Peptide gene expression profiles in response to fasting and re-feeding in hoarding and non-hoarding titmice

Doctor of Philosophy

Thesis submitted in fulfilment for the requirements of the degree of Doctor of Philosophy School of Natural and Environmental Sciences, Newcastle University

Newcastle University

United Kingdom

By

Bedour Alsayegh

Dedication

I would like to dedicate my thesis to my gorgeous mother and brothers who have been there with me since the start giving me love and support.

I would also like to say thanks to my lovely supervisors Tim & Tom who have guided and supported me all the way through my Ph.D.

Bedour Alsayegh

January 2021

Declaration

This thesis is submitted to Newcastle University for the degree of Doctor of Philosophy in (Peptide gene expression profiles in response to fasting and re-feeding in hoarding and non-hoarding titmice). The research detailed within was performed from 2016 to 2019 and was conducted in Newcastle University laboratories under the supervision of both Dr. Timothy Boswell and Dr. Tom Smulders.

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other sources were used, they have been acknowledged.

Bedour Alsayegh

January 2021

Acknowledgment

In the first place, I would like to praise Allah for his guidance and help for this accomplishment. I am very grateful to my sponsors at Kuwait University and especially; Dr. Salem Al-Mohana; Dr. Amani Al-Zaidan and Dr. Lolwa Al-Gaith for supporting and helping me, but most importantly of all having faith in my abilities by giving me the chance to peruse my dream to study for a PhD at Newcastle University.

Words cannot give enough credits to my dearest and loveliest mother Khalida Alsayegh who has been and still is the main engine in my life. It is through her endless love and support that I have been sailing through this pathway. She was there for better and worse, endure endless complaints, and been there when I am happy. Also, I cannot forget my brothers who have been a shoulder to me in every single step through my PhD.

I am also very grateful for my supervisors Dr. Tim and Dr. Tom for their unlimited support, guidance and understanding, for always being there to help whenever I need it even beyond this thesis.

Finally, a special thanks to my colleagues Liam, Sam, Amy and Chloe for being the best thing that happens to me providing company, love and friendship throughout this 4-year PhD.

Abstract

The avian appetite regulatory system has been continuously studied over the last decades but it is less well understood than the mammalian system. It has also been studied much more in domestic birds than in wild passerine species. This PhD aims to investigate the role of different neuropeptides as well as gut peptides in controlling and regulating the ingestive behaviours of songbirds. My aim was to pinpoint candidate peptide genes that may differentiate a hoarding from a non-hoarding bird species and I used non-hoarding great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) to make comparisons with a closely-related hoarding species, the coal tit (*Periparus ater*) In this context, I used molecular techniques combined with video analysis to quantify selected peptide gene mRNAs suspected from the literature to play a major role in controlling both food intake and hoarding behaviour. By identifying candidate peptide genes that respond to an individual's nutritional state, I was able to make some distinctions between hoarding and non-hoarding species. I also established for the first time in passerines the tissue distribution of gene expression in the gut for cholecystokinin (CCK), proglucagon (GCG), insulin and peptide YY. Overall, this study suggests that proglucagon (GCG) both in the gut and the hindbrain, as well as hypothalamic agouti-related protein (AGRP) and pro-opiomelanocortin (POMC) gene expression could be used as neural signals reporting the nutritional state of titmice. Moreover, hypothalamic AGRP and POMC, and hindbrain GCG and POMC seem to be involved in the regulation of food hoarding in coal tits. These observations support observations from the hamster literature that peptides that are known to control and regulate food intake are also involved in food hoarding.

Table of Contents

Glossary of terms

Number	Abbreviation	Definition
1	ACTH	Adrenocorticotropic hormone
2	AgRP	Agouti-related protein
3	AMPK	AMP-activated protein kinase
4	Ant	Antrum
5	AP	Area postrema
6	ARC	Arcuate nucleus
7	BBB	Blood brain barrier
8	BT	Blue tit
9	Cae	Caecum
10	CaMKKβ	Calmodulin dependent protein kinase β
11	CART	Cocaine- and amphetamine regulated transcript
12	CCK	Cholecystokinin
13	CNS	Central nervous system
14	CRH	Corticotropin releasing hormone
15	CT	Coal tit
16	CVO	Circumventricular organ
17	Dist.IL	Distal Ileum
18	Dist.Jej	Distal Jejunum
19	DMN	Dorsomedial nucleus
20	DMNX	Dorsal motor nucleus of the vagus

21	Duo	Duodenum
22	GCG	Pre-pro-glucagon
23	GHRL	Ghrelin
24	GHS-R	Growth hormone receptor
25	Gizz	Gizzard
26	GLP-1	Glucagon like peptide 1
27	GLP-2	Glucagon like peptide 2
28	GRs	Glucocorticoid receptors type II
29	GT	Great tit
30	HPA	Hypothalamo-pituitary-adrenal
31	ICV	Intracerebroventricular
32	IL	Ileum
33	INS	Insulin
34	IP	Intraperitoneal
35	IR	Insulin receptor
36	JAK	Janus kinase
37	LBR	Lamin B receptor
38	LEP	Leptin
39	LEPR	Leptin receptor
40	LHA	Lateral hypothalamic area
41	LRN	Lateral reticular nucleus
42	MCR	Melanocortin receptors
43	MRs	Glucocorticoid receptors type I (Also called: Mineralocorticoid)

44	NPY	Neuropeptide Y
45	NTS	Nucleus of the solitary tract
46	Ob	Obese gene
47	Pan	Pancreas
48	PFA	Perifornical area
49	PKA	Protein kinase A
50	POMC	Pro-opiomelanocortin
51	PP	Pancreatic polypeptide
52	Prov	Proventriculus
53	Prox.Jej	Proximal Jejunum
54	PVN	Paraventricular nucleus
55	PYY	Peptide YY
56	Rect	Rectum
57	STAT3	Signal transduction and activator of transcription 3
58	TRH	Thyrotropin-releasing hormone
59	VMN	Ventromedial nucleus
60	YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monoooxygenase activation protein zeta
61	α, β, γ-MSH	Melanocortin stimulating hormone
62	B-EP	Beta endorphin

Chapter 1. General introduction

In nature, the severity, duration, and the amplitude of extreme weather as well as the fluctuation and the unpredictability of food sources is a consistent threat to the fitness and survival of animals. Those fluctuations provide selection pressure that favours the ability to either store energy internally as fat or externally as food hoards (Keen-Rhinehart et al. 2013). Those adaptations act as a buffer against variability in food supplies thus allowing individuals to maximise their reproductive success (Schneider 2006). So, animals combat food shortage by using a wide array of survival strategies to insure increasing their survivability rate (Munn et al. 2010). Typically, animals that are confronted with conditions that challenge their limitations or tolerance levels, will counter them with evolved physiological responses (Brattstrom 1968). One adaptation is daily torpor and hibernation. Both behaviours involve a controlled reduction in metabolic rate and body temperature that help the individual to decrease its daily energetic requirement (Munn et al. 2010). Hibernation is associated with large fat deposits and typically lasts for more than 24h, with deep depression of metabolic rates. In contrast, daily torpor bouts are very short (shorter than 24h) are less effective at reducing energy expenditure and are associated with lower fat deposition (Geiser and Ruf 1995). Another strategy to face food unpredictability is migration where individuals move seasonally from an unfavourable environment to a more favourable place where the weather conditions are more suitable and food sources are more abundant. By doing so, individuals expand their home range size by dispersing elsewhere and may become non-territorial floaters (Cooper et al. 2015). Another adaptation/strategy is food hoarding, a focus of this thesis. In principle, animals are faced with two decisions when encountering a food source, either eat it or store it. The process of decision making depends largely on the current energetic status of the animal and its life history. Non- hoarding animals (the vast majority of species) do not store food and only ever eat the items they encounter.

Considerable efforts have been devoted to investigating the internal as well as external forces driving an individual to partake in a certain behaviour during its life. In this case, the appetite regulatory system has been the focus of research for more than half a century (Woods 2013). The approach taken in this thesis is that by understanding the signals that drives the animal to feed, similar principles could be applied for food hoarding assuming that both behaviours are controlled and regulated by the same factors.

1.1 Food intake

Food intake a highly complex process that involves both internal and external factors that interact to produce signals sent to the brain. Most of our knowledge regarding how appetite system is regulated comes from the mammalian literature. The current view of how food intake is regulated is that the brain is a higher control centre that both sends and receives a dual innervating signal coming mainly from the autonomic system by the parasympathetic (cholinergic) nervous system that includes the vagal and pelvic nerves and the sympathetic (noradrenergic) nervous system represented by the splanchnic nerves. The cholinergic

innervation includes pre-ganglionic nerves that function to control the motor activity of the gut thus regulating the secretory activities of the visceral circulation (Konturek et al. 2004). On the other hand, the noradrenergic nerves terminate on the postganglionic cholinergic neurones to inhibit acetylcholine from binding to its receptors thus effecting the intestinal smooth muscles cells which in turn effect the motility of the gut or via α_2-presynaptic receptors (Langley 1994).

The brain as an organ is divided into different areas each specialized for a specific function. The hypothalamus is one of the most intensely studied areas within the brain when looking at appetite regulation. Its importance was historically established when Mohr and colleagues first coined the term "hypothalamic obesity" to describe the condition of a 57-year-old woman who got extremely obese one year before her death. Autopsy showed a hypophysial tumour that was large enough to distort and compress the base of the brain including the hypothalamus (Brobeck et al. 1943). This led to a research focus on the role of the hypothalamus as the centre of appetite regulation mostly involving stimulation and lesion studies. Hetherington and Ransome (1942) induced electrolytic lesions in several different central hypothalamic regions in rats. They observed that symmetrical lesions that bilaterally destroyed most of the ventromedial hypothalamic nuclei (VMN) led to overeating and obesity. Thus, it was suggested that the VMN acts as a satiety centre, which is consistent with more recent knowledge of inhibitory circuits within the hypothalamus.

The hypothalamus contains a discrete number of neural populations that are especially connected with neural circuits that are linked to appetite control (Kalra et al. 1999) including the arcuate nucleus (ARC), paraventricular nucleus (PVN), ventromedial nucleus (VMN) and dorsomedial nucleus (DMN) of the hypothalamic nuclei, the ARC, which is situated adjacent to the floor of the third ventricle and above the pituitary gland, is known to be one of the crucial neural populations for the control of appetite. Its location is very important because, in this position, the ARC receives a rich supply of blood and is situated where neurons can directly receive signals from the blood borne nutrients and metabolic hormones (Boswell 2010). Within the ARC, there are two major subpopulations of cells that have been found to contribute to integrating signals that both reports nutritional status and influence energy homeostasis in laboratory rodents (Cone et al. 2001). It is becoming more and more evident that some of those hormonal signals are directly correlated with the level of body fat stores, reporting directly to the central nervous system (CNS) via negative feedback loops to control energy balance. This means that body adiposity is regulated by circulating factors released in response to adipose tissue mass that act in specific areas within the brain to maintain energy homeostasis (Morton and Schwartz 2001). Within the ARC, there are two pathways involving two populations of neurons that were found to interact with internal and external signals to produce a net effect on appetite and energy homeostasis (Morton and Schwartz 2001). The first pathway is termed "anabolic" where neurons in this pathway stimulate food intake and reduce energy expenditure. This consists of neuropeptide Y (NPY) and agouti-related peptide (AgRP), co-expressed within the same neurons. The second pathway is the opposite to the first one and is called "catabolic" and as the name implies this pathway promote negative energy balance by inhibiting food intake and increasing energy expenditure. The neurons

involved co-express cocaine- and amphetamine- regulated transcript (CART) and pro-opiomelanocortin (POMC) mRNAs. Those two pathways are very responsive to adiposity signals: leptin and insulin. Under fasting conditions when circulating leptin and insulin are reduced, the anabolic pathway is activated leading to increased food intake while shutting down the catabolic route. In contrast, the catabolic pathway is activated and the anabolic pathway is inhibited when insulin and leptin are high (Morton and Schwartz 2001). Therefore, activation of the NPY/AgRP neurons allows depleted fat stores to be replenished, whereas activation of CART/POMC neurons initiates satiety. The α-melanocyte stimulating hormone (α-MSH) peptide encoded by the POMC gene and AgRP peptide both compete to access the melanocortin receptors (MCR) that are expressed in different hypothalamic areas to exert their effect. The two pathways will be explained in the following section.

1.1.1 Anabolic pathways

As mentioned previously, this pathway includes the NPY/AgRP population and functions to stimulate feeding. Although both NPY and AgRP mRNA are co-localized within the ARC and respond similarly to fat depot fluctuation due to insulin and leptin secretion, the magnitude and time course of feeding response differs between them. For instance, NPY is one of the most abundant and potent orexigenic neuropeptides that is synthesised in different brain areas but mainly in the ARC (Lopaschuk et al.2010). It exerts its effect on energy expenditure via the NPY Y1 and Y5 receptors. Intracerebroventricular (ICV) administration of NPY stimulates feeding in rodents (Clark 1984). In addition, chronic administration of NPY into the PVN leads to hyperphagia and sustained weight gain (Neary et al. 2004) while injecting selective receptor antagonists (Y1 and Y5) will inhibit feeding (Wójcik-Gładysz and Szlis 2016). The levels of NPY rapidly increase before meal time in the PVN suggesting that it might play a crucial role in initiating eating (Neary et al.2004).

As for AgRP, it is exclusively expressed in the ARC, and its mechanism of action is mainly by its antagonism to the MCR receptors (MC3 and MC4) leading to the blockade of the inhibition of the anorexigenic effect of α-MSH thus causing increased food intake. Central administration of AgRP stimulates food intake and that effect can last up to one week (Rossi et al. 1998). So, while the NPY signalling is considered a more potent short-term acting neuropeptide, AgRP has a longer lasting orexigenic effect that is not similar to any other orexigenic peptides (Morton and Schwartz 2001). It is the NPY/AgRP neuron dynamics functioning by both increasing food intake and body weight via NPY signalling system and inhibition of the melanocortin signalling in the hypothalamus that the stimulating part of appetite occurs.

1.1.2 Catabolic pathway

The pathway consists of neuron populations that function to inhibit feeding. It includes the CART/POMC neurons that are also co-expressed in the ARC. Similar to the anabolic neurons, those populations respond to adiposity signals but in an opposite fashion. This route is activated upon feeding and aid in initiating satiety (Elmquist et al. 1998). When individual eats, glucose levels increase, thus more insulin is secreted to counter

that spike in glucose, removing it from the circulation and storing it as glycogen and fat, increasing leptin at the same time. It is the increase of both leptin and insulin that activates the CART/POMC pathway thus reducing feeding and increasing energy expenditure. POMC is a common precursor to several melanocortin peptides. The POMC peptide most implicated in feeding is αMSH which binds to the melanocortin receptors MC3and MC4 which are expressed in the PVN (Mercer et al.2013). ICV and central injection of αMSH inhibits feeding and reduces body mass (Neary et al. 2004). When first sequenced, CART function was unknown. However along with POMC, it is co-expressed in the ARC. Its expression is responsive to the organism's nutritional state, as it increased with feeding and reduced with fasting. ICV infusion of CART results in appetite reduction (Lambert et al. 1997). Elias et al. (1998) demonstrated that circulating leptin targets the CART neurons as those neurons express c-fos like immunoreactivity as a response to ICV injection of leptin.

1.2 Anabolic and catabolic pathway interaction effects on food intake

Both NPY/AgRP and CART/POMC neural populations exert opposing influences on food intake, metabolic rate and body mass by sending their projections to other sets of neurons located in the PVN where other anorexigenic substances such as thyrotropin-releasing hormone (TRH) and corticotropin-releasing hormone (CRH) are produced, but also to the lateral hypothalamic area (LHA) and the perifornical area (PFA) where orexins are produced (Valassi et al, 2007). Those 2 sets of distinct neuron populations along with various satiety and hunger peptides act as sensors responding to internal as well as external stimuli that reflects body fat content and energy expenditure, such as leptin for example (Cowley et al. 2001). Thus, it is apparent that the CNS through the dual action of both sets of neural populations generates motivational states to promote the seeking and the ingestion behaviour that is very important for the individual to restore and replenish its depleted fat stores in times of lower energetic state (Harley and Johnson 2014). In addition, how strong a motivation is to perform a certain behaviour (in this case either eat or stop eating) also depends largely on the amount of activity in a specific excitatory or inhibitory centre (e.g., ARC) in the hypothalamus.

So how do the different pathways within the hypothalamus integrate the different stimuli to generate responses that control the appetite system? The different nuclei of the hypothalamus receive their signals via two routes: the short-term route (satiety system) that functions to transmit meal-related information to the central system, and the long-term route (adiposity system) that supplies the central system with body fat content status.

1.2.1 The short-term system

The short-term process is initiated when food is swallowed, entering the digestive system. It is the presence of food in the gastrointestinal tract that first stimulates the release of the regulatory peptides that control gut motility but also sends signals to the central feeding systems to terminate feeding by initiating satiety. The gut-brain axis has afferent fibres and vagal nerves that run in the preganglionic vagal and pelvic nerves

representing major routes that regulate the enteric nervous system during digestion. The afferent fibres of the gut-brain axis run through the afferent vagal and spinal nerves transmitting information to the central nervous system about the signals produced from the different sensors of the gut (mechanical (contraction) chemical (nutrient) and neuro-hormonal (gut peptides)) (Holzer 1992). The excitation of the gastrointestinal receptors can initiate short distance signalling to cause vascular and secretory reflexes or can act over a longer route reaching either prevertebral ganglia or the CNS through vagal afferents via the pelvic nerve to enter the brainstem (Wingate and Ewart 1980). The digestive phase includes cephalic, gastric and intestinal phases. All those phases send different stimuli to the brain to regulate food intake. Most of the afferent vagal fibres that innervate the gastrointestinal tracts project to the nucleus of the solitary tract (NTS) in the brainstem where there are a vast number of neural populations that are responsive to meal related stimuli including noradrenergic neurons that project to the PVN in the hypothalamus.

The rostral NTS relays gustatory signals to the forebrain for food flavour perception, while the caudal part integrates homeostatic signals and regulate hunger and satiety. The short-term satiety signals (peptide YY, cholecystokinin and glucagon-like peptide 1) are conveyed to the NTS via the vagal afferent fibres that share a monosynaptic connection with the NTS and function to excite the NTS by glutamatergic transmission. Those satiety peptide receptors have not only been identified on the vagal
nerve fibres (Williams et al. 2016) but also in the CNS (Richards et al. 2014). In addition to using the vagal afferent ascending fibres, those signals are also able to regulate directly the activity of the NTS neurons in the hindbrain (Rui 2013). Those NTS neurons are exposed to circulating nutrients and satiety hormones due to the absence of the BBB. NTS neurons are considered a heterogenous population as they include complete set of neurons that express a variety of peptides such as glucagon -like peptide 1 (GLP-1), catecholamine and POMC.

As mentioned earlier, the digestive phase is divided into three phases, each generates signals that contribute to the overall appetite regulation system. First, the cephalic phase which refers to a set of food intake-associated autonomic and endocrine responses that lead to the stimulation of the oro-pharangeal cavity (Zafra et al. 2006). Most of the cephalic responses are mediated via the vagus nerve, although some brain centres are involved. For instance, Konturek (2000) showed that leptin secretion increased steadily in response to stimulating the oropharyngeal cavity. Since leptin is synthesized in the adipose tissues and in the gastric mucosa, and its release is controlled via vagus nerve stimulation (Sobhani et al. 2002), Konturek (2000) concluded that stomach leptin might contribute to the satiety process. In fact, Buyse et al. (2001) was able to detect both the long Ob-Rb and short Ob-Ra leptin receptor isoforms the rat nodose ganglion which contains the cell bodies of the vagal efferent neurons. Additionally, the continuous stimulation of the oropharyngeal cavity leads to the endocrine pancreatic secretion of hormones (Zafra et al. 2006), and according to Pavlov (1910) those pancreatic secretions are results of direct vagal stimulation of the secretory cells. The cephalic phase stimulates the production of pancreatic peptides in steps. When food is seen, smelled or tasted, glucagon is produced first (Secchi et al. 1995). Ten minutes after the meal starts, an increase in pancreatic polypeptide (PP) is observed stimulating the cephalic receptors (Taylor et al. 1978).

Insulin is secreted last and was proposed to have an anticipatory function during the cephalic phase (Proietto et al. 1987). The production of those hormones is mediated via vagus nerve and contribute to the cephalic phase (Zafra et al. 2006).

According to Breit et al. (2018), the vagus nerve has both a motor and sensory function in controlling and modulating the gut-brain axis. The vagal afferents that are distributed throughout the digestive system originate from the dorsal motor nucleus of the vagus (DMNX) (Powely 2000). Those vagal afferents are activated via the upper brain centres (in turn activated by the oropharyngeal activities, smell, sight or taste of the food) (Giduck et al. 1987). Kalia and Sullivan (1982) showed that the DMNX neurons receive visceral information either directly by vagal afferents or indirectly via the NTS neurons. According to Powely et al. (1992) the DMNX are located parallel to the NTS and share profuse dendritic branches establishing many synaptic contacts.

Both the gastric and intestinal phase, unlike the cephalic phase, occur as a result of the food and its components coming into direct contact with the stomach and the intestine respectively (Schneeman 2002). It is the interactions between the nutrients and the different parts of the gastrointestinal tract that cause signals to be generated, leading to peptides being secreted to cause appetite suppression.

There are multiple types of gut peptides that are released in response to the presence of ingested food particles. Those peptides are mostly known to be secreted from certain endocrine cells that are found lining the GI tract. Over the years many studies have proven that those peptides are not only localised in the gut but are also found in the central nervous system and are hence known as gut-brain peptides (Emson et al, 1980). Those signals are crucial to carry metabolic information between the brain and gut forming the typical foundation of the gut-brain axis. So how does the brain receive and integrate signals from the gut peptides thus initiating or ending a meal?

1.2.1.1 Cholecystokinin (CCK)

Cholecystokinin is one of the most well-known satiety signals, primarily secreted in several forms after post-translational processing of different peptide sizes such as: CCK-33, CCK8, CCK39, CCK58, CCK12 and CCK4 from the I-cell of the duodenal and jejunal mucosa (Polak et al. 1975) but also widely distributed in different parts of the brain such as hypothalamus, hippocampus, midbrain and the cerebral cortex (Emson et al. 1982). It has two receptors: one that is localised in the GI tract (pyloric sphincter, vagus nerve and pancreas) (CCKAR, also known asCCK-1R) (Innis and Snyder 1980) and the other (CCKBR also known as CCK-2R) in the brain especially in the NTS, posterior nucleus accumbens and the hypothalamic dorsomedial nucleus where it was found to mediate the satiety effects of CCK (Herranz 2003, Ritter 1999). In other words, the peripheral and central CCK receptors are different. Homogenate binding studies demonstrate that brain CCK receptors are not able to distinguish between CCK or gastrin (the second member of gastrin/cholecystokinin family) or other CCK analogues the same way that peripheral pancreatic and gastric CCK receptor can. Gibbs et al. (1973) showed that IP administration of CCK in rats reduced their meal size. This suppressing action of the CCK could be partially explained by the fact that CCK acts as a gate keeper in

a sense that when present it stimulates the synthesis of the anorexigenic peptides α-MSH and CART. Additionally, when centrally administered, it also causes the animal to reduce its meal size (Zhang et al, 1986). Several reports showed that food intake was increased significantly after the injection of selective CCK receptor antagonist in several mammalian species (reviewed in Boswell and Li 1998). For instance, Ebenezer et al. (1990) demonstrated that the injection of MK-329 (which is a CCKAR antagonist) increased food intake in pigs trained to make operant response for food reinforcement. In general, peripherally circulating CCK is very metabolically active (i.e., once produced it is degraded very quickly by catalytic enzymes to regulate the CCK-8 levels in the brain)) (Deschodt-Lanckman et al. 1981) and do not cross the BBB, suggesting that peripheral and central CCK represents different functional pools (Moran et al. 1986). CCK interacts with CCKAR receptors on the vagal sensory fibres that project to the NTS (Crop et al. 1993). Within the NTS, POMC neurons are activated via the presence of CCKBR receptors on the vagus nerves, thus aiding in suppressing feeding and terminating a meal (Fan et al. 1997). In the ARC, POMC projects to leptin dependent areas such as the DMH and the PVN where the action of leptin is to stimulate the neuronal activity of POMC thus also causing satiety (Millington 2007).

1.2.1.2 Peptide YY (PYY)

PYY belongs to the pancreatic peptide (PP) family that also includes pancreatic polypeptide Y (PPY) and neuropeptide Y (NPY) all of which mediate their effect through NPY receptors subtypes (Y1, Y2, Y4 and Y5). There have been conflicting reports regarding the distribution of PYY, but in *situ hybridization* studies confirmed the presence of PYY mRNA mainly in the gastrointestinal tract, pancreas and the hindbrain (Broome et al, 1985). It exists in two endogenous forms: PYY_{1-36} and PYY_{3-36} both of which are mainly secreted from the endocrine L-cells of the gut. The first form binds and activates the Y1, Y2 and Y5 receptors promoting feeding, whereas the latter is more specific and only binds to Y2 and inhibits feeding (Nonaka et al.2003). Studies have shown that upon feeding, the PYY_{3-36} isoform increases within 15min and causes the reduction of both dark-phase feeding and re-feeding after fasting in rodents (Batterham and Bloom 2003). Since PYY_{3-36} exerts its effect via binding to the Y2 receptor, distribution studies of the Y2 were performed to map its location. Gustafson and colleagues in 1997 found that Y2 mRNA is localised in the ARC, DMN and the preoptic nucleus. In addition, it is also found in different brainstem regions such as the NTS and the lateral reticular nucleus which provides both ascending innervation to the hypothalamus and descending innervation to the spinal cord (Batterham and Bloom 2003). The BBB with its high selectivity features serves as a major regulator of communication between CNS and the peripheral system. PYY is able to cross this barrier bi-directionally by means of non-saturable processes (Banks and Kastin 1985). The distribution pattern of theY2 receptor combined with its high affinity to PYY_{3-36} and the knowledge of PYY function all fit to the story of how this peptide regulates food intake. Thus, upon feeding, $PYY_{3-36\ is}$ released into circulation modulating the activity of NPY and POMC in the ARC of the hypothalamus by binding to the Y_2 receptor. This binding results in a decrease in NPY expression while increasing the expression of

POMC, which is consistent with the Y2 receptor's role as an inhibitory auto receptor on NPY neurons, thus decreasing feeding (Batterham and Bloom 2003).

1.2.1.3 Glucagon (GCG)

Glucagon is a member of the glucagon superfamily that includes a variety of metabolic hormones that are not only found in the gut but also in the central nervous system (Bell 1986). Members of the glucagon superfamily function as both hormones and as neurotransmitters. The diversification of the different peptides occurs at the level of the alternative post-transitional processing of proglucagon (Drucker et al. 1986). The peptide is cleaved via enzymatic actions to multiple different products (glucagon, glucagon like peptide-1 (GLP-1), and GLP-2) that are specifically expressed in different cell types such as pancreatic islets, distal ileum, large intestine and certain brain areas such as the NTS and other hindbrain nuclei of (Jin 2008). This alternative processing reflects a dichotomy between the hormones that are essential for glucose metabolism in fed vs. fasted states. Glucagon functions to mobilize glucose from the peripheral tissues to maintain glucose levels in fasting conditions. GLP-1 (which was found to increase in immunoreactivity from the proximal to distal parts of the gut of mammals (Goke et al. 1992) is secreted during feeding to increase glucose-dependent insulin release to promote satiety (Kieffer and Habener 1999). GLP-1 is also present in the NTS of the brainstem where it exerts an ileal break function effect via sensory neurons relying in the hypothalamus to inhibit gastric emptying and contributing to satiety (Holst 2007).

Glucagon exerts its function by binding to its receptors which are expressed in brain, small and large intestine, pancreas, liver and adipose tissue (Jelinek et al. 1993). Glucagon's prominent physiological role is gluconeogenesis (glucose production), thereby maintaining a state of euglycemia during rapid glucose utilization to meet energy demands during fasting in laboratory conditions or periods of food scarcity in wild populations (Woods et al.2006) . So, how does glucagon cause satiety when it increases during food deprivation/ fasting conditions? During a fasting condition, the combination of reduced insulin levels, and a declining glucagon/ insulin molar ratio maximises hepatic glucose production which rapidly increases glycogenolysis (Unger and Orci 1976). When glycogen stores are depleted, the increase in ketone production provides an alternative fuel for the central nervous system. Thus, it seems that the anorexigenic effect of glucagon seems to be mediated via the liver-brain axis. Geary (1998) showed that different systemic routes of glucagon administration such as intramuscular and intraperitoneal lead to meal size reduction in rats. However, hepatic-portal infusions were shown to be the most effective method to elicit a very rapid, dose-dependent decrease in food intake (Geary et al, 1993). The most likely pathway taken by glucagon is that it acts on the sensory afferents in the liver and the gastrointestinal tract and then the satiety signal is relayed to the brain via the vagus nerve (Geary 1993). The hepatic branch of the vagus nerve conveys satiety signals to the area postrema (PA) and the NTS, and from there to the hypothalamus (Al-Massadi et al. 2019). Mechanistic studies have deciphered the neural pathways that regulate the anorexigenic actions of glucagon. According to Quinones et al. (2015) the process occurs in the ARC where glucagon receptor is expressed. When glucagon binds to its receptor in the ARC, protein kinase A (PKA) is activated and calmodulin

dependent protein kinase-β (CaMKKβ) is decreased affecting activated protein kinase (AMPK) (a metabolic sensor that responds to nutrients). AMPK has been implemented in food intake and energy homeostasis (Large et al. 2008). The activation of PKA inhibits the action of AMPK causing satiety (Large et al. 2008). Parker and colleagues (2013) demonstrated that peripherally administered glucagon induced c-Fos immunoreactivity at the NTS which as mentioned previously receives its signals directly from the vagus nerves originating from the liver and the gastrointestinal tract (Leslie et al, 1982). It is also worth mentioning that low levels of glucagon receptors mRNA are found in the hypothalamus and brainstem of rodents indicating direct glucagon action via those area to suppress food intake (Hoosein and Gurd 1984).

1.3 The long-term system

In the previous section, the molecular signals comprising the short-term satiety system were considered and it was established that although most of them (CCK and PYY) increase with a meal and their administration reduces meal size, some increase their expression with fasting thus providing an emergency mechanism for fast energy release (via glucose) such as GCG. Those peptides provide very short-lived effects in terms of hunger and satiety. On the other hand, since the regulation of energy homeostasis is crucial to an animal's survival, particularly considering the huge day-to-day variation in food resources and energy expenditure (Murphy and Bloom 2004) it is very important to have a longer-term signal strategy to report energy and fat stores.

In contrast to satiety signals that are secreted during meals and are short-lived, adiposity signals are tonically active, long-lived and provide continuous input to the brain in proportion to the body fat store content (Woods et al. 2006). The most reliable signals used to report continuously about body fat content are several peptide hormones released into circulation from peripheral organs and transported to the brain. These have been termed adiposity signals (Woods and Seeley 2000). In general, for a signal to be considered as an adiposity signal it should be secreted in proportion to body fat, have access to appropriate areas within the nervous system and lastly affect both food intake and body weight in a predictable manner (Woods et al. 1998). There are several peptides that have been heavily investigated: leptin (mostly secreted from adipose tissues), insulin (secreted from B cells of the pancreas) and ghrelin (from the stomach).

