of obesity is a resistance to leptin, a hormone generated by adipose tissue and causes a decrease in food intake [6]. Understanding these endogenous factors and the hypothalamic mechanisms mediating their effects could lead to novel ways to treat conditions like obesity caused by overeating, as well as other conditions associated with under-consumption of calories, such as anorexia nervosa.

Both exogenous factors and endogenous factors can affect appetite, so understanding and comparing the two can be beneficial in finding ways to treat appetite-related issues. Within the brain, the hypothalamus is responsible for the production and release of hormones via the neuro-endocrine system to control many bodily functions such as appetite, body temperature, emotions, and more. It is also responsible for integrating signals originating from the rest of the body so it may respond appropriately to manage whole body energy homeostasis. Exogenous factors, such as feed additives, as well as endogenous factors, such as hormones produced in the gut, can be responsible for those signals that reach the hypothalamus and ultimately affect appetite.

The aim of this thesis is to evaluate the effects of two factors, ferulic acid and oxyntomodulin, exogenous and endogenous factors, respectively, on appetite within avian models. Chapter 2 is a review of literature relating to the origin, physiological

capabilities, known mechanisms, and established effects on food intake of ferulic acid and oxyntomodulin. The aim of Chapter 3 was to examine the effects of ferulic acid on broiler chick food intake, determine if the effect is direct or a result of an aversive behavior, and elucidate the hypothalamic mechanism and nuclei responsible for mediating its effects. The research conducted in Chapter 4 is an evaluation of the effects of oxyntomodulin on food intake in Japanese quail, an avian model that has not undergone intense selection like the broiler chicken, as well as the hypothalamic appetite-related nuclei mediating oxyntomodulin's effects. Chapter 5 will summarize and conclude the implications of the novel findings presented.

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Chapter 2: Literature Review

1. Ferulic Acid, a Plant-Based Chemical

1.1 Sources of Ferulic Acid

Ferulic acid is a naturally occurring polyphenol found within the plant cell well. Polyphenols, a type of phytochemical, are present in a vast amount of food items and contain multiple identification classes, and many are biologically active [1]. Through their presence in plant-based foods, humans consume between 150 mg to 1 g/d depending on their diets [2, 3], and many provide antioxidative effects [4]. Polyphenols can be divided into different groups, including flavonoids and non-flavonoids, like phenolic acids [5]. Phenolic acids are compounds that have two separate categories based on the type of acid they are derived from: benzoic acid and cinnamic acid [6]. Cinnamic acid has multiple derivatives, however the main ones are caffeic, sinapic, *p*-coumaric, and ferulic acid [7]. These cinnamic acid derivatives are found in many different foods, the main sources being coffee, fruits, vegetables, and cereal grains [8].

Ferulic acid was first isolated in 1866 from the *Ferula foetida* plant [9], a perennial plant that has traditional medicinal uses in treating digestive issues and other various ailments [10]. After its isolation, it was chemically synthesized in 1925 [11]. Ferulic acid provides rigidity to plant cell walls by crosslinking with polysaccharides and lignin [12]. As a natural compound, ferulic acid can be found in a wide range of different food items, broad categories being grains, fruits, vegetables, nuts, leaves, and more. Some food sources with a higher concentration of ferulic acid compared to others are bamboo shoots, sugar-beet pulp, popcorn, and various corn and wheat bran products [3, 13]. Within plant tissues, it can be in a free or conjugated form [3]. Once ingested, ferulic acid can then be

absorbed from the stomach [14], as well as the intestine, specifically the jejunum [15] and the ileum [16]. After absorption, possibly via passive diffusion [14], Na^+ dependent carrier-mediated transport [15], or a H^+ dependent transport system [17], ferulic acid directly enters the portal circulation and can be conjugated and/or metabolized via enzymes such as sulfotransferases and UDP glucuronosyl transferases within the liver [14]. Those metabolites can then be found within the plasma, or in free form if there is oversaturation of the conjugating enzymes [18], and can travel through circulation, with serum albumin as a carrier [19], to reach other tissues such as the kidney, spleen, heart, uterus, and brain [20, 21]. Ferulic acid can then be mainly excreted via the urine [22, 23], as well as through bile [20, 24], with the half-life of ferulic acid estimated to be from 10 to 30 min in rats on a dose dependent basis [18, 21].

1.2 Physiological Effects of Ferulic Acid

Through years of study, ferulic acid has been found to have many beneficial physiological effects, the most notable being its antioxidative capabilities. Ferulic acid is known as a free-radical scavenger, which is what enables it to have antioxidative effects [25]. A free radical is a molecular species that contains an unpaired electron, for example, reactive oxygen species (ROS) and reactive nitrogen species (RNS) [26]. Endogenously, they can be created within cells when an electron leaks from an electron transport chain, or enzymatically, where the species can donate that electron or take on another electron [27]. Exogenously, ROS's and RNS's can be generated from factors like air pollution, smoking, radiation, and alcohol or drug use, where the compounds enter the body then decompose into free radicals [28]. These free radicals can be beneficial when in low concentration levels, for example, free radicals are responsible for regulating intracellular

signaling cascades in some nonphagocytic cells [29], as well as being capable of inducing mitogenic responses [30]. However, when free radicals are over-produced, they can cause oxidative stress that can lead to cellular damage [31]. Ferulic acid has a phenolic hydroxyl group from which it can donate a hydrogen atom to create a resonance stabilized phenoxy radical to prohibit any damaging effects of free radicals [25, 32]. As a free-radical scavenger, ferulic has antioxidant capabilities to prevent diseases that can result from free radicals, such as cancer [33].

In addition to its antioxidant potential, ferulic acid has many other beneficial physiological functions. In terms of anti-carcinogenic effects, it has been shown to have inhibitory effects on colon cancer cell growth [34], a tumor-suppressive effect on breast cancer cells [35], as well as potential for reducing side effects of chemo- and radiotherapy [36]. Ferulic acid is able to decrease hyperglycemic-induced over production of free radicals that cause oxidative stress in a diabetic state, thereby resulting in a decrease in blood glucose levels [37]. Oxidative damage resulting from free radical creation via nicotine use can be moderated by ferulic acid's free radical scavenging, preventing damage to cell membranes [38]. Ferulic acid is an active component of *Cimicifuga heracleifolia*, a plant that is frequently used in anti-inflammatory Japanese medicine [39]. There is a reduction in neutrophils as well as interleukin-8 levels, both pro-inflammatory-associated, in mice, when ferulic acid was orally administrated [40], lending support to the idea that ferulic acid has anti-inflammatory actions, although it is unclear if they are the result of or independent of its anti-oxidative properties.

A few studies have demonstrated how ferulic acid exerts anti-depressant effects in animal models. Mice that had ferulic acid administered via oral gavage were found to have

significant decreases in immobility time during a tail suspension test as well as a forced swimming test [41]. In that same study, it was found that ferulic acid administration resulted in higher serotonin and norepinephrine levels within the hippocampus and the frontal cortex, along with a decrease in monoamine oxidase-A activity, an enzyme responsible for the breakdown of the previously mentioned monoamines, within the same regions [41]. Another study observing the effects of ferulic acid administered via oral gavage also found a reduction in immobility time by 60% in mice during a tail suspension and forced swimming test [42]. In mice with a corticosterone (CORT)-induced depression-like state, ferulic acid treatment reduced the CORT-induced immobility time in a tail suspension- and grooming during a splash-test, as well as mitigating the oxidative stress caused by the CORT treatment [43].

1.3 Ferulic Acids Effects on Food Intake

The effects of ferulic acid in rodent models fed an obesogenic diet have been studied, however direct effects on food intake have not been assessed. Rats fed a high-fat (HF) and high-fructose diet with 0.05% ferulic acid for 13 weeks showed a decreased total body weight gain compared to control rats, however food intake, which was measured once daily, was not affected by ferulic acid supplementation within the diet [44]. In a similar study, mice that were fed a HF diet that contained 0.5% ferulic acid for a total of 7 weeks showed a reduction in weight gain compared to control mice, but similarly there was no difference in food intake which was measured once daily [45]. Likewise, there was a reduction in body weight gain of mice fed a HF diet and drinking water with 0.05% ferulic acid for 15 weeks, however in this case both weekly food and water intake were

reduced [46]. Thus, from these rodent studies it is unclear whether ferulic acid has a direct effect on feeding behavior.

2. Oxyntomodulin, an Endogenous Hormone

2.1 The Gut-Brain Connection

The gastrointestinal tract has a connection with the central nervous system that is donned the "gut-brain axis". This connection is able to regulate food intake and other processes by the exchange of signals between the gut and the brain [47]. Within the gastrointestinal tract there are enteroendocrine cells that can release hormones, known as gut hormones, in response to the ingestion of nutrients [48]. These peptide hormones released from the gut can then enter blood circulation and travel to areas in the brain such as the hypothalamus to reach appetite-related nuclei [49]. In particular, the arcuate nucleus (ARC) of the hypothalamus is strategically positioned close to a circumventricular organ (lacking a blood-brain barrier), the median eminence, making it ideally situated to receive a vast mixture of peripheral and central signals [50]. The hormonal signals from the gut will reach anorexigenic or orexigenic neurons which then integrate those peripheral and central signals to regulate feeding behavior [51]. Additionally, factors released in the gut have the potential to communicate with the brain via the nervous system, in particular vagal afferents [52].

2.2 Oxyntomodulin as a Proglucagon-Derived Peptide

One of the groups of peptides produced within the gastrointestinal tract are proglucagon-derived peptides which are encoded in the proglucagon gene, which through sequential cleavages of the pre-pro-glucagon protein generates bioactive peptides [53]. This group includes glucagon, glucagon-like peptide 1 (GLP-1), glucagon-like peptide 2 (GLP-2),

and oxyntomodulin [54]. Oxyntomodulin is a 37 amino acid peptide and contains glucagon's entire 29 amino acid sequence plus an additional 8 residues on the C-terminus [55] that is created by the conversion of proglucagon by prohormone convertase 1/3 into glicentin to be further cleaved into oxyntomodulin [56]. When nutrients are ingested, oxyntomodulin is secreted from intestinal enteroendocrine L-cells [51] into the circulation [57]. Oxyntomodulin was named for its ability to target oxyntic glands in the gastric mucosa [58] where it inhibits gastric acid secretion [59, 60]. While a specific receptor has not been identified, oxyntomodulin is able to exert cellular effects via the GLP-1 receptor (GLP-1R) or the glucagon receptor [61].

Oxyntomodulin is capable of stimulating insulin secretion from islet beta cells in the pancreas [62, 63]. Similar to other agonists of the GLP-1R, oxyntomodulin lowers blood glucose and stimulates cAMP formation in kidney cells after oral or intraperitoneal (IP) administration of glucose [64]. Intravenous infusion of oxyntomodulin in rats resulted in significant increases in intestinal glucose uptake capacity [65].

2.3 Appetite-Related Effects of Oxyntomodulin

The effects of oxyntomodulin on food intake and energy expenditure are reported. When administered intracerebroventricularly (ICV), oxyntomodulin inhibited food intake in fasted rats for up to 4 hours post injection [66]. It is also worth noting in this same study that ICV oxyntomodulin reduced food intake in non-fasted rats at the beginning of the dark cycle, the rat's natural feeding time, and had no effect on food intake when co-administered with a GLP1R antagonist [66]. Oxyntomodulin administered via IP injection also reduced food intake in rats that had been fasted, as well as rats at the onset of the dark phase [67]. Similar to rats, fasted mice administered ICV oxyntomodulin

showed a reduction in food intake up to 8 hours after injection, however interestingly, when fasted mice were IP administered oxyntomodulin there was no effect on food intake, dissimilar to rats [68]. In broiler chickens, oxyntomodulin was effective in reducing food intake after ICV injection [69], however there was no effect on food intake when oxyntomodulin was administered via IP or IV injection [70], similar to mice. Human subjects that were IV-administered oxyntomodulin had lower levels of energy intake compared to control subjects, as well as suppressed appetite levels after treatment [71]. When oxyntomodulin was administered subcutaneously in overweight or obese human subjects three times a day for a total of four weeks, body weight as well as energy intake of treated subjects was reduced compared to subjects only administered saline [72]. In a similarly designed study, overweight or obese human subjects were subcutaneously administered oxyntomodulin three times daily for a total of four days. As a result, participants treated with oxyntomodulin had reduced energy intake, reduced body weight, and increased total energy expenditure [73].