1.3.1 Leptin (LEP)

Leptin has been considered as an important energy regulator providing negative feedback to the brain, aiding in the inhibition of body fat accumulation via its actions on food intake and energy expenditure (Gardner et al. 1998). Secreted exclusively from adipose tissues in mammals, leptin servers as a lipostatic signal transmitting critical information regarding the metabolic state of an individual (Bjorbaek and Kahn 2004).

It was originally identified as the protein product of the mouse *obese* (ob) gene that is expressed predominantly in the adipocytes (Zhang et al. 1994), but is also found in different tissues such as: placenta (Masuzaki et al. 1997) and stomach (Bado et al. 1998). A mutation in the *Ob* gene that causes the absence of leptin protein in the circulation leads to hyperphagia in mice which can be reversed by leptin administration (Halaas et al.1995). The leptin receptor (LEPR) has a single transmembrane domain and belongs to the

cytokine receptor family and has multiple isoforms due to mRNA splice variation (Chua et al. 1997). The LEPR is divided into three classes: long (LEPRb), short (LEPRa, LEPRc, LEPRd) and secreted (LEPRe) (Chua et al. 1997). The long form is differed by having a long intracellular domain that binds to both janus kinases (JAK) and to signal transduction and activator of transcription 3 (STAT3) transcription factors, which are required for the action of leptin on appetite (Lee et al. 1996).

The circulating leptin gains access to the brain across the BBB via saturable processes which has a great advantage in being responsive to physiological stimuli (Kastin and Pan 2008). The LEPRb receptor is expressed within the hypothalamus (ARC, LHA and DMH) (Fei et al. 1997), and its mRNA was found to be expressed by NPY/AgRP (Mercer et al.1996) and POMC/CART neurons (Cheung et al. 1997). NPY/AgRP neurons are inhibited by leptin, and are therefore activated in conditions of reduced leptin (producing an orexigenic effect) (Stephens et al. 1995). On the other hand, leptin activates the anorexigenic CART/POMC neurons (Thornton et al. 1997). In normal conditions, leptin levels in the circulation falls when an animal is in a state of negative energy balance (such as fasting) which is coordinated with energy conservation and foraging behaviour. The suppressive effect of leptin on food intake is reduced by the administration of MCR4 antagonists, demonstrating that the melanocortin pathway is a crucial downstream mediator of leptin signalling (Seeley et al. 1997). Similar to the ARC, the NTS contains LEPRb (Mercer et al .1998) and administering leptin to the fourth ventricle reduces food intake and body weight gain (Grill and Kaplan 2002). Peripheral administration of leptin also results in the activation of the NTS, which means that leptin exerts its effect on appetite via both the hypothalamus and the brainstem (Wynne et al. 2005).

1.3.2 Insulin (INS)

Insulin is the predominant controller of glucose levels, and its secretion from beta cells in the pancreas is largely controlled by the surrounding glucose levels in the circulation. Nonetheless, the sensitivity of those beta cells to glucose is proportional also to body fat (Bagdade et al. 1967), so higher insulin levels reflect both the metabolic needs and how much fat is present in the body (Woods and Seeley 2000). Insulin is considered too large to cross the BBB (Woods et al. 2006). Insulin receptors (IR) are expressed in variety of tissues such as liver, muscle, fat and neurons of the CNS (Bru¨ning et al. 2000). IRs in the brain displays a very specific expression pattern, specifically in the olfactory bulbs, hypothalamus and the pituitary (Marks et al. 1990). So how does insulin gain access to the brain where its receptor is? Circulating insulin is able to access the brain via areas with a reduced BBB where there are highly permeable capillaries that serve as an alternative route that sense signals coming through blood and pass the information using their neural circuitry to different brain areas. Those permeable capillaries are termed circumventricular organs (CVO) . Insulin molecules, by using receptor mediated transport processes, will move to cells within a CVO to be released to the brain's interstitial fluid (Schwartz 1992). Generally, insulin receptors are expressed in brain areas that function in energy control such as the ventromedial nucleus of the hypothalamus (VMN) (Crop et al.1986), but also in the ARC which was found to have the highest density of insulin receptors (Marks et al. 1990). The ARC, as mentioned previously, functions in regulating food intake via the two neural-

subpopulations (NPY/AgRP- POMC/CART), and both populations were demonstrated to express IR (Morton and Schwartz 2001, Hill et al. 2010). So, we can conclude that the effect of the insulin on food intake is via central mechanisms that are linked to acting via those populations especially by inhibiting the NPY/AgRP subpopulation in the ARC (Karla et al. 1999). After consuming a meal, insulin levels are elevated in the body and function to clear the blood of the absorbed fuels by stimulating their storage (Strubbe and Steffens 1993). When exogenous insulin was administered directly into the ARC, rats ate less and lost weight and that response is dose-dependent (Van Dijk et al. 1997). Similarly, when insulin was administered via intracerebroventricular injection in rodents, meal size was reduced, and the animals lost body mass (Air et al. 2002).

1.3.3 Ghrelin

First discovered as an endogenous ligand of the growth hormone secretagogue receptor (GHS-R), ghrelin was purified from rat stomach (Kojima et al. 1999). Studies have recognised ghrelin as the only orexigenic gut peptide in mammals (Nakazato et al. 2001). Cummings (2006) demonstrated that the effect of ghrelin on food intake is very rapid, as food intake was observed to elevate as fast as 5-10 min after its systemic administration. Thus, it seems that ghrelin brain accessibility is relevant for the unique function of that hormone. Ghrelin cell bodies are found in the central nervous system filling the spaces of the paraventicular areas sending their projections to the lateral hypothalamus (Carlini et al. 2004). Carlini et al. (2002) showed that icv injection of ghrelin to the central nervous system induces an anxiogenic effect in rats. The action of ghrelin is facilitated by the fact that ghrelin-producing neurons are located in the hypothalamus integrating the orexigenic properties of ghrelin. Ghrelin has the ability to bind to its receptor (GHS-R1) which is expressed the ARC, PVN and VMH of the hypothalamus and the peptide is able to penetrate the blood brain barrier (Pan et al. 2006). The signalling pathways to the hypothalamus is via three routes: 1. Penetration to the ARC by blood stream and active transport through the blood brain barrier which is largely dependent on how saturable ghrelin is and that saturability is species dependent. The clearance of bioactive ghrelin via the BBB is very important in the regulatory mechanism of energy homeostasis (Banks et al. 2002). 2. Activation of the GHS receptors on the vagal afferents, signalling to the NTS that in turn communicate to the hypothalamus. This happens via the suppressing action of ghrelin to the vagal afferent discharge. One of those vagal afferents transmits CCK satiety signals between gut and the brain. Suppressing those fibres gives ghrelin its orexigenic property (Date et al. 2002) 3. Small amounts of ghrelin could be produced in the hypothalamus itself via ghrelin containing axons that have terminal endings that make synapses with neurons at the ARC (Cawley et al. 2003) and are able to activate NPY/AgRP neurons and neurons in the LHA (Huda et al. 2006). Plasma ghrelin levels are elevated during fasting and are reduced after a meal, consistent with its action as an orexigenic hormone (Toshinai et al 2001). Both intracerebroventricular and peripheral administration of ghrelin in rodents increase food intake, adiposity and weight gain (Nakazato et al. 2001). The mechanism behind that orexigenic action is that NPY/AgRP neurons are localised in the ARC (which is

the main target for hypothalamic ghrelin). In the ARC, ghrelin stimulates both NPY and AgRP transcription promoting their release in the PVN (Schneeberger et al. 2014).

1.4 The stress system

Energy management in animals is attributed mostly to internal factors such as hormonal signals. As mentioned previously, living in a fluctuating challenging environment imposes difficulties for an individual's daily life. Having to survive adverse environmental conditions can cause stress to an animal. Stress is a challenge to the natural homeostasis of an organism, which means, in turn, those individuals need to react to stress by producing a physiological response to regain equilibrium lost by the impact of the stressor (Yvonne and Potenza 2013). After being subjected to an acute stress, the hypothalamo-pituitary-adrenal axis (HPA) is rapidly activated to elevate blood glucose concentration to ensure an adequate substrate for both the brain and muscles for life-saving situations (Dallman et al. 2004). One of the main products of the HPA activation are glucocorticoid hormones (Dallman et al. 2004). There is a close relationship between glucocorticoids secretion and feeding and energy storage (Dallman et al. 2004). Long-term chronic stress that is associated with elevated levels of glucocorticoids has a negative effect on individuals and is associated with neural cell death and reduced neurogenesis (Sapolsky 1996). Individuals living in temperate zones which are continuously subjected to seasonal environmental fluctuations respond by rapidly elevating the glucocorticoids levels in response to stressors (Wingfield et al. 1997). By suppressing unnecessary physiological function and enhancing critical processes, the elevated glucocorticoids serve to increase the animal's probability of survival (Wingfield et al. 1997). According to Asheimer et al. (1992) the increasing levels of corticosterone that come with being exposed to short term seasonal fluctuations such as snowstorms or sudden drop in temperatures or even food deprivation, elicit changes in behaviour that are considered emergency life history stages. The emergency life history stages are defined as: responses to sudden events that disrupts the normal life cycle of an individual causing them to re-direct their behaviour and physiology to survive (Pravosudov et al. 2001). Glucocorticoids have been demonstrated to have a major effect on food intake. Studies have shown that rats subjected to different doses of glucocorticoids showed a decrease in both food intake and body weight, and it was suggested that the appetite suppression action of glucocorticoids occurs via inhibiting the activation of appetite stimulating neurons (NPY/AgRP) (Liu et al. 2011). The activation of glucocorticoids occurs via binding to type I (mineralocorticoid receptors (MRs)) and type II (glucocorticoid receptors (GRs)). While type I is tonically activated by low basal levels of glucocorticoids, the type II receptors require higher glucocorticoids levels that are usually linked to the action of stresors (Temple and Leibowitz 1994). Both receptors show a very distinct brain distribution. Aronsson et al. (1988) using immunocytochemistry, demonstrated that GR expression is abundant in the ARC, a receptor distribution patten that overlaps with the distribution of AgRP and POMC neurons. Moreover, studies have shown that both POMC and NPY neurons express GR (Cintra et al. 1991) which suggests that their neuropeptides neuropeptide expression is modulated via the action of glucocorticoids. Direct evidence for this was provided by Lu et al. (2002) where a peak of corticosterone was seen before the start of the active

phase which coincide with the peak in AgRP mRNA expression. There was also a positive correlation between the circulating corticosterone and AgRP mRNA in the ARC. Furthermore, the fluctuation of AgRP expression coincides with the feeding rhythms of rats, elevating before the active phase of feeding that peak 4 h after dark onset (Lu et al. 2002). The authors suggested that one candidate to explain the fluctuation of AgRP mRNA is corticosterone since it exhibits a similar single peak just before the active feeding period.

In rodents, food ingestion was found to elevate glucocorticoid secretion (Sander and Porter 1988). This contributes to the control of whole-body homeostasis and the response of animals to stress conditions by stimulating the release of energy stores by promoting glucose mobilization (Harvey et al., 1986). After being subjected to an acute stressor, an individual experiences a rapid increase in the HPA axis activity which interacts with elevated levels of glucagon and insulin (Dallman et al. 2004). Perley and Kipnis (1966) found that perfused rat's pancreas that has been subjected to a long-term administration of glucocorticoids showed an increased in insulin levels. So, we can safely assume that the appetite suppression effect of glucocorticoids is a result of the elevation of insulin. Having said that, studies also suggested that there is a link between the activation of the HPA axis on one hand and the orexigenic effect of ghrelin that contributes to the fat accumulation effect caused by increased food intake (Tung et al. 2004). According to Orth (1992), in humans an increase in appetite was associated with excess glucocorticoids. Conversely, adrenalectomy (removal of the adrenals) prevents obesity induced by chronic central administration of NPY in normal rats (Sainsbury et al. 1997). This inhibition action occurs because once glucocorticoids enter the brain, they have a permissive action on the transport of NPY in the hypothalamus (Ahima et al. 2008).

1.5 Avian appetite system

Considerable efforts have been made to understand the appetite system in mammals. In contrast, the appetite control system in birds has been less investigated and is not well understood. However, the avian appetite system is arranged similarly to the mammalian one in terms of functionality (Figure 1). Similar to mammals, birds consume food in discrete meals and the meal frequency is much dependent on the overall state of the individual at a specific time point during the day (Martínez del Rio et al. 2001). Based on that fact, much of the research into the regulation of food intake by peptides has been on the assumptions that the mechanisms in birds are similar to those in mammals. Although there are examples of evolutionary conservation of these mechanisms, taxonomic differences in the effects of some of the peptides involved have also been revealed.

1.5.1 Hypothalamic neuropeptides

As mentioned earlier, like mammals, the central nervous system plays a vital role in controlling and regulating feeding behaviour in chickens (Kuenzel 1989). It has been long established that both the ventromedial nucleus and the lateral hypothalamus are considered as important satiety centres in birds as they are in mammals. In addition, the paraventricular nucleus and the arcuate nucleus (historically referred to in birds as the infundibular nucleus) are also involved in regulating feeding (Tachibana and Tsutsui 2016).

The regulatory process of energy homeostasis functions to match energy intake to energy expenditure to aid in maintaining stability in energy stores (usually fat) (Boswell 2005). Thus, in conditions where energy expenditure exceeds energy intake, the body uses fat stores to supply the immediate energy need resulting in a drop in body mass to levels below the homeostatic ranges (Boswell 2005). As in mammals, lesioning studies of the ventromedial nucleus in birds induced obesity, while damaging the lateral hypothalamus prevented eating (Kuenzel 1982). Thus, the ventromedial nucleus was historically termed "the satiety centre", while the lateral hypothalamus was proposed as "the feeding centre", demonstrating the importance of the hypothalamus as a brain region of importance in the regulation of body energy (Boswell 2005).

Just like in mammals, in the hypothalamus there exists a neural network that is vital in the regulation of food intake and energy expenditure (Saper et al. 2002). This neural network which is in the ARC which in birds and mammals has two opposing neural populations: NPY/AGRP and POMC/CART.

In birds, NPY is one of the most widely studied hypothalamic neuropeptides and has been implicated in energy balance regulation. Research in mammals demonstrated that the neuroendocrine response of energy depletion (usually accompanied by food restriction/ fasting) is coordinated by the action of NPY (Schwartz et al. 1995). Thus, food deprivation stimulates NPY expression in the ARC leading to the increase of NPY peptide in the paraventricular nucleus (Karla et al. 1982). Similarly, Boswell et al. (1999) showed that food restriction and deprivation increased hypothalamic NPY mRNA in chickens.

Denbow et al. (1988) demonstrated that icv administration of NPY elevated food intake in chickens. That stimulatory effect of NPY on food intake has been confirmed in other avian species including chickens and white-crowned sparrows (*Zonotrichia leucophrys*) (Richardson et al. 1995) and in ring doves (*Streptopelia risorii*) (Strader and Buntin 2001). Those experiments demonstrated that the magnitude and time course effect of NPY on food intake are similar to those reported in rodents. The similarity between birds and mammals in NPY functionality is also extended to NPY distribution. In *situ hybridization* studies showed that NPY mRNA is localized in the hypothalamus of both chickens and Japanese quail (Boswell et al. 2002). It is worth noting that unlike mammals, the site of action where NPY might exert its effect on food intake in birds is still unknown. However, Zhou et al. (2005) found that the ARC of broiler chickens had the highest NPY content suggesting that the NPY content is most likely related to NPY neuron distribution with more NPY being synthesised in regions containing many NPY neurons. That result indicates that NPY plays different roles in different hypothalamic regions. Holmberg et al. (2002) were able to detect both Y1 and Y5 NPY receptor mRNA in the chicken ARC. And since in mammals the binding of NPY to Y1 and Y5 in the ARC was suggested to play a role in appetite regulation (Durkin et al. 2000), this suggests a general conservation in binding properties and neuro-anatomical localization of avian NPY receptors (Y1 and Y5) with mammals, which most likely suggests that the function of the receptor is also conserved (Holmberg et al. 2002). Furuse et al. (2002) demonstrated that it is in fact the NPY Y5 receptor that mediates feeding behaviour in neonatal chicks.

AGRP mRNA is co-expressed in the same neurons as NPY in birds (Boswell et al. 2002) and has a similar anabolic (stimulatory) action on food intake when activated. AgRP is considered extremely sensitive to the nutritional status of individuals (Boswell and Dunn 2017). This sensitivity arises as counter-regulatory mechanism for situations of negative energy balance (such as food deprivation). Studies in adult Japanese quail showed that when food deprived for 24h, an increase in AGRP mRNA was observed (Philips-Singh et al. 2003). A similar response was also seen in domestic broiler chicks when fasted for 24-48h (Higgins et al. 2010). And according to Fang et al. (2014) the AGRP levels return to base line after 24h of re-feeding.

Unlike NPY's direct action on its receptor, AGRP functionality arises from the fact reviewed above for mammals that it is an endogenous antagonist to the melanocortin system receptors (MC-R) specifically MC3-R and MC4-R acting in a paracrine manner to regulate the MC-R function (Ollmann et al. 1997). Thus, the overall function of the melanocortin signalling system is regulated via the opposing actions of both AGRP and the different POMC products that increase and decrease food intake respectively as also demonstrated in birds (Tachibana et al. 2001).

POMC is strongly expressed in the ARC of both chickens and Japanese quail (Gerets et al. 2000; Phillips-Singh et al 2003) and its distribution is therefore equivalent to that of the mammals (Phillips-Singh et al. 2003). Contrary to NPY/AGRP neurons, the stimulation of POMC neurons initiates a cascade of catabolic effects inhibiting food intake. However, while the action of POMC is clearer in mammals, in birds contradictory results were found in terms of POMC actions (Boswell and Dunn 2017). Some studies were not able to detect any differences in the POMC mRNA levels even after 24h-48h of food deprivation (Japanese quail, Phillips-Singh et al. 2003; and broiler chicken Song et al. 2012), which was in accordance with similar studies in mammals where no differences in POMC expression ere noted (Adam et al. 2002). While others reported significant decrease in POMC expression levels in broiler chicks fasted for either 24-48h (Higgins et al. 2010). This suggests in general a relatively weak contribution of decreased POMC expression to increased food intake observed after fasting. Since POMC encodes many peptides including adrenocorticotropic hormone (ACTH) and melanocyte- stimulating hormones (α-, β- and γ- MSH), it was necessary to pinpoint which product is responsible for regulating food intake in birds. Kawakami et al. (2000) showed that in Japanese quail, it was α-MSH peptide in the infundibular nucleus that inhibits food intake when administered in the brain. The administration of β- endorphin (β-EP) (another POMC product) in chickens and pigeons stimulated food intake which is similar to the effect seen in rats. Dutia et al. (2012) demonstrated that a single i.c.v injection of (β-EP) stimulated food intake in rats for 2-6h. On the other hand, Song et al. (2012) reported no change in POMC mRNA levels in Japanese quails experiencing 24-48h food deprivation. This shows that the effect of the melanocortin system is yet to be fully explored (Boswell 2005).

1.5.2 Hindbrain neuropeptides

While the hypothalamus is responsible for monitoring the periphery for signals alerting central circuits of the depleted energy store, the hindbrain receives oral and gastrointestinal information of the amount and the quality of food that is being ingested (Broberger 2005). Thus, the hypothalamus functions as a long-term meal initiator, while the hindbrain is a short-term meal terminator. Unlike the vast variety of hypothalamic neuropeptides, only a small number of hindbrain peptides have been linked to the avian appetite system.

Glucagon like peptide- 1 (GLP-1) is one of the glucagon superfamily members that have been found to be expressed in the medulla oblongata of neonatal chicks (Tachibana et al. 2005). Studies have shown that food deprivation/fasting was found to decrease GLP-1 mRNA levels which suggests that endogenous GLP-1 are related to food regulation in neonatal chicks (Tachibana et al. 2005). Moreover, i.c.v administration of GLP-1 was found to decrease food intake in both neonatal chicks (Furuse et al. 1997) and Japanese quails (Shousha et al. 2007). According to Tachibana and Tsutsui (2016), the mechanism whereby GLP-1 exhibits its suppressing action on appetite is the activation of neurons in the ventromedial nucleus (VMN) since an i.c.v injection of GLP-1 in young chickens increases Fos expression in this nucleus

1.6 Gut peptides

1.6.1 Cholecystokinin (CCK)

Cholecystokinin is one of the best studied gut peptides in birds. Belonging to the gastrin/cholecystokinin family, it is conserved between vertebrate groups, and is likely to have arisen due to a duplicate event early in the vertebrate lineage (Johnsen 1998). CCK overall has a physiological role in in peripheral signalling to regulate appetite, digestive organ activity such as: gallbladder contraction, gut motility and gastric emptying (Miyasaka and Funnakoshi 2003) as well as in emotion (Ballaz 2017). Similar to mammals, CCK is produced from the small intestine of chickens and ostrich (Jonson et al. 2000) as well as in the brain. For example, Reid et al. (2018) showed that the primary expression location in the chicken for neural CCK is the basal hypothalamus, while peripheral CCK was mainly expressed around the proximal half of the ileum. As mentioned previously, CCK is structurally conserved among vertebrates and this extends to its effect on food intake in birds. For example, Savory and Gentle (1983) demonstrated that iv injection of CCK in chickens increased their latency to feed. Richardson et al. (1993), showed that CCK-8 administered intraperitoneally to white-crown sparrows' dose-dependently reduced food intake and that the satiety effect seen is mostly mediated through the CCK-A receptor since injecting MK-329 (CCK-A receptor antagonist) attenuated the anorexic effect of CCK and significantly increased meal size. In broiler chickens when administered peripherally, CCK was shown to reduce food intake via acting on the vagal nerve (Covasa and Forbes 1994). CCK longevity in the circulation is short-lived (Liddle et al. 1985). The rapid dissociation of CCK in the circulation is due to the delay in its transcriptional processing. Thus, the differences in the mRNA translation activity are mainly dependent on differential post-translational processing rather than different expression

levels (Sayegh et al. 2014). Consistent with this, Reid et al. (2018) was not able to detect any differences in CCK gene expression in the gut between fed and fasted chickens.

1.6.2 Glucagon (GCG)

Unlike mammals, birds (best studied in chickens) have a two proglucagons transcribed from the glucagon gene that express multiple mRNA transcripts by alternate promoter and alternate first axon usage (Honda 2016). Those transcripts are tissue specific. The first class are called PGA: the translation of that class of mRNA yields preproglucagon A which is specific to the pancreas and through the process of cleavage by the action of prohormone convertase 1 (PC1) produces glucagon. The second class are called PGB: that class of mRNA translates to preproglucagon B collection that are specific to the brain and gut and again through the action of prohormone convertase 2 (PC2) are cleaved to generate glicentin, oxyntomodulin, glucagon-like peptide 1 and 2 (GLP-1, GLP-2) (Honda 2016). Thus, the splicing of the two proglucagons yields; proglucagon mRNA transcripts encoding glucagon and GLP-1 in the pancreas, and the second splice results in the three products in the intestine (Irwin and Wong 1995). This means that the alternative splice mechanism is different between mammals and birds in that one yields more product.

Despite the differences underlying the mechanisms involved in the translation of glucagon gene, the amino acid sequence of glucagon is highly conserved between mammals and birds (Honda 2016). The anorexigenic effect of glucagon in mammals was similarly found in chickens. ICV injection of GCG in chicks decreased food intake (Honda et al. 2007). That effect was suggested to be mediated via the hypothalamo-pituitary-adrenal axis (HPA) (Honda et al. 2012) because glucagon administration increased the plasma levels of corticosterone and also hypothalamic corticosterone releasing factor suggesting that corticosterone acts as a downstream molecule for the glucagon suppressing appetite pathway (Honda 2016). Another explanation for the suppressive effect of glucagon is through the induction of hyperglycaemia because intravascular administration of glucose significantly reduced food intake in chicks (Honda et al. 2007), and the administration of phentolamine an α-adrenergic receptor antagonist significantly attenuated glucagon induced hyperglycaemia in both rats (Marubashi et al. 1985) and chicks (Honda et al. 2012). Therefore, it is likely that the hyperglycaemia-mediated pathways are also involved in the anorexigenic action of glucagon in chicks. In birds, the greatest aggregation of GCG receptors was detected in the hypothalamus of chickens but more investigations are needed to localise the targeted areas within the hypothalamus (Wang et al. 2008).

GLP-1 is considered a potent gastrointestinal incretin (i.e., A group of metabolically active factors that stimulate a decrease in blood glucose). Evidence from chickens suggests that it is mainly secreted from the L-cells of the distal parts of the small intestine in particular it is distributed in the whole jejunum and the ileum (Monir 2014). Furuse et al. (1997) showed that ICV administration of chicken GLP-1 supressed food intake in neonatal chicks. The suppressing action of GLP-1 comes from the induced gastric acid and pancreatic juice secretion as well as slowing down of the gastric emptying process, in addition to enhancing insulin release (MacDonald et al. 2002). An immunohistochemical study by Nishimura (2013) demonstrated that GLP-1 has a physiological role as one of the most common peptides secreted by L-cells, and according

to Monir (2014) the occurrence of GLP-1 immunoreactive cells were decreased in food deprived chicken. This is consistent with a catabolic role for GLP-1 in promoting insulin secretion thus stimulating glucose uptake (Carless et al. 2017).

Co-localized with GLP-1, GLP-2 is also produced in the brain and gut. Thus, it could be classified as an anorexigenic neurotransmitter in mammals. In birds, a report by Shousha et al (2007) demonstrated that ICV injection of rat GLP-2 has no effect on food intake in Japanese quail. However, Mo et al. (2014) reported the GLP-2 receptors are expressed in the chicken telencephalon, and various gut tissues such as: proventriculus, jejunum, duodenum and ileum. ICV administration of chicken GLP-2 supresses food intake in chicks, and the anorexigenic mechanism by which it acts appears to be through the decrease of blood glucose (Honda et al. 2015) rather than by affecting plasma corticosterone concentration as in the case of glucagon. Those findings suggested that, overall, GLP-2 is a strong anorexigenic peptide that exerts its effects through the CNS in domestic chicks. However, GLP-2 has also been found to act peripherally on appetite via binding to its receptor at the target tissues. Intravascular administration of chicken GLP-2 significantly reduced food intake in chicks (Honda et al. 2015). In contrast, as mentioned above, peripheral administration of mammalian GLP-2 did not influence meal size in both chickens and Japanese quail (Shousha et al. 2007). However, mammalian GLP-2 shares only 51-55% amino acid identity with chicken GLP-2 (Honda 2016) indicating the importance of using native avian peptides for investigations.

1.6.3 Peptide YY (PYY)

Peptide YY was isolated from the small intestine of chickens in 1992 (Conlon and O'Harte 1992) and when analysing its amino acid sequence, it was found that there is an additional N-terminal alanine residue. The intestinal extracts of the chicken did not contain PYY_{3-36} which is the major form of PYY in mammals. Reid et al. (2017) showed that unlike mammals that showed higher PYY tissue distribution in the large intestine and distal ileum (Miyachi et al. 1986), chicken PYY mRNA is most abundant in the mid- to distal jejunum. An in *vivo* binding assay study demonstrated that chicken PYY binds to Y2 receptors which are expressed in the brain and peripheral tissues (Salaneck 2000). PYY mRNA levels were higher in chickens under *ad libitum* feeding in comparison to a 12h fasting condition (Aoki et al. 2017). In addition, PYY levels were significantly higher in satiated broiler layer hybrid chicken in comparison to individuals subjected to 11h fasting (Reid et. al. 2017). However, Kuenzel et al. (1974) demonstrated that i.c.v administration of PYY dramatically increased food intake in chicks together with an increase in crop size. This observation suggests that PYY has an additional function of reducing the rate of food passage through the gastrointestinal tract.

1.6.4 Leptin (Lep)

For over 15 years, the existence of avian leptin was challenged by some researchers (Boswell and Dunn 2015). Although studies in birds suggested that mammalian leptin induced physiological and behavioural effects, no leptin-like genes were identified in avian genomes until 2014. Once leptin genes were identified, gene expression studies indicated that leptin is expressed over a wider range of tissues in birds than mammals where it is primarily produced in the adipose tissues. Thus, avian leptin was discovered to be

expressed mainly in the hypothalamus and pituitary of zebra finches (Huang et al. 2014), in liver, testis and ovary of rock doves (Friedman-Einat et al. 2014) and in hypothalamus, adrenal glands and embryonic testis and ovaries in chicken (Seroussi et al. 2016) .One shared characteristic between the different avian leptin sequences is the very high G-C content (~70%) which might explain why all these years it was hard to find from genome sequencing as it has very high melting point that requires specialised polymerase chain reaction (PCR) adapted conditions. It is worth mentioning that avian leptin shows only 30% amino acid sequence similarity to its mammalian counterparts (Boswell and Dunn 2015). The leptin receptor long form that mainly functions in appetite regulation is expressed in the chicken hypothalamus (Horve et al. 2000). Lohmus et al. (2003) peripherally administered recombinant chicken leptin (which in fact was of mammalian origin) to great tits and observed that leptin reduced food intake which is the same effect in chicken when injected intracerebroventricularly (Denbow et al. 2000). However, there is truly little evidence till this day that leptin regulates ARC neuropeptides like it does in mammals, because the expression of leptin receptor has not been detected yet in avian ARC (Boswell and Dunn 2017).

1.6.5 Ghrelin (GHRL)

Avian GHRL was first isolated from chicken proventriculus and was found to only share 54% amino acid sequence similarity with rat and human GHRL (Kaiya et al. 2002). Following its initial discovery, GHRL was subsequently identified in goose, quail and duck (Kaiya et al. 2008), Similar to mammals, plasma GHRL was found to increase with fasting in birds. Shousha et al. (2005) demonstrated that Japanese quail fasted for 24h showed a 5-fold increase in plasma ghrelin which then decreased after 3h of re-feeding. Domestic layer hens also showed an increase in plasma GHRL after experiencing 12h fasting which then returned to the baseline after 6h re-feeding (Kaiya et al. 2007). However, while GHRL administration in mammals stimulat food intake, that appears not to be the case in birds. Chen et al. (2008) showed that i.c.v administration of chicken GHRL in different doses inhibited food intake in 8-week-old broiler chickens in a dose-dependent manner and that suppression effect lasted for 2-7h. A similar effect was also seen in Japanese quail injected i.c.v with rat ghrelin whereby food intake was inhibited for 12h (Shousha et al. 2005). Ideas have been proposed to explain the suppressing effect of ghrelin in birds. According to Saito et al. (2002) vocalisation and stepping behaviour was observed in chicks receiving i.c.v injection of GHRL. Those behaviours are signs of hyperactivity in chicks. Furuse et al. (1997) observed similar behaviour in neonatal chicks receiving i.c.v injection of corticotropin releasing factor CRF), and furthermore ic.v administration of CRF suppresses food intake in neonatal chicks (Zhang et al. 2001). It therefore makes sense to hypothesise that GHRL exerts its appetite suppression effects in birds by acting on the CRF system via activating the hypothalamo-pituitary-adrenal axis that results in the release of corticosterone from the adrenal glands (Kaiya et al. 2009). This hypothesis is supported by the fact that i.c.v injection of GHRL in neonatal chickens increases plasma corticosterone (Saito et al. 2005). Another explanation for the anorexigenic action of ghrelin in birds is related to NPY. It is well known that in rats, NPY plays a vital role in mediating the orexigenic function of ghrelin (Mondal et al. 2005). However, although NPY has orexigenic actions in birds too (Ando et al. 2001)

it is unlikely that the inhibiting action of ghrelin is associated with NPY. In neonatal chicks, hypothalamic NPY mRNA did not change after 30 minutes of receiving i.c.v injection of ghrelin (Saito et al. 2005). This suggest that the suppressing action of ghrelin is not mediated via the activation of NPY neurons in birds, rather its more likely that ghrelin activates CRF neurons releasing corticosterone which might be involved in appetite suppression.

1.6.6 Insulin (INS)

Peripheral administration of insulin has been shown to suppress food intake in chickens (Smith et al. 1974). In addition, insulin in blood circulation was observed to decrease with fasting in birds (Christensen et al. 2013). Honda et al. (2007) demonstrated that central administration of insulin decreases food intake in one-day old male chicks. In mammals, insulin central action is mainly governed by the upregulation of mRNA expression of POMC/CART to suppress the food intake (Porte et al. 2002). Similarly, Shiraishi et al (2008) proved that the inhibiting effect of insulin on food intake in day old male chicks was significantly reduced after the ICV administration of selective melanocortin antagonist such as SHU9119 or HS014. This attenuated effect is mainly due to the fact that ICV injection of insulin stimulates the expression of POMC, while suppressing NPY (Shiraishi et al. 2008). Shiraishi et al. (2001) demonstrated that NPY neurons expressed insulin receptors mRNA in domestic chicks and given that generally AgRP is co-expressed with NPY, we might expect those receptors to affect the expression of AgRP as well.

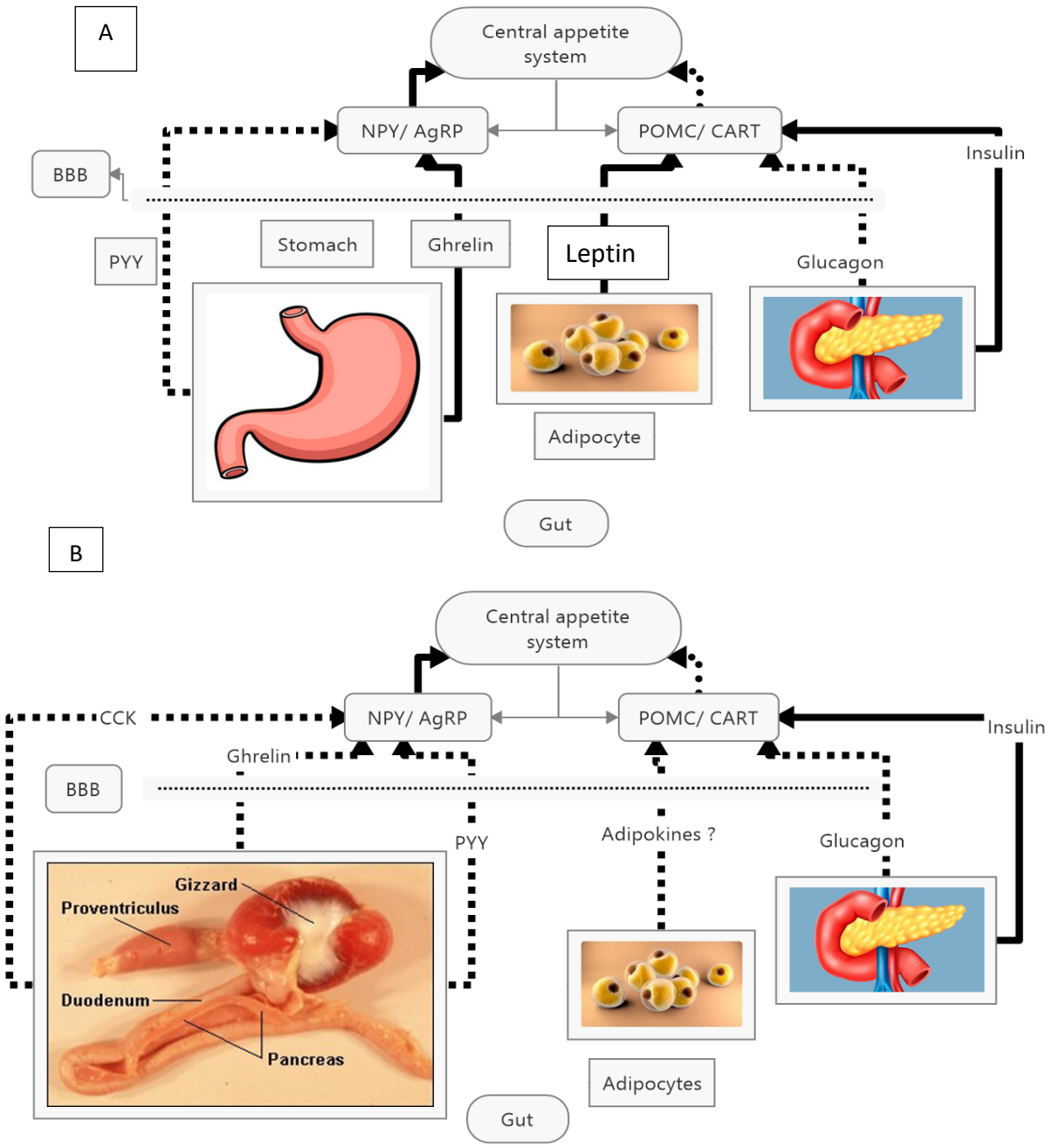


Figure 1: Hypothalamic neuro and gut peptide comparison between A. mammals, B. birds and their interaction and correlation with the appetite system (gut). Black dashed lines illustrate inhibitory effect of peptides, while the black continuous lines represent stimulatory effect of peptide. The abbreviations are as follow: Blood brain barrier (BBB), cholecystokinin (CCK), neuropeptide Y (NPY), agouti related protein (AgRP), Proopiomelanocortin (POMC), Cocaine- and amphetamine-regulated transcript (CART), peptide YY (PYY).

1.7 Avian stress system

In birds, the hypothalamus plays a pivotal role in interpreting environmental factors such as stressors to generate appropriate response in food intake (Richards et al. 2010). Glucocorticoids in general have a profound effect on carbohydrates, lipids and protein metabolism (Bamberger et al. 1996). They are involved in body homeostasis and the response of individual to stress by stimulating the release of energy store by

promoting glucose mobilization and lipolysis (Harvey et al. 1986). Remage-Healey and Romero (2001) reported that in birds both insulin and corticosterone interact to regulate glucose and triglycerides levels during stress situations. Similarly, studies in poultry have shown that corticosterone reduced food intake (Yuan et al. 2008). Additionally, El-Lethey et al. (2001) reported that corticosterone increases food intake in chickens. Liu et al. (2012) demonstrated that in corticosterone exposed laying hens, CCK levels reduced food intake.

Unlike their effect in mammals, El-Lethey et al. (2001) demonstrated that corticosterone increases food intake in chickens. Moreover, plasma corticosterone was found to be elevated after 24h of food deprivation in adult chickens (Scanes et al. 1980). Having said that, it should be noted that the response of corticosterone to fasting varied depending on the individual energy reserves as well as their past experience (Webster 2003). Liu et al. (2012) demonstrated that corticosterone exposed hens showed decreasing levels of POMC mRNA. Furthermore, Byerly et al. (2009) showed also that chickens treated with corticosterone had lower POMC levels in the hypothalamus. On the other hand, gut peptides (e.g., CCK) in 7-day old chicks was found to be unchanged even after receiving icv injection of corticosterone which suggest that gut peptides in poultry might not be the target of glucocorticoids for peripheral control of food intake (Liu et al. 2012).

1.8 Food hoarding

Hoarding (also called caching/storing) is the act of storing food items either in one place or in different locations for times when food sources are scarce (Sherry 1985). By storing surplus food items, individuals will ensure having a reliable food source for times of high energetic demands (Morse 1980). As reviewed by Vander Wall (1990) food hoarding has been observed in several animal phyla: thirty mammalian families such as shrews, foxes, monkeys, tigers and bears, in avian species (titmice, hawks, owls, woodpeckers and jays) and also in Arthropoda (bees, spiders, ants and several species of wasps).

Animals are faced every day with energetic challenges that they need to cope with to ensure both the survivability of their offspring and increasing their reproductive fitness. The environment around individuals fluctuates annually. Thus, they need to adapt with those changes in order to survive to the next day. For instance, in the northern hemisphere regions, winter seasons are characterised by very long nights combined with drastic drop of temperature. Surrounded by those factors, the danger of death by starvation is elevated particularly for animals that are smaller in size. Some might argue that the immediate consumption of food items once found are the best way to eliminate the chances of starving. However, sometimes it might not be possible to consume all the food that is found due to stomach capacity constraint.

Moreover, as mentioned above having food stashed away assures food availability for times when environmental conditions change and might restrict an animal's access to food sources (Vander Wall 1990). In addition, hoarding itself is more energetically efficient in a sense that unlike eating, it does not require the act of digestion to convert the food to lipid deposits that would need to be mobilise from its site of storage to be utilise once needed (energetically costly) (Bartness et al. 2011). Also, having an external energy source

means that the individual could avoid the increase of body mass (that comes with increased lipid stores) which means being more agile in avoiding predators. On the other hand, hoards face the danger of spoilage and theft by other animals (Vander Wall 1990).

The pattern of hoarding is divided into two types: those who concentrate their stashes into one place and are called larder hoarders, and those who spread and disperse food into several locations and are so-called scatter hoarder (Vander Wall 1990). The first type employs the animal spending more energy to protect and relocate their food items (Jenkins et al. 1995), while the second type depends heavily on having a strong spatial memory to remember where the food is stored. Sherry and Vaccarino (1989) were able to demonstrate that similar to mammals, lesioning of the hippocampus (the main part of the brain linked to memory) of chickadees lead them to perform poorly in trials of cache recovery. They would store food items similarly to intact birds, but their retrieval attempts were not accurate.

Over the years, investigations have been undertaken to try and understand why some species hoard while others do nor and how hoarding behaviour has evolved? Theories have emerged in the late 1970s to explain the rationale behind scatter hoarding. Those were mostly based on the assumptions that the individual should at least retrieve a sufficient proportion of its stored hoard in order to repay the effort spent to hide it for it to be beneficial thus have the potential to evolve (Vander Wall and Jenkins 2003). The functional theory looks at the conditions when food hoarding is adaptive. Roberts (1979) have suggested several points to summarise how food storing behaviour might have been selected: a. in species that reside in seasonal environments. As mentioned above, in northern temperate latitudes that experience strong seasonality, changes in daylight and temperature combined with food shortage favours caching behaviour (Fretwell 1972). This comes from the fact that species that do not migrate are obligated to find ways to survive harsh winter. b. hoarders are mostly territorial. According to Brown (1964) storing behaviour is costly and adaptations are necessary to increase the benefits to buffer those costs. Being territorial is cost effective in a sense that it protects stored food from other animals. c. hoarders developed characteristic behaviours that enhances the retrieval of stored food. This statement could be explained in accordance with smaller co-adaptations showed by the species linked to their life histories (Roberts 1979). For instance, acorn woodpeckers are likely to store one or few acorns usually in the centre of their territory, and according to Ritter (1938) they tried several holes before finding the perfect one where they could wedge several items so competitors would find it hard to get, and apparently no other species showed this behaviour (McRoberts and McRoberts 1976).

The mechanistic explanation of food hoarding looks mainly at the mechanism behind food hoarding. One aspect is the deficit hypothesis. This hypothesis was proposed by Morgan et al. (1943). According to his theory, the animal would hoard in accordance to growing energetic shortage that would eventually reach a threshold after which food hoarding is triggered. This hypothesis seems to hold a similar principle to the lipostatic hypothesis, which states that food intake is stimulated and driven by shortage of fat stores (Kennedy 1953). Although the deficit hypothesis might explain some hoarding events, Bartness and Day

(2003) viewed hoarding behaviour from a metabolic control perspective, even though hoarding and food intake are not mutually exclusive. They proposed that the signals that trigger hoarding/foraging are generated mainly from neuropeptides, gut hormones, as well as other circulating factors that are associated with the movement of nutrients from one organ to the target tissue to be oxidised. Their theory implicated the involvement of external and internal cues that affect food-hoarding behaviour. So why do birds hoard?

For most animals living in higher latitudes, the winter season entails many problems. Along with the increasing energy requirements due to the drop in temperature, food sources start to decline as well as the foraging and feeding time windows shortens (due to short photoperiod). It becomes curtail for those animals that reside in their environment to undergo not only physiological but also behavioural changes to survive those periods (Broggi et al. 2003). During harsh conditions, fat is the best fuel source to be used as it releases far more energy once metabolised. On the other hand, it is quite costly and risky to carry the maximum amount of fat stores because it might make them more vulnerable to predators as well as causing restricted locomotion (Wells 1993). Since storing energy internally is inefficient in this case, having an external source (as hoards) provides another option. And unlike larder hoarding, having a food source dispersed means that if it was taken by other individual the loss is negligible (Brodin 2010). Hurly (1992) suggested that individuals that are energetically stressed tends to increase hoarding behaviour. In addition, it was found that Carolina chickadees and tufted titmice store food more as their body weight fall (Lucas 1994).

1.8.1 Photoperiod effects

It is believed that short days stimulate food hoarding behaviour in many animals living in the mid to high latitudes (Barry 1976). That effect was assumed to be mediated via sex hormones (testosterone and estrogen). It is known that sex steroids increase in the circulation as the gonads develop to prepare the individual for breeding, but then declines as those gonads regress after the breeding season (Vander Wall 1991). According to Nyby et al. (1973), the elevation of the sex hormones exerts behavioural effects in that higher levels supresses hoarding in Mongolian gerbils. Similar findings were also observed in birds. Increasing day length is associated with the onset of breeding and when autumn/winter approaches an increase in storing behaviour is found as gonads regress (Gwinner 1989). That been said, chickadees housed in long days stored less food in comparison to those housed in short days regardless of their reproductive status (MacDougall-Shackleton et al. 2003). That finding implies that gonadotrotropin and sex steroid hormones, contribute to controlling seasonal changes in reproductive state, but do not play a central role in regulating seasonal changes in food storing behaviour (MacDougall-Shakleton et al. 2003). However, that does not mean that we could rule out the possibility that the reproductive state itself may affect caching behaviour.

1.8.2 Temperature

In boreal forests, temperatures are extremely low for a very long time. On top of that, small passerines have a larger surface area for heat exchange which means they need to increase their metabolism in order to

maintain a stable body temperature (Brodin et al. 2017). To meet those higher energy requirements, they need to store more fat, which is not ideal since it decrease their agility and ability to escape predators (McNamara and Huston 1990). It is because of the higher metabolic rates that birds increase their foraging routine not only to feed but also to build an energy source (hoards) that could be use in later times to protect themselves from starvation especially at night (Pravosudov and Grubb 1997).

1.8.3 Food availability

It appears that food availability is the strongest stimulus for hoarding behaviour in nature (Vander Wall 1990), and is the factor most easy to manipulate in the laboratory when studying the underling neural and physiological mechanisms of food hoarding. Having said that, hoarding also seems to occur when food sources are in excess. Thus, the surplus food is stored for periods when energetic demands exceed available recourses and that is when additional food is needed so the individual can sustain itself (Vander Wall 1990). The energetic demands of an individual fluctuate according to behavioural states, reproductive and circadian cycles. Although some might argue that food deprivation conducted in laboratory experiment does not occur naturally for animals, one could envision that there might be times spent by a foraging animal searching for food (Bartness et al. 2011). Food availability changes in free-living conditions, it increases with pulse production and decrease because of the action of microbes and foragers (Vander Wall 1990).

Most of our understanding of the control of food hoarding behaviour comes mainly from the mammalian literature and specifically from studies in hamsters. Much research has been done in the last decade in regard to how ingestive behaviour is regulated in rodents because they are more similar to humans. The roots of hoarding studies extend as far back as 1939 when Wolfe demonstrated that this behaviour is quantifiable. Although he used rats in his experiments (which are known to carry food), there was some controversies as to whether rats could be classified as “hoarders”. Soon after this study, Morgan et al. (1943) proposed the deficit hypothesis to explain what trigger hoarding. He suggested that the main force behind hoarding is not dependent on the usage of metabolic fuel; rather it is based on the overall decrease of the fuel used.

Hoarding behaviour appears to occupy a vital position in Siberian hamster’s energetic repertoire (Wood and Bartness 1996). Living in the temperate zone, Siberian hamsters show a variety of responses triggered by the extreme changes in the environmental conditions. While in summer, they actively breed and show peaks in body mass, body fat and food intake (Bartness and Wade 1985), in winter they are reproductively quiescent, have lower body mass, fat and food intake (Wade and Bartness 1984). Additionally, hamsters show physiological and behavioural responses to changes in food availability by increasing food hoarding. Similar to food intake, hoarding is mainly controlled by the signals exchanged between the central and peripheral systems. Nutrients affect the CNS, while peripheral hormones are release affecting the brain directly or through sensory efferents projecting to the brain (Bartness et al. 2011). Changes in neuropeptide secretions can affect the periphery causing changes in adiposity, hormone release and behaviour.

1.9 Central control of food hoarding

Unlike laboratory rats and mice (Bartness and Demas 2003), hamsters do not overeat when food deprived (Bartness et al. 1995), instead they increase food hoarding (Silverman and Zucker 1976). The mechanisms underlying this fasting-induced increase in food hoarding has been the focus of many studies, especially the central control aspects of it. Food deprivation seems to alter a wide array of neuropeptides that are involved with energy balance. The most well studied neural populations that were found to change in response to food deprivation are: 1) NPY and AGRP (hunger neurons) 2) POMC and CART (satiety neurons) that both reside in the ARC of the hypothalamus. Schwartz et al. (1995) demonstrated that in Siberian hamster's food deprivation increased NPY and AGRP expression. On the other hand, fasting seems to inhibit the expression of POMC (Reddy et al. 1999) and CART (Khorooshi et al. 2008) in hamsters. It should be noted that those changes in the gene expression do not necessarily translate into changes in the protein synthesis or release, but seems to be consistent with such change (e.g., food deprivation increases the release of NPY from the PVN) (Bartness et al. 2011). Day et al. (2005) demonstrated that 3rd ventricular injection of NPY significantly increased food hoarding in Siberian hamsters (300%-1100%). The explanation for such a large increase is related to the fact that central injection of NPY mimics the pattern of elevated food intake after re-feed following a fast (Marín Bivens et al. 1999). The action of NPY is mainly mediated by the action of its receptor subtype Y1, as an injection of Pro34 (NPY Y1 receptor agonist) into 3rd ventricular increased food hoarding in hamsters, while DTrp34 (NPY Y5 receptor agonist) increased food intake (Day et al. 2005). Dailey and Bartness (2009) investigated the brain area whereby NPY exert its effect on food deprivation induced increase in hoarding. They found that microinjection of BIBO 3304 (NPY Y1 receptor antagonist) into PFA but not the PVH completely blocked the food deprivation induced increase in food hoarding. Co-expressed in same ARC neurons as NPY, AGRP expression was found to increase with food deprivation in Siberian hamsters (Mercer et al. 2000). Moreover, as mentioned in the food intake section, it is in fact the competition between the AGRP and the α-MSH for the melanocortin 3- and 4-receptors (MC3/MC4-Rs) that the action of AGRP is exerted. According to Day and Bartness (2004), a single 3rd ventricular injection of AGRP had a marked effect on several behaviours in Siberian hamsters. These included, increased foraging (75~400%), food intake (100~150%) but most notably food hoarding (200~1200%). These results suggest a greater contribution of the melanocortin system in inducing food-seeking behaviour, and in the case of Siberian hamster the outcome of foraging is increased hoarding rather than food intake (Day and Bartness 2004). This apparent difference in ingestive behaviour fits the well-known response of hamsters to re-feeding after a fast of increasing food hoarding (Day and Bartness 2003). Since AGRP gene expression is elevated in the ARC due to food deprivation in Siberian hamsters (Mercer et al. 2000), it is more likely that AGRP has a significant role in the post fast-induced increase in food hoarding and foraging. It is noteworthy that, the AGRP effect on stimulating food hoarding is prolonged (5-7 days) which is similar to its ability to elicit longer sustained increase in food intake in laboratory rats (Hagan et al. 2000). On the other hand, NPY causes early but short-lived increase in food hoarding (1-2 days) (Dailey and Bartness 2009).

It is well-known that food deprivation/fasting is a potent stimulator for food hoarding and is accompanied by an increase in γ-aminobutyric acid (GABA), which is a major inhibitory neurotransmitter in the hypothalamus (Kamatchi and Rathanaswami, 2012) and nucleus accumbens (Meena et al., 2009). The relationship between food storing and GABA was established by Mogenson and Wu (1988) in male rats (not natural hoarders) where it was found that an injection of GABA into the subpallidal region decreased food hoarding. Like hamsters, Mongolian gerbils do not overeat when food deprived. Instead, they over hoard (Schneider et al. 2007).

1.10 Peripheral control of food hoarding

In the previous section, we focused on the CNS control of food hoarding, but it is very important to establish a distinction between the peripheral and central controls that aid in the regulation of hoarding behaviour. Similar to food intake, there is a wide array of gut peptides that are secreted in response to both environmental factors as well as internal cues within the body reporting the energetic status of an individual. Indeed, those peripheral release hormones cause local and global effects on the overall behaviour of an animal.

1.10.1 Gut peptides

The predominant form of CCK in rodents is either CCK-33 (Linden et al. 1989) or CCK-58 (Reeve et al. 2003). Studies had shown that the administration of CCK-33 inhibited both food intake and food hoarding of Siberian hamsters (Teubner and Bartness 2010). While it is known that CCK exerted its satiety effect through the inhibition of gastric emptying via its binding to its receptor at the vagus nerve (Raybould 2007), the mechanism by which it effects hoarding behaviour is unclear. It could be via a similar route, but it could also be due to a more different process. We are yet to fully understand the different aspects regulating and controlling hoarding.

It is worth noting that till this date, we are still lacking data regarding the actions of other gut peptides on food hoarding (e.g., PYY, insulin and glucagon) in both mammals and birds.

1.10.2 Stress system

The mechanisms whereby glucocorticoids exert their effect on neuro/ and gut peptides in hoarding birds is yet to be understood. It is well-known that chronic stress and elevated levels of corticosterone are associated with negative effects on cognitive abilities (Sapolsky 1996). However, there are situations when stress is considered beneficial and a survival mechanism where it increases the fitness of an individual. Short-term food deprivation was shown to cause increasing levels of corticosterone (Harvey et al. 1984). Hurly (1992) showed by using a functional model, that it is adaptive for individuals to hoard food when food source is unpredictable. Having said that, it should be noted that there is no mechanistic link between unpredictable food source and food hoarding. So, the elevation in corticosterone levels is undoubtedly a response to the variability in food sources (Pravosudov and Lucas 2001). According to all those observations, it is tempting to think that the unpredictability of food produces internal signals that increase corticosterone levels leading

to increased food hoarding behaviour. Yang et al. (2014) demonstrated that female Mongolian gerbils that have high tendencies to hoard exhibit this behaviour because of being stressed as they were food deprived for 22h. The stress induced by fasting led to the activation of the HPA axis. The net result of HPA activation is the release of glucocorticoids (Tsigos and Chrousos, 2002). Studies showed that food deprivation increases circulating corticosterone in both rodents (McGhee et al. 2009) and birds (Pravosudov 2003). This was further proven in the Mongolian gerbil experiment (Yang et al. 2014). The authors showed that females with high hoarding tendencies had a higher level of corticosterone in comparison to those with low hoarding tendencies suggesting that corticosterone are in fact involved in facilitating food hoarding behaviour. Silverin (1998) observed that in resident parids that cache food when food sources are limited/unpredicted, an increase in corticosterone levels were recorded. He noted that willow tits (*Poecile montanus*) (small caching bird) showed twice as high baseline corticosterone during harsh winter months when foraging trips were most energetically demanding. Similarly, Pravosudov (2003) demonstrated that the moderate elevation of corticosterone in mountain chickadees (*Poecile gambeli*) facilitated food hoarding and enhanced food retrieval. Additionally, referring back to hierarchy and food hoarding, there is a mixed view in the parids literature on dominant / subordinate dynamics and food storing. On one side, it is predicted that because subordinate individuals face greater uncertainty in foraging success thus having higher corticosterone, this leads them to store more (Pravosudov and Grubb, 1997). On the other end, some studies showed that dominants store more (Pravosudov 1985). Indeed, if food is scarce, the bird that can access the food (the dominant birds) will be the only ones who have enough of it to hoard.

Whether limited food source cause corticosterone levels to rise or the elevation comes from increased foraging behaviour remains to be answered.

To this point, most of the research in the field of the neural mechanisms underlying hoarding behaviours has been done in hamsters. And bird, basic knowledge of mechanisms regulating food intake has come from studies of domestic chickens. Less is known about how brain and gut peptides behave in wild small songbirds in relation to changes in the nutritional status of the individuals. However, wild songbirds are one of the best studied groups in the fields of ecology, behaviour and physiology related to hoarding in comparison to other hoarding animals (Brodin 2010). In particular, unlike hamsters, there are closely related species in families such as the Paridea that exhibit either hoarding or non-hoarding behaviour. This provides the opportunity for informative comparative studies. Thus, the aim of this PhD is to: 1. compare the presence and distribution of gene expression of neuropeptides and gut peptides between hoarding and non-hoarding titmice with other avian species. 2. Establish how neuropeptide and gut peptide gene expression is influenced by fasting and re-feeding. 3. Investigate whether there is a correlation between gene expression and different ingestion behaviour. 4. Identify candidate peptides genes that could be responsible to distinguish hoarding from non-hoarding birds. By subjecting individuals to unpredictable food treatments (fasting and re-feeding), recording their behaviour, quantifying candidate peptide mRNAs that control appetite/or hoarding and then

comparing the outcome of the video analysis and statistical analysis we hope to pinpoint key peptide genes that might standout as different between hoarding vs. non-hoarding species.

In the following chapters we will:

- Investigate the distribution patterns of brain and gut peptides that regulate the appetite system in relation to other avian species (chapter 2).
- Compare peptide gene expression in response to a fasting and re-feeding regime between hoarding vs. non-hoarding species (chapter 3).
- Establish whether there is a correlation between different ingestive behaviours and brain and gut gene expression (chapter 4).

Chapter2. Distribution patterns of different brain and gut peptides in great tits *Parus major*

2.1 Avian appetite system

For resident passerine birds that spend winter in the temperate zone and do not migrate, fat reserves are considered one of the most crucial fuels that serve as an important buffer against starvation during harsh conditions (Witter and Cuthill 1993). One would speculate that those small birds would maximise their energy reserves as body fat to counteract the fluctuating environmental conditions. Between the increasing risk of starvation, and their need to reach certain evening mass, individuals are expected to enhance their foraging efforts early in the morning to buffer against the loss of foraging opportunities in the afternoon (Lilliendahl 2002). However, individuals maintain a much lower fat reserve (Witter and Cuthill 1993) which implies the possibility of a cost for maintaining or carrying larger body mass. One example of a risk of carrying much fat reserve is predation (Lima 1986). Having said that, it is well documented that small birds use between 5-10% of their body mass in fat (King 1972). To do so, small songbirds need to have a well-orchestrated appetite system to ensure appropriate signal flow between the external and the internal environment. While chickens and Japanese quail can survive periods of low food availability due to their size, passerine birds have a higher metabolic rate, thus it is expected that their appetite system might be regulated differently even though peptides regulating feeding behaviour and energy balance might be conserved between avian taxa. This different regulation may extend to the expression of hoarding behaviour in some passerine species such as titmice.

2.1.1 Hypothalamic neuropeptide

Studies has shown that NPY stimulates food intake in several avian species: chickens (Kuenzel and McMurtry 1988), white-crowned sparrows (*Zonotrichia leucophrys gabelii*) (Richardson et al. 1995) and ring doves (*Streptoelia risorii)* (Strader and Buntin 2001). Those studies demonstrated that the dose range, magnitude of induced change, and time course effect of NPY's stimulatory effect on food intake in those birds are comparable to those of laboratory rodents. We predict that in great tits, NPY levels would increase with food deprivation.

AGRP is co-expressed with NPY and was shown to exert a similar orexigenic effect on food intake with its gene expression increasing with fasting/ food deprivation in Japanese quail (Philips-Singh et al. 2001). I.C.V injection of AGRP notably increased food intake in domestic chicks (Tachibana et al. 2001) and ring doves (Strader et al. 2003). McConn et al. (2019) demonstrated that Japanese quail fasted for 3h showed an increase in AGRP mRNA levels. The stimulating effect of AGRP on appetite is through its antagonism to the

melanocortin receptor (MC4R) (Ueno and Nakazato 2016). Having said that, little information is known about the action of AgRP action in passerine birds. We predict that in great tits, food deprivation would elevate AgRP gene expression.

Finniss et al. (2000) demonstrated that in rats POMC mRNA is decreased following food deprivation. That decrease is parallel to the changes that occurs on the levels of its peptides α- and γ- melanocortin stimulating hormones and the firing frequency of POMC neurons. The action of POMC is mediated via bind to its receptor MC4R (Ling et al. 2004). There is limited consistent information regarding the contribution of POMC in food intake in birds overall and passerines specifically. However, the inhibitory effect of POMC on food intake in chicken and ring doves has been confirmed to be due to POMC being a potent agonist competing with AGRP to bind to NMC4R (Strader et al. 2003).

2.1.2 Hindbrain neuropeptide

In neonatal chicks, proglucagon mRNA has been found in the medulla oblongata (Tachibana et al. 2005). Food deprivation was found to downregulate the mRNA gene expression of the proglucagon in the medulla oblongata which suggests that the endogenous proglucagon derived products are responsible to regulate and control feeding behaviour in neonatal chicks (Tachibana et al. 2005). Studies in neonatal chicks (Furuse et al. 2007) and Japanese quail (Shousha et al. 2007) demonstrated that i.c.v administration of GLP-1 supress food intake by the activation of the ventromedial nucleus (VMN) Fos expression (Tachibana et al. 2004). However, little is known about the involvement of proglucagon in passerine birds. We predict that similar to chicks and Japanese quail, proglucagon would be elevated in fed individuals.

There are a few peptides that are classified as having dual actions both in the brain and the gut and CCK is one of those peptides (Vanderhaeghen et al. 1975). Localised in the hindbrain, CCK there is evidence that it contributes to memory, learning and satiation (Herness and Zhao 2009). According to Herness and Zhao (2009) the central action of CCK neuropeptide on the appetite system might be due to its expression in the taste buds where it contributes to the bitter transduction cascade. This suggests that CCK participate in tastant information processing in the mouth. So, we could assume that through CCK bitter taste processing while having a meal that satiation signals are generated. Having said that, very little is known regarding the central actions of CCK in passerine. However, we predict that fed individuals would have higher CCK mRNA levels.

We are not aware of any information on the action and gene expression of POMC in the hindbrain in any avian species. However, we could speculate from rodent studies (Fan et al. 2004) that hindbrain POMC suppresses appetite just as for hypothalamic POMC

2.1.3 Gut peptide

2.1.3.1 Cholecystokinin (CCK)

In the previous chapter, we explicitly talked about CCK tissue distribution, function, and the mechanism behind the CCK satiety effect in mammals and poultry. However, less is known about the action of CCK in passerines which is the main core of this chapter.