Oxyntomodulin is able to exert its anorexigenic effects via the ARC within the hypothalamus [74]. In rats, IP injection of oxyntomodulin resulted in an increase in c-fos immunoreactivity, an indication of neuronal activation, within the ARC [67]. In this same study, a direct injection of oxyntomodulin into the ARC resulted in a significant reduction in food intake [67]. In chicks, there was also an increase in c-fos immunoreactivity within the ARC after ICV administration [70]. Rats that first had a GLP-1R antagonist injected directly into the ARC after which oxyntomodulin was IP injected showed no reduction in food intake as an IP administration typically would in the rat [67]. Mice with an inactivated GLP-1R gene, GLP-1R$^{-/-}$ mice, that were ICV

administered oxyntomodulin showed no reduction in food intake compared to control GLP-1R$^{-/-}$ mice [68], further supporting oxyntomodulin inhibits food intake via the GLP-1R.

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Chapter 3: Ferulic acid, a phytochemical with novel anorexigenic effects in birds

Abstract

Ferulic acid (FA) is a phenolic acid found within the plant cell wall. It is used extensively in food, cosmetic, and pharmaceutical industries, and has physiological benefits as an antioxidant and anti-obesity agent. Although metabolic roles are described for FA in mammalian species, to our knowledge there are no reports of effects on appetite regulation. Thus, our objective was to determine if FA affects food intake, using chicks (*Gallus gallus*) as a model. At 4 days post-hatch, chicks were intraperitoneally (IP) injected with 0 (vehicle), 12.5, 25, or 50 mg/kg of FA. Chicks treated with 50 mg/kg of FA consumed 70% less food than controls at 30 min post-injection, with no effects observed thereafter. Water intake was not affected at any time point. In a behavior analysis, FA-treated chicks defecated fewer times than vehicle-injected chicks, while other behaviors were not affected. There was an increase in c-Fos immunoreactivity within the hypothalamic arcuate nucleus (ARC) of FA-treated chicks. No differences were detected within other nuclei. mRNA abundance was measured in the whole hypothalamus and the ARC. There was decreased hypothalamic galanin, ghrelin, melanocortin receptor 3, and pro-opiomelanocortin (POMC) mRNA in FA-treated chicks. Within the ARC, there was an increase in c-Fos mRNA and a decrease in POMC mRNA in response to FA. Thus, it is likely that the mechanism responsible for mediating FA's transient effects on food intake originates within the ARC, possibly involving POMC.

Practical Application

These data demonstrate the direct anorexigenic effects of peripherally-administered ferulic acid in birds, although the hypothalamic molecular mechanism requires further investigation. Ferulic acid's anti-obesity effects in birds and mammals could be utilized in developing strategies to

combat compulsive feeding behavior and weight gain in humans, as well as to influence body weight gain and composition in agriculturally-relevant species such as the chicken. Because of its widespread distribution in plants and industrial uses, understanding its physiological effects in animals is important for developing supplementation strategies and incorporating it into food products.

Introduction

Ferulic acid (FA), or 4-hydroxy-3-methoxycinnamic acid, is a plant cell wall-based polyphenol. FA was isolated for the first time from the *Ferula foetida* plant in 1866 [1], and its chemical synthesis was reported in 1925 [2]. It can be found in varying amounts in many fruits, vegetables, seeds, and grains [3] and has applications in food, cosmetic, and pharmaceuticals industries [4, 5]. FA has antioxidant [6-8], anti-inflammatory [9, 10], and anti-cancer [11, 12] effects. FA supplementation lowers blood glucose levels [13], contributes to gut microbiome maintenance [14], and has anti-obesity and anti-diabetic effects in rodents [15-17]. In relation to FA's effects on the brain, it has been repeatedly reported to demonstrate anti-depressant-like effects. FA administered via oral gavage led to increased serotonin and norepinephrine levels while also inhibiting monoamine oxidase A activity in mice, in the hippocampus and frontal cortex, but not in the hypothalamus [18]. FA treatment also reduced immobility that was a result of an induced-depressive state [19, 20].

There are multiple reports of body weight gain-lowering effects of supplementary FA in the presence of an obesogenic or high-fat (HF) diet. Total body weight gain was reduced in rats that were fed a HF and high-fructose diet (HFFD) supplemented with 0.05% FA for 13 weeks, although daily food intake values did not differ [21]. Similarly, mice that were fed a HF diet with 0.5% FA, for 7 weeks, gained less weight and had reduced blood glucose concentrations, but

daily food intake values were not reported [13]. Body weight gain reductions, as well as overall decreases in food and water intake, were also observed in mice that were fed a HF diet for 15 weeks with drinking water containing 0.05% FA [15].

While anti-obesity effects of orally-supplemented FA have been demonstrated in rodent models, there have not been any studies, to our knowledge, to assess its physiological effects in birds and whether it directly affects feeding behavior, which might explain some of its anti-obesity effects in animals fed a HF diet. Thus, the objective of this study was to evaluate effects of peripheral administration (intraperitoneal; IP) of FA on food and water intake and hypothalamic molecular physiology in chicks. Many studies have employed designs that involve oral administration, and FA is able to cross the blood brain barrier from its ability to affect hippocampal mRNA levels [22], prevention of injury as a result of cerebral ischemia [23], and protection against cell apoptosis resulting from cerebral ischemia [24, 25]. Based on this knowledge and that natural entry of FA into the animal would be through the diet, we utilized the IP route of administration.

Materials and Methods

Animals

On day of hatch, unsexed Hubbard x Cobb-500 broiler chicks (*Gallus gallus*) were obtained from a nearby commercial hatchery and group-caged in a room maintained at 30 ± 1 °C and 50 ± 5% relative humidity with a 24-hr light schedule. Food (22% crude protein and 3,000 kcal ME/kg) and water were provided ad libitum [26]. Chicks were caged individually on day 1 post-hatch. One day prior to each experiment, chicks were weighed and assigned to treatments using a randomized complete block design. All experiments were conducted on day 4 post-hatch. All experimental procedures were performed according to the National Research Council

Publication, Guide for Care and Use of Laboratory Animals and were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Experiment 1: Effect of IP FA on food and water intake

Chicks were randomly assigned to receive 0 (vehicle comprised of 53% dimethyl sulfoxide [DMSO] and 47% phosphate buffered saline [PBS]), 12.5, 25, or 50 mg/kg of FA by IP injection. Doses were based on (Mhillaj et al., 2018), where volume injected per chick was based on individual body weight. After injection, chicks were returned to their original cages and given ad libitum access to food and water. Food and water intake were measured (0.01 g) every 30 minutes (min) for a total of 180 min post-injection, with water weight (g) converted to volume (1 g = 1 mL). At the end of each experiment, chicks were sexed by visual inspection of the gonads post-euthanasia. Data were analyzed using analysis of variance (ANOVA) within each time point by the GLM procedure in SAS 9.3 (SAS institute, Cary, NC, USA), with the statistical model including the main effect of dose. Sex and interaction of FA dose was also included in the model, but no interaction was found between sex and FA. Tukey's method of multiple comparisons was used to separate the means within each time point. Differences were considered significant at $P < 0.05$.

The minimum required amount of DMSO was used to fully dissolve the FA. We conducted a preliminary test to compare this vehicle to a PBS-only control. There was no difference in food or water intake, over the course of 3 hours (hr) post-injection, in chicks administered either solution (data not shown).

Experiment 2: Behavioral analysis

Chicks were randomly assigned to receive 0 or 50 mg/kg of FA (dose selected based on results from Experiment 1) through IP injection. Once the injection was performed, chicks were

immediately placed (1 chick per arena) in a 290 mm X 290 mm acrylic recording arena with food and water in the diagonal corners. Chicks were simultaneously and automatically recorded from 3 angles for a total of 30 min post-injection on DVD. Data were analyzed in 5 min intervals using ANY-maze behavioral analysis software (Stoelting Co., Wood Dale, IL).

At 30 min post-injection, food intake was measured. Locomotion (m traveled), the amount of time (in seconds; s) spent perching, preening, in deep rest, standing, sitting, and the number of steps, food pecks, water pecks, jumps, defecations, exploratory pecks, and escape attempts 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 > 5 s (its time starting 5 s after eye closure and ending when eyes reopened). Preening was defined as trimming or dressing down with the beak. Because of non-heterogeneous variance, behavior data were analyzed by the Mann-Whitney *U* test. Differences were considered significant at $P < 0.05$.

Experiment 3: c-Fos immunohistochemistry

Chicks were randomly assigned to receive 0 or 50 mg/kg of FA (dose selected based on results from Experiment 1) through IP injection, then were given ad libitum access to water, but food was restricted to prevent any c-Fos immunoreactivity associated with food consumption. At 60 min post-injection, which is the time point with the highest expected c-Fos expression [27], the chicks were decapitated, and heads were 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 the skulls, post-fixed for 1 hour in the same solution, then placed through a series of sucrose incubations, consisting of 20% and 30% in 0.1 M PB until they sank. Several 60 μm coronal sections that contained appetite-related nuclei, based on published

anatomies [28], were collected in 0.02 M PBS containing 0.1% sodium azide using a cryostat at -10°C. The area postrema (AP) and the nucleus of the solitary tract (NTS) were collected corresponding to interaural -6.08 mm, the arcuate nucleus (ARC) at 5.4 mm, the dorsomedial nucleus (DMN) at 5.4 mm, the lateral hypothalamus (LH) at 8.0, 7.4, and 6.8 mm, the paraventricular nucleus (PVN) was collected at 8.0 and 7.4 mm, and the ventromedial nucleus (VMN) was collected at 6.8 mm.

Procedures for the immunohistochemistry assay were performed as we described and validated previously [29] using rabbit polyclonal anti-c-Fos at a dilution of 1:5,000 (AB429, Abcam, Branford, CT, USA). The anatomy of the sections on slides were compared with the chicken stereotactic atlas [28] to confirm that the desired interaural position had been collected. Digital micrographs were captured for each section with a Nikon Eclipse 80i microscope, 4x objective and DS-Ri1 color camera using NIS-Elements Advanced Research Software (Nikon). Overlays containing the respective nucleus boundaries were digitally merged with each micrograph. The number of c-Fos immunoreactive cells within each respective nucleus were then counted by a researcher blind to treatments. Data were analyzed via a two-sample *t*-test using SAS 9.4 (SAS Institute). Differences were considered significant at $P < 0.05$.

Experiment 4: Hypothalamic mRNA abundance

Chicks were randomly assigned to receive 0 or 50 mg/kg of FA (dose selected based on results from Experiment 1) through IP injection, and then given ad libitum access to water, but food was not provided to ensure that there were no molecular changes associated with differences in food consumption. At 60 min post-injection, consistent with the c-Fos assay (Experiment 2), chicks were decapitated, and the brains removed and snap-frozen in liquid nitrogen for 9 s. Cuts were then made visually according to the following anatomy: perpendicular to the midline suture a cut

was made at the septopallio-mesencephalic tract and at the third cranial nerves. Two cuts were made 1.8 mm parallel to the midline. The final dorsal cut was made from the anterior commissure to 0.8 mm ventral to the posterior commissure [30], and the resulting block (comprised primarily of the hypothalamus) was immediately stored in RNAlater (Applied Biosystems, Carlsbad, CA, USA).