Richardson et al. (1993) demonstrated that, similar to mammals, CCK has an anorexic effect in white-crowned sparrows. This anorexigenic effect was dose dependent and specific to food intake but not water intake. The suppressing effect of CCK in mammals is mostly due to its role in increasing gallbladder contraction, gut motility and gastric emptying (Miyasaka and Funnakoshi 2003), and its binding to its receptor CCL-1R in the vagus nerve. We predict that CCK mRNA expression levels would elevate with increased food intake.

2.1.3.2 Glucagon (GCG)

In the general introduction, we detailed the process of glucagon cleaving and showed how the products are distributed in the body and the function of each peptide within specific tissues. However, it is worth mentioning that there are no data in the passerine literature that addresses the splicing product of GCG which is GLP-1.

Glucagon is a well-known satiety peptide (Honda et al. 2007) increasing when individuals are food deprived in poultry (Dupont et al. 2008). Being food restricted means that birds are expected to rely heavily on the catabolic pathways to replenish their depleted energy stores (Totzke et al. 2000). Thus, in challenging situations, glucagon becomes overly sensitive switching to its hyperglycaemic and lipolytic actions (Mialhe et al. 1997). Once stimulated glucagon starts increasing circulating glucose, free fatty acids and β-hydroxybutyrate levels, increasing fat deposition, signalling satiety and insure higher survival rate during longer nights in garden warblers (Totzke et al. 2000). As for passerines, we predict that glucagon gene expression would be elevated in fed individuals.

2.1.3.3 Peptide YY (PYY)

Peptide YY was first isolated from the small intestine of chickens in 1992 (Conlon and O'Harte 1992). PYY immunoreactive cells were detected in both the duodenum and jejunum of chickens (El-Salhy et al. 1982). PYY mRNA levels were higher in chickens under *ad libitum* feeding in comparison to a 12h fasting condition (Aoki et al. 2017). In addition, PYY levels were significantly higher in satiated broiler layer hybrid chicken in comparison to individuals subjected to 11h fasting (Reid et. Al 2017). To this date nobody has studied PYY gene expression in songbirds. However, we predict that in great tits, PYY mRNA levels would increase in fed individuals.

2.1.3.4 Insulin (INS)

Peripheral administration of insulin has been shown to suppress food intake in chickens (Smith et al. 1974). In addition, insulin in blood circulation was observed to decrease with fasting in birds (Christensen et al.

2013). Honda et al. (2007) demonstrated that central administration of insulin decreases food intake in one-day old male chicks. Shiraishi et al (2008) proved that the inhibiting effect of insulin on food intake in day old male chicks was significantly reduced after the ICV administration of selective melanocortin antagonist such as (SHU9119 or HS014). This attenuated effect is mainly due to the fact that ICV injection of insulin stimulated the expression of POMC, while suppressing NPY gene expression (Shiraishi et al. 2008). Shiraishi et al. (2011) demonstrated that NPY neurons expressed insulin receptor mRNA in Japanese quail, and given that generally AgRP is co-expressed with NPY, we might expect those receptors to be expressed there as well, hence the insulin effect on appetite is governed. We predict similar results in insulin mRNA gene expression in great tits. Insulin mRNA would increase with increased food intake and decrease with fasting.

As a first step for us to understand how peptides involved in the regulation of food intake may also control hoarding behaviour, we need to identify peptides involved in the appetite regulation of titmice in general for us to know if they are involved in hoarding behaviour as well.
Thus, we started by studying great tits (*Parus major*) because they are not only closely related to the hoarding birds we are interested in, but also because their genome sequence information is readily available in databases making it easy for us to design the correct primers to measure expression of mRNAs of interest in response to nutritional manipulation. We predict that the same brain and gut peptide as in chickens and quails are sensitive to reduced food availability, and that sex differences should not influence how peptides respond to changes in the nutritional state of individuals given that male and female titmice are of a similar size.
Thus, we hypothesise that both NPY and AgRP gene expression will be increased with food deprivation, since their encoded peptides have been well established as hunger signals in birds (Richardson et al., 1995). In contrast, we predict that hypothalamic POMC, and hindbrain POMC, CCK and GCG mRNAs (Shousha et al., 2007) are decrease during fasting because their encoded peptides signal satiety. Similarly for gut peptides, CCK, PYY, GCG and INS are all satiety signals expected to decrease with feeding. However, in the pancreas, we expect GCG expression to increase during fasting because of the role of pancreatic glucagon peptide in energy release.

2.2 Methods

2.2.1 Animals

Sixteen great tits (*Parus major)* (10 females and 6 males) were caught in woodland near Newcastle upon Tyne, UK, under a permit from Natural England (2017-31219-SCI-SCI) between October/ November 2017, using mist nets. The birds were transported after capture to climate chambers at Newcastle University where they were placed into aviaries where light and temperature were controlled. Each bird received a distinct colour ring for identification. Once in captivity, birds were placed on a 11L:13D light/dark cycle lights (on at 10:00) and with temperature maintained at 15°C. Birds were fed *ad libitum* with a pre-made food mixture (pine nuts, sunflower seeds, crushed peanuts, mealworms, wax worms and Orlux Insect Patee) with water available for drinking and bathing. On average the birds were kept for two weeks (the overall median was 13 days and the range was 10-22 days) before being humanely killed for dissection. The different captivity duration was due to the birds being captured and brought into captivity at different times. The choice of 2 weeks habituation period was based on previous studies done in our lab where it was shown this period was enough for small wild songbirds to show normal foraging and eating behaviour and to respond to unpredictable food by increasing food intake and hoarding (Henderson et al. 2018). Thus, when the birds were brought at first to the aviaries, they were given food ad lib. However, the food source was made unpredictable once placed in the cages to mimic the conditions in their environment. So, we predict that the stress of bringing birds from the field to laboratory conditions might not subject them to drastic stress because those songbirds exhibit strong resilience when it comes to drastic changes in their environment.

Birds were humanely killed using a United Kingdom Home Office Schedule 1 method of lethal intraperitoneal injection of sodium pentobarbital (Euthatal) either immediately before lights on (fasted) having not fed during the night, or after being allowed to feed for 4h after lights on (fed). Birds were dissected in batches of four at intervals between October and early November, 2017, with each batch being balanced across both the sex and treatment. The body mass of the birds was recorded immediately before dissection.

2.2.2 Tissue collection

Tissue samples were dissected from each individual and were stored in RNAlater (Sigma-Aldrich) at -80°C until further analysis. The tissue analysed were hypothalamus, hindbrain, proventriculus, gizzard, antrum, duodenum, pancreas, proximal jejunum, distal jejunum, ileum, caecum and rectum (Figure 1). For the gizzards, they were cut and inspected to determine whether it was full or empty to decide the feeding state of the individual at the time of dissection.

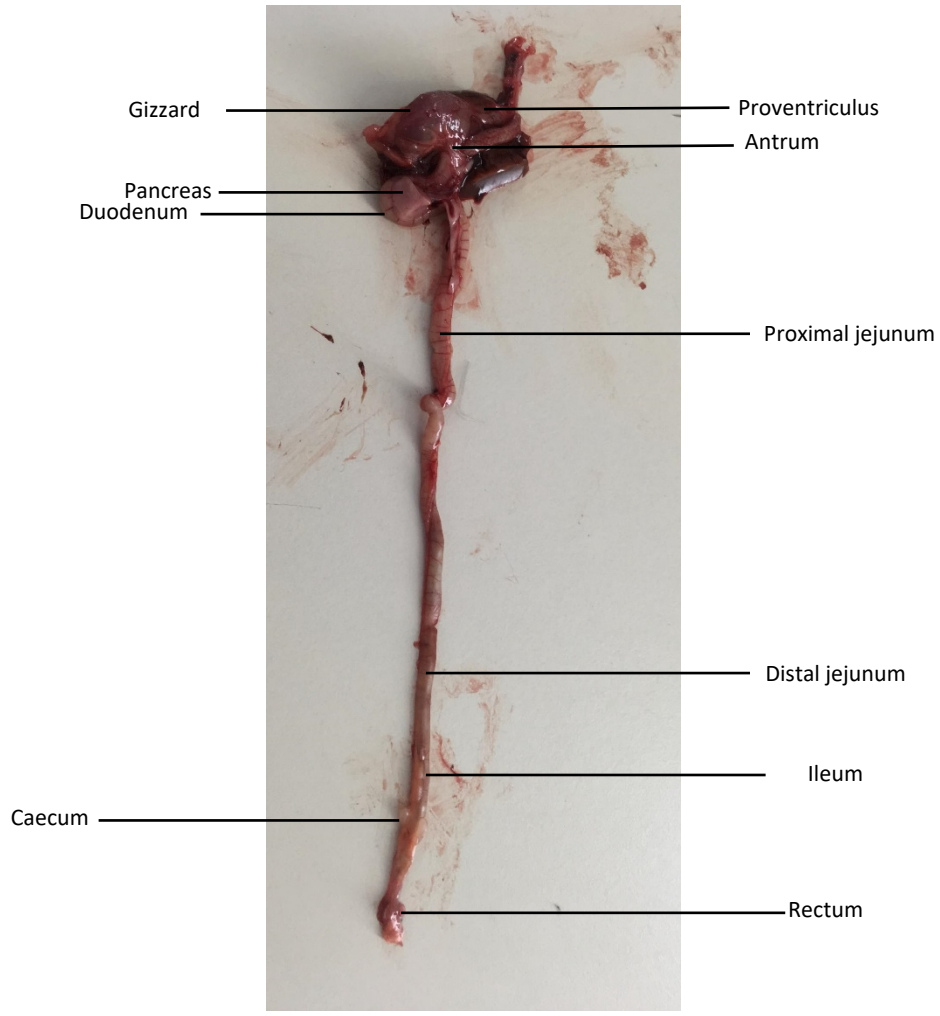


Figure 1: Photograph of a great tit digestive system showing the different parts of the gut dissected.

Table 1: Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) listing the extracted tissues, number of fasted/ fed individuals, mean RNA concentration for each tissue and mean 260/280 ratio

Tissue	Sample size fed	Sample size fasted	Mean RNA concentration (ng/µl)	Mean 260/280 ratio
Hindbrain	6	6	739.25	1.96
Hypothalamus	6	6	418.96	1.95
Proventriculus	6	6	1505.25	1.90
Gizzard	6	6	1349.12	1.93
Antrum	6	6	1637.11	1.93
Duodenum	6	6	3433.13	1.90
Pancreas	6	6	1362.28	1.86
Proximal Jejunum	6	6	1314.50	1.92
Distal jejunum	6	5	1399.15	1.94

Distal ileum	6	5	1039.63	1.95
Caecum	6	4	866.48	1.94
Rectum	6	4	1365.10	1.95

2.2.3 RNA extraction and reverse transcription

Total RNA was extracted from each tissue. Samples were homogenised in 1ml TRIsure (Bioline, London, UK) with 2mm Bashing Beads (Zymo Research, Cambridge Bioscience, Cambridge, UK) using a FastPrep FP120 Instrument (MP Biomedicals, Fisher Scientific, Leicester, UK). The aqueous phase was loaded onto Direct-zol RNA miniprep columns (Zymo Research) following the manufacturer's instruction that included on-column DNase treatment. The RNA concentration eluted was measured using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Cramlington, UK) and the RNA was stored at -80℃ for later analysis.

The total RNA was reverse transcribed using a SensiFast cDNA Synthesis Kit (Bioline) following the manufacturer's protocol. In brief, the master mix was prepared using 1µg of RNA, 1µl of reverse transcriptase, 4µL of 5×TransAmp buffer and the appropriate volume of RNase/DNase free water was added to create a total volume of 20µl per reaction. The tubes were placed in a Thermo Scientific Arktik PCR machine programmed as follows: 25℃ for 10min, 42℃ for 15min, 85℃ for 5min to yield cDNA which was diluted with 15µl water to a final volume of 35µl before being stored at -80℃ for the later gene quantification.

2.2.4 PCR primers

For each gene of interest, National Centre for Biotechnology Information (NCBI)- primer- BLAST software was used to design the primers using great tit genome reference sequences (Table 1).

Table 2: Primers used for real-time PCR designed using great tit (*Parus major*) genome sequences for tyrosine *3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta* (*YWHAZ*), *peptide YY* (*PYY*), *cholecystokinin* (*CCK*), *preproglucagon* (*GCG*), *insulin* (*INS*), *agouti-related protein* (*AGRP*), *pro-opiomelanocortin (POMC)* and *neuropeptide Y (NPY*).

Gene	Accession	Primers	Product size (bp)
YWHAZ	XM_015617837	Forward: GGAGCCCGTAGGTCATCTTG Reverse: GCAACCTCAGCCAAGTAACG	242
PYY	XM_015616308	Forward: TCACGGTTCGATGACGACTC	125

		Reverse: GAGTCGTCATCGAACCGTGA	
CCK	XM_015649630	Forward: TCCAGCAAGCCAGAAAAGGT Reverse: GCGTCCAAAATCCATCCAGC	122
GCG	XM_015633925	Forward: GCAATGCAATCTCCAAGCGT Reverse: TCCATTCACCAACCAAGCGA	125
INS	XM_015629749	Forward: CCACTTGGTGGAAGCTCTGT Reverse: CAGGTGTTGTGGCAGCATTG	192
AGRP	XM_015639942	Forward: CTCTCTGCTTGGAGAGGTCAC Reverse: ACACCTCCTTGGCTTTCCTC	203
POMC	XM_033518242	Forward: GGAATCTCCGTTCCTTCCCG Reverse: GGGTTTTCCCCATCGGAAGT	238
NPY	XM_015620265	Forward: CAACCTCATCACCCGACAGAG Reverse: ACTGGGGATGACGCTATGATT	214

2.2.5 Sequencing of PCR products

The PCR products were sent for sequencing at the DBS sequencing facility at Durham University following their sequencing guidelines: 15µL of PCR product was mixed with 6µL of ExoSap-IT PCR-product clean up solution (Thermo Fisher). Both forward and reverse primers were used at a concentration of 3.2 pmol/µL. Sequencing confirmed amplification of the PCR products expected. This step was also done for amplification products from blue and coal tits to validate the use of the same primers across the three species.

2.2.6 Real time quantitative PCR

Real-time quantitative (qPCR) PCR was performed using the absolute quantification standard curve method. DNA standards were prepared by gel purification of PCR products. These were generated by end-point PCR performed in 25µL reactions including the following: 12.5µL 2× MyFi Taq polymerase (Bioline), 1µL of forward and reverse primers (0.4µM final concentration) and 2µL of cDNA from the sample. Four 25µL reactions were loaded onto a 2% agarose gel and, following electrophoresis, the PCR products were cut using a scalpel blade. A QIAquick Gel Extraction kit (Qiagen, Manchester, UK) was used to purify the PCR product DNAs according to the manufacturer's protocol. The purified PCR standard DNAs were then quantified using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific).

Real-time PCR reactions were set up in 20 µL volumes including the following: 0. 4µM primers (Final concentration), 5 µL cDNA and 10µL 2× Sensifast SYBR No Rox solution (Bioline).

The qPCR reactions were carried out using a CFX Connect real-time PCR machine (Bio-Rad, Oxford, UK) with the following three-step cycling conditions: 95℃ for 2min, followed by 40 cycles of 95℃ for 5s, 60℃ for 10s and 72℃ for 15s. No- template controls were run in duplicate, and cDNA samples were run in singlicate for each gene within a single 96 well plate. Standards were used in duplicate to create the standard curve. Six 5-fold standard dilutions were used for each standard curve. Results were analysed using CFX Manager Software (Bio-Rad). Melting curve analysis confirmed the presence of a single peak for all the designed primer pairs. PCR reaction efficiencies for the used primers ranged from 97-107%.

Gene expression values in gut tissues were calculated as a ratio of the reference gene tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) measured in the same individual samples due to its reliability in previous avian studies including gut tissues (Olias et al., 2014, Reid et al. 2017). For brain samples values were corrected by using the reference gene lamin B receptor (LBR) as we have shown this to be expressed stably in previous studies of gene expression in the brain (Dunn et al. 2015).

The percentage coefficient variation (CV%) was calculated as shown for the example below which indicated that variation for the duplicate standards in the qPCR measurements, I made in this thesis was less than 1% which means that we can assume that this level of variation would also apply to my samples had they been performed in duplicate.

	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
CT replicate 1	12.93	15.11	17.89	20.23	22.74
CT replicate 2	13.10	15.26	18.03	20.49	23.05
Mean	13.02	15.19	17.96	20.36	22.90
Standard deviation (SD)	0.12	0.11	0.10	0.18	0.22
CV%	0.92	0.70	0.55	0.90	0.96

Table 1: Experimental group distribution across the different treatments according to the gizzard fullness at the time of the dissection

Sex/ condition	**Males/ empty**	**Males/ full**	**females/ empty**	**Females/ full**
Numbers	**3**	**3**	**6**	**4**

2.2.7 Data analysis

Before the beginning of the analysis where it was relevant, a histogram plot was generated for the residual values to ensure the normality of our data when using parametric analysis.

The gene expression levels of PYY, CCK, GCG and INS were calculated using log10 gene ratios to the YWHAZ reference genes as used in similar gut tissue gene expression studies in chickens (Reid et al., 2017). The power of 10 was chosen for each gene according to the smallest number. So, CCK (102), GCG (102), PYY (103), INS (102) and GCG/INS ratio (103).

While NPY, POMC and AGRP were calculated using log10 gene ratios of the reference gene (LBR) (The choosing process is the same as YWHAZ). Similarly, to quantify CCK, GCG and POMC in the hindbrain we used LBR as a reference gene. It should be noted that the analysis was done after eliminating outliers. The outliers were identified as such: after doing log (gene/YWHAZ) for all individuals, occasional extreme values (products of technical errors) that were more than two standard deviations away from the mean and clearly lay outside the normal range of variation were termed outliers. Because technical errors are unavoidable, it was inappropriate to include those values in the analysis and this step is part of the standard quality control process we applied routinely. Statistical analysis was performed using the Generalized Estimating Equations function in the SPSS statistical package (IBM 25). To correct for post hoc test, we used Bonferroni correction method to account for the multiple comparison. Most of the times interactions between different factors were included in the analysis, while in few they were removed due to their non-significance. The rule to remove the non-significant interactions was (specifically the 3-way): in a stepwise fashion, interactions were removed if $p>0.1$. No 2-way interaction were removed if the 3-way interaction was

significant. Therefore, unless mentioned within the results, reader should assume that interactions were not included in the model due to their insignificance.

Two groups of individuals were compared based on gizzard fullness (full versus empty) rather than fed or fasted feeding condition because one bird had an empty gizzard when sampled during the day. So, in the end our sample size was as follows: 6 fasted females, 4 fed females, 3 fasted males, 3 fed males.

2.3 Results

2.3.1 Body mass

Body mass recorded immediately before dissection did not differ significantly between males and females (female, mean=13.63±2.42, males, mean=12.28±3.12, $\chi 2$ (1) = 0.084, p=0.772). There was also no main feeding effect on the body mass (empty gizzard, mean=13.08±2.75, full, mean=13.03±2.75, $\chi 2$ (2) = 0.0, p=0.990).

2.3.2 Housekeeping gene testing

Hypothalamus LBR

Neither sex (females, mean= 9.31E-015 ± 1.03E-15, males, mean= 8.56E-015± 1.33E-15, □2(1) =0.192, p= 0.661), nor gizzard fullness (empty, mean= 9.39E-015 ± 1.19E-15, full, mean= 8.48E-015± 1.19E-16, □2(1) =0.287, p= 0.592) had a significant effect on LBR gene expression in the hypothalamus. Additionally, there was no significant interaction between sex and gizzard fullness (□2(1) =3.16, p= 0.075).

Hindbrain LBR

Neither sex (females, mean= 1.74E-014 ± 1.16E-15, males, mean= 1.41E-014± 1.1.50E-15, □2(1) =3.07, p= 0.080), nor gizzard fullness (empty, mean= 1.51E-014 ± 1.34E-15, full, mean= 1.65E-014± 1.34E-15, □2(1) =0.515, p= 0.473) had a significant effect on LBR gene expression in the hindbrain.

Additionally, there was no significant interaction between sex and gizzard fullness (□2(1) =0.695, p= 0.405).

Gut peptide YWHAZ

Main effect

Neither sex (females, mean= 6.90 ± 0.07, males, mean= 6.69± 0.07, □2(1) =3.74, p= 0.053), nor gizzard fullness (empty, mean= 6.84 ± 0.07, full, mean= 6.76± 0.07, □2(1) =0.535, p= 0.464) had a significant effect on YWHAZ gene expression. However, there was a significant difference in YWHAZ gene expression between the different gut tissues (□2(9) = 26.98, p=0.001) (Figure 2).

Interactions

There was neither a significant interaction between sex and gizzard fullness (□2(1) =0.239, p= 0.625), nor sex with tissue (□2(9) =9.46, p=0.396). Also, no significant interaction between gizzard fullness and tissue was detected (□2(9) =14.06, p=0.120). Finally, no significant 3-way interaction was observed (□2(9) =3.75, p=0.927).

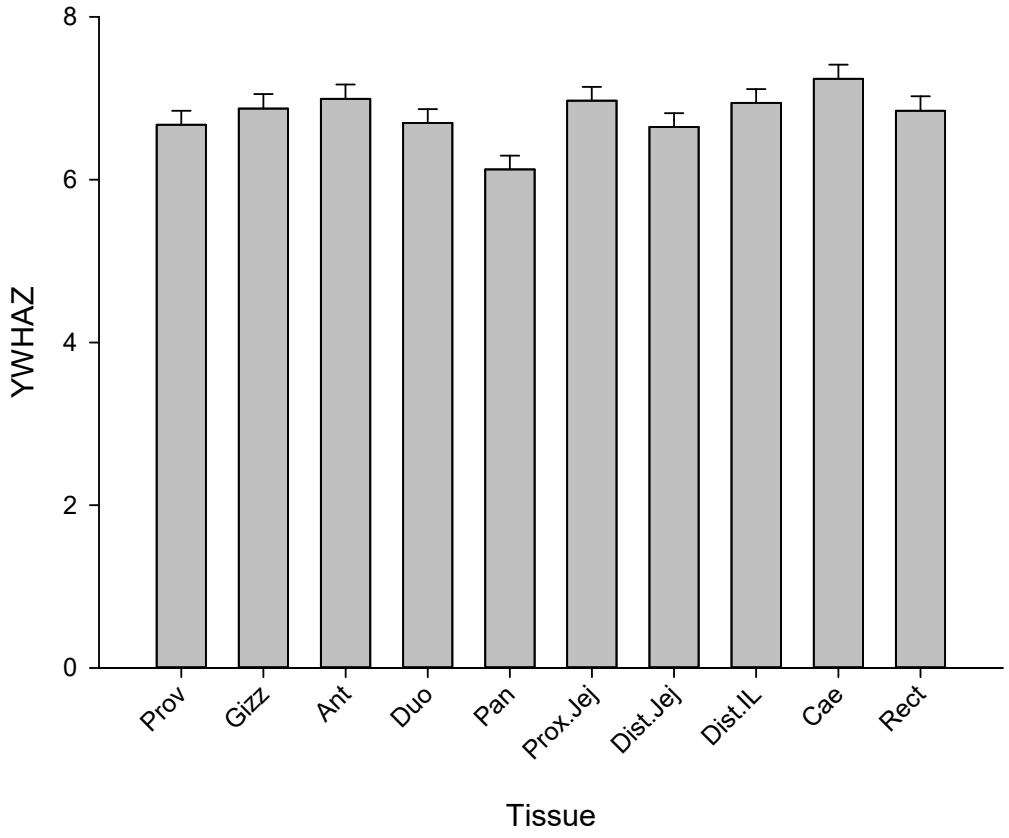


Figure 2: Pairwise comparison of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta ***YWHAZ housekeeping gene across different gut tissues. Each bar represent mean ±SEM.***

Table 2: Pairwise comparison of YWHAZ gene expression. Refer to the glossary for abbreviation. Green rectangles represent non-significant p-value

	Prov	Gizz	Ant	Duo	Pan	Prox.Jej	Dist.Jej	Dist. IL	Cae	Rect
Prov		p=1.000	p=1.000	p=1.000	p=0.981	p=1.000	p=1.000	p=1.000	p=0.940	p=1.000
Gizz	p=1.000		p=1.000	p=1.000	p=0.113	p=1.000	p=1.000	p=1.000	p=1.000	p=1.000
Ant	p=1.000	p=1.000		p=1.000	p=0.016	p=1.000	p=1.000	p=1.000	p=1.000	p=1.000
Duo	p=1.000	p=1.000	p=1.000		p=0.763	p=1.000	p=1.000	p=1.000	p=1.000	p=1.000
Pan	p=0.981	p=0.113	p=0.016	p=0.763		p=0.019	p=1.000	p=0.029	p<0.001	p=162
Prox.Jej	p=1.000	p=1.000	p=1.000	p=1.000	p=0.019		p=1.000	p=1.000	p=1.000	p=1.000
Dist.Jej	p=1.000	p=1.000	p=1.000	p=1.000	p=1.000	p=1.000		p=1.000	p=0.680	p=1.000
Dist. IL	p=1.000	p=1.000	p=1.000	p=1.000	p=0.029	p=1.000	p=1.000		p=1.000	p=1.000
Cae	p=0.940	p=1.000	p=1.000	p=1.000	p<0.001	p=1.000	p=0.680	p=1.000		p=1.000
Rect	p=1.000	p=1.000	p=1.000	p=1.000	p=0.162	p=1.000	p=1.000	p=1.000	p=1.000	

Look at the appendix page 228 to look at the stepwise deletion for each neuropeptide and gut peptide.

2.3.3 Neuropeptides

2.3.3.1 Hypothalamus

In the hypothalamus, we quantified the expression of the arcuate nucleus neuropeptide genes NPY, AgRP and POMC.

There was no significant difference in NPY levels between males and females (females, mean= 5.12 ± 0.09, males, mean= 4.94± 0.11, □2(1) =1.42, p= 0.233). Similarly, NPY levels were not affected by gizzard fullness (empty, mean= 4.99 ± 0.10, full, mean= 5.06± 0.10, □2(1) =0.205, p= 0.651) (Figure 3-A). AgRP gene expression was not different between males and females (females, mean= 1.55 ± 0.11, males, mean= 1.23± 0.14, □2(1) =3.10, p= 0.078). Gizzard fullness did not affect AgRP levels (empty, mean= 1.46 ± 0.12, full, mean= 1.32± 0.12, □2(1) =0.575, p= 0.448) (Figure 3-B). Sex did not interact significantly with gizzard fullness (□2(1) =2.44, p= 0.118).

The expression levels of hypothalamic POMC were not affected by sex (females, mean= 1.92 ± 0.11, males, mean= 1.65± 0.14, □2(1) =2.25, p= 0.133). In addition, POMC levels were not different between empty and full individuals (empty, mean= 1.78± 0.12, full, mean= 1.79± 0.12, □2(1) =0.002, p= 0.967) (Figure 3-C).

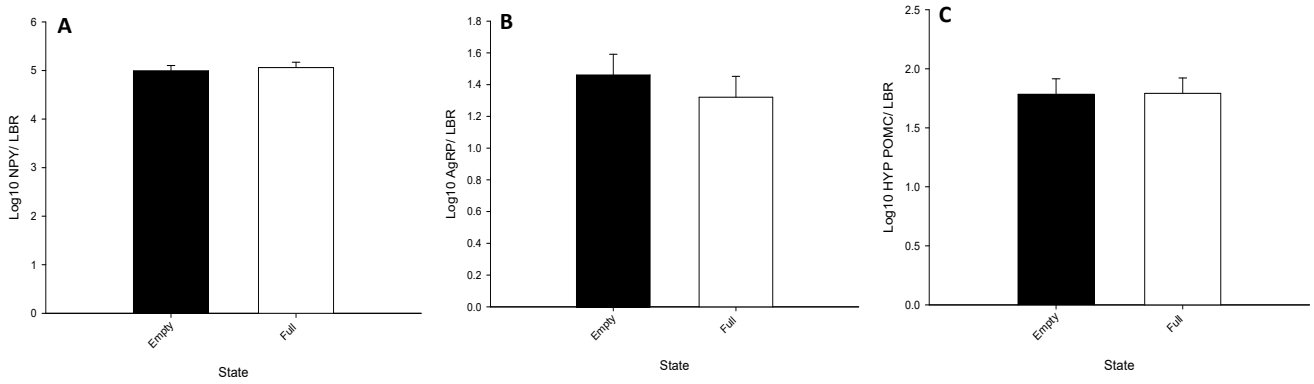


Figure 3: Comparison between the expressions of neuropeptide Y (NPY) (A), agouti-related protein (AgRP) (B) and pro-opiomelanocortin (POMC) (C) in the hypothalamus. Values are presented as log10 ratio to lamin B receptor (LBR) using gizzard fullness as a variable. Empty (black bars), full (white bars). Each bar shows mean ± SEM.

Both AgRP and POMC producing neurons are located in distinct non-overlapping area within the ARC and compete for the same receptor. Studies in rats had shown that an elevation in AgRP/POMC ratio was detected in ad lib and pair-fed groups showcasing the importance of the ratio as a crucial stimulator for the central melanocortin signalling pathway regulating and controlling the appetite system (Korner et al. 2003). Thus, we examined the ratio between those hypothalamic neuropeptides to investigate whether similar increase in the ratio would be detected in fed individuals. The ratio was not significantly different between males and females (females, mean= 3.61 ± 0.16, males, mean= 3.43± 0.21, □2(1) =0.442, p= 0.506). Gizzard fullness did not affect the expression of AgRP/ HYP POMC (empty, mean= 3.69± 0.19, full, mean= 3.35± 0.19, □2(1) =1.43, p= 0.231) (Figure 4). However, there was a significant interaction between sex and gizzard fullness (□2(1) =5.18, p= 0.023) but Bonferroni-corrected post-hoc test did not indicate effect of gizzard fullness in either sex

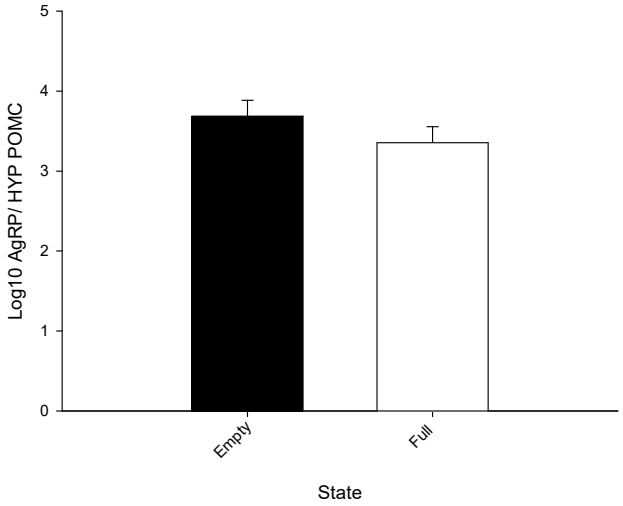


Figure 4: Pairwise comparison showing the expression ratio between agouti-related protein (AgRP) and pro-opiomelanocortin (POMC) (AgRP/ POMC) in the hypothalamus as log10 sing gizzard fullness as a variable. Empty (black bars), full (white bars). Each bar shows mean ± SEM.

2.3.3.2 Hindbrain

In the hindbrain, there was no significant difference in CCK levels between males and females (females, mean= 2.34 ± 0.11, males, mean= 2.22± 0.14, □2(1) =0.466, p= 0.495). Gizzard fullness did not affect the expression of CCK in hindbrain (empty, mean= 2.16± 0.12, full, mean= 2.40± 0.12, □2(1) =1.90, p= 0.168) (Figure 5-A).

The expression of GCG gene was not different between males and females (females, mean= 2.91 ± 0.13, males, mean= 2.75± 0.14, □2(1) =0.601, p= 0.438). Gizzard fullness did not affect the expression of GCG in hindbrain (empty, mean= 2.80± 0.14, full, mean= 2.86± 0.14, □2(1) =0.110, p= 0.741) (Figure 5-B).

The levels of POMC in the hindbrain was not different between males and females (females, mean= 2.57 ± 0.14, males, mean= 2.60± 0.18, □2(1) =0.012, p= 0.911). POMC gene expression did not significantly differ between empty and full individuals (empty, mean= 2.80± 0.16, full, mean= 2.37± 0.16, □2(1) =3.21, p= 0.073) (Figure 5-C). Sex did not interact significantly with gizzard fullness (□2(1) =2.78, p= 0.095).

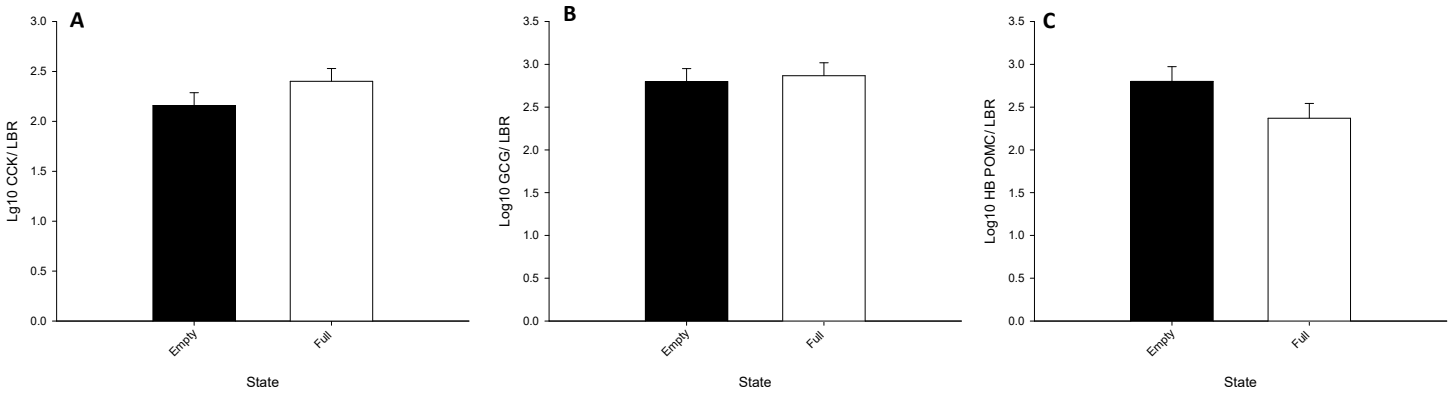


Figure 5: Comparison between the expression of cholecystokinin (CCK) (A), glucagon (GCG) (B) and pro-opiomelanocortin (POMC) (C) in the hindbrain. Values are presented as log10 ratio to lamin B receptor (LBR) using gizzard fullness as variable: empty (black bars), full (white bars). Each bar shows mean ± SEM.

2.3.4 Gut peptides

In total, ten gut tissues were analysed per individual to quantify the expression of the following genes: CCK, GCG, PYY and INS along with the GCG/INS ratio

2.3.4.1 CCK

Main effect

Neither sex (females, mean= 1.00 ± 0.10, males, mean= 1.08± 0.03, □2(1) =0.434, p= 0.510), nor gizzard fullness (empty, mean= 1.10± 0.09, full, mean= 0.97± 0.06, □2(1) =1.31, p= 0.252) had a significant effect on CCK levels. However, CCK gene expression was significantly different between the gut tissues (Figure 4, Table 1) (□2(9) =2.55E+10, p<0.001) mainly the antrum, caecum, distal jejunum and ileum, duodenum and proximal jejunum.

Interactions

There was no significant interaction between sex and gizzard fullness (□2(1) =1.22, p=0.269). However, sex interacted significantly with tissue (□2(9) =7779371258, p<0.001) mostly seen in both the duodenum and the rectum where males showed higher CCK in both tissues in comparison to females (Duodenum; females, mean= 1.06 ± 0.18, males, mean= 1.49± 0.08, p= 0.035; Rectum; females, mean= 0.34 ± 0.06, males, mean= 1.11± 0.33, p= 0.024). Tissue also interacted significantly with gizzard fullness (□2(9) =78.24, p<0.001) (Figure 6). However, Bonferroni-corrected analysis did not indicate any effect of the state among the different tissues. There was a significant 3-way interaction (□2(9) =111.04, p<0.001) due to the proventriculus and the caecum of males: while the full state proventriculus showed higher CCK than when empty (empty, mean= 0.16± 0.02, full, mean= 0.35± 0.08, p= 0.035), empty state caecum expressed more CCK in comparison to the full state (empty, mean= 1.33± 0.05, full, mean= 1.14± 0.02, p= 0.003). On the other hand, females did not show any difference between full and empty states in CCK levels in both tissues

(Proventriculus; empty, mean= 0.16± 0.02, full, mean= 0.94± 0.54, p= 0.149; Caecum; empty, mean= 1.11± 0.14, full, mean= 1.14± 0.32, p= 0.940).

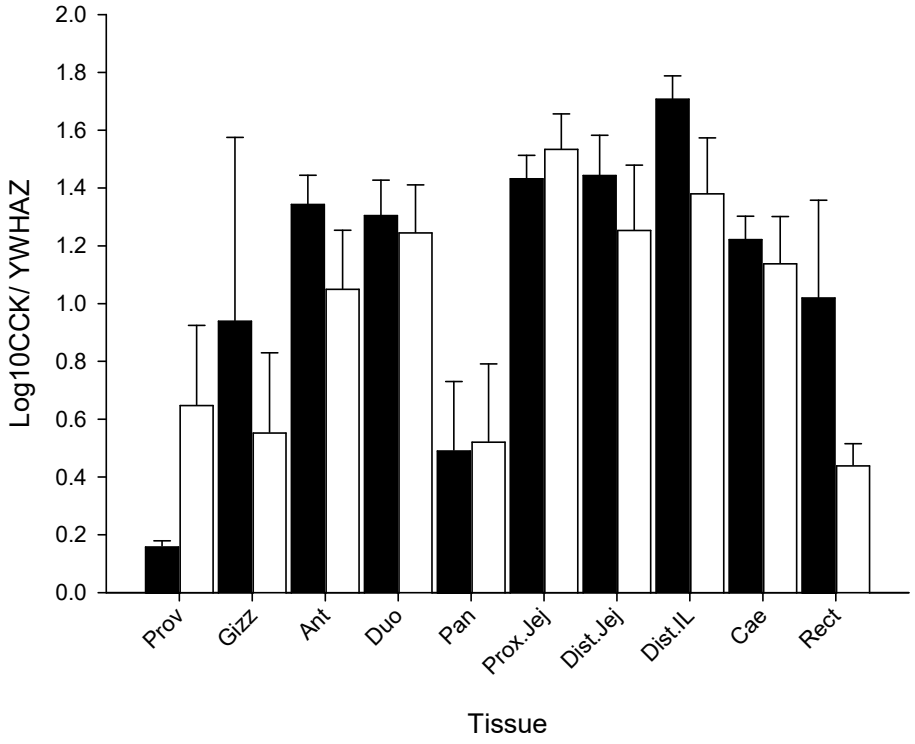


Figure 6: Pairwise comparison of cholecystokinin (CCK) gene expression across different gut tissues in as log10 ratio to tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) using Gizzard fullness as a variable. Refer to the glossary for the abbreviation. Black bars represent empty gizzard while white ones show full gizzard state. Each bar represent mean ± SEM.

Table 3: Pairwise comparison of cholecystokinin (CCK) gene expression in the different tissues. Tissues are arranged in order of dissection. Refer to the glossary for the abbreviation. Green colour rectangles show non-significant p value.

	Prov	Gizz	Ant	Duo	Pan	Prox.Jej	Distal.Jej	Distal.IL	Cae	Rect
Prov		p=0.288	p<0.001	p<0.001	p=0.418	p<0.001	p<0.001	p<0.001	p<0.001	p=0.133
Gizz	p=0.288		p=0.208	p=0.133	p=0.479	p=0.032	p=0.098	p=0.030	p=0.182	p=0.965
Ant	p<0.001	p=0.208		p=0.521	p=0.002	p=0.010	p=0.265	p=0.016	p=0.918	p=0.029
Duo	p<0.001	p=0.133	p=0.521		p=0.004	p=0.163	p=0.095	p<0.001	p=0.587	p=0.009
Pan	p=0.418	p=0.479	p=0.002	p=0.004		p<0.001	p=0.004	p<0.001	p<0.001	p=0.384
Prox.Jej	p<0.001	p=0.032	p=0.010	p=0.163	p<0.001		p=0.444	p=0.697	p=0.001	p<0.001
Distal.Jej	p<0.001	p=0.098	p=0.265	p=0.095	p=0.004	p=0.444		p=0.033	p=0.396	p=0.004
Distal.IL	p<0.001	p=0.030	p=0.016	p<0.001	p<0.001	p=0.697	p=0.033		p=0.053	p<0.001
Cae	p<0.001	p=0.182	p=0.918	p=0.587	p<0.001	p=0.001	p=0.396	p=0.053		p=0.018
Rect	p=0.133	p=0.965	p=0.029	p=0.009	p=0.384	p=0.018	p=0.004	p<0.001	p=0.018	

2.3.4.2 GCG

Main effect

Neither sex (females, mean= 1.55 ± 0.04, males, mean= 1.52± 0.04, □2(1) =0.220, p= 0.639), nor gizzard fullness (empty, mean= 1.56± 0.03, full, mean= 1.51± 0.15, □2(1) =0.644, p= 0.422) had a significant effect on GCG levels. However, GCG gene expression was significantly different between the gut tissues (□2(9) =5756816.35, p<0.001) with the pancreas having the highest levels.

Interactions

Sex did not interact significantly with gizzard fullness (□2(1) =0.009, p=0.924). On the other, sex had a significant interaction with tissue (□2(9) =11977.55, p<0.001) but Bonferroni-corrected post-hoc test did not indicate any sex difference among the tissues. There was a significant interaction between tissue and gizzard fullness (□2(9) =3.29E+11, p<0.001). However, Bonferroni-corrected analysis did not indicate any effect of the state among the different tissues (Figure 7). Finally, there was a significant 3-way interaction (□2(9) =2.05E+11, p<0.001). While in females empty state duodenum showed higher GCG levels in comparison to the full state (empty, mean= 1.82± 0.08, full, mean= 1.55± 0.10, p= 0.044), males expressed higher GCG in their antrum when they were in full state in comparison to empty state (empty, mean= 2.01± 0.27, full, mean= 2.70± 0.14, p= 0.027). On the other hand, the expression of GCG was neither different in the duodenum of males (empty, mean= 1.46± 0.07, full, mean= 1.74± 0.19, p= 0.171), nor the antrum of females (empty, mean= 1.97± 0.29, full, mean= 2.00± 0.34, p= 0.942).

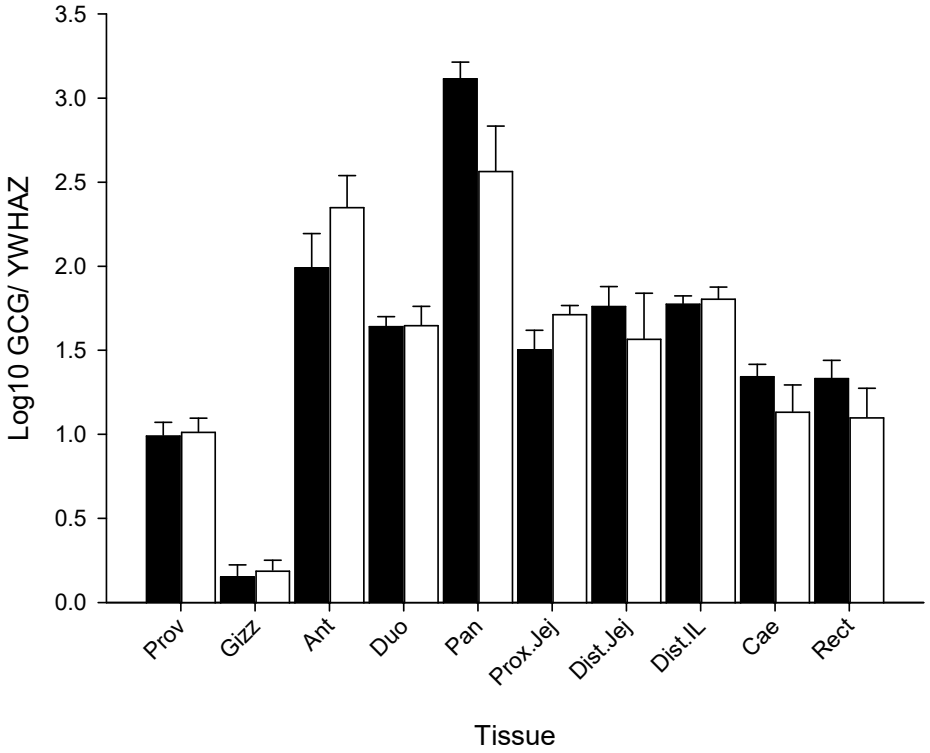


Figure 7: Pairwise comparison of preproglucagon (GCG) gene expression across different gut tissues as log10 ratio to tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) using Gizzard fullness as a variable. Refer to the glossary for the abbreviation. Black bars represent empty gizzard while white ones show full gizzard state. Refer to the glossary for the abbreviation. Each bar represent mean ± SEM.

Table 4: Pairwise comparison of preproglucagon (GCG) gene expression in the different tissues. Refer to the glossary for the abbreviation. Green colour rectangles show non-significant p value.

	Prov	Gizz	Ant	Duo	Pan	Prox.Jej	Distal.Jej	Distal.IL	Cae	Rect
Prov		p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p=0.010	p=0.099
Gizz	p<0.001		p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
Ant	p<0.001	p<0.001		p<0.001	p=0.002	p<0.001	p=0.041	p=0.001	p<0.001	p<0.001
Duo	p<0.001	p<0.001	p<0.001		p<0.001	p=0.598	p=0.922	p=0.025	p<0.001	p=0.004
Pan	p<0.001	p<0.001	p=0.002	p<0.001		p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
Prox.Jej	p<0.001	p<0.001	p<0.001	p=0.598	p<0.001		p=0.717	p=0.014	p<0.001	p=0.004
Distal.Jej	p<0.001	p<0.001	p=0.041	p=0.922	p<0.001	p=0.717		p=0.466	p=0.036	p=0.004
Distal.IL	p<0.001	p<0.001	p=0.001	p=0.025	p<0.001	p=0.014	p=0.466		p<0.001	p<0.001
Cae	p=0.010	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p=0.036	p<0.001		p<0.001
Rect	p=0.099	p<0.001	p<0.001	p=0.004	p<0.001	p=0.004	p=0.004	p<0.001	p=0.854	

2.3.4.3 PYY

Main effects

Females had a higher PYY expression level in comparison to males (females, mean= 0.95 ± 0.06, males, mean= 0.43± 0.04, $\chi^2(1) = 49.86$, $p<0.001$). Similarly, PYY levels were significantly different between the different gut tissues ($\chi^2(9) = 12143.16$, $p<0.001$) with the antrum, duodenum, pancreas and the proximal jejunum having the highest levels. On the other hand, the nutritional state of the individual did not affect PYY gene expression (empty, mean= 0.66± 0.04, full, mean= 0.72± 0.05, $\chi^2(1) = 0.721$, $p= 0.396$).

Interactions

Sex had no significant interaction with gizzard fullness ($\chi^2(1) = 0.512$, $p=0.474$). On the other hand, sex interacted significantly with tissue ($\chi^2(9) = 1.45E+12$, $p<0.001$) where females showed higher PYY levels in comparison to males in the following tissues (Table 3):

Table 5: pairwise comparison of peptide YY (PYY) gene expression differences between males and females across different tissues.

Tissue	Females: Mean ± Standard deviation	Males: Mean ± Standard deviation	P-value
Antrum	1.44±0.15	0.71±0.07	P<0.001
Caecum	0.20±0.04	0.01±0.00	P<0.001
Distal jejunum	1.43±0.10	0.38±0.10	P<0.001
Distal ileum	0.37±0.07	0.02±0.01	P<0.001
Duodenum	1.85±0.23	0.91±0.11	P<0.001
Pancreas	1.75±0.05	0.86±0.00	P<0.001
Proventriculus	0.62±0.11	0.23±0.14	P=0.033
Proximal jejunum	1.55±10	0.98±0.13	P<0.001

Tissue also interacted significantly with gizzard fullness ($\chi^2(9) = 3.86E+12$, $p<0.001$). However, Bonferroni-corrected analysis did not indicate any effect of the state among the different tissues (Figure 8). There was also a significant 3-way interaction ($\chi^2(9) = 34.70$, $p<0.001$). But Bonferroni-corrected test did not indicate an effect of gizzard fullness on the sex*tissue interaction.

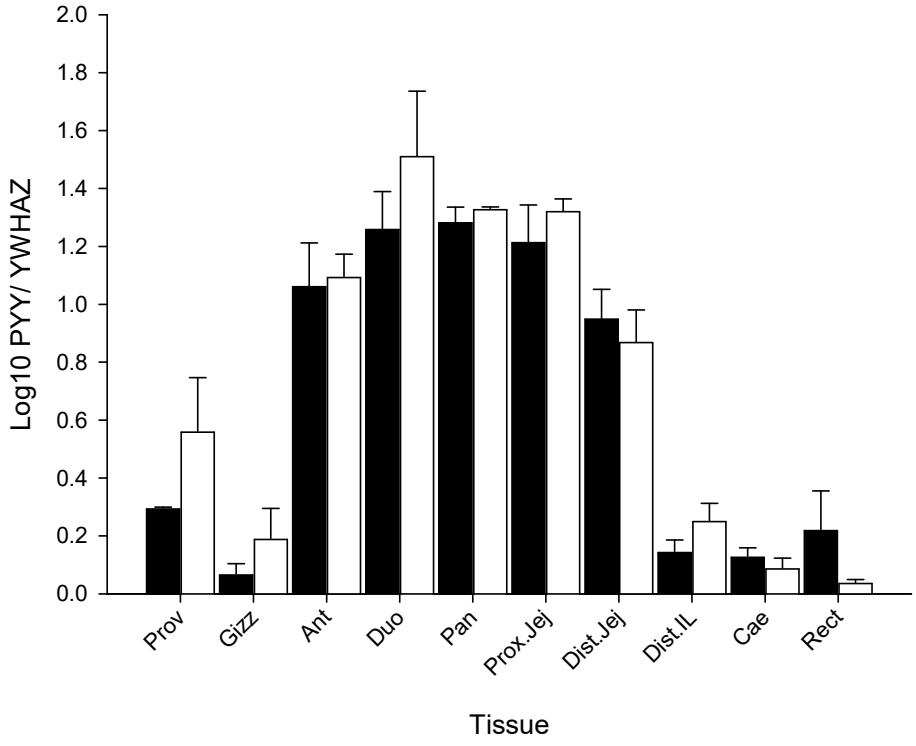


Figure 8: Pairwise comparison of peptide YY (PYY) gene expression as log10 ratio to tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) using gizzard fullness as a variable. ***Refer to the glossary for the abbreviation.*** **Each bar represent mean ± SEM.**

Table 5: Pairwise comparison of peptide YY (PYY) gene expression in the different tissues. Tissues are arranged in order of dissection. Refer to glossary for abbreviation. Green colour rectangles show non-significant p value.

	Prov	Gizz	Ant	Duo	Pan	Prox.Jej	Distal.Jej	Distal.IL	Cae	Rect
Prov		p=0.009	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p=0.004	p<0.001	p=0.008
Gizz	p=0.009		p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p=0.366	p=0.689	p=0.993
Ant	p<0.001	p<0.001		p=0.009	p=0.011	p<0.001	p=0.024	p<0.001	p<0.001	p<0.001
Duo	p<0.001	p<0.001	p=0.009		p=0.540	p=0.221	p<0.001	p<0.001	p<0.001	p<0.001
Pan	p<0.001	p<0.001	p=0.011	p=0.540		p=0.601	p<0.001	p<0.001	p<0.001	p<0.001
Prox.Jej	p<0.001	p<0.001	p<0.001	p=0.221	p=0.601		p<0.001	p<0.001	p<0.001	p<0.001
Distal.Jej	p<0.001	p<0.001	p=0.024	p<0.001	p<0.001	p<0.001		p<0.001	p<0.001	p<0.001
Distal.IL	p=0.004	p=0.366	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001		p=0.067	p=0.367
Cae	p<0.001	p=0.689	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p=0.067		p=0.756
Rect	p=0.008	p=0.993	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p=0.367	p=0.756	

2.3.4.4 INS

Main effect

Neither sex (females, mean= 3.41 ± 0.14, males, mean= 3.46± 0.10, □2(1) =0.077, p= 0.781), nor gizzard fullness (empty, mean= 3.47± 0.09, full, mean= 3.40± 0.15, □2(1) =0.115, p= 0.735) had a significant effect on INS levels. However, INS gene expression was significantly different between the gut tissues (□2(9) =2.04E+13, p<0.001) with the pancreas having the highest levels.

Interaction

Sex had no significant interaction with gizzard fullness (□2(1) =0.020, p=0.888). On the other hand, sex interacted significantly with tissue (□2(9) =2.80E+11, p<0.001), however Bonferroni-corrected post-hoc test did not indicate any sex difference across the tissues. There was a significant interaction between tissue and gizzard fullness (□2(9) =2.72E+11, p<0.001) clearly seen in both the (empty, mean= 3.35± 0.43, full, mean= 2.36± 0.22, p= 0.045) and distal ileum (empty, mean= 3.68± 0.27, full, mean= 2.71± 0.33, p= 0.025). On the other hand, full state antrum showed higher INS levels in comparison to empty state (empty, mean= 3.77± 0.61, full, mean= 5.46± 0.19, p= 0.009) (Figure 9).

Finally, there was a significant 3-way interaction (□2(9) =4.49E+12, p<0.001) mostly clear in males where INS levels were higher in full state antrum (empty, mean= 3.62± 0.90, full, mean= 5.76± 0.13, p= 0.019) and duodenum (empty, mean= 1.82± 0.42, full, mean= 3.57± 0.52, p= 0.010). On the other hand, male's empty distal ileum had higher INS levels than full one (empty, mean= 4.13± 0.24, full, mean= 1.67± 0.61, p<0.001). Females did not show difference in INS expression levels in those tissues: (Antrum; empty, mean=

3.92± 0.82, full, mean= 5.16± 0.36, p= 0.169; Distal ileum; empty, mean= 3.24± 0.49, full, mean= 3.75± 0.27, p= 0.368; Duodenum; empty, mean= 4.13± 0.90, full, mean= 3.31± 0.95, p= 0.534).

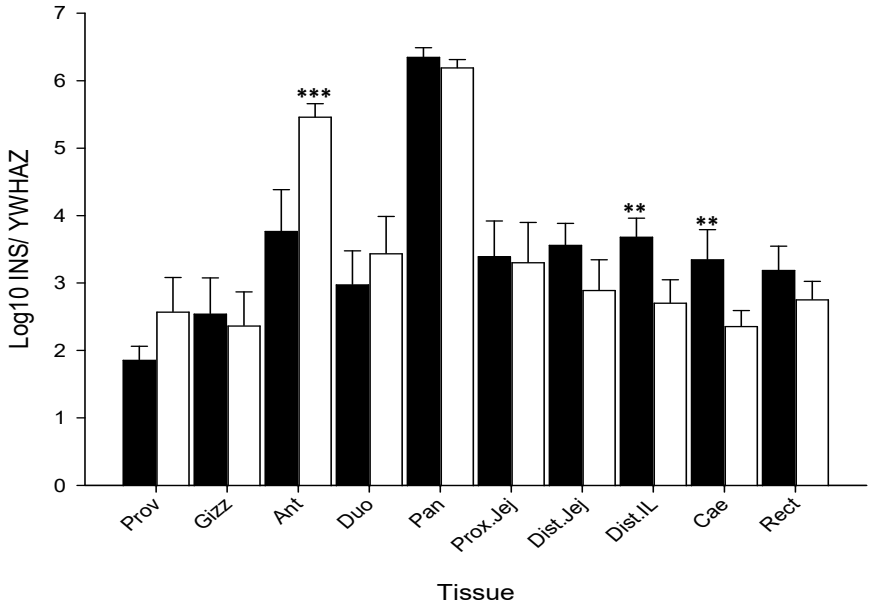


Figure 9: Pairwise comparison of insulin (INS) gene expression across different gut tissues as log10 ratio to YWHAZ using gizzard fullness as a variable. Black bars represent empty gizzard while white ones show full gizzard state. Refer to the glossary for the abbreviation. The asterisk represents significant difference between the two species ((*) p<0.05, (**) p<0.01 (***) p<0.001). Each bar represent mean ± SEM.

Table 6: Pairwise comparison of insulin (INS) gene expression in the different tissues. Refer to glossary for abbreviation. Green colour rectangles show non-significant p value.

	Prov	Gizz	Ant	Duo	Pan	Prox.Jej	Distal.Jej	Distal.IL	Cae	Rect
Prov		p=0.520	p<0.001	p=0.066	p<0.001	p=0.040	p=0.006	p=0.024	p=0.132	p=0.004
Gizz	p=0.520		p<0.001	p=0.230	p<0.001	p=0.211	p=0.123	p=0.162	p=0.265	p=0.177
Ant	p<0.001	p<0.001		p<0.001	p<0.001	p=0.016	p<0.001	p<0.001	p<0.001	p<0.001
Duo	p=0.066	p=0.230	p<0.001		p<0.001	p=0.704	p=0.952	p=0.959	p=0.396	p=0.590
Pan	p<0.001	p<0.001	p<0.001	p<0.001		p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
Prox.Jej	p=0.040	p=0.211	p=0.016	p=0.704	p<0.001		p=0.736	p=0.630	p=0.325	p=0.416
Distal.Jej	p=0.006	p=0.123	p<0.001	p=0.952	p<0.001	p=0.736		p=0.921	p=0.343	p=0.496
Distal.IL	p=0.024	p=0.162	p<0.001	p=0.959	p<0.001	p=0.630	p=0.921		p=0.325	p=0.526
Cae	p=0.132	p=0.265	p<0.001	p=0.396	p<0.001	p=0.325	p=0.343	p=0.325		p=0.645
Rect	p=0.004	p=0.177	p=<0.001	p=0.590	p<0.001	p=0.416	p=0.496	p=0.526	p=0.645	

2.3.3.5 GCG / INS ratio in the pancreas only

The ratio was not significantly difference between males and females (females, mean= 0.80 ± 0.10, males, mean= 0.85± 0.13, □2(1) =0.066, p= 0.797). Gizzard fullness did not affect the GCG/INS expression (empty, mean= 0.84± 0.10, full, mean= 0.81± 0.13, □2(1) =0.039, p= 0.843).

2.4 Discussion

As a part of numerous efforts to pinpoint peptides that are responsible to transduce and translate the different signals that report both the nutritional state and energetic needs from and to the brain, we have investigated a group of gut and neuropeptides profiles that have been studied extensively in the literature and were proven to change according to the state of the individual (fed or fasted). To our knowledge, this study is the first to explore and report tissue distribution as well as the effect of gut fullness on the gene expression of gut peptides in small wild songbirds. Although in general no effect of nutritional state was detected on gene expression, different gut tissues showed a clear pattern in tissue distribution of the different peptides. However, no particular tissue showed a difference in gene expression between the two-treatment group.

In our experiment, we recorded the body mass of individuals immediately before dissection and noticed that overall, the body mass of great tits was not significantly affected by the nutritional status of the individuals. The reasoning behind this might likely be because there was variability in the body mass which did not allow us to detect the little bit extra mass due to the gizzard content. As for the fat reserves, there may not have been sufficient time during the day to cause them to increase. However, we did not record fat scores in this experiment. According to Moiron et al. (2018) the diurnal mass gain in wintering great tits in the field was the highest in the hours after dawn and declined slowly over the course of the day with very little mass gained during the second half of the day. In that Moiron et al. (2018) study, great tits showed an increase in foraging in the morning then decreased their feeding throughout the day only to terminate their feeding at sunset. According to the proposed pattern, we could predict that the initial increase in body mass is due to the early feeding immediately after lights-on (when the energy depots are depleted due to overnight fasts) and the termination of feeding before sunset and under daylight conditions might imply that the bird reached its satiation threshold (Houston and McNamara 1993). If we were to explain the variability, we observed in our study according to Morion et al's (2018) explanation they proposed that the individual variability in body mass could be due to differences in the feeding activities. This means that each individual would forage according to their own metabolic needs.

2.4.1 Neuropeptides

Hypothalamus

We investigated the gene expression of selected hypothalamic peptides that are known to change their expression levels according to the energetic status and nutrient demands of individuals. Overall, we did not observe any significant difference in the expression of NPY, AGRP and POMC in relation to the gizzard fullness. Previous studies by Boswell et al. (1999) demonstrated that NPY mRNA levels in the hypothalamus increased in food restricted broiler chickens in comparison to fed birds. McConn et al. (2019) showed that in 5-day old Japanese quail fasted for 3h-6h there was an increase in NPY and Y2 receptor subtype mRNA expression, which implies that this receptor is highly sensitive to fasting in Japanese quail. That effect mirrors findings by Boswell et al. (2002) where they demonstrated that a 24h fast increased NPY expression

not only in the ARC but also in the whole hypothalamus of quail thus increasing meal size. As for AGRP, Phillips-Singh et al (2003) demonstrated that in adult Japanese quail, a 24h fast led to an increase in AgRP mRNA levels. Also 24-48h fasting produced similar effects in broiler chicks (Fang et al. 2014). Caughey et al. (2018) showed that in 12-week-old chickens re-fed for 2 days after being food restricted had higher AgRP mRNA levels.

On the other hand, hypothalamic POMC expression shows more contradictory results in the literature. Some studies were not able to detect any differences in the POMC mRNA levels even after 24h-48h of food deprivation (Japanese quail, Phillips-Singh et al. 2003; and broiler chicken Song et al. 2012), which was in accordance with similar studies in mammals where no differences in POMC expression was noted (Adam et al. 2002). However, others reported a significant decrease in POMC expression levels in broiler chicks fasted for either 24-48h (Higgins et al. 2010). The variability in detecting changes in POMC mRNA and expression magnitude may reflect a limited importance of POMC regulatory control on energy balance (Boswell and Dunn 2017). Instead, the balance of signalling between AGRP and POMC during fasting appears to depend predominantly on the antagonism action of AGRP.