The hypothalamus was homogenized using stainless steel beads (Qiagen), Tri-Reagent (Sigma Aldrich, St. Louis, MO, USA) and a Tissue Lyser II (Qiagen). The Direct-zol™ RNA MiniPrep Kit and RNase-free DNase I (Zymo Research, Irvine, CA, USA) were used for total RNA purification. The concentration and purity of the total RNA was 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) 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 in 10 µL reactions containing 5 µL Fast SYBR Green Master Mix (Applied Biosystems), 0.25 µL each of 5 µM primers, 1.5 µL nuclease-free water, and 3µL 10-fold diluted cDNA using a 7500 Fast real-time PCR System (Applied Biosystems). The conditions for real-time PCR were 95°C for 20 s and 40 cycles of 90°C for 3 s plus 60°C for 30 s. A dissociation step consisting of 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s was performed at the end of each PCR reaction to ensure amplicon specificity. Real-time PCR data were analyzed using the ΔΔCT method with β-actin as the reference gene and the average of the vehicle chicks as the calibrator sample. Relative quantities

calculated as $2^{-\Delta\Delta CT}$ were used for statistical analysis. Data were analyzed via a two-sample *t*-test using JMP Pro 15 (SAS Institute). Differences were considered significant at $P < 0.05$.

Experiment 5: Nucleus mRNA abundance

Procedures were similar to Experiment 2, the difference being that the chicks were decapitated and perfused via the carotid artery with 1.5 mL of RNA stabilizing buffer (13.3 mM EDTA, 16.7 mM sodium citrate, and 3.3 M ammonium sulfate; pH = 5.2). Within 30 min of perfusion, brains were sectioned in a cryostat at -10°C into 500 µm thick coronal sections from rostral to caudal. The ARC was chosen based on results from Experiment 3. Sections that corresponded to 5.4 (ARC) intraneural were collected and punches performed. Punches were collected using sterile disposable biopsy punch instruments (1 mm, Integra, York, PA, USA) and immediately placed in RNA lysis buffer (Norgen Biotek) with 1% beta-mercaptoethanol (Millipore Sigma, Burlington, MA, USA), vortexed, snap-frozen in liquid nitrogen, and stored at -80°C. The remaining brain section was photographed, and punch accuracy was verified via overlays from the chicken stereotactic atlas [28].

The punches were vortexed vigorously and incubated at room temperature for 5 min before adding 70% molecular biology-grade ethanol. Total RNA was isolated following the manufacturer's instructions for the Total RNA Purification Micro Kit and RNase-Free DNase I (Norgen Biotek). The concentration and purity of total RNA was assessed as described in Experiment 3. Subsequent reactions were performed under the same conditions as described in Experiment 3, except that 100 ng of total RNA was used for reverse transcription and 5-fold diluted cDNA was used for real-time PCR. Real-time PCR data were analyzed using the ΔΔCT method with β-actin as the reference gene and the average of the vehicle chicks as the calibrator sample. Relative quantities calculated as $2^{-\Delta\Delta CT}$ were used for statistical analysis. Data were

analyzed via a two-sample *t*-test using JMP Pro 15 (SAS Institute). Differences were considered significant at $P < 0.05$.

Results and Discussion

Previous studies have assessed body weight and some metabolic changes induced by FA supplementation. It is possible some of these effects might be associated with increased satiety, although this has not been directly addressed in any species. We tested a range of doses, based on previous studies, and administered FA intraperitoneally to observe the direct effect of FA on food intake. At 30 min post-injection, chicks injected with 50 mg/kg of FA consumed less food (70% less) than the vehicle-injected chicks (Fig. 3.1). While the low and middle doses (12.5 mg/kg and 25 mg/kg) resulted in food intake values that were numerically lower than those of the vehicle-injected chicks, only those that received the highest dose ate significantly less than the control group. The effect was both potent (FA-injected chicks ate about one-fourth the amount of vehicle-injected individuals) and transient, with the effect disappearing after 30 min. There was no effect on daily food intake of a 0.05% FA-supplemented HFFD diet that was fed for 13 weeks to rats, although body weight gain was reduced [21]. Similarly, a 0.5% FA-supplemented HF diet fed for 7 weeks to mice did not alter food intake but mitigated weight gain [13]. Similar results were observed with the same concentration of FA supplemented in the drinking water of mice for 15 weeks [15]. In the same experiment, net food and water intake (on a weekly basis) were reduced in response to FA supplementation [15]. Collectively, results from rodent studies demonstrate anti-obesigenic effects of oral FA supplementation, however because only one study found a reduction in total food intake, the techniques used to measure feeding behavior may not have been sensitive enough to detect the direct effects of FA. This idea is supported by the present findings, that when directly administered into the animal, there was a

potent, but very transient suppression of appetite, which might explain detectable differences when measured over the long-term (weekly/monthly basis) and lack of detectable differences on a daily basis in other studies.

Water intake was not affected by FA treatment in the present study (Fig. 3.2). Rats that were administered 50 mg/kg of FA/day via an intragastric tube for 30 days did not differ in either food or water intake from control animals [31]. However, net water intake (mL per week) was reduced in mice that were given drinking water supplemented with FA for 15 wks [15]. In chicks, peripheral FA treatment does not affect water ingestion, further supporting that it has a specific, direct effect on food intake. That water intake neither increased or decreased also supports that the effect on food intake was appetite-related and not an indirect effect of malaise.

A behavioral analysis was conducted to determine whether the reduction in food intake was a result of a competitive behavior, such as increased deep rest, rather than a direct effect on satiety. At 5, 15, 20, and 25 min post-injection, chicks treated with FA had fewer defecations than vehicle treated chicks (Table 2). Other behaviors that were measured, such as distance traveled, steps, jumps, escape attempts, feed pecks, water pecks, exploratory pecks, time spent standing, sitting, in deep rest, preening, or perching were not affected by FA. In rats, oral administration of a range of doses of FA (50-200 mg/kg), was associated with a dose-dependent increase in gastric transit and emptying rate [32], contrary to what would be expected with fewer defecations observed in the present study, although it is important to note that the effects measured herein occurred on a short time scale (within 30 min). Rats treated with FA (10-80 mg/kg) via oral gavage did not differ in locomotor activity from control individuals [18]. However, there was reduced immobility time in rats treated with 20, 40, or 80 mg/kg of FA during a forced

swimming test [18]. In summary, results suggest that effects of FA on chick food intake are a direct effect on appetite regulation rather than an indirect effect induced by another behavior.

To identify hypothalamic and/or brainstem regions responsible for these effects, we detected the expression of c-Fos, an indicator of neuronal activity, within a variety of nuclei known to be associated with appetite regulation. Samples were collected at 60 min, because this is the time at which c-Fos expression is assumed to be the most robust, consistent with all of our other food intake studies. In chicks injected with FA, we observed an increase in the number of c-Fos immunoreactive cells (approximately 43% increase) in the ARC compared to the vehicle-injected chicks (Fig. 3.3A and 3.3B). No differences were detected in the NTS and AP of the brainstem, or other hypothalamic nuclei including the LHA, PVN, DMN, and VMN. The ARC contains first order orexigenic (NPY and AgRP) and anorexigenic (POMC and CART) neurons that integrate a variety of peripheral and central signals to modulate energy intake [33], thus activation of the ARC could imply that a variety of signaling pathways were induced in response to FA to suppress appetite. It is important to note that molecular effects were measured at 60 min, whereas food intake effects disappeared after 30 min, thus effects on gene expression could be the direct effect of FA on ARC activation, or a feedback response associated with a return to homeostatic feeding.

Based on the conclusion that the ARC was activated in response to FA, we measured gene expression of appetite-related factors in the ARC as well as in whole hypothalamus in order to capture factors that may have changed but were not ARC-specific. In the whole hypothalamus, chicks treated with FA had less galanin (GAL), ghrelin (GHREL), melanocortin receptor 3 (MC3R), and pro-opiomelanocortin (POMC) mRNA relative to chicks injected with the vehicle (Table 4). The mRNA abundance of AgRP, CART, CRF, GHSR, LEPR, MC4R, NPY, NPY1R,

NPY5R, or OREX was not affected by FA treatment. POMC is a protein that when cleaved will result in multiple peptides that can exert anorexigenic (the majority of the peptides) or orexigenic [34, 35] effects. Thus, increased expression of POMC can lead to a greater production and release of those anorexigenic peptides, for example, α-MSH, which can activate the MC3R to induce anorexigenic effects [36, 37]. In chicks, GHREL inhibits food intake [38], contrary to rodents, where GHREL is hunger-stimulating via NPY receptor activation [39]. The decreased expression of GHREL in whole hypothalamus but not the ARC suggests that another nucleus was responsible for the down regulation. GAL was also down regulated in the hypothalamus but not the ARC. GAL has food intake-stimulatory capabilities via the DMN and PVN [40, 41], and central administration led to increased c-Fos immunoreactivity in the ARC of chicks [42].

In the ARC, chicks injected with FA expressed more c-Fos and less POMC mRNA in the ARC as compared to chicks that received the vehicle (Table 5). The mRNA abundance of AgRP, CART, GAL, GHREL, MC3R, MC4R, NPY, NPY1R, or NPY5R was not affected by FA treatment. That c-Fos expression was increased, this corroborates our findings at the immunohistochemical level, and further demonstrating that FA-induced changes in feeding behavior likely involve signaling pathways mediated via the ARC. However, aside from the c-Fos, the only other gene that we measured that was affected was POMC, with mRNA being down-regulated, also consistent with what we observed at the whole hypothalamic level, suggesting that FA-induced satiety involves ARC and POMC-derived neuropeptide signaling.

Conclusions and Implications

In conclusion, results demonstrate that IP injection of FA induces a short, but potent anorexia in chicks. That effects dissipated quickly, and that water intake and most behaviors were not affected, suggests that this effect was specific to suppression of appetite. This was further

confirmed by the potent activation of the ARC, demonstrated at both the mRNA and protein level. Changes in POMC mRNA in both the whole hypothalamus and ARC suggest that FA-induced satiety is mediated via POMC-derived peptide signaling originating in the ARC. Further exploration of the mechanisms underlying the anti-obesogenic effects of FA in birds and mammals may facilitate the development of a novel strategy to combat compulsive feeding behavior and weight gain.

Conflicts of Interest

There are no conflicts of interest to declare by any author.

Table 3.1. **Primers for real-time PCR.**

Gene	Accession No.	Sequence (forward/reverse)
AgRP	XM_004950992.1	GGTTCTTCAACGCCTTCTGCTA/TTCTTGCCACATGGGAAGGT
β-actin	NM_205518.1	GTCCACCGCAAATGCTTCTAA/TGCGCATTTATGGGTTTTGTT
CART	XM_003643097.2	GCTGGAGAAGCTGAAGAGCAA/GCACCTGCCCGAACTT
c-Fos	NM_205508.1	TGTTCCTGGCAATATCGTGTTC/CTTTCCCCCCCACGTAAGA
CRF	NM_001123031.1	TCAGCACCAGAGCCATCACA/GCTCTATAAAAATAGGTGACATCAGA
GAL	NM_001159678.1	CGAATTTCTGACTTACTTGCATCTTAA/AAGGTTTGTTTCCTCTGGTGAAG
GHREL	NM_001001131.1	GAAGCACTGCCTAACGAAGACA/GGATGCTGAGAAGGAGAATTCCT
GHSR	XM_015291631.1	TCTGCGAGCGAAGGTGATC/AGACGGCCCAGAGGATGAG
LEPR	NM_204323.1	GCAAGACCCTCTCCCTTATCTCT/TCTGTGAAAGCATCATCCTGATCT
MC3R	XM_004947236.1	GCCTCCCTTTACGTTCACATGT/GCTGCATGCGCTTCAC
MC4R	NM_001031514.1	CCTCGGGAGGCTGCTATGA/GATGCCCAGAGTCACAAACACTT
NPY	NM_205473.1	CATGCAGGGCACCATGAG/CAGCGACAAGGGGAAAGTC
NPY1R	NM_001031535.1	TAGCCATGTCCACCATGCA/GGGCTTGCCTGCTTTAGAGA
NPY5R	NM_001031130.1	GGCTGGCTTTGTGGGAAA/TTGTCTTCTGCTTGCGTTTTGT
OREX	NM_204185.2	CCAGGAGCACGCTGAGAAG/CCCATCTCAGTAAAAGCTCTTTGC
POMC	NM_001031098.1	GCCAGACCCCGCTGATG/CTTGTAGGCGCTTTTGATGAT

Abbreviations: AgRP, agouti-related peptide; CART, cocaine and amphetamine-regulated transcript; CRF, corticotropin-releasing factor; GAL, galanin; GHREL, ghrelin; GHSR, growth hormone secretagogue receptor; LEPR, leptin receptor; MC3R and 4R, melanocortin receptors 3 and 4, respectively; NPY, neuropeptide Y; NPYR1 and R5, NPY receptor sub-type 1 and 5, respectively; OREX, orexin; POMC, pro-opiomelanocortin.

Table 3.2. Behavior Analysis: count-based behaviors.

Behavior (n)	TRT	Time Post-Injection (min)					
		5	10	15	20	25	30
Distance Traveled (m)	Vehicle	2.6±0.3	4.6±0.5	7.2±0.9	8.9±1.1	10.9±1.6	12.7±2.1
	FA	2.0±0.5	3.7±0.6	6.0±0.8	7.9±0.8	9.5±1.0	10.9±1.2
Steps	Vehicle	116.3±9.5	199.8±12.7	302.7±22.0	377.2±27.7	450.8±42.5	520.4±61.1
	FA	93±20.2	172.6±28.9	273.2±37.5	370.1±40.9	442.6±49.0	504.7±56.3
Jumps	Vehicle	0.9±0.6	2.0±0.7	3.1±0.9	3.8±0.9	4.6±1.1	5.0±1.2
	FA	0.8±0.2	1.7±0.4	3.2±0.6	3.9±0.7	4.6±0.8	4.8±0.9
Defecations	Vehicle	0.8±0.1	0.9±0.1	1.3±0.1	1.6±0.1	1.9±0.2	2.0±0.1
	FA	0.4±0.1*	0.7±0.1	1.0±0.1*	1.0±0.1*	1.2±0.1*	1.5±0.2
Escape Attempts	Vehicle	1.2±0.8	2.6±1.0	3.5±1.3	4.1±1.3	4.9±1.4	5.3±1.5
	FA	1.0±0.3	2.0±0.6	3.7±1.0	4.3±1.3	5.0±1.4	5.6±1.7
Feeding Pecks	Vehicle	8.6±4.7	52.3±26.2	101.2±33.5	141.3±35.2	148.8±36.8	194.4±48.1
	FA	10.6±6.0	51.9±26.2	100.7±42.1	158.9±42.1	236.7±51.5	310.4±73.4
Water Pecks	Vehicle	0	0	0.3±0.2	0.3±0.2	0.3±0.2	0.3±0.2
	FA	0	0	0	0.7±0.5	1.6±1.4	2.0±1.8
Exploratory Pecks	Vehicle	42.5±6.6	80.2±12.1	121±14.3	157.5±16.0	193.9±19.2	216.5±22.1
	FA	35.9±7.1	75.0±12.2	121.1±17.2	164.2±21.6	200.4±25.9	241.3±38.3

Four-day-old broiler chicks (*Gallus gallus*) were administered 0 (vehicle) or 50 mg/kg of ferulic acid (FA) through intraperitoneal (IP) injection. Chicks were observed for 30 min post-injection and their count-based behaviors recorded. Values are means ± standard errors. Values marked with an asterisk (*) are significantly different from vehicles ($P < 0.05$. For this experiment, 12-13 chicks per treatment (TRT) were available for analysis.

Table 3.3. Behavior Analysis: time-based behaviors.

Behavior (s)	TRT	Time Post-Injection (min)					
		5	10	15	20	25	30
Standing	Vehicle	293.8±2.2	573.7±13.3	846.1±24.2	1079.3±54.0	1295.4±86.0	1505.9±11.7
	FA	292.6±2.4	574.4±8.3	851.6±15.1	1111.5±29.0	1360.7±47.5	1593.1±70.0
Sitting	Vehicle	4.4±2.1	23.3±13.4	45.3±24.5	94.8±47.0	135.8±65.2	196.0±85.1
	FA	4.6±2.2	20.2±8.7	39.4±15.8	73.9±29.0	119.3±46.4	172.1±63.8
Deep Rest	Vehicle	0	0	0	13.5±13.5	52.1±36.8	71.1±48.9
	FA	0	0	0	0.9±0.9	1.9±1.9	14.4±8.1
Preening	Vehicle	0.3±0.3	1.4±0.7	7.0±3.6	10.6±4.9	14.8±6.7	24.9±11.6
	FA	0.7±0.6	3.1±1.7	6.4±3.6	9.3±4.9	13.8±6.3	15.9±6.7
Perching	Vehicle	0	0	0	0.1±0.1	0.1±0.1	0.3±0.3
	FA	0	0	0.3±0.3	1.9±1.1	1.9±1.1	2.0±1.1

Four-day-old broiler chicks (*Gallus gallus*) were administered 0 (vehicle) or 50 mg/kg of ferulic acid (FA) through intraperitoneal (IP) injection. Chicks were observed for 30 min post-injection and their time-based behaviors recorded. Values are means ± standard errors. Values marked with an asterisk (*) are significantly different from vehicles ($P < 0.05$. For this experiment, 12-13 chicks per treatment (TRT) were available for analysis.

Table 3.4. Hypothalamic mRNA abundance.

	AgRP	CART	CRF	GAL	GHREL	GHSR	LEPR
Vehicle	1.11±0.23	1.04±0.08	1.03±0.08	1.02±0.05	1.00±0.11	1.02±0.05	0.98±0.04
FA	1.29±0.21	0.94±0.08	0.84±0.08	0.82±0.05	0.59±0.11	0.98±0.05	0.96±0.04
P-value	.56	.36	.12	**.02***	**.02***	.67	.80
	MC3R	MC4R	NPY	NPYR1	NPYR5	OREX	POMC
Vehicle	1.02±0.06	0.97±0.04	1.01±0.04	1.00±0.04	1.00±0.02	1.02±0.06	1.03±0.06
FA	0.79±0.06	1.06±0.04	0.96±0.04	0.91±0.03	0.94±0.02	0.91±0.06	0.81±0.06
P-value	**.01***	.13	.44	.11	.11	.22	**.02***

Four-day-old broiler chicks (*Gallus gallus*) were administered 0 (vehicle) or 50 mg/kg of ferulic acid (FA) through intraperitoneal (IP) injection. The hypothalamus was collected at 1-hr post-injection. Values are means ± standard errors; *P*-values marked with an asterisk (*) highlight significant differences ($P < 0.05$) within that gene. For this experiment, 9-12 chicks per treatment were available for analysis.

Abbreviations: AgRP, agouti-related peptide; CART, cocaine and amphetamine-regulated transcript; CRF, corticotrophin-releasing factor; GAL, galanin; GHREL, ghrelin; GHSR, growth hormone secretagogue receptor; LEPR, leptin receptor; MC3R and 4R, melanocortin receptors 3 and 4, respectively; NPY, neuropeptide Y; NPYR1 and R5, NPY receptor sub-type 1 and 5, respectively; OREX, orexin; POMC, pro-opiomelanocortin.

Table 3.5. Arcuate nucleus mRNA abundance.

	AgRP	CART	c-Fos	GAL	GHREL	MC3R	MC4R
Vehicle	1.10±0.27	1.28±0.24	1.04±0.18	1.69±0.42	0.99±0.14	1.46±0.30	1.79±0.27
FA	0.78±0.25	1.14±0.22	1.58±0.16	1.78±0.40	0.88±0.12	1.78±0.27	1.55±0.26
P-value	.40	.68	**.03***	.88	.55	.43	.52
	NPY	NPYR1	NPYR5	POMC			
Vehicle	1.68±0.35	1.70±0.22	1.75±0.24	1.49±0.18			
FA	1.26±0.33	1.45±0.21	1.53±0.22	0.93±0.16			
P-value	.38	.45	.49	**.03***			

Four-day-old broiler chicks (*Gallus gallus*) were administered with 0 (vehicle) or 50 mg/kg ferulic acid (FA) through intraperitoneal (IP) injection. The arcuate nucleus was collected at 1-hr post-injection. Values are means ± standard errors; *P*-values marked with an asterisk (*) highlight significant differences ($P < 0.05$) within that gene. For this experiment, 14-17 chicks per treatment were available for analysis.

Abbreviations: AgRP, agouti-related peptide; CART, cocaine and amphetamine-regulated transcript; GAL, galanin; GHREL, ghrelin; MC3R and 4R, melanocortin receptors 3 and 4, respectively; NPY, neuropeptide Y; NPYR1 and R5, NPY receptor sub-type 1 and 5, respectively; POMC, pro-opiomelanocortin.

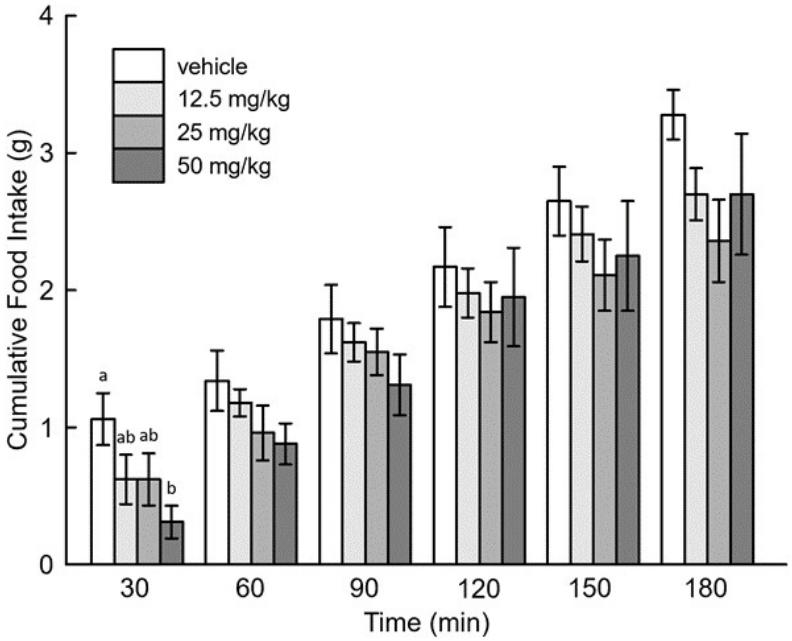

Figure 3.1. Cumulative food intake following intraperitoneal injection of ferulic acid in broiler chicks.

Cumulative food intake following intraperitoneal (IP) injection of 0, 12.5, 25, or 50 mg/kg ferulic acid in four-day-old broiler chicks (*Gallus gallus*). Values are means ± standard errors; bars with unique letters are significantly different from each other ($P < 0.05$) within that time period. For this experiment, 9-10 chicks per treatment were available for analysis.