Collectively, the most likely explanation as to why we could not detect any significant effect of gut fullness on the expression of the hypothalamic neuropeptide is related mostly to body mass. Studies in which a change in expression in hypothalamic neuropeptides has been observed after fasting or food restriction are associated with a reduction in body mass. For example, in Japanese quail showing increased NPY mRNA after 24h fast, body mass was reduced in the fasted group by 12% (Boswell et al. 2002). It is therefore possible that the expression of the neuropeptides will only change if the body mass and fat stores fall below a particular threshold. So, since we were not able to detect differences in the body mass of great tits, the changes in gene expression may not have been triggered.

An alternative explanation might be related to circadian rhythms in neuropeptide expression. In our study, great tits were killed at different times of the day, so gene expression might be affected. Mishra et al. (2016) explored the daily rhythms of NPY in redheaded buntings to elucidate the involvement of daily NPY mRNA oscillation and its relation to physiology and behaviour. Similar to our experiment set up, the investigators did not food restrict the birds in any way; they kept them on an ad lib food regime and took samples at different time points. They were able to confirm the presence of a daily rhythm as well as tissue-specific patterns in NPY gene expression in both central and peripheral tissues. The daily peak of NPY mRNA in the hypothalamus was observed at the end of the light phase and it was lowered at the end of dark phase. If great tits show the same pattern, it is possible that NPY expression was increasing in birds we sampled four hours into the light phase and that this might have hidden increased NPY mRNA that might have been associated with an empty gut at the end of the dark phase.

Mercer et al. (2000) reported that both AgRP and POMC mRNA respond inversely to photoperiodic manipulation of seasonal appetite in the hypothalamus of Siberian hamsters. One would expect AgRP as an

orexigenic peptide to peak in situations when the animal is fasted and in the case of rodents it would be during the light phase, but that was not the case for Lu et al. (2002). They investigated the diurnal rhythm of AgRP in adult male Sprague Dawley rats housed in 12 light: 12 dark cycles. They found that AgRP levels showed a significant peak during the dark cycle when the animals are active, while it reaches a nadir during the day. The low diurnal expression of AGRP is in accordance with the overall feeding rhythms in rats, where it rises before the starting of their active phase peaking as the dark cycle reach its peak (Lu et al. 2003). This observation is contrary to the physiological pattern seen in AGRP/NPY neurons that are activated when the individual is fasting. This suggests the involvement of other factors other than the nutritional state of the animal when it comes to regulating the appetite system centrally.

One explanation might be in relation to adrenal glucocorticoids. It has been well established that glucocorticoids have an important role in the regulation of feeding. Laboratory rodent studies have shown that glucocorticoids exhibit a 24h diurnal secretion with levels highest between the beginning and termination of the active feeding period (Dallman et al. 1995). Green et al. (1992) demonstrated that exogenous glucocorticoids when administered centrally are able to stimulate feeding whereas adrenalectomy decreases food intake. The function of glucocorticoids is mediated through their binding to two steroid receptors mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) (Lu et al. 2002). The MRs are tonically activated by low levels of glucocorticoids, while GR require much higher levels of glucocorticoids, which normally are present in stress conditions (Tempel and Leibowitz 1994). The distribution of both MR and GR are distinct in the brain. In particular, the GR mRNA expression and binding sites are numerous in the Arc which happens to overlap with the AgRP neurons (Aronsson et al. 1988). Given all those facts, it is possible that the daily activity of AgRP neurons might be under glucocorticoid modulation. Going back to Lu et al. (2002), the drop in AgRP levels might be related to levels of glucocorticoids because both decreases happened around the same period (dark phase). Many studies in birds have documented the presence of a diurnal rhythm in the plasma concentration of glucocorticoids such as in Japanese quails (Boissin and Assenmacher 1970), white throated sparrows (Meier et al. 1978) and domestic chickens (Beuving and Vonder 1977), with a peak during the night period. Having said that, we did not measure glucocorticoids in the great tits so we could not be sure whether there is any relation between their plasma concentration and AgRP expression, but we can predict from other avian studies that the glucocorticoid levels are low just until before the birds wake up.

The expression of the hypothalamic neuropeptides was also not significantly different between male and female great tits. Studies in rodents have generally agreed that the central nervous system of rodents is different between the two sexes mostly due to the expression of gonadal steroid hormones during the differentiation stage (Phoenix et al. 1959). According to Acosta-Martinez et al. (2007) the individual's metabolic state is profoundly affected by its reproductive state. For example, oestrogen was found to reduce food intake via the ventromedial hypothalamus (Butera 2010). This effect is mediated by oestrogen receptors in POMC neurons (Xu et al. 2011). So, during ovulation when oestrogen is high, the binding of the hormone

to its receptors, which are expressed in the POMC neurons, would lead to the reduction of food intake. Thus, body weight is decreased in rats (Shimizu and Bray 1993). This effect is also seen in cattle (Imakawa et al., 1986), chacma baboons (Bielert and Busse, 1983) and rhesus monkeys (Kemnitz et al., 1989). Having said that, although overall, food deprivation resulted in increased food consumption in both male and female rats (Wang et al. 2006), research showed that after 12h of fasting, females showed a greater increase in food intake than males, and that increased food intake is mostly due to the fact that food deprivation induced an elevation in plasma ghrelin levels in females more than males (Gayle et al. 2006).

On the other hand, a study in 12-week-old chickens reported that overall, males tended to express more *NPY*, *AgRP* and *POMC* in comparison to females, and according to the authors those higher levels in male adult chickens add more evidence to the idea that the expression levels of neuropeptides in a growing chicken's hypothalamus is a good indication of the bird's growth potential (Caughey et al. 2018). However, in our study the male great tits were already adults and are not growing anymore, so we rule out the possibility of growth as a factor controlling the hypothalamic neuropeptide expression.

Overall, not seeing differences in gene expression in our study might be due to the smaller sample size or the great variation in hypothalamic peptide levels between the individuals.

Hindbrain

We explored GCG mRNA in the hindbrain of great tits and found that an individual's nutritional state did not change the expression of GCG. GLP-1 immunoreactive perikarya were found in chicken NTS (Tachibana et al. 2005) supporting the idea that GCG expression we observed in the hindbrain reflects synthesis of GLP-1. Our findings in great tits differ from the observations found in the literature. Tachibana et al. (2005) demonstrated that GCG mRNA levels decreased in the brain stem of chicks after 24h of fasting. Next, we investigated CCK expression pattern in the hindbrain of great tits. CCK mrNA levels were not significantly different between males and females. Additionally, the bird's nutritional state did not affect CCK levels in the hindbrain. Reid and Dunn (2018) looked at CCK gene expression in the brain of chickens and concluded that it was highest in the basal hypothalamus. In our study, we could have measured CCK mRNA in the hypothalamus of great tits, but due to time constraints we were not able to do so. However, the significance of the hypothalamic CCK is yet to be understood, hence investigating its expression in the hindbrain was prioritised. But if we were to measure CCK mRNA in the hypothalamus, we expect to find higher levels because many immunoassay studies were able to detect higher CCK immunoreactivity in the hypothalamus (Benfield et al. 1981, Beinfeld and Palkovits 1981) reinforcing not only its vital role as a neuropeptide in birds but also is consistent with the broad distribution of the active CCK (Rehfeld 2017). We already know the CCK is not only present in the gut and acts to suppress appetite via the vagus nerve, but also is present in the brain as neurotransmitter and work as a satiety factor via its receptor CCK-A in the brain.

Finally, although much of our knowledge about the role of POMC neurons in energy balance comes mainly from studies on the POMC neural population that resides within the ARC of the hypothalamus (De Jonghe et al. 2012), according to Palkovits and Eskay (1987) POMC neurons are also highly expressed in the NTS of the hindbrain in laboratory rodents. In our study, POMC mRNA levels neither differed between males and females nor were affected by the nutritional state of individuals.

Perello et al. (2007) showed that in male Sprague-Dawley rats that were fasted for 65h, hindbrain (NTS) POMC expression was decreased, which suggests that fasting not only down-regulates POMC gene expression, but also decreases the biosynthesis of its protein in the hindbrain. We did not observe decreased hindbrain POMC mRNA after the overnight fast in our great tits but this is likely to be because the rodents were subjected to more extreme fasting periods than we used for our experiments. However, our observations of POMC gene expression in the hindbrain are the first that we are aware of in birds. This suggests that hindbrain POMC signalling may influence the regulation of feeding in birds as it does in mammals.

2.4.2 Gut peptides

Glucagon (GCG)

In our experiment, we generally found high levels of GCG mRNA in the pancreatic tissues of great tits in comparison to other gut tissues. Richards and McMurtry (2008) confirmed the presence of high expression levels of GCG mRNA in the pancreas of male broiler chickens. The similarity observed in the site of expression of GCG across these different species shows evolutionary conservation in both distribution and function of the GCG peptide as an energy supplier in demanding conditions.

Overall, we did not detect any significant effect of either nutritional state or sex on the expression of GCG in the pancreas of great tits. Nonetheless, the physiological role of glucagon has been well documented in poultry. For example, 3-week-old broiler chickens that were fasted either 24h or 48h showed a strong and significant increase in plasma glucagon, which then declined upon 24h of refeeding (Richards and McMurtry 2008). However, the findings of that study differ from ours in that their results showed only significant increase in plasma glucagon protein but no effect on GCG gene expression in the pancreas. Chen et al. (1989) reported that in rats the pancreatic expression of glucagon mRNA increased with a 96h fast. So, the increase in glucagon peptide is not always associated with an increase in GCG mRNA and there may be species differences in the relationship between gene transcription and protein secretion.

After the pancreas, the antrum, duodenum, distal ileum, proximal and distal jejunum, rectum, and caecum expressed GCG mRNA the most. In some of these tissues, we did observe effects of gizzard fullness in at least one of the sexes. In particular, females had higher GCG levels in the empty state duodenum, while males expressed higher levels of GCG in the full state antrum This observation is more likely to reflect the production of glucagon like peptide-1 (GLP-1) because the mRNA distribution matches the distribution of GLP-1 immunoreactivity in the intestine. The distribution pattern of GLP-1 seems to differ between

mammals and birds. While those GLP-1 cells are more densely clustered in the large intestine of rodents (Fridolf et al. 1991), the number of GLP-1 producing cells in the small intestine of chicken seems to be higher in more proximal regions, reaching a maximum in the distal ileum (Hiramatsu 2020). In mice, the distal colon and the rectum showed higher expression levels of GLP-1, while in rats, it is the distal ileum and in pigs, it is the caecum (Kuhre et al. 2014). Contrarily, GLP-1 immunoreactive cells were found in the mucosa of the jejunum and ileum of chickens and ostriches (Hiramatsu et al. 2003). The GLP-1 tissue distribution seen in our study is the first report in passerines and is indeed in agreement with what is found in other bird orders. Therefore, this difference in GLP-1 site of expression is more likely to indicate that proglucagon gene expression regulates digestion processes differently between mammals and birds (Hiramatsu 2020). The high expression of GCG in male great tits with full gizzards fits an established role of GLP-1 peptide in suppressing appetite in chickens (Furuse et al. 1997) and Japanese quail (Shousha et al. 2007). A study by Ronveaux et al. (2014) in male Wistar rats showed that GLP-1 supresses food intake in re-fed rats rather than the fasted ones. Similarly, Richards and McMurtry (2008) found higher plasma GLP-1 in refed chicks. However, the pattern seen in female great tits might reflect their higher sensitivity to the elevated glucose levels as result of food deprivation thus stimulating the production of insulin on one hand while depressing glucagon on the other (Smits et al. 2016).

Insulin

In the present study, insulin gene expression was highest in the pancreas of great tits. This result was not at all surprising considering the fact that insulin is a very well-known pancreatic hormone that is exclusively expressed in the beta cells of the islet of Langerhans that are strategically positioned to sense the nutritional state of the individual. Those islets form dense networks that are highly vascularised, receiving ~10 times the amount of blood than other exocrine regions (Fu et al. 2013). Insulin is released into the circulation principally in response to elevated glucose levels in birds and mammals (Simon and Rosselin 1978). Having said that, in our study, overall insulin mRNA levels did not change according to the nutritional state of the individual. We might have expected that insulin expression should be lower when individuals were food deprived because plasma insulin was found to be lower after fasting in chickens (Simon et al. 2011). However, in our study we did not measure the peptide, so it is difficult to make conclusions about how insulin transcription is related to the circulating peptide. Our study appears to be the first to measure insulin mRNA in birds in response to food deprivation.

Studies in rodents demonstrated the importance of insulin in the development of sex organs as early as before birth (Nef et al. 2003). For example, insulin receptors are required for the development of the testis in mice (Nef et al. 2003). On the other hand, although insulin receptors are present in the oocytes in females, their role seems to be the regulation of meiotic progression. Having said that, in general, it appears that the relationship between insulin and sex hormones is mostly that sex hormones enhance the respond of tissues to insulin (Ortiz-Huidobro et al. 2021). In our study, male insulin levels were higher in full state antrum and duodenum, and empty state distal ileum, with no difference observed in the same gut tissues of females in

either state. While the metabolic actions of insulin are more understood in the liver adipose tissue and skeletal muscles in relation glucose homeostasis, little is known about its function in other gut tissues. However, Desjeux et al. (1979) suggested that insulin secretion is regulated by hormonal and nervous signals arising from the duodenum. So, the stimulation of the villi of the duodenum would cause the secretion of insulin. Having said that, insulin stimulation and action vary between males and females mostly due to their specific hormonal profile as well as adiposity differences (Ortiz-Huidobro et al. 2021).

Because insulin and glucagon expression are linked in the pancreas, we measured the ratio between the two peptides mRNA expression levels. We demonstrated that the GCG/INS mRNA ratio was neither significantly different between the two sexes nor the two feeding states. Numerous studies have implicated a tight relationship between insulin and glucagon in the regulation of appetite and glucose metabolism. It is a very well-known fact that glucagon has the ability to stimulate insulin secretion, thus causing a rise in insulin levels (Song et al. 2017). For instance, it was found that the administration of glucagon substantially increased insulin levels with a peak concentration achieved within one minute in rats (Song et al. 2017). Samlos et al. (1966) showed that in normal humans, the administration of glucagon rapidly increased insulin levels independently of glucose via acting on the pancreatic β-cells. Similar observations were also found in chickens. Honey and Weir (1979) showed that in isolated perfused chicken pancreas-duodenum tissue, the infusion of 20,000 μU/ml of insulin caused a rapid suppression of glucagon secretion. All those studies imply that the higher levels of insulin mRNA seen in great tits might be a response to elevated glucose levels that are mainly induced by the high levels of glucagon in the empty state individuals. So, we could assume that glucagon can enhance prehepatic insulin secretion, thus the increased in circulating insulin achieved by glucagon causes the elevation of insulin secretion. The fact that insulin expression was higher in the empty state individuals in comparison to the full state in some tissues, meant that we expected the GCG/INS ratio to be higher in the empty state but that was not the case. The expression of GCG/INS ratio was not affected by the nutritional state.

One possibility for the lack of difference between the two nutritional state is the circadian rhythm. Little is known about the diurnal changes in insulin in birds, but the mammalian literature suggests that insulin is higher at the end of the inactive phase (Van Cauter et al. 1991). Assuming that this is the case in great tits, we hypothesis that insulin rhythmicity could mask the effect of an empty gizzard in reducing insulin expression. Studies have shown that the size of glucose and insulin response after a meal is dependent on the time of the day (Hara and Saito 1980). The rhythm of insulin secretion is mainly driven by the spike of blood glucose that is a consequence of feeding behaviour (Kalsbeek and Strubbe 1998). Limited numbers of overnight studies in fasted humans showed that insulin concentration/secretion is increased in a small but significant manner towards the end of the dark period (Bolli et al. 1984). Similar diurnal patterns were also observed in rats and mice (Qian and Scheer 2016). However, it should be noted that the situation is reversed in rodents since they are nocturnal animals. According to la Fleur et al. (2001), glucose uptake in rats showed a clear peak at the beginning of the dark period irrespective of their feeding regime. This increase in glucose

uptake coincides with the elevation of glucose concentration that occurs as the light period ends. The same situation occurs in human as well, where glucose production and concentration increase at the beginning of the activity period (equal to the dark period in rodents) (la Fleur et al. 2001). Therefore, the elevation of plasma glucose levels before the onset of an activity in general, is due to an increase in glucose production from glycogen stores and a drop in glucose utilisation. So, the peak in insulin production is directly related to an increase in glucose uptake over the circadian cycle. The speculation as to why a night-time fast cause increased insulin levels alongside satiety was proposed by (Sinha et al. 1996). He reported that a pronounced elevation in leptin concentration was reported in humans during the overnight fast thus causing appetite suppression during the sleeping period. Another explanation for the rise in insulin levels was interpreted as the increased demand for insulin in the early morning (Van Cauter et al. 1997). Linking those studies to our experiment, since we did not measure neither leptin levels nor glucose levels, we can only speculate that the higher insulin expression levels that were observed in the fasted state individuals is an indication of more release of glucose to supply energy. Thus, more insulin should be produced to counteract the elevation of glucose to promote more glucose uptake to clear the circulation.

It is worth also mentioning that insulin expression was unexpectedly high in the antrum which has not been observed before in either birds or mammals. However, according to (Ariyachet et al. 2016) the antrum and the stomach have a common origin in development and the cells in both tissues are more similar than expected in their patterns of gene expression. It is therefore possible that insulin could be synthesized in the antrum in birds. This has been documented in the green headed tanager where cells showing insulin like immunoreactivity was found in the antrum (Cardoso et al. 1999).

Peptide YY

In our study, great tits showed higher PYY expression levels mainly in the antrum, duodenum, pancreas and proximal jejunum. This is broadly consistent with recent observation of the distribution of PYY gene expression in chicken gut tissues where high levels were observed in the jejunum. For instance, Aoki et al. (2017) found that in chickens PYY is mainly distributed in the small intestine in comparison to the large intestine. Additionally, PYY like immunoreactive cells have also been observed in the duodenum and jejunum of chickens (El-Salhy 1982). The main difference between the distribution of PYY expression in our study and the chicken studies of Reid et al. (2017) and Gao et al. (2017) was the much lower PYY expression in the pancreas of great tits. In our study expression in the pancreas was similar to the levels in the small intestine whereas in the chickens it was the main site of PYY expression in the gut. This may reflect taxonomic and perhaps body size differences in metabolic regulation by PYY in the pancreas between chickens and great tits.

We next investigated the expression pattern of PYY in response to gizzard fullness. Overall, no gut tissue showed a significant effect of gizzard fullness on PYY mRNA gene expression. This is in contrast to work in chickens, where PYY expression levels in the small intestine were higher in fed individuals in comparison to

the ones under 12h fasting conditions (Aoki al. 2017). El-Salhy et al. (1982) reported higher PYY immunoreactive cells in the duodenum and jejunum of chickens. Aoki et al. (2017) also found that PYY levels in the jejunum increased as short as 2h after re-feeding, and that an intravenous administration of PYY decreased food intake in chickens in a dose dependent manner. Short-term (11h) fasting also reduced pancreatic PYY expression in chickens (Reid et al. 2017). So why did we not observe these patterns? We speculate that the lack of difference in PYY expression might be due to circadian rhythm. Although there is no information on the diurnal changes in PYY gene expression in mammals or birds, Moghadam et al. (2017) reported in rats that circulating PYY peptide levels were highest in the light phase (which is when rats are asleep). If the circadian pattern of PYY secretion is equivalent in birds, it would suggest that PYY expression may be higher at night. If so, the lack of difference could be due to higher expression in the birds sampled at the end of the dark phase (compared to fasted birds during the active phase), which would mask any differences in expression related to fed and fasted state.

CCK

In the present study, we were able to demonstrate that CCK gene expression was significantly higher in the antrum, caecum, distal jejunum and ileum, duodenum and proximal jejunum in comparison to other parts of the gut. Previous distribution studies of CCK in mammals showed that CCK was higher in the proximal ileum of rats (Larson and Reheld 1978), while in humans CCK was higher in the duodenum and jejunum (which is similar to our finding) with different forms of CCK localised in certain parts of the gut (Maton et al. 1984). Reid and Dunn (2018) demonstrated that in chickens, CCK levels were highest around the proximal half of the ileum which is similar to the murine CCK distribution (Fakhry *et al. 2017)*. On the other hand, CCK was lower but detectable in the proventriculus and the boundaries within the antro-duodenal regions in chickens. Thus, CCK was expressed over a broader range of gut tissues in great tits, particularly in the more anterior part of the intestine.

Upon closer inspection to the tissue distribution, we found that in males, the full state proventriculus had higher CCK expression, while the empty state caecum expressed more CCK. No such difference was seen in females in either tissue. The pattern seen in the proventriculus follows a typical biological direction in terms of the physiological role of CCK as a satiety hormone. However, it is still not clear as to why we observed higher CCK levels in tissues that do not typically express CCK. Studies had shown that CCK is a hormone that is mostly known to be produced in the small intestine and functions in stimulating gastric secretion (Langlois 2003). Eastwood et al. (1998) demonstrated that in rats where their proximal jejunum was used for in vitro study, endogenous CCK plays a vital role in transducing nutrient signals to the sensory afferents. Those afferent fibres have their terminals in the proximal jejunum mucosa thus conveying the information to the brain stem via the vagus nerve pathways initiating satiety (Richards et al. 1996). It is worth mentioning that unlike our experiment, many studies did not directly compare CCK expression profile across the different tissues in response to different feeding states. For instance, Reid and Dunn (2018) looked at the CCK expression in chickens either fasted for an hour or had been reintroduced to food after 3h from

removing it and given 2.5h to feed before dissection to investigate how CCK expression response to short-term feed restriction. Although they were not able to detect any significant difference between the two treatments (fasted vs. fed), CCK hybridisation signals between the two groups showed that CCK anticipatory expression might be different if the two groups were under longer nutritional challenges. Comparing to our study, Reid and Dunn (2018) only looked at the gastric antrum and the proximal ileum where CCK showed a peak expression, but they did not directly compare the mRNA levels within each tissue in response to different feeding treatment. So, it is hard to predict to what magnitude the different tissue was affected by fasting and re-feeding and to what extent CCK levels fluctuated among the different gut parts.

Generally, the well-established role of CCK in digestion is through stimulating gall bladder contraction and pancreatic exocrine secretion to signal satiety by activating the vagal afferents (Owyang 1996). Those functions are perfectly associated with the fact that CCK is primarily secreted from tissues of the lower intestine (Fakhry et al. 2017), hence it is expected to see peak expressions within those areas. Our speculation as to why some gut tissues showed a CCK response in the opposite direction to that expected might be linked to PYY gene expression. Lin et al (2000) investigated the correlation between distal gut release of PYY and CCK in dogs. The authors found that PYY release increases dose dependently in response to intravenous injection of CCK but was blocked when a CCK-A receptor antagonist was introduced. Additionally, a decrease in the postprandial PYY effect was observed when a segment of the proximal jejunum was removed (higher levels of CCK are documented in that area (Eastwood et al. 1998)). It is well established that in mammals the intestinal CCK mRNA levels change parallel to CCK in the circulation (Kanayama and Liddle 1991) and decrease with fasting (Suominen et al. 1998). On the other hand, plasma PYY levels were observed to increase after 15 days of fasting in blue foxes.

This could be explained as being due to the down regulation of unnecessary physiological processes that are suppressed by PYY such as gastric acid secretion, colonic motility and endocrine and exocrine action of the pancreas, during a prolonged fasting period (Mustonen et al. 2005). In contrast in humans, PYY decreases after a 3 day fast (Beer et al. 1989). Thus, the different PYY response to fasting seems to be species specific, dependent on fasting time course, feeding habits and life histories (Mustonena et al. 2005). Given the correlation between CCK and PYY, when comparing tissue responses to gizzard fill and the expression of both CCK and PYY in the great tit it was noticeable that PYY levels were high in the following tissues in the empty state: gizzard, proventriculus and rectum. CCK was also showed the highest expression in the empty state of the following tissues: distal jejunum, distal ileum, duodenum, gizzard and the proventriculus. So, two tissues are in common: the gizzard and the proventriculus. Our hypothesis of a correlation between CCK release and PYY release might not be the entire reason for the different expression pattern seen in CCK levels, but it is a good starting point.

When we compared CCK expression levels between sexes it was apparent that males had significantly higher CCK levels in comparison to females especially in the duodenum and the rectum. Several studies have

reported the possibility of a link between different sexes and food intake physiology. Putting aside sexual dimorphism, individuals are limited by their digestive processing capabilities such as the transit time of nutrient particles. In a study done by Markman et al. (2006) on Palestine sunbirds, the authors found that males had longer transit time in comparison to females when both were fed on an equicaloric diet even after correcting for body mass differences and food intake. This finding suggests that males had lower digestive capacities which might result in different abilities in food consumption. In the Palestine sunbird case, the different digestion abilities might aid in reducing the competition between males and females on nectar sources as each one would feed according to their digestion abilities. Additionally, we should not forget to mention the assimilation efficiency (the nutrient consumed that are actually digested and absorbed) that depends on the nutrient content of the food, gut surface area and volume, gut transit time and enzyme activity. This plays a major role in how any individual could maximise its net energy gain to benefit from what it eats, and one would expect that different sexes would vary in their assimilation rates independent of their size (Markman et al. 2006). All that considered, we speculate that the fact that males have longer transient time while digesting their food might be responsible for higher CCK expression levels, which gives them more assimilation time causing more CCK to be secreted during the time course thus increasing their satiety levels.

Having said that, and to our knowledge, no previous study has been done to compare CCK gene expression between different sexes in response to manipulation of the nutritional state. So, this is the first recording of the different CCK mRNA levels between males and females in avian species and more future experimentation needs to be done to explore why that difference was observed.

Why we were able to see significant difference in CCK mRNA levels in the duodenum and rectum in male great tits only but in the opposing direction from expected (higher in empty rather than full state) could be possibly explained by circadian rhythms of CCK secretion. Xu et al. (2017) investigated the effect of reversing of the light: dark cycle for 7 days on the expression of CCK in the duodenum and the pancreas of male Wistar rats. The authors found that those rats showed peak expression of CCK during the light phase (resting phase). This observation is consistent with what we observed in our great tits study. We noticed a significant peak of CCK levels in the duodenum and rectum during the dark phase which is equivalent to the light phase of those rats. Those similar findings implicate that clock genes might be the main driving force behind circadian gene expression through the regulation of the promoter activity of the clock-controlled gene leading to the activation of downstream genes at specific times of the day (Brown and Schibler 1999). Although to this date, no evidence was found proving that CCK is under the control of clock genes, it could be still be regulated by circadian systems via indirect pathways. Therefore, our experimental design to collect tissues at different times in the circadian cycle may have obscured the effect of gut full on the expression levels of CCK, because it is confounded with the circadian cycle.

2.5 Conclusion

In conclusion, overall, although both mammalian and the limited bird literature showed that both neuro and gut peptides play a significant role in appetite regulation, in the present study, our PCR quantification data showed otherwise. Most of the selected peptide's levels did not fluctuate according to the nutritional state of the individual. This is not to say that these peptides are not involved in signalling hunger or satiety, this might just imply the fact that measuring mRNA is not a direct indication of the actual gene expression, and that measuring protein content (using Enzyme linked immunosorbent assay (ELISA) or immunohistochemistry for instance) might be a better and more accurate approach to detect differences in peptide synthesis within the tissues. In addition, there are still are some unanswered questions regarding how peptide gene expression is linked to circadian rhythms in birds as most of the known information in that area comes from mammalian studies, but also how to control the variability in fasting/feeding times. Those question indeed led to the next chapter where we tried to avoid the confounding effect of both fasting and circadian rhythm.

Chapter 3. A comparison of gene expression profile in response to unpredictable food sources in hoarding vs. non-hoarding songbirds

In the first chapter, we had a general overview about the differences in hoarding behaviour between mammals and birds and we hypothesised that we could start understanding the mechanisms behind food storing behaviour if we had a full understanding of how the appetite system operates. Assuming that both food intake and hoarding are regulated by similar external and internal factors, the mechanisms regulating the food hoarding system might be a modification of the appetite regulation system. Having said that, there is one difference separating hoarding from non-hoarding animals, and that is hoarding animals tend to forage more than the non-hoarding animals because at the end of the day, they forage to both eat and hoard.

However, in this chapter we aim to focus more on the peptide gene expression differences between hoarding and non-hoarding wild songbirds in response to fasting vs. re-feeding state.

Paridae are a group of closely related songbirds that includes tits, titmice and chickadees. This group includes both hoarding and non-hoarding species (Vander Wall 1990). We have already established in the general introduction chapter that living in a fluctuating environment poses many challenges to small birds. In particular, food availability and predictability are proving to be a particularly challenging factor considering how small these individuals are. Thus, songbirds have evolved different techniques to counter act those harsh conditions to insure increasing survivability chances and reproductive success. For instance, migration has been hypothesised to be an adaptation to escape adverse conditions that may impose constraints on the individual's life (Fryxell and Sinclair 1988). Having said that, not all passerines migrate during winter. Residential birds need to find an alternative to meet their higher metabolic rate demands, and although storing fat might seem the perfect solution since its release provide a good energy source (Haftorn 1989), it is not ideal since fat can hinder the bird's flight ability and decrease their manoeuvrability. So, there is a trade-off between increasing body mass and risking agility (Lima 1986). Thus, another adaptation has evolved which is storing food. For hoarding birds, it is better to store food in a physical sense rather than having energy as lipid stores because it will increase their body weight, which would eventually, hamper their flight ability (Brodin 2000). Having food stores for times when foraging trips might be energetically costly, makes hoarding behaviour one of the most efficient ways to conserve energy (Koenig, 1991).

The appetite regulation literature has long established that fasting, food restriction and unpredictability has been found to be the main drivers for eliciting a variety of ingestive behaviours in mammals (Wood and Bartness 2007) and birds (Furuse et al. 1997). The unpredictability of food sources (natural conditions) or food deprivation (laboratory conditions) engenders a variety of central and peripheral changes to the

metabolic hormones within the individual (Keen-Rhinehart et al. 2009). Those internal changes are transduced into the main centre (brain) causing the animal to attain a certain behaviour as a result to obtain energy and support its existence. And as we have established to this point, the ARC in the hypothalamus particularly housed a number of neuropeptides that have been shown to play vital roles as regulators of feeding and energy expenditure (Cone 2005). For instance, Li et al. (2000) demonstrated an increase in AGRP peptide release from the hypothalamus of fasted rats. Thus, the increase of AGRP is considered the main driver to increase food intake. On the other hand, with increased feeding a wide array of gut peptide release is stimulated initiating satiety. Batterham et al. (2002) showed that the gut peptide hormone PYY decrease food intake and support weight gain in rodents. The same effect of peptide was observed in birds whereby chronical food restriction was shown to elevate AGRP expression of broiler hens (Dunn et al. 2013). Whereas an intravenous administration of PYY decrease food intake in chickens in dose dependant manner (Aoki et al. 2017). Thus, it seems that appetite regulating peptide's function are conserved between vertebrates.