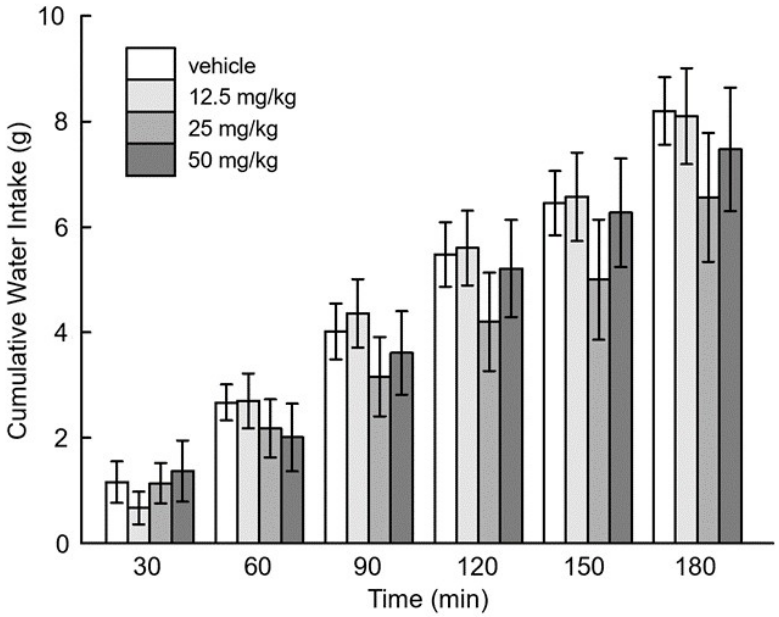

Figure 3.2. Cumulative water intake following intraperitoneal injection of ferulic acid in broiler chicks.

Cumulative water intake following intraperitoneal (IP) injection of 0, 12.5, 25, or 50 mg/kg ferulic acid in four-day-old broiler chicks (*Gallus gallus*). Values are means ± standard errors; bars with unique letters are significantly different from each other ($P < 0.05$) within that time period. For this experiment, 9-10 chicks per treatment were available for analysis.

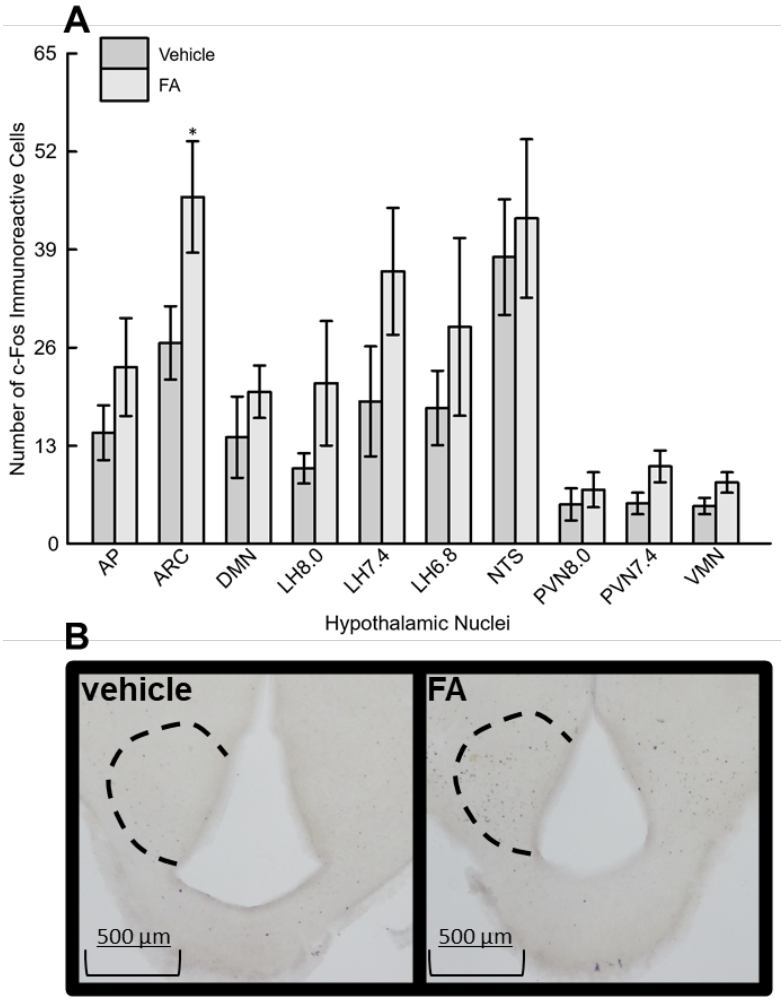

Figure 3.3. Effect of intraperitoneal injection of ferulic acid in broiler chicks on c-Fos immunoreactivity.

Effect of intraperitoneal injection of 0 (vehicle) or 50 mg/kg of ferulic acid (FA) in four-day-old broiler chicks (*Gallus gallus*) on the number of c-Fos immunoreactive cells at 60 min post-injection. Coronal sections were collected corresponding to intraneural anatomies described by Kuenzel and Masson [28]. The area postrema (AP) and the nucleus of the solitary tract (NTS) were collected corresponding to interaural -6.08 mm, the arcuate nucleus (ARC) and the dorsomedial nucleus (DMN) at 5.4 mm, the lateral hypothalamus (LH) at 8.0, 7.4, and 6.8 mm,

the paraventricular nucleus (PVN) was collected at 8.0 and 7.4 mm, and the ventromedial nucleus (VMN) was collected at 6.8 mm. (A). Values are means ± standard errors; bars with an asterisk (*) are significantly different from the vehicle-injected group within that nucleus ($P < 0.05$). For this experiment, 8-9 chicks per treatment were available for analysis. Also shown are representative photomicrographs of c-Fos staining in the ARC (B).

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Chapter 4: Oxyntomodulin induces satiety and activates the arcuate nucleus of the hypothalamus in Japanese quail

As published in Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology in 2020 (247: 110721)

Abstract

Oxyntomodulin (OXM) is a proglucagon-derived peptide that suppresses hunger in humans. There are some differences in its food intake-inhibitory effects among species. The central mechanisms are unclear and it is unknown if OXM is more efficacious in a gallinaceous species that has not undergone as much selection for growth as the chicken. The objective was thus to determine the effects of OXM on food and water intake and hypothalamic physiology in Japanese quail. At 7 days post-hatch, 6-hour-fasted quail were injected intracerebroventricularly (ICV) or intraperitoneally (IP) with 0.32, 0.65, or 1.3 nmol of OXM. All doses decreased food intake for 180 minutes post-ICV injection. On a cumulative basis, water intake was not affected until 120 minutes, with the lowest and highest doses decreasing water intake after ICV injection. The two highest doses were anorexigenic when administered via the IP route, whereas all doses were anti-dipsogenic starting at 30 minutes post-injection. In hypothalamic samples collected at 1-hour post-ICV injection, there was an increase in c-Fos immunoreactivity, an indicator of recent neuronal activation, in the arcuate nucleus (ARC) and dorsomedial nucleus (DMN) of the hypothalamus in OXM-injected individuals. Results suggest that quail are more sensitive than chickens to the satiety-inducing effects of OXM. The central mechanism is likely mediated through a pathway in the ARC that is conserved among species, and through activation of the DMN, an effect that is unique to quail. Such knowledge is

critical for facilitating the development of novel, side effect-free anti-eating strategies to promote weight-loss in obesity.

Key words: anorexigenic, arcuate nucleus, dorsomedial nucleus, food intake, hypothalamus, oxyntomodulin, quail

Introduction

Proglucagon-like peptides are produced in the gastrointestinal (GI) tract and pancreas in response to digestion products, and include glucagon, glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), and oxyntomodulin (OXM) [1]. OXM, a 37 amino acid peptide, is released with GLP-1 from the GI tract in response to nutrient intake and activates both the glucagon and GLP-1 receptors (GLP-1Rs) [2]. The amino acid sequence of OXM contains the entirety of glucagon's sequence, as well as 8 additional residues at the C-terminus. Prohormone convertase 1/3 processes proglucagon into glicentin within the enteroendocrine L-cells, in which it is then cleaved into OXM [3, 4]. Thereafter, OXM, as the name implies, targets oxyntic glands [5] and reduces gastric acid secretions [6-8]. The enzyme dipeptidyl-peptidase IV inactivates OXM, an interaction that can be exploited in the design of structural analogs that have an extended half-life [9, 10].

As reviewed, OXM potently induces satiety in humans and promotes weight loss, and appetite-related effects are mediated via the hypothalamus, in particular the arcuate nucleus (ARC) through GLP-1Rs [11]. However, there are some conflicting behavioral effects reported among studies and species. For instance, in rats, both intracerebroventricular (ICV) [12] and intraperitoneal (IP) [13] injection of OXM reduce food intake, whereas in mice, the IP route of administration did not influence

consumption [14]. In broiler (meat-type) chicks, ICV [15] but not IP or intravenous (IV) injection of OXM inhibited food intake [16]. Water intake was also reduced in ICV-injected broiler chicks [16] and IP-injected rats [13], but not IV-infused humans [17]. It is unclear if the effects in chickens are species-specific or influenced by the fact that chickens have been intensely selected for growth-related traits that have led to correlated responses in food intake. Thus, it would not be surprising that selection for growth and consequently the increase in inherent appetite would alter anorexigenic tone and influence the sensitivity to appetite-suppressants. Japanese quail (*Coturnix japonica*) are also gallinaceous and well-adapted to a captive environment, but are not as extensively selected and may thus better resemble a more "wild-type" bird. Thus, the objective of the present study was to characterize responses in food and water intake to centrally and peripherally administered OXM in quail, and to identify associated activation in hypothalamic nuclei.

Materials and Methods

Animals

All protocols were approved by the Institutional Animal Care and Use Committee at Virginia Tech. Japanese quail were bred, hatched, and grown in our facility. After hatching, quail were group-caged in a brooder at 37 ± 1°C, and 50 ± 5% humidity for 3 days, with lights on for 14 hours a day. The quail were then weighed and individually caged in a room at 32 ± 1°C and 50 ± 5% humidity with 14-hour lighting, in wire cages that provide visual and auditory contact with the other birds. Unless stated otherwise, quail had *ad libitum* access to diet (2,900 kcal ME/kg and 24% crude protein) and water.

Birds were handled twice daily to reduce the effects of stress on the day of data collection. Details of the handling procedure are as we previously described [18].

ICV injection procedure

An injection method was used that appears to not induce any physiological stress [18, 19]. The chick's head was placed into the restraint device. Injection coordinates were 2 mm anterior to the coronal suture, 0.75 mm lateral from the sagittal suture, and 1.5 mm deep targeting the left lateral ventricle. Depth of injection was controlled by a plastic tube sheath that was placed on the needle. The needle was held in position for a few seconds post-injection to reduce backflow. Bovine OXM (AnaSpec Inc.) was dissolved in artificial cerebrospinal fluid [20] as a vehicle for a total injection volume of 5 µl containing 0.1% Evans Blue dye to facilitate injection site localization. After data collection, the chick was decapitated and then the head was sectioned along the frontal plane, exposing the injection site. Chicks without dye in the ventricle system were excluded. Sex was determined visually through dissection after decapitation. Results represent a single trial for each experiment and numbers of chicks used for each analysis are provided in Figure captions.

Experiment 1: effect of ICV OXM on food and water intake

Japanese quail, 7 days old, were fasted for 6 hours prior to injection. Chicks were randomly assigned to receive 0 (vehicle), 0.32, 0.65, or 1.3 nmol (n= 10-12 per treatment) OXM by ICV injection, where doses used were based on [16]. On the day prior to the experiment, chicks were weighed to ensure that each treatment group had similar mean body weights. Chicks were injected, then returned to original cages and given *ad libitum* access to food and water. Food and water intake were measured (0.01 g) every 30

minutes post-injection for a total of 180 minutes, with water weight converted to volume (1 g = 1 mL). Data were analyzed using analysis of variance (ANOVA) within each time point by the GLM procedure in SAS 9.3 (SAS institute, Cary, NC, USA), 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. Significance was set at $P < 0.05$ for all experiments.

Experiment 2: effect of IP OXM on food and water intake

Procedures were the same as described in Experiment 1, except that OXM was diluted in avian saline and injections were administered into the peritoneum (IP), at a volume of 200 mL, of the fasted chick.