Studies had shown that the motivation to hoard is driven by the similar factors to feeding (Keen-Rhinehart and Bartness 2005) such as fasting and food deprivation, so we assume that there should not be a huge difference in hormones that control feeding in hoarding and non-hoarding individuals since both systems appeared to be are controlled by the same internal factors. For instance, AGRP expression was recorded to be the highest in fasted rats. On the other hand, although AGRP increased food intake in Siberian hamsters substantially (100-150%), its effect on food hoarding was even more dramatic increasing the behaviour up to 1200% does dependently (Day and Bartness 2003). Similarly, CCK injected intraperitonially into fasted rats produced a large related suppression in food intake (Gibbs et al. 1973). Similar effects were observed in hamsters were food deprivation induced increase in food intake was inhibited by the administration of CCK.

Birds, like mammals, express genes encoding neuro and gut peptides that control food intake. For instance, NPY has been shown to be a potent orexigenic agent in chickens when administered centrally (Kuenzel et al. 1987). On the other hand, although ghrelin is a potent stimulant for appetite in mammals (Murakami et al. 2002), studies have shown that in birds opposing effect of ghrelin were found. Lindsay et al. (2018) demonstrated that ghrelin injected peripherally reduces mass gain (which is a proxy of food intake) in coal tits.

Collectively, besides ghrelin that showed opposite function in mammals than in birds, most of gut and brain peptides that controls appetite seems to expert the same effect on food intake in hoarding and non-hoarding individuals. This further confirms our assumptions that the system controlling food intake might also be responsible for regulating food hoarding behaviour.

Thus, because there is little information available regarding the appetite regulation of wild songbirds in the literature, we are aiming in this chapter to compare peptide expression in the hypothalamus, hindbrain and the gut of both blue and coal tits, either fasted for 18 hours or after a 1.5h of re-feeding following a 17h fast

as an effort to gain more insight regarding appetite regulation peptides gene expression differences between two closely related species with two different responses to energy deficit .Our prediction is that in both species we would find an increase in genes responsible for reporting hunger (e.g. NPY and AGRP) , while those that report satiety (CCK, GCG,INS,PYY and POMC) are expected to be elevated in re-fed individuals.

3.2 Methodology

3.2.1 Animal capture and housing

Nineteen blue tits (Cyanistes caeruleus) and nineteen coal tits (Periparus ater) were caught in woodland near Newcastle Upon Tyne, UK, under a permit from Natural England (2018-37524SCI-SCI) between October-December 2018, using mist nets. The birds were transported after capture to climate-controlled chambers at Newcastle University. Each species was housed in separate enriched aviaries where light and temperature were controlled. Each individual received a distinct numbered ring for easy identification. Once in captivity, all the birds were maintained on constant temperature of 15°C and on naturally changing photoperiod until it reached 8:16 light: dark cycle respectively (mimicking short days) after which it was held constant. All birds were fed ad libitum and supplied with pre-made food mixture of sunflower seeds, crushed peanuts, pine nuts, mealworm, wax worms and Orlux insect patee with water bowls available for drinking and bathing.

On average the birds were kept in aviaries for two weeks based on previous studies done in our lab where it was shown to be enough for small wild songbirds to show normal foraging and eating behaviour and to respond to unpredictable food by increasing food intake and hoarding (Henderson et al. 2018).

3.2.2 Sexing the birds

Before assigning each group, blood samples were taken from each individual for sexing. A small blood sample was obtained (between 80-100µl) using a standard heparinised capillary tube after a puncture of the branchial vein with the aid of 25-gauge needle. The whole blood sample was placed in a tube of 100% ethanol which was stored at room temperature until further analysis.

The DNA was extracted from the blood samples using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Manchester, UK) following the manufacturer's protocol. The primer pair P2/P8 recommended for sexing a variety of avian species were used (Griffiths et al. 1998). The PCR amplification reaction was carried out in a total volume of 25µl. The final condition of the reaction was as followed: 1µl of each primer (P2/P8) with a concentration of 10µM, 2µl extracted DNA (50 ng) and 12.5µl of MyFi mix (Bioline). Thermal cycling was carried in CFX Connect real-time PCR machine (Bio-Rad, Oxford, UK) programmed as following: 94°C/2 min, 30 cycles of (49°C/40 s, 72°C/40 s and 94°C/30 s, 49°C/1 min, 72°/5 min. The PCR products were then separated by electrophoresis on a 2% agarose gel stained with ethidium bromide. The reaction ran in the gel for 60 mins at 100 volts. The gel was then examined, if two bands were observed the individual was identified as female, while the male sample had a single band.

3.2.3 Experimental design

Prior to the beginning of the experiment, two climate chambers (lights were programmed to allow 1h difference in when they turned on or off (i.e., chamber 1(Ch1): lights on at 9:30am; off 5:30pm, chamber 4 (Ch4): lights on at 10:30 and off at 6:30) were prepared with metal cages supplied with enrichment branches, automated balances and sliding dividers. Each chamber had two large cages (90 (w) × 46 (d) × 80 (h) cm) with dividers placed in the middle during the day (for 3h) for the first 3 days, and then at night dividers were placed at the end of the cage providing a contained compartment till the next day (for the remainder of the study). After allowing the birds to acclimatize in the aviaries for 2 weeks, 4 blue tits (BT) and 4 coal tits (CT) were matched based on sex, selected and put in a separate compartment in the cages (Figure 1).

The enrichment branches were placed in the middle two quarters of the cage and four food dishes were distributed in the middle section such that each individual had free access to the food, while water dishes were placed in the furthest side of the cage. Each bird was locked in its one quarter compartment where there is a perch at the top, connected to a balance. We separated each individual in its compartment so we could video their behaviour.

No enrichment was placed in the further sides of the cages to draw the birds to sit on the only perch. In each chamber, both species and sex were balanced, and either were placed in the top or bottom cage. So, in chamber 1 the BT were always at the top while the CT were at the bottom, in chamber 4 it was the opposite (CT at the top; BT at the bottom). Initially, each species had black (CT) or blue (BT) numbered ring around their leg for identification purpose, a second ring (either red or yellow) was placed in the other leg for each individual to facilitate their identification while video recording them.

For the first 3 days of the study, the birds were left in the cages to acclimatize and adapt to the new environment and individual housing for several hours during day and night.

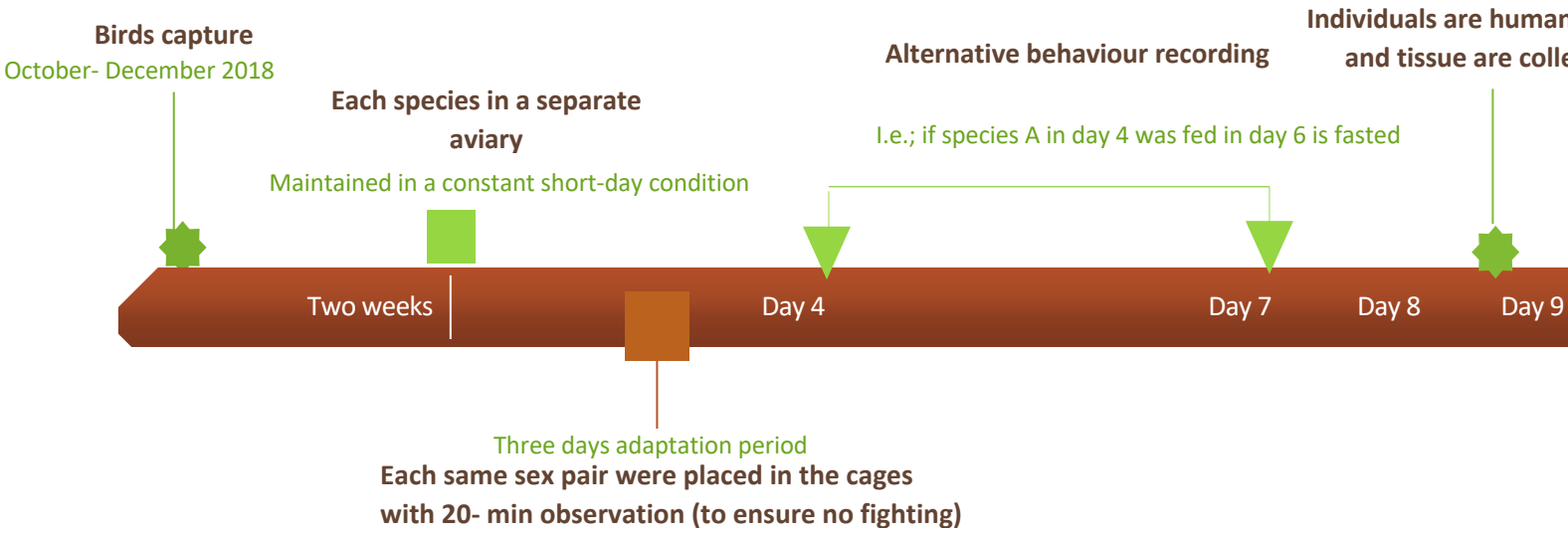


Figure 1: Detailed diagram showing the time line of the experiment

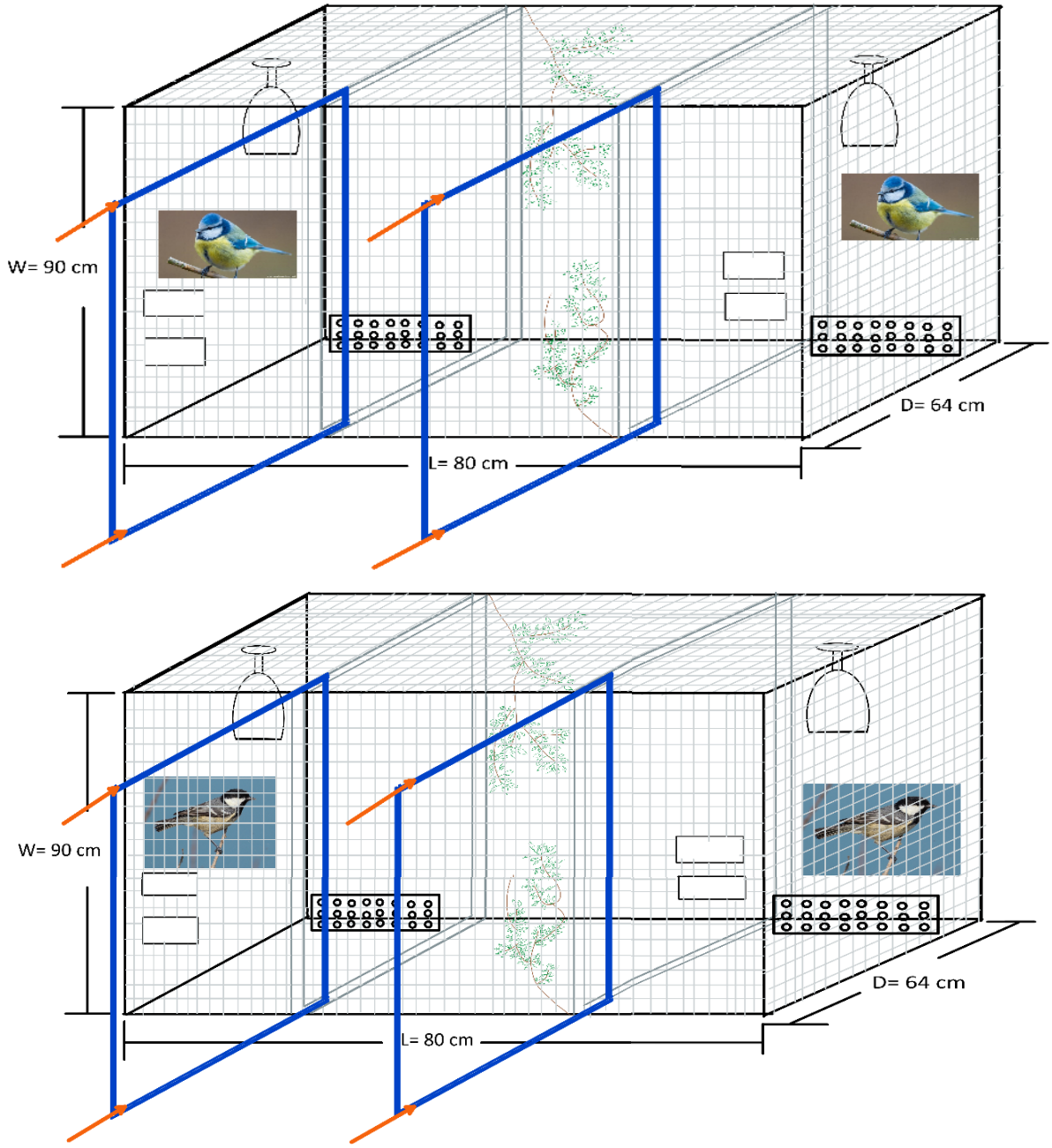


Figure 2: Detailed diagram showing how were positioned during the experiment in chamber 1, and the same set up in chamber 4, however, coal tits are up and coal tits were at the bottom.

Day 1

The birds were captured and put in the cages before the lights on in each chamber, with the dividers placed in the middle for the first three hours after lights on and each individual received its own Orlux insect patee and water bowl at their compartment along with another food bowl in the middle. The dividers were removed after 3h and the individuals were observed interacting for 15-20 minutes. Thirty minutes before lights off in each chamber, the dividers were put back. As such each individual only had a quarter cage space, along with a bowl of crushed peanuts and water in its compartment.

Day 2

Before lights on in each chamber, the peanuts bowl was taken after removing the dividers and Orlux patee (total diet) were supplied. Thirty minutes before lights off the dividers were put back in along with a bowl of crushed peanuts for each individual in its compartment.

Day 3

Two hours after lights on in each chamber, the bowls of peanuts were removed along with the dividers, so the pair had free access to total diet. During the day, 3.5h after lights on (Ch1: 1:00 pm; Ch4: 2:00 pm) the cages were cleaned, and food was removed from the top and bottom cages for 1.5h and was given back after that. Thirty minutes before lights off the birds were again separated into their individual quarter cages, and crushed peanuts were placed for all the four individuals. The crushed peanuts bowls were then removed from BT (top cage Ch1) and CT (top cage Ch4) only after the lights went off.

Day 4

Each individual in a pair was assigned to either fast or fed treatment. Top cages the fed birds were given a bowl of crushed peanuts along with a hoarding block (30.4cm wide, 9cm high and 4cm deep with a hundred holes) 1h after lights on in each chamber. Fasted birds were given food and a block 2h after lights on and their behaviour was recorded using Sony Handycam (HDR-CX240E) mounted on a tripod for 1h. As for the fed birds, their behaviour was recorded (for the same duration 1h) after being given a fresh bowl of crushed peanuts 1.5h hours after the first bowl.

On the other hand, birds that were in the bottom cages received similar treatment as to day 3 as follows: 2h after lights on, both dividers and the bowl of crushed peanuts were removed allowing the birds free access to the total diet. During the day, 3.5h after lights on where the cages were cleaned, and food was removed for 1.5h and was given back after that. Thirty minutes before lights off birds were again separated into their individual quarter cages; and crushed peanuts were placed for all the four individuals in the cages. Thirty minutes later the crushed peanuts were removed from CT (bottom cage Ch1) and BT (bottom cage Ch4).

Day 5

The bottom cages were treated in the same way as the top cages had been treated on day 4, and the top cages like the bottom cages on day 4. At the end of the day, crushed peanut bowls were removed from BT (top cage Ch1) and CT (top cage Ch4).

Day 6

Similar protocol to day 4 except that the individual that was fed at that day is fasted and vice versa (the one that was fasted become fed)

Day 7

Similar to day 5 except that the individual that was fed at that day is fasted and vice versa (the one that was fasted become fed). Thirty minutes before lights off, crushed peanuts were placed for all the four

individuals in the cages. Thirty minutes later the balances were switched off and crushed peanuts were removed from BT (top cage Ch1) and CT (top cage Ch4).

Day 8

The birds at the top cages were sacrificed on the 8th day. Fed BT and CT were given a bowl of crushed peanuts 1h after lights on. The fasted BT was killed 2h after lights on, 30 minutes later the fed BT was killed. And the same applied for the CT in Ch4 (fasted kill 2h after lights on, fed 2.5h after lights on). As for the individuals at the bottom cages, crushed peanuts and the dividers were removed giving them free access to the total diet. Thirty minutes before lights off, crushed peanuts were given to the remaining individuals and balances were switched on. Thirty minutes later the balances were switched off and crushed peanuts were removed from CT (bottom cage Ch1) and BT (bottom cage Ch4).

Day 9

One hour after lights on, the fed CT and BT were given a bowl of crushed peanuts. As for the fasted CT it was killed 2h after lights on (11:30 am), 30 minutes later the fed CT was killed. And the same applied for the BT in Ch4 fasted kill 2h after lights on, fed 2.5h after lights on).

3.2.4 Tissue collection

Before being sacrificed, each individual was both weighed, and their fat score was evaluated according to European Science Foundation (ESF) and Biometric Working Group (BWG) system with a score of 0 (no fat) to 8 (most fat) (British Trust for Ornithology 2020).

Birds were euthanized using an intra-peritoneal overdose injection of pentobarbital. Tissue samples of up to 100 mgs were collected from each individual and placed in tubes with 1ml of RNAlater: the entire hypothalamus, hindbrain and proventriculus; a small part of the gizzard, antrum, duodenum, pancreas, proximal jejunum, distal jejunum, ileum, caecum and rectum. Gizzard fullness was noted for each individual as such: either empty (no food inside) or full (if it had food inside). Those samples were initially stored at 4ºC for two days then transferred to -80ºC for later analysis.

3.2.5 RNA extraction and reverse transcription

Details about how RNA was treated and extracted from tissue samples are detailed in chapters 2.

3.2.6 PCR primers

Details about primers sequence are listed in chapter 2, methods section.

3.2.7 Sequencing of PCR products

The PCR products were sent for sequencing at the DBS sequencing facility at Durham University following their sequencing guidelines: 15µL of PCR product was mixed with 6µL of ExoSap-IT PCR-product clean up solution (Thermo Fisher). Both forward and reverse primers were used at a concentration of 3.2 pmol/µL. Sequencing confirmed amplification of the PCR products expected.

.

3.2.8 Real time PCR

Step by step details on how the samples were prepared and treated for real-time PCR are listed in chapter 2, methods section.

3.2.9 Notes

We originally started with sixteen coal tits and sixteen blue tits, divided as such: eight empty and eight full gizzard individuals balanced across the two species. However, during the recording session some birds succeeded in escaping from their home cage, thus having access to food when they should not. Those individuals were classified as full state because they had access to food and did manage to feed before being caught and returned to their cage. So, our earlier distribution of individuals to the different treatment group changed as follows:

Table 2: Experimental group distribution across the different treatment as a result of individuals escaping from their home cage.

Sex/ condition	Blue tits Males/ empty	Blue tits Males/ full	Blue tits Females/ empty	Blue tits Females/ full	Coal tits Males/ empty	Coal tits Males/ full	Coal tits Females/ empty	Coal tits Females/ full
Numbers	**3**	**8**	**2**	**3**	**4**	**5**	**2**	**5**

3.2.10 Data analysis

Before the beginning of the analysis where it was relevant, a histogram plot was generated for the residual values to ensure the normality of our data when using parametric analysis.

The gene expression levels of the gut peptides (PYY, CCK, GCG and INS) were calculated using log10 gene ratios to the YWHAZ reference genes, while neuropeptides (NPY, CCK, GCG, POMC and AGRP) were calculated using log10 gene ratios of the reference gene (LBR). The calculation was done as follows: the ratio to LBR was multiplied by a power of 10 then log 10 was applied to the result. The power of 10 was

chosen for each gene according to the smallest number. So, for NPY (1014), AGRP (1017) and hypothalamic POMC (1017). Hindbrain peptides: CCK (101), GCG (102) and POMC (104).

The analysis was done after eliminating the outliers. The outliers were identified as such: after doing log (gene/YWHAZ) for all individuals, occasional extreme values (products of technical errors) that were more than two standard deviations away from the mean and clearly lay outside the normal range of variation were termed outliers. Because technical errors are unavoidable, it was inappropriate to include those values in the analysis and this step is part of the standard quality control process we applied routinely. Statistical analysis was done using the Generalized Estimating Equations (GEE) function for gut peptides and generalized Linear Models (GLM) for brain neuropeptides in the SPSS statistical package (IBM 25). To correct for post hoc test, we used Bonferroni correction method to account for the multiple comparison The rationale behind using two different models when analysing our data was where we had repeated measures it was appropriate to use GEE, and GLM for non-repeated measures.

When analysing neuropeptides in both the hypothalamus and the hindbrain, we used 2×2 analysis (species × gut full) including the two-way interaction. As for gut peptide expression quantification, we used 2×2×10 analysis (species ×gut full× tissue) with all interactions included initially. In occasions where significant 3-way interaction was found, a follow up separate analysis was done using the 'split file' function in SPSS and choosing the variable that we want to investigate further. Non-significant interactions were removed from the model in a stepwise fashion, starting with 3-way interaction and working our way down. Interactions were removed if $p>0.1$. No 2-way interaction were removed if the 3-way interaction was significant. Therefore, unless mentioned within the results, reader should assume that interactions were not included in the model due to their insignificance.

The reader should note that gizzard fullness (aka. gut fullness) is the terminology we used to describe the nutritional state of the individuals (where empty= fasted, full= fed) rather than experimental treatment because during the course of the experiment, some individuals managed to escape from their cages and feed.

Table 1: Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) for full state blue tits listing the extracted tissues, mean RNA concentration for each tissue and mean 260/280 ratio.

Tissue	Sample size: full	Mean RNA concentration (ng/μl)	Mean 260/280 ratio
Hindbrain	7	375.6	1.97
Hypothalamus	9	207.3	1.99
Proventriculus	7	1730.4	1.96
Gizzard	7	1085.4	1.95
Antrum	7	1668.3	1.96
Duodenum	7	1599	1.95
Pancreas	7	1284.4	1.87
Proximal Jejunum	7	1700	1.93
Distal jejunum	7	1543.2	1.97
Ileum	7	1145.6	1.99
Caecum	7	443.4	1.89
Rectum	7	498.7	1.96

Table 2: Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) for empty state blue tits listing the extracted tissues, mean RNA concentration for each tissue and mean 260/280 ratio.

Tissue	Sample size: empty	Mean RNA concentration (ng/μl)	Mean 260/280 ratio
Hindbrain	4	288.2	1.98
Hypothalamus	4	241.7	2
Proventriculus	9	1340.1	1.97

Gizzard	9	918.9	1.98
Antrum	9	1525.6	1.96
Duodenum	9	1274	1.98
Pancreas	9	1376.8	1.82
Proximal Jejunum	9	1543.2	1.94
Distal jejunum	9	1512	1.98
Ileum	9	937.1	2
Caecum	9	424.7	1.91
Rectum	9	608	1.95

Table 3: Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) for full state coal tits listing the extracted tissues, mean RNA concentration for each tissue and mean 260/280 ratio.

Tissue	Sample size: full	Mean RNA concentration (ng/μl)	Mean 260/280 ratio
Hindbrain	10	403.1	1.96
Hypothalamus	10	184.5	2.01
Proventriculus	10	1534.5	1.92
Gizzard	10	1263.4	1.97
Antrum	10	1309.4	1.99
Duodenum	10	1580.5	1.96
Pancreas	10	1412.3	1.8
Proximal Jejunum	10	1552.7	1.96
Distal jejunum	10	1171	1.99
Ileum	10	629.8	1.95
Caecum	10	310.3	1.94
Rectum	10	451.3	1.93

Table 4: Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) for empty state coal tits listing the extracted tissues, mean RNA concentration for each tissue and mean 260/280 ratio.

Tissue	Sample size: empty	Mean RNA concentration (ng/μl)	Mean 260/280 ratio
Hindbrain	6	343	1.96
Hypothalamus	6	191	2.02
Proventriculus	6	1656	1.96
Gizzard	6	1073.3	1.94
Antrum	6	1418	1.97
Duodenum	6	1553.1	1.95
Pancreas	6	1442.6	1.83
Proximal Jejunum	6	1412.9	1.85
Distal jejunum	6	1446.8	1.98
Ileum	6	795.7	1.99
Caecum	6	341.8	1.79
Rectum	6	456.7	1.93

3.3 Results

3.3.1 Body mass

On average, blue tits were heavier in comparison to coal tits (BT, mean= 10.12g ±0.11, CT, mean= 8.73g±0. 11, χ^2 (1) = 72.02, p<0.001). Gizzard fullness had a significant effect on body mass with full gut individuals being slightly heavier than the empty gut ones (empty, mean= 9.20 ±0.12, full, mean= 9.65±0.10, χ^2 (1) = 7.69, p=0.006). However, no significant interaction was seen between species and gizzard fullness (χ^2 (1) = 0.932, p=0.334) (Figure 2).

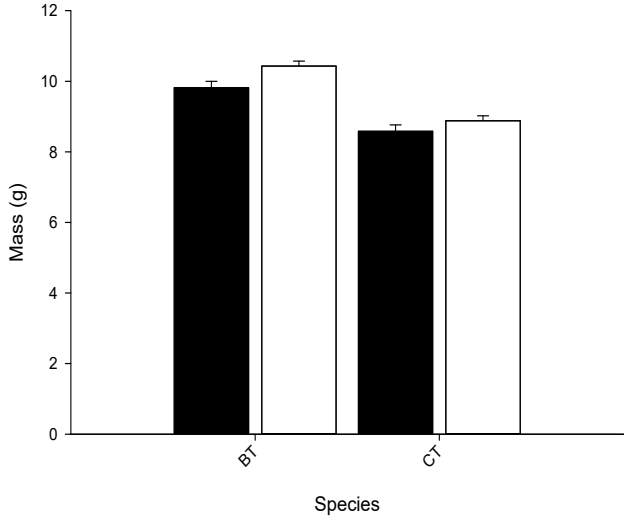


Figure 2: Pairwise comparison illustrating body mass difference between blue tits (BT) and coal tits (CT) using gizzard fullness as a variable where empty (black) and full (white). Each bar shows mean ± SEM.

3.3.2 Fat score

Fat score did not significantly differ between the two species (BT, mean= 3.40 ±0.14, CT, mean= 3.28±0. 11, χ^2 (1) = 0.332, p=0.564). Gizzard fullness had a significant effect on fat scores with full gut individuals showing higher scores than empty gut individuals (empty, mean= 2.83 ±0.16, full, mean= 3.85±0.12, χ^2 (1) = 25.22, p<0.001). However, no significant interaction was observed between species and gizzard fullness (χ^2 (1) = 1.14, p=0.284).

3.3.3 Housekeeping gene testing

Hypothalamus LBR

Hypothalamic LBR was not significantly different between blue and coal tits (BT, mean= 4.38E-015± 3.45E-16, CT, mean= 4.80E-015± 3.53E-16, $\chi^2(1) = 0.718$, p=0.397). There was no difference in LBR levels between males and females (females, mean= 4.65E-015 ± 3.74E-015, males, mean= 4.53E-015± 3.23E-16, $\chi^2(1) = 0.059$, p= 0.808). The treatment has no effect on LBR expression (empty, mean= 4.48E-015 ± 3.90E-16, full, mean= 4.69E-015± 3.03E-16, $\chi^2(1) = 0.184$, p= 0.668). Additionally, there was neither significant interaction between species*sex ($\chi^2(1) = 0.038$, p= 0.846), species*gizzard fullness ($\chi^2(1) = 1.14$, p= 0.285), sex*gizzard fullness ($\chi^2(1) = 1.26$, p= 0.261), nor a significant 3-way interaction ($\chi^2(1) = 3.02$, p= 0.082).

Hindbrain LBR

The gene expression of LBR in the hindbrain was neither different between the two species (BT, mean= 9.63E-015± 6.36E-16, CT, mean= 8.63E-015± 6.04E-16, $\chi^2(1) = 1.39$, p=0.238), nor between males and females (females, mean= 9.60E-015 ± 7.03E-015, males, mean= 8.67E-015± 5.46E-16, $\chi^2(1) = 1.13$, p= 0.287). LBR levels was not affected by treatment (empty, mean= 8.66E-015 ± 7.25E-16, full, mean= 9.60E-015± 5.26E-16, $\chi^2(1) = 1.12$, p= 0.288).

Gut peptide YWHAZ

Main effect

The expression of YWHAZ was neither different between the two species (BT, mean= 12.86± 0.08, CT, mean= 12.77± 0.10, $\chi^2(1) = 0.388$, p=0.534), nor between males and females (females, mean= 12.76 ± 0.09, males, mean= 12.87± 0.09, $\chi^2(1) = 0.663$, p= 0.416). Similarly, YWHAZ levels was not affected by the treatment (empty, mean= 12.86 ± 0.10, full, mean= 12.77± 0.08, $\chi^2(1) = 0.436$, p=0.509). However, gut tissues differ significantly in YWHAZ expression levels ($\chi^2(9) = 597.44$, p<0.001) (Figure 3).

Interactions

Tissue interacted significantly with species ($\chi^2(1) = 40.99$, p<0.001), however Bonferroni-corrected pairwise comparison analysis did not indicate any species difference for any tissue. Species also interacted significantly with gizzard fullness ($\chi^2(1) = 4.55$, p= 0.033) but Bonferroni-corrected post-hoc test did not indicate a gizzard fullness effect on both species. There was also a several significant 3-way interactions between: tissue*species*sex ($\chi^2(1) = 22.41$, p= 0.008). However, Bonferroni-corrected pairwise comparison indicated that there were no tissues in either species that showed a significant sex difference. There was also a significant tissue*species*gizzard fullness interaction ($\chi^2(1) = 42.86$, p<0.001), but Bonferroni-corrected

test indicated that there were no tissues in either species showed a significant effect of gizzard fullness. Tissue also interacted significantly with both sex and gizzard fullness ($\square^2(1) = 19.39$, $p = 0.022$). However, Bonferroni-corrected pairwise comparison indicated that there were no tissues in either sex that showed a significant treatment effect. In addition, there was a significant 4-way interaction ($\square^2(1) = 18.15$, $p = 0.033$). However, Bonferroni-corrected pairwise comparison showed that gizzard fullness did not have a significant effect on any interaction combination of sex/species, sex/ tissue, and species/tissue.

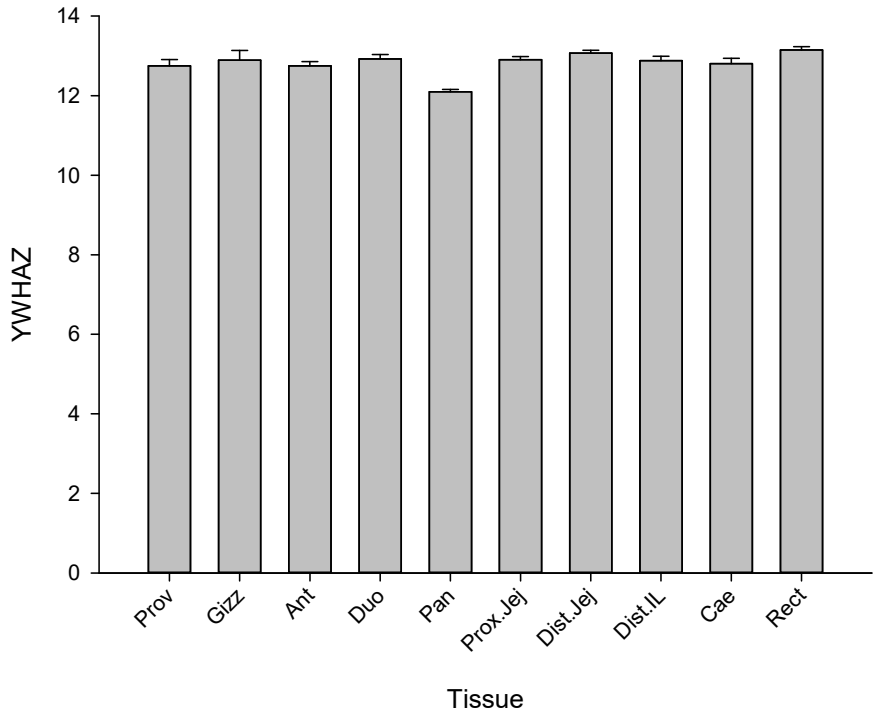


Figure 3: Pairwise comparison of YWHAZ housekeeping gene across different gut tissues. Refer to the glossary for abbreviation. Each bar represents mean ±SEM.