Experiment 3: c-Fos immunohistochemistry

At 7 days post-hatch, chicks fasted for 6 hours were randomly assigned to receive 0 (vehicle) or 0.32 nmol (n= 15-17 per treatment) of OXM (dose selected based on results of Experiment 1) through ICV injection. C-Fos expression is induced in association with neuronal firing, and as such is commonly used as an indirect marker of neuronal activation. 60 minutes post-injection, the time point at which the most c-Fos induction is anticipated [21], the chicks were deeply anesthetized using sodium pentobarbital (30 mg/kg body weight) via cardiopuncture and then decapitated. The brain was fixed with 2% paraformaldehyde and 0.1% glutaraldehyde solution via the left carotid artery. The brain was then placed in 30% sucrose in phosphate-buffered saline at 4°C for 48 hours. Brains were sectioned at 40 μm using a cryostat, from brain areas containing the dorsomedial nucleus (DMN), lateral hypothalamus (LH), ventromedial hypothalamic nucleus (VMH), ARC, and paraventricular nucleus (PVN). Procedures for the c-Fos

immunohistochemistry assay were performed as we described and validated previously [22] using rabbit polyclonal anti-c-Fos at a dilution of 1:5,000 (AB429, Abcam, Cambridge, MA, USA). Reactive cells were counted from the injected side of the brain in 0.2 mm^2 areas in the center of the respective nucleus using light microscopy. For each chick, two sections of the desired area were counted and averaged by a researcher blind to sample information. Data were analyzed using ANOVA within each nucleus.

Results

Experiment 1: effect of ICV OXM on food and water intake

On a cumulative basis, all ICV doses reduced food intake at all time points (30 through 180 minutes post-injection; Fig. 4.1A). On a non-cumulative basis, all doses reduced food intake at 30, 150, and 180 minutes post-injection, with the 1.3 nmol dose also reducing intake at 60 minutes (Fig. 4.1B).

When expressed cumulatively, water intake was reduced by the 0.32 and 1.3 nmol ICV dose at 120 through 180 minutes post-injection (Fig. 4.2A). Non-cumulatively, the 1.3 nmol dose reduced water intake at 90 through 180 minutes post-injection, and the 0.65 nmol dose at 120 and 180 minutes (Fig. 4.2B).

Experiment 2: effect of IP OXM on food and water intake

Quail that received IP injections of the 0.65 and 1.3 nmol doses of OXM consumed less food at 30 through 90 minutes post-injection, with the 1.3 nmol dose also reducing food intake at 120 minutes, on a cumulative basis (Fig. 4.3A). On a non-cumulative basis, the 0.65 and 1.3 nmol doses of OXM reduced food intake at 30 minutes post-injection (Fig. 4.3B). At 180 minutes post-IP injection, the quail that were injected with 0.65 nmol had

consumed more than chicks injected with the 1.3 nmol dose, during the preceding 30 minutes (Fig. 4.3B).

On a cumulative basis, water intake was reduced by all IP-injected doses at 30 minutes, and by the 0.65 and 1.3 nmol doses at 60 and 90 minutes post-injection (Fig. 4.4A). When expressed non-cumulatively, all doses decreased water intake at 30 minutes (Fig. 4.4B). The 0.65 nmol dose was associated with reduced water intake relative to 0.32 nmol-injected birds at 60 minutes, and increased water consumption at 150 and 180 minutes post-injection. Chicks that received the 1.3 nmol dose drank more relative to vehicle-injected individuals at 120 and 150 minutes post-injection (Fig. 4.4B).

Experiment 3: c-Fos immunohistochemistry

Relative to vehicle-injected quail, there were increased numbers of c-Fos immunoreactive cells in the ARC and DMN (Fig. 4.5). No differences among treatment groups were detected in the LH, PVN, and VMH.

Discussion

In this study, exogenous OXM potently suppressed feeding in young quail, irrespective of route of administration. When injected centrally, all doses were effective, with a rapid suppression of feeding that persisted through the entirety of the experiment. When the same doses were administered peripherally, only the two higher doses were efficacious and effects dissipated between 30 and 60 minutes earlier. This robust reduction in food intake is similar to that observed with broiler chicks, where ICV injection of 0.67, 1.34, and 2.68 nmol to 3-hour-fasted chicks decreased food intake, an effect that persisted for the 180 minute observation period [16]. However, the lowest dose, 0.67 nmol, did not affect food intake at 30 minutes post-injection, and was thus described as the lowest

effective dose, which was why a lower dose was not further tested in that study [16]. In the present research, preliminary experiments (data not shown) revealed that an ICV dose lower than 0.67 was effective at reducing food intake in quail, thus in the experiments reported herein, we used a lowest dose of 0.32 nmol. Interestingly, the reduction in food intake after IP injection that we observed in quail was not detected in broiler chicks [16]. In that study, they tested a wide range of doses: 0.11 to 31.7 nmol, and two routes of peripheral administration, both IP and intravenously via the brachial vein [16]. Thus, it appears that quail might be more sensitive to the appetite-suppressive effects of OXM, although it should be noted that we fasted quail for 6 and not 3 hours prior to injection, as we identified this as a fasting duration in previous studies that produces effects that are similar in magnitude to a 3 hour fast in broiler chicks [23]. It is unclear whether differences between quail and chick are related to a reduction in anorexigenic tone in broilers because of intense, long-term genetic selection for growth-related traits that led to a correlated response in increased homeostatic food intake.

The anorexigenic effect of OXM appears to be highly conserved between birds and mammals. In 24-hour-fasted rats, both ICV and intra-PVN administration suppressed food intake [12]. Via the ICV route, a dose as low as 0.3 nmol reduced food intake at 2 hours, and 1 nmol as early as 1 hour (there were no earlier time points at which food intake was recorded), and effects were sustained at 4 hours post-injection [12]. Via the intra-PVN route, 1 nmol of OXM reduced food intake and the effect persisted for even longer – 8 hours [12]. Inhibition of food intake was also observed in non-fasted rats at the beginning of the dark phase, with 3 nmol having a potent effect at 1 hour post-ICV injection [12].

As alluded to earlier, a unique OXM receptor has not been identified and it is presently assumed that central effects of OXM are mediated via the GLP-1R. In rats, food intake-suppressive effects of OXM were similar to GLP-1, and OXM-induced satiety was blunted by co-administering a GLP-1 agonist (exendin(9-39)), although in the same study it was demonstrated that OXM has a much lower affinity for GLP-1R than its other ligand GLP-1 [12]. OXM also has a lower affinity for the glucagon receptor than glucagon and can mediate physiological effects through either the glucagon receptor or GLP-1R; in a rat study, an OXM analogue was developed that has a greater potency at both receptors than native OXM [24]. This analogue, OXM-SR, was subcutaneously administered and robustly increased energy expenditure (as assessed in metabolic cages) similar to glucagon, but had variable effects on food intake; authors attributed this to the peptide's affinity for both glucagon receptor and the GLP-1R and varying effects depending on the dose [24]. Perhaps the most compelling data to support that OXM-induced satiety is GLP-1R-mediated are from a study with glucagon receptor and GLP-1R knockout mice [14]. In that study, ICV but not IP OXM inhibited food intake, an effect that was blocked by central injection of exendin(9-39). This appetite-suppressive effect was similar in glucagon receptor$^{-/-}$ mice but not those in which GLP-1R function was absent (GLP-1R$^{-/-}$). In the GLP-1R$^{-/-}$ mice, central OXM failed to elicit an inhibitory effect on food intake, convincing evidence that appetite regulatory effects of OXM are mediated through GLP-1R [14].

Unlike chickens [16] and mice [14], but similar to the present study with quail, peripherally-administered OXM reduced food intake in rats [13]. After 24 hours of fasting, 30 and 100 nmol/kg BW OXM (but not 3 or 10) reduced food intake at 1 hour

post-injection, with the effect of the lower effective dose sustaining through 4 hours and the higher dose through 24 hours post-injection [13]. There were also reductions in food intake after injection in non-fasted rats during the dark phase [13]. In the same study, twice-daily injections of OXM reduced food intake and body weight gain [13]. Similarly, intravenous infusion to human subjects (3.0 pmol/kg/min) reduced energy intake at a buffet meal by 19% and the feeling of hunger [17]. Self-administration of OXM via subcutaneous injection for 4 weeks (3 times daily, 30 minutes prior to each meal) similarly inhibited energy intake and led to weight loss in healthy and overweight subjects [25].

Anorexigenic responses to exogenously administered peptides can sometimes be difficult to interpret as there is the question of whether the effect is a direct modulation of appetite versus a secondary effect to something else, such as malaise. Malaise can be difficult to define and identify in animal models, however, evidence that OXM induces satiety without causing malaise, is that healthy humans infused with OXM reported a decrease in their hunger without having any symptoms of nausea or perceiving a decline in the palatability of the food [17]. In healthy and overweight subjects who self-administered daily injections of OXM for 4 weeks, 3% of all injections were associated with a short-lived nausea, while most were associated with no such effect, and subjects reported a decline in hunger and weight loss without a change in the palatability of food. [25]. Differences in the food intake response among studies, including dose threshold and duration of response, and injection route-dependency could be attributed to a variety of factors, one being the OXM sequence utilized in non-human studies. For instance, we used bovine OXM (84 % sequence identity to chicken OXM) in broiler chicks [16].

Another group used chicken OXM and observed that ICV doses of 0.1, 0.3, and 1 nmol were efficacious at reducing food intake in both layer-type and broiler-type chicks at 8 days post-hatch, with slight differences between the genetic stocks [15]. Layers responded to all doses with a reduction that did not become significant until 60 minutes post-injection, whereas broilers responded to the 0.3 and 1 nmol doses at 30 minutes and the 0.1 nmol dose at 1 hour, with the effect of the two higher doses sustained at 120 minutes post-injection (final time point reported in the study) [15]. Thus, both mammalian and chicken-specific OXM are potently anorexigenic in chickens, supporting the use of bovine OXM in the present study.

Water intake was also reduced by OXM administration in this study, but interestingly, the response varied depending on the route of injection. When administered centrally, reductions in drinking were not observed until 2 hours after injection, whereas when injected peripherally, the response was observed earlier and had dissipated by 2 hours post-injection. Similarly, fasted broiler chicks that were ICV-injected with OXM drank less and the effect occurred later than for feeding, with reductions observed at 60 minutes post-injection [16]. In the chick study, it was hypothesized that the latency to reduce water intake suggested that it was an effect that was secondary to food intake, thus they repeated the experiment and withheld food but not water after injection, revealing that OXM did not affect water intake [16]. In rats, daily (twice) IP injections (50 nmol/kg BW) caused reductions in water intake during the first 2 days of injection, but increases thereafter that restored levels of intake to those of the controls [13], and intravenous infusions to healthy human subjects had no effect on water intake [17]. These effects differ from the related GLP-1, which reduced drinking and renal salt excretion in

intravenously-infused human subjects [26]. Thus, although OXM-injected quail drank less at some time points in response to certain doses, the physiological significance of this anti-dipsogenic effect is unclear.

To identify hypothalamic regions associated with OXM-induced satiety, we quantified expression of c-Fos, a commonly used marker for neuronal activation. We observed that two key appetite-related nuclei, the ARC and DMN, displayed increases, suggestive of activation in response to OXM. In broiler chicks, there was also activation of the ARC, but no change in the DMN, and a decrease detected in the LH [16]. In mice, IP injection of OXM (as well as a GLP-1R agonist) activated the PVN, and two nuclei of the brainstem – the nucleus of the solitary tract (NTS) and the area postrema (AP) [14]. In rats, IP (50 nmol/kg BW) injection of OXM was associated with increased c-Fos-reactivity in the ARC, but no changes in the PVN, DMN, VMH, NTS, or AP at 90 minutes post-injection [13]. Intra-ARC administration of OXM also suppressed food intake in rats, and interestingly, prior intra-ARC administration of GLP-1R antagonist prevented IP-OXM-induced reductions in food intake, suggesting that appetite-associated effects of OXM are mediated via ARC-expressed GLP-1Rs [13]. Consistent with this hypothesis, incubation of rat hypothalamic explants with OXM (100 nM) prompted a release of the highly anorexigenic peptide, α-melanocyte-stimulating hormone (α-MSH) [13]. In birds, α-MSH is also anorexigenic and elicits changes in gene expression in hypothalamic nuclei in chicks [27] and quail [18]. Thus, it appears that activation of the ARC is observed and conserved across species, except in the mouse, although it should be noted that OXM was administered IP in the mouse study and the IP route of administration does not have an effect on food intake in mice [14].