Table 3: Pairwise comparison of YWHAZ gene expression across different gut tissues. Refer to glossary for abbreviation. Green colour rectangles illustrate non- significant p-values.

	Prov	Gizz	Ant	Duo	Pan	Prox.Jej	Dist.Jej	IL	Cae	Rect
Prov		p=1.000	p=1.000	p=1.000	p=0.021	p=1.000	p=1.000	p=1.000	p=1.000	p=0.158
Gizz	p=1.000		p=1.000	p=1.000	p<0.001	p=1.000	p=1.000	p=1.000	p=1.000	p=1.000
Ant	p=1.000	p=1.000		p=1.000	p<0.001	p=1.000	p=0.005	p=1.000	p=1.000	p<0.001
Duo	p=1.000	p=1.000	p=1.000		p<0.001	p=1.000	p=1.000	p=1.000	p=1.000	p=1.000
Pan	p=0.021	p=0.008	p<0.001	p<0.001		p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
Prox.Jej	p=1.000	p=1.000	p=1.000	p=1.000	p<0.001		p=0.079	p=1.000	p=1.000	p=0.719
Dist.Jej	p=1.000	p=1.000	p=0.005	p=1.000	p<0.001	p=0.079		p=0.068	p=0.481	p=1.000
IL	p=1.000	p=1.000	p=1.000	p=1.000	p<0.001	p=1.000	p=0.068		p=1.000	p=0.732
Cae	p=1.000	p=1.000	p=1.000	p=1.000	p<0.001	p=1.000	p=0.481	p=1.000		p=0.410
Rect	p=0.158	p=0.732	p<0.001	p=1.000	p<0.001	p=0.719	p=1.000	p=1.000	p=0.410	

Look at the appendix page 228 to look at the stepwise deletion for each neuropeptide and gut peptide.

3.3.4 Neuropeptides

Hypothalamus

In the hypothalamus, we quantified the expression of the ARC neuropeptides *NPY, AGRP* and *POMC.*

There was no significant difference in NPY gene expression between males and females (females, mean= 3.07 ± 0.12, males, mean= 3.29± 0.10, $\chi^2(1) = 0.197$, p= 0.160). However, NPY gene expression was significantly affected by species, blue tits had higher NPY levels in comparison to coal tits (Blue tits (BT), mean= 3.37 ± 0.11, coal tits (CT), mean= 2.99± 0.10, $\chi^2(1) = 5.68$, p=0.017). No effect of gizzard fullness was detected on NPY expression (empty, mean= 3.20 ± 0.13, full, mean= 3.16± 0.09, $\chi^2(1) = 0.056$, p= 0.813). There was no significant interaction between species and gizzard fullness ($\chi^2(1) = 0.482$, p=0.488) (Figure 4-A).

AGRP levels were significantly higher in BT than CT (BT, mean= 3.42± 0.18, CT, mean= 2.86± 0.18, $\chi^2(1) = 4.65$, p=0.031). Empty gut individuals had higher AGRP in comparison to full ones (empty, mean= 3.42± 0.20, full, mean= 2.86± 0.15, $\chi^2(1) = 4.57$, p= 0.032). There was a significant species* gizzard fullness

interaction ($\chi^2(1) = 5.12$, $p = 0.024$) mostly clear in coal tits where empty gut individuals had higher AGRP gene expression than full ones (empty, mean= 3.43± 0.29, full, mean= 2.29± 0.22), while no difference was detected in blue tits (empty, mean= 3.41± 0.29, full, mean= 3.44± 0.21) (Figure 4-B). On the other hand, no significant difference in AGRP levels were observed between males and females (females, mean= 3.29 ± 0.20, males, mean= 2.99± 0.16, $\chi^2(1) = 1.43$, $p = 0.231$).

POMC expression was higher in BT than CT (BT, mean= 4.39± 0.12, CT, mean= 2.03± 0.11, $\chi^2(1) = 184.481$, $p<0.001$). Males had higher POMC levels in comparison to females (females, mean= 3.01 ± 0.13 males, mean= 3.40± 0.11, $\chi^2(1) = 4.95$, $p = 0.026$). Additionally, species interacted significantly with sex ($\chi^2(1) = 5.39$, $p=0.020$): where male coal tits had higher POMC in comparison to females (females, mean= 1.63 ± 0.17, males, mean= 2.43± 0.16, p=0.006), but no sex difference was seen in blue tit (females, mean= 4.40 ± 0.19, males, mean= 4.39± 0.16, p=1.000). Gizzard fullness did not affect POMC gene expression (empty, mean= 3.15± 0.14, full, mean= 3.27± 0.10, $\chi^2(1) = 0.497$, $p = 0.488$). No significant interaction was seen between species and gizzard fullness ($\chi^2(1) = 0.031$, $p=0.859$) (Figure 4-C).

Finally, we looked at the AGRP/POMC ratio in the hypothalamus. Coal tits had significantly higher AGRP/POMC ratio in comparison to blue tits (BT, mean= 3.05± 0.31, CT, mean= 4.70± 0.32, $\chi^2(1) = 13.31$, $p<0.001$) (Figure 4-E). Empty gut individuals had higher AGRP/POMC gene expression in the hypothalamus in comparison to full ones (empty, mean= 4.42± 0.36, full, mean= 3.32± 0.26, $\chi^2(1) = 6.09$, $p = 0.014$) (Figure 1-D). However, the ratio was not significantly different between males and females (females, mean= 4.17 ± 0.34 males, mean= 3.57± 0.28, $\chi^2(1) = 1.84$, $p = 0.174$).

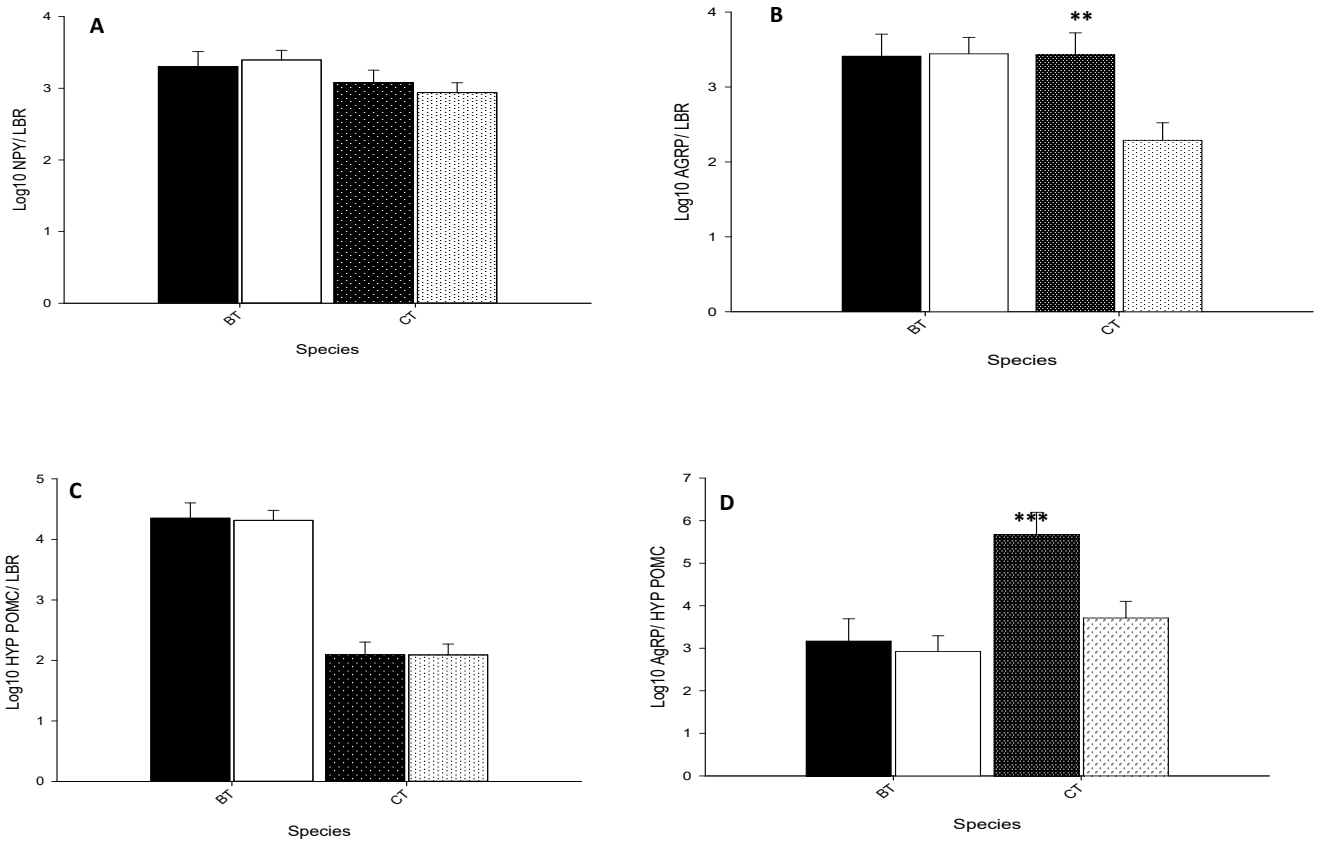


Figure 4: Comparison between the expressions of neuropeptide Y (NPY) (A), agouti-related protein (AgRP) (B) and pro-opiomelanocortin (POMC) (C) and the ratio between (AGRP/HYP POMC) (D) in the hypothalamus. Values are presented as log10 ratio to lamin B receptor (LBR). All graphs represent the interaction between species and gizzard fullness: blue tits (BT) (solid bars) and coal tits (CT) (dotted bars). Empty (black bars), full (white bars). Each bar shows mean ± SEM. The asterisk represents significant differences ((*) p<0.05, (**) p<0.01 (***) p<0.001).

Hindbrain

The second brain area investigated was the hindbrain, where we quantified CCK, GCG and POMC.

There was no significant difference in CCK gene expression between BT and CT (BT, mean= 1.25± 0.05, CT, mean= 1.30± 0.04, $\square^2(1) = 638$, p=0.424). Neither sex (females, mean= 1.27 ± 0.05 males, mean= 1.28± 0.04, $\square^2(1) = 0.05$, p= 0.815) nor gizzard fullness (empty, mean= 1.34± 0.05, full, mean= 1.21± 0.04, $\square^2(1) = 3.68$, p= 0.055) had any significant effect on CCK levels. Additionally, no significant interaction was observed between species and gizzard fullness ($\square^2(1) = 1.85$ p= 0.173) (Figure 5-A).

As for GCG, there was a strong difference in the gene expression between the two species with CT showing higher levels in comparison to BT (BT, mean= 2.96± 0.16, CT, mean= 3.88± 0.15, $\square^2(1) = 16.26$, p<0.001). On the other hand, neither sex (females, mean= 3.28 ± 0.17, males, mean= 3.56± 0.14, $\square^2(1) = 1.51$, p= 0.218) nor gizzard fullness (empty, mean= 3.29± 0.18, full, mean= 3.55± 0.13, $\square^2(1) = 1.32$, p= 0.251) had any significant effect on GCG levels. However, even though there was a significant interaction between species and gizzard fullness ($\square^2(1) = 4.87$, p= 0.027) (Figure 5-B), Bonferroni post-hoc test indicated that neither species showed a significant effect of gizzard fullness.

Hindbrain POMC was significantly higher in BT in comparison to CT (BT, mean= 3.35± 0.09, CT, mean= 2.04± 0.08, $\square^2$ (1) =111.94, p<0.001). Neither sex (females, mean= 2.64 ± 0.09 males, mean= 2.76± 0.07, $\square^2$(1) =0.877, p= 0.349) nor gizzard fullness (empty, mean= 2.68± 0.09, full, mean= 2.71± 0.07, $\square^2$(1) =0.072, p= 0.788) had any significant effect on POMC levels. There was no significant interaction between species and gizzard fullness ($\square^2$(1) =0.519, p= 0.471) (Figure 5-C).

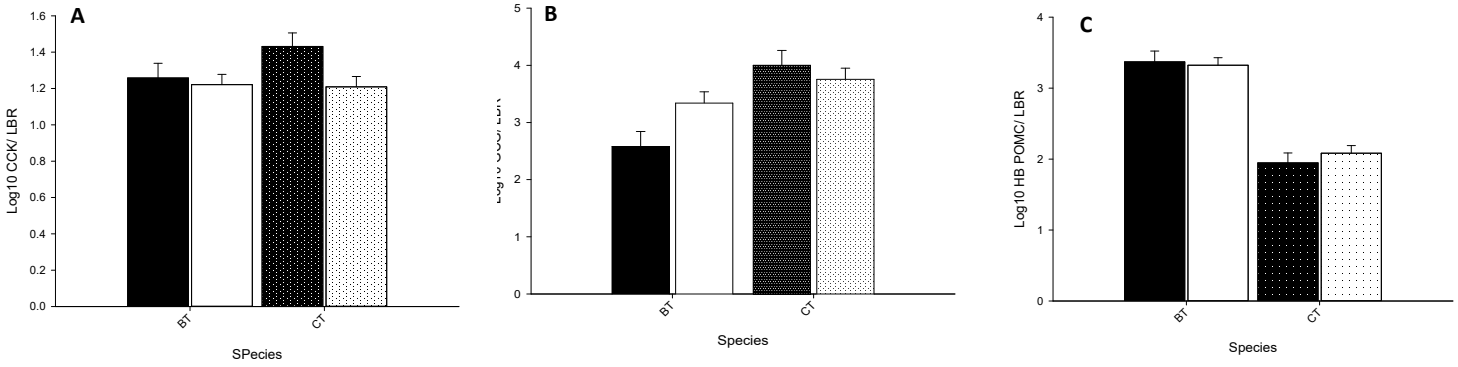


Figure 5: Comparison between the expressions cholecystokinin (CCK) (A), glucagon (GCG) (B) and pro-opiomelanocortin (POMC) (C) in the hindbrain. Values are presented as log10 ratio to lamin B receptor (LBR). All graphs illustrate the species* gizzard fullness interaction. Blue tits (BT) (solid bar) and coal tits (CT) (dotted bar). Empty (black bars), full (white bar). Each bar shows mean ± SEM.

3.3.5 Gut peptides

3.3.5.1 CCK

Main effect

The expression levels of CCK did not differ between males and females (females, mean= 3.24 ± 0.08 males, mean= 3.31± 0.12, $\square^2$(1) =0.188, p= 0.665). Similarly, neither species (BT, mean= 3.22± 0.09, CT, mean= 3.33± 0.11, $\square^2$ (1) =0.606, p=0.436) nor gizzard fullness (empty, mean= 3.20± 0.11, full, mean= 3.35± 0.09, $\square^2$(1) =0.899, p= 0.343) had a significant effect on CCK levels. The different gut tissues showed significant differences in CCK expression, with the highest levels in the proximal and distal jejunum, duodenum and ileum ($\square^2$(9) =1518.83, p<0.001).

Interactions:

Although a significant interaction between sex and tissue ($\square$2(1) =21.90, p= 0.009) was found, Bonferroni-corrected pairwise comparison analysis did not indicate any sex differences for any tissue. Additionally, despite a significant interaction between species and tissue ($\square$2(9) =25.29, p=0.003), post-hoc Bonferroni-corrected analyses did not indicate any species difference among any tissue. Tissue interacted significantly with gizzard fullness ($\square$2(9) =19.38, p=0.022), however, upon looking to the pairwise comparison we could

not detect any gut tissue that showed a clear difference in CCK levels in response to gizzard fullness (Figure 6). There was also a significant 3-way interaction between sex*species* tissue (□2(9) =22.24, p=0.008) where female coal tits had slightly higher CCK in their caecum in comparison to female blue tits (BT, mean= 2.98± 0.12, CT, mean= 3.81± 0.11, p=0.001) but there was no species difference for the males (BT, mean= 3.61± 0.19, CT, mean= 3.471± 0.27, p=1.000). There was also a significant species* tissue*gizzard fullness (□2(9) =18.50, p=0.030), however, Bonferroni- corrected pairwise comparison indicated that there were no tissues in either species showed a significant effect of gizzard fullness. There was a significant 4-way interaction (□2(9) =18.60, p=0.029). Empty state female coal tits had higher CCK in the gizzard than full state ones (empty, mean= 2.75± 0.00, full, mean= 1.72± 0.18, p<0.001). On the other hand, no such difference in CCK expression was seen in the gizzards of male coal tits (empty, mean= 1.85± 0.33, full, mean= 1.70± 0.15, p= 1.000), nor male (empty, mean= 1.92± 0.15, full, mean= 2.05± 0.16, p=1.000) or female (empty, mean= 1.99± 0.14, full, mean= 2.36± 0.17, p= 1.000) blue tits gizzards.

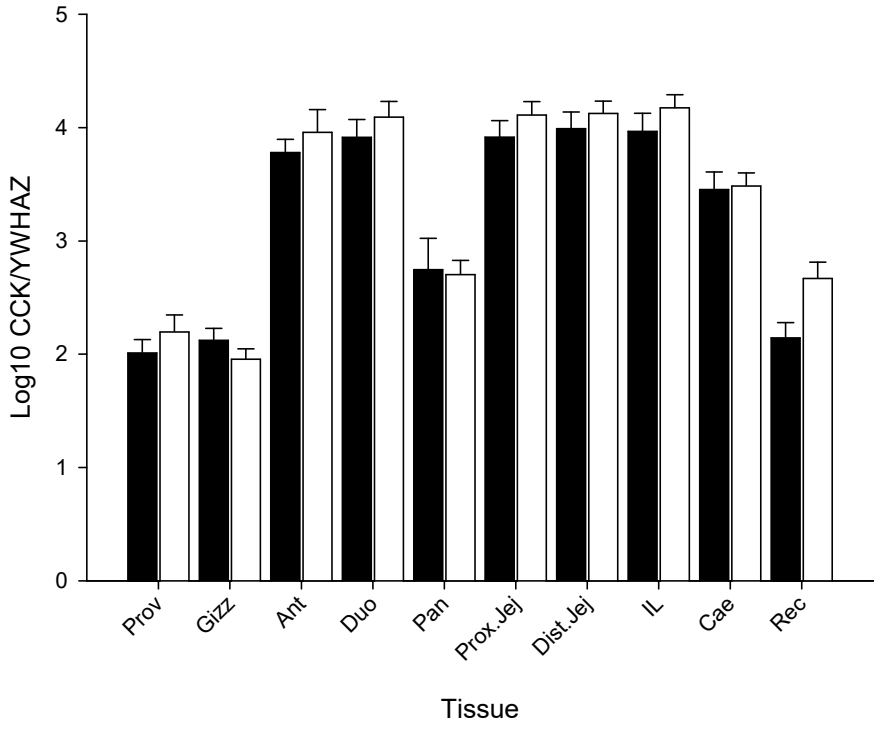


Figure 6: Pairwise comparison of cholecystokinin (CCK) gene expression across different gut tissues in as log10 ratio to tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) using Gizzard fullness as a variable. Refer to glossary to see the abbreviation. Black bars represent empty gizzard while white ones show full gizzard state. Each bar represent mean ± SEM.

Table 2: Pairwise comparison of cholecystokinin (CCK) gene expression in the different tissues. Refer to glossary to see the abbreviation. Green colour rectangles show non-significant p value.

	Prov	Gizz	Ant	Duo	Pan	Prox.Jej	Dist.Jej	IL	Cae	Rect
Prov		**p=1.000**	**p<0.001**	**p<0.001**	**p=0.002**	**p<0.001**	**p<0.001**	**p<0.001**	**p<0.001**	**p=0.005**
Gizz	**p=1.000**		**p<0.001**	**p<0.001**	**p<0.001**	**p<0.001**	**p<0.001**	**p<0.001**	**p<0.001**	**p=0.003**
Ant	**p<0.001**	**p<0.001**		**p=1.000**	**p<0.001**	**p=1.000**	**p=0.890**	**p=1.000**	**p=0.009**	**p<0.001**
Duo	**p<0.001**	**p<0.001**	**p=1.000**		**p<0.001**	**p=1.000**	**p=1.000**	**p=1.000**	**p<0.001**	**p<0.001**
Pan	**p=0.002**	**p<0.001**	**p<0.001**	**p<0.001**		**p<0.001**	**p<0.001**	**p<0.001**	**p<0.001**	**p=1.000**
Prox.Jej	**p<0.001**	**p<0.001**	**p=1.000**	**p=1.000**	**p<0.001**		**p=1.000**	**p=1.000**	**p<0.001**	**p<0.001**
Dist.Jej	**p<0.001**	**p<0.001**	**p=0.890**	**p=1.000**	**p<0.001**	**p=1.000**		**p=1.000**	**p<0.001**	**p<0.001**
IL	**p<0.001**	**p<0.001**	**p=1.000**	**p=1.000**	**p<0.001**	**p=1.000**	**p=1.000**		**p<0.001**	**p<0.001**
Cae	**p<0.001**	**p<0.001**	**p=0.009**	**p<0.001**	**p<0.001**	**p<0.001**	**p<0.001**	**p<0.001**		**p<0.001**
Rect	**p=0.005**	**p=0.003**	**p<0.001**	**p<0.001**	**p=1.000**	**p<0.001**	**p<0.001**	**p<0.001**	**p<0.001**	

3.3.5.2 PYY

Main effect

The expression levels of PYY were not significantly different between males and females (females, mean= 4.22 ± 0.15, males, mean= 3.97± 0.13, □2(1) =1.52, p= 0.217). The levels of PYY were not different between BT and CT (BT, mean= 4.14± 0.08, CT, mean= 4.05± 0.18, □2 (1) =0.187, p=0.666)

The individual's gizzard fullness did not affect PYY levels (empty, mean= 4.16± 0.16, full, mean= 4.04± 0.12, □2(1) =0.334, p= 0.564). However, different gut tissue expressed PYY differently (□2(9) =247.003, p<0.001) with the antrum, duodenum, pancreas and the proximal jejunum having higher levels in comparison to the other tissues. Despite the presence of a strong interaction between species and tissue (□2(9) =26.666, p=0.002), the Bonferroni-corrected pairwise comparison showed no significant species differences for any of the tissues (Figure 7).

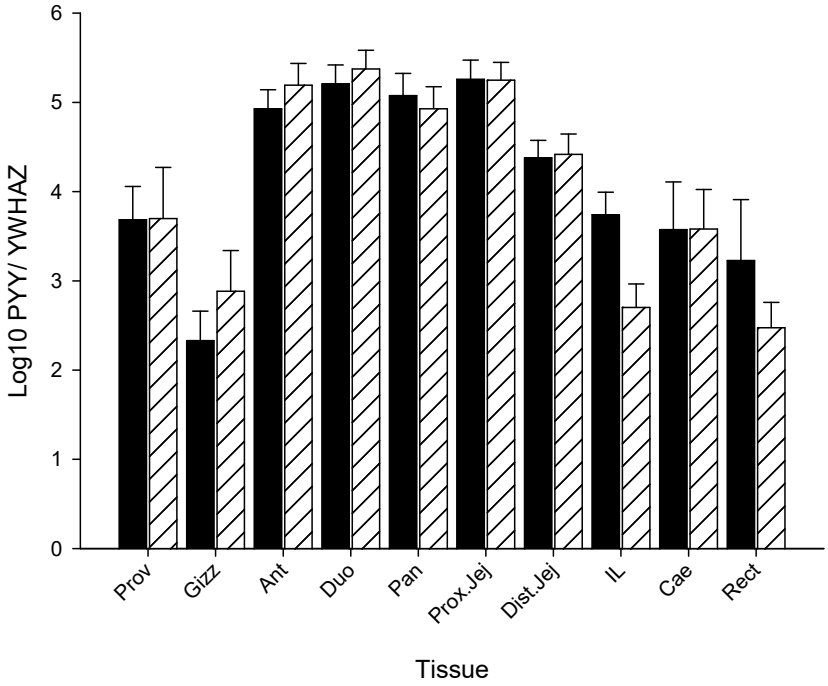


Figure 7: Pairwise comparison of peptide YY (PYY) gene expression between blue tits (solid bar) and coal tits (dashed bar) as log10 ratio to tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) using species as a variable. Refer to glossary to see the abbreviation. Each bar represent mean ± SEM.

Table 3: Pairwise comparison of peptide YY (PYY) gene expression in the different tissues. Refer to glossary to see the abbreviation. Green colour rectangles show non-significant p value.

	Prov	Gizz	Ant	Duo	Pan	Prox.Jej	Dist.Jej	IL	Cae	Rect
Prov		p=0.712	p=0.034	p=0.003	p=0.075	p=0.010	p=1.000	p=1.000	p=1.000	p=1.000
Gizz	p=0.712		p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p=1.000	p=1.000	p=1.000
Ant	p=0.034	p<0.001		p=1.000	p=1.000	p=1.000	p<0.001	p<0.001	p=0.059	p<0.001
Duo	p=0.003	p<0.001	p=1.000		p=0.219	p=1.000	p<0.001	p<0.001	p=0.005	p<0.001
Pan	p=0.075	p<0.001	p=1.000	p=0.219		p=0.460	p<0.001	p=<0.001	p=0.157	p<0.001
Prox.Jej	p=0.010	p<0.001	p=1.000	p=1.000	p=0.460		p<0.001	p<0.001	p=0.011	p<0.001
Dist.Jej	p=1.000	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001		p<0.001	p=1.000	p=0.001
IL	p=1.000	p=1.000	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001		p=1.000	p=1.000
Cae	p=1.000	p=1.000	p=0.059	p=0.005	p=0.157	p=0.011	p=1.000	p=1.000		p=1.000
Rect	p=1.000	p=1.000	p<0.001	p<0.001	p<0.001	p<0.001	p=0.001	p=1.000	p=1.000	

3.3.5.3 GCG

Main effect

The expression of GCG was not different between males and females (females, mean= 4.01 ± 0.09, males, mean= 4.03± 0.10, □2(1) =0.023, p= 0.880). BT and CT did not differ in GCG levels (BT, mean= 3.92± 0.09, CT, mean= 4.11± 0.11, □2 (1) =1.77, p=0.183). Individual's nutritional state did not affect GCG gene expression (empty, mean= 4.00± 0.11, full, mean= 4.04± 0.08, □2(1) =0.096, p= 0.757). On the other hand, different gut tissues expressed GCG differently (□2(9) =2100.13, p<0.001) with the pancreas expressing the highest levels of GCG among the other tissues (mean= 5.57± 0.09).

Interactions:

There was a significant species* tissue interaction (□2(9) =105.12, p<0.001) (Figure 8), however, Bonferroni- corrected pairwise comparison did not show any significant species differences for any of the tissues.

There was also a significant tissue* gizzard fullness interaction (□2(9) =46.54, p<0.001). But again, post-hoc Bonferroni- corrected analysis did not detect a nutritional state difference for any of the tissues. There was a significant 3-way interaction between sex*species*gizzard fullness (□2(9) =4.98, p=0.026), sex*tissue*gizzard fullness (□2(9) =43.79, p<0.001), species*tissue*gizzard fullness (□2(9) =35.61, p<0.001). However, Bonferroni-corrected pairwise comparison showed that gizzard fullness did not have a significant effect on any interaction combination of sex/species, sex/ tissue, and species/tissue. Finally, there was a significant 4-way interaction between sex*species*tissue*gizzard fullness (□2(9) =27.28, p=0.001) mostly due to the females of the two species. Empty gut female BT had higher GCG in their antrum in comparison to full ones (empty, mean= 4.98± 0.26, full, mean= 3.57± 0.10, p= 0.002). On the other hand, empty gut female CT had higher GCG in their gizzards in comparison to fed ones (empty, mean= 1.23± 0.00, full, mean= 2.35± 0.13, p<0.001). Having said that, no such difference in GCG expression was found in the antrum of male BT (empty, mean= 3.80± 0.29, full, mean= 4.15± 0.09, p= 1.000) nor in the gizzards of male CT (empty, mean= 2.22± 0.38, full, mean= 2.31± 0.23, p= 1.000).

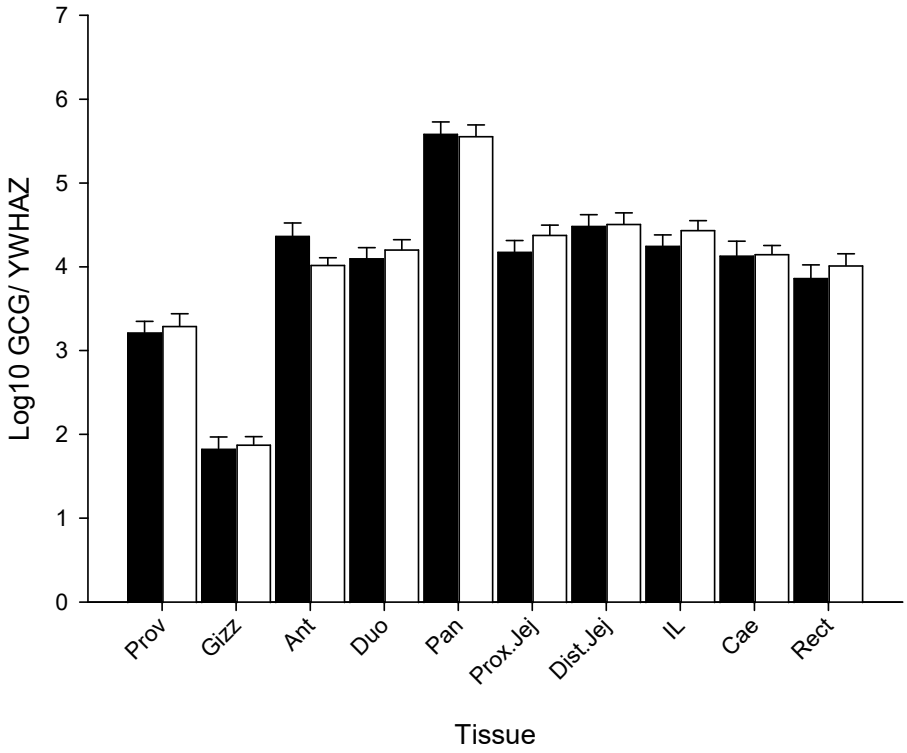


Figure 8: Pairwise comparison of preproglucagon (GCG) gene expression across different gut tissues as log10 ratio to tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) using Gizzard fullness as a variable. Black bars represent empty gizzard while white ones show full gizzard state. Refer to glossary to see the abbreviation. Each bar represent mean ± SEM.

Table 4: Pairwise comparison of preproglucagon (GCG) gene expression in the different tissues. Refer to glossary to see the abbreviation. Green colour rectangles show non-significant p value.

	Prov	Gizz	Ant	Duo	Pan	Prox.Jej	Dist.Jej	IL