There was also increased expression of c-Fos in the DMN of OXM-injected quail. At least in mammals, preproglucagon gene expression in the central nervous system is restricted to a subset of neurons in the NTS, which cleave preproglucagon into bioactive peptides, including OXM [28]. These OXM-producing neurons project to hypothalamic nuclei, including the PVN and DMN, which are highly immunoreactive for GLP-like peptides and have known roles in reducing food intake, maintaining body weight, and regulating thirst [28]. Thus, the DMN is likely to contain GLP-1Rs and may contribute to the anorexigenic response of OXM via its projections to neurons in the PVN. Future studies should attempt to quantify gene expression and production of appetite-related peptides in hypothalamic nuclei in response to central and peripheral OXM administration.

Conclusions

In conclusion, similar to other species, OXM elicits a potent reduction in food intake in quail. This effect occurs in response to both central and peripheral administration of the peptide. Water intake is also reduced, but the effect is of a much lesser magnitude than that of appetite. The increased expression of c-Fos in the ARC and DMN implies that the actions of OXM are mediated via these nuclei. The activation of the ARC is consistent with what is observed in other species, however, activation of the DMN is a novel finding. Because of the potent appetite-suppressive and weight-loss effects of OXM reported over a range of species, there is considerable interest in OXM as an anti-obesity agent. However, targeting OXM and its associated molecular pathways requires a greater understanding of its mode of action in different species, especially where some physiological effects differ.

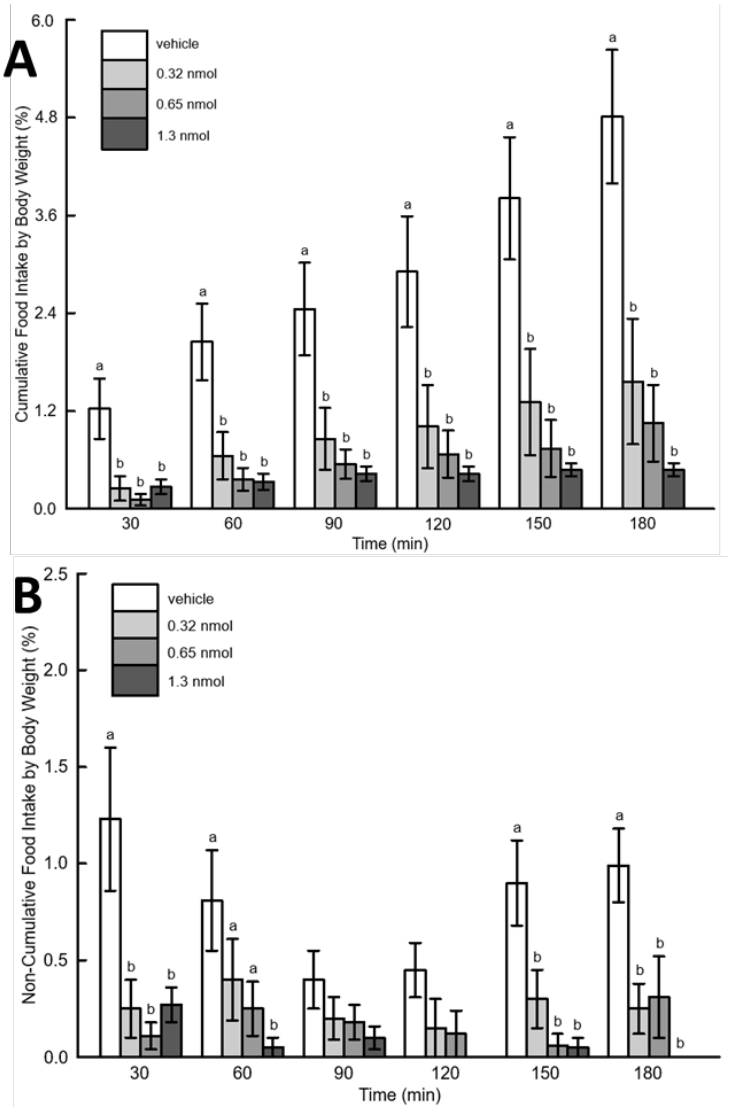

Fig 4.1. Cumulative and non-cumulative food intake as a percent of body weight following intracerebroventricular injection of oxyntomodulin in Japanese quail. Cumulative (A) and non-cumulative (B) food intake as a percent of body weight following intracerebroventricular injection of OXM in 7-day post-hatch Japanese quail that were fasted for 6 hours prior to injection. Values are means ± standard error; bars with unique letters are different from each other within a time ($P < 0.05$). For this experiment, 10 to 12 chicks per treatment were available for analysis.

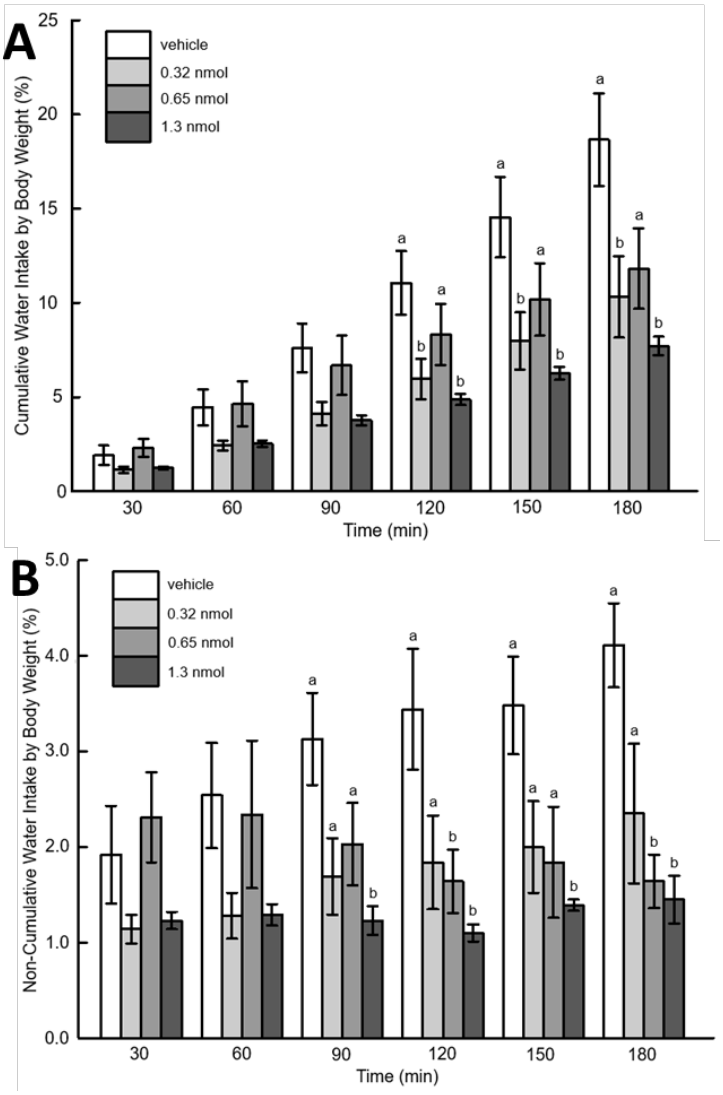

Fig 4.2. Cumulative and non-cumulative water intake as a percent of body weight following intracerebroventricular injection of oxyntomodulin in Japanese quail. Cumulative (A) and non-cumulative (B) water intake as a percent of body weight following intracerebroventricular injection of OXM in 7-day post hatch Japanese quail that were fasted for 6 hours prior to injection. Values are means ± standard error; bars with unique letters are different from each other within a time ($P < 0.05$). For this experiment, 10 to 12 chicks per treatment were available for analysis.

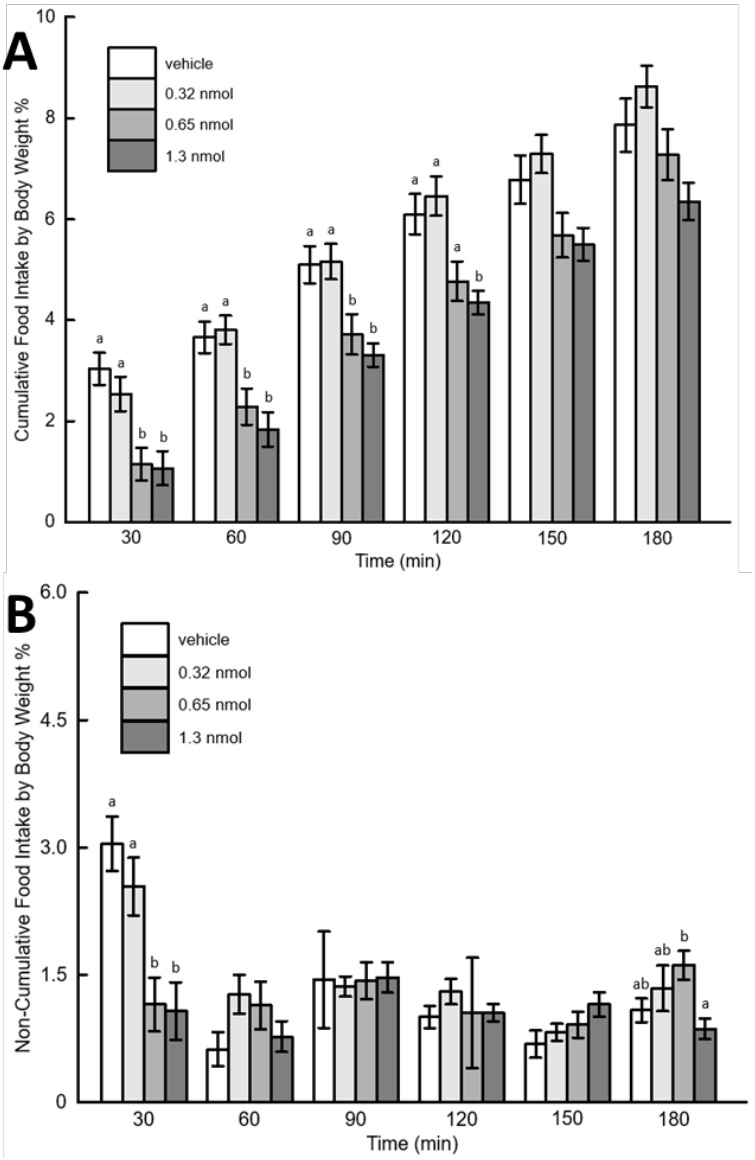

Fig 4.3. Cumulative and non-cumulative food intake as a percent of body weight following intraperitoneal injection of oxyntomodulin in Japanese quail.

Cumulative (A) and non-cumulative (B) food intake as a percent of body weight following intraperitoneal injection of OXM in 7-day post hatch Japanese quail that were fasted for 6 hours prior to injection. Values are means ± standard error; bars with unique letters are different from each other within a time ($P < 0.05$). For this experiment, 10 to 12 chicks per treatment were available for analysis.

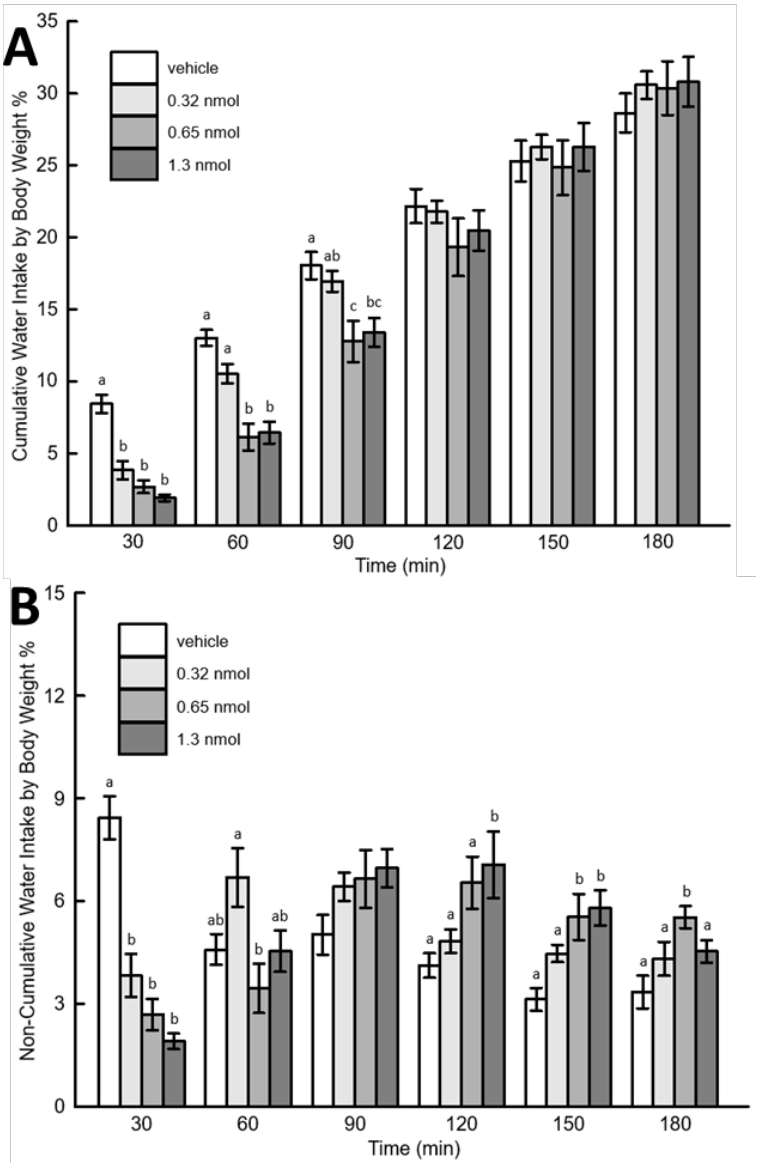

Fig 4.4. Cumulative and non-cumulative water intake as a percent of body weight following intraperitoneal injection of oxyntomodulin in Japanese quail.

Cumulative (A) and non-cumulative (B) water intake as a percent of body weight following intraperitoneal injection of OXM in 7-day post hatch Japanese quail that were fasted for 6 hours prior to injection. Values are means ± standard error; bars with unique letters are different from each other within a time ($P < 0.05$). For this experiment, 10-12 chicks per treatment were available for analysis.

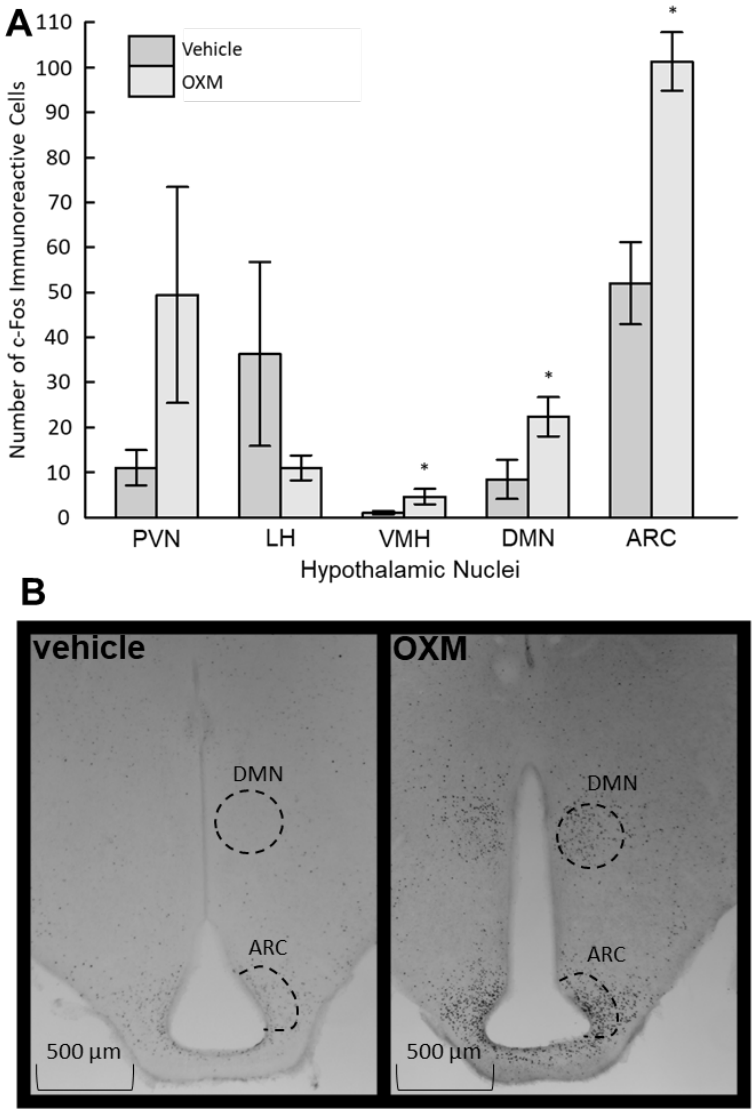

Fig 4.5. Effect of intracerebroventricular oxyntomodulin on c-Fos immunoreactivity in Japanese quail.

Number of c-Fos immunoreactive cells in the paraventricular nucleus (PVN), lateral hypothalamus (LH), ventromedial hypothalamic nucleus (VMH), dorsomedial nucleus (DMN), and arcuate nucleus (ARC) of the hypothalamus (A), and representative photomicrographs that display the ARC and DMN (B). At 7 days post-hatch after 6 hours of fasting, 0.32 nmol of oxyntomodulin (OXM) was intracerebroventricularly injected

and hypothalamic collected at 1 hour post-injection. Values are means ± standard error, * different from vehicle-injected within nucleus ($P < 0.05$). For this experiment, 15-17birds per treatment were available for analysis.

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Chapter 5: Epilogue

FA was effective at reducing food intake when administered peripherally into the broiler chick. When treated with the highest dose of 50 mg/kg, which was determined to be the lowest effective dose, chicks consumed 70% less food compared to control birds for up to 30 min post-injection. Its effects were potent, however quickly diminishing as there was no effect after 30 min. The behavioral analysis revealed FA's effects on food intake are direct. The only behavior altered by FA treatment were the number of defecations, where chicks administered with FA defecated fewer times than those who received no FA. The fact that no aversive behavior was the cause of the decrease in food intake is encouraging for the possible use of FA in animal agriculture or humans. At one-hour post-injection, chicks treated with FA showed an increase in c-Fos immunoreactivity within the ARC, indicating there was neuronal activation at the time of brain collection. Within the whole hypothalamus, we found a decrease in GAL, GHREL, MC3R, and POMC mRNA in FA treated chicks, where specifically within the ARC there was a decrease in POMC mRNA and an increase in c-Fos mRNA. These data show that FA's effects on food intake in the chick are likely mediated within the ARC and may involve POMC.

While the effects of OXM on appetite were understood in rodents, humans, and the broiler chick, its effects on an avian model that had not undergone intense selection were unknown. When administered via ICV injection, OXM treated chicks had a reduction in food intake in response to all doses, for a total of three hours post-injection, with the lowest dose-treated birds consuming 80% less than control birds. An IP injection also resulted in a decrease in food intake, however only the two highest doses were effective for up to two hours post-injection. Also, at one-hour post-injection, OXM-treated chicks

showed an increase in c-Fos immunoreactivity within the ARC and the DMN. These findings were similar to those in broiler chicks and rodents treated with OXM, where the ARC is likely the nucleus responsible for mediating the mechanism for OXM's effects. However, the activation within the DMN is a novel finding, so it is also possible that OXM could activate the nucleus of the solitary tract within the brain stem, which can then project signals to the DMN.

Being selected for increased growth rates and meat output has resulted in the tendency for the broiler to eat nearly continuously to achieve the desired rapid growth. Essentially, the desire to eat has been bred into these broilers, as well as the breeder lines responsible for producing those broilers. However, the purpose of the breeder lines is not rapid growth but in fact fertility and consistent egg-laying. Like any other animal or human, these birds are able to become obese. In the females, while restriction of food access enhances egg production [1], allowing ad libitum feeding results in lower levels of egg production [2]. Leaner male birds had increased sperm concentrations and a higher number of normal sperm compared to a fat chicken line [3]. Similar concepts apply to humans, where obese women can suffer from infertility, increased risk of miscarriages, and overall impairment of ovarian function via insulin resistance which causes androgen excess [4]. Reducing food intake in either poultry or humans affects many physiological aspects.

Because the broiler chicken has undergone such intense genetic selection, this has led to the idea that their appetite circuitry has been indirectly affected. Our lab demonstrated that OXM had similar but also somewhat different effects in the broiler when compared to other models. Broilers were not affected by IP or IV administration of OXM [5], where

rats demonstrated a reduction in food intake via IP administration [6], and humans had reduced food intake via IV administration [7]. Our utilized model, the Japanese quail, provides an insight to an avian model that has not experienced such dramatic artificial selection. We demonstrated the quail had reduced food intake in response to IP administration, as well as the DMN showing activation within the hypothalamus, neither of which were found in broilers. Every possible effect of the dramatic artificial selection on the broilers should be understood to be able to fully maximize production capabilities. Similarly, FA's overall effects in any poultry model should be further explored to compare to the results in the quail, as well as it's multiple beneficial physiological effects, only briefly examined in poultry [8].

While we uncovered the novel effects of OXM on the quail and FA on broilers, the true hypothalamic mechanisms responsible for mediating both FA and OXM's effects need to be further studied. We were able to locate the hypothalamic nuclei responsible for both FA and OXM's effects in broiler chicks and quail chicks, respectively, however more needs to be done to understand what mechanism is directly responsible for mediating their effects within the ARC. Further studies should be conducted to evaluate FA and OXM's effects in a few areas:

1. FA's effects should also be observed in Japanese quail to demonstrate any possible differences compared to the broiler because of any indirect effects resulting from genetic selection.
2. A behavioral analysis could also be performed to evaluate if OXM is the cause of any unknown behavioral changes.

3. Both FA and OXM's hypothalamic mechanisms need to be studied in broilers and in the Japanese quail.

4. Both FA and OXM's effects on layer chickens should be studied for their possible use in reducing food intake in breeder lines to maximize fertility and reduce reproductive issues.

5. The possible use of FA in humans could be explored further through many routes of administration, likely beginning with oral supplementation, as well as IV and subcutaneous, similar to OXM's known effects.

These findings demonstrate how both FA and OXM show promise for appetite-regulatory use in animal agriculture and/or human medicine. FA provides a potential exogenous source of a compound that is able to reduce food intake, while OXM provides a potential endogenous source. OXM is naturally produced within the body in response to digestion, and FA is naturally found within plants and is already consumed on a daily basis via various foods. Either could be utilized in poultry breeder birds to negate the need for feed-restriction because of the tendency of the chicken to continuously feed, or in humans to combat the desire to consume an excessive number of calories. Similarly, once fully understood, FA and OXM's effects could be reversed to encourage eating behavior in broiler birds where rapid growth is critical, or in humans suffering from an insufficient consumption of calories because of anorexia nervosa.

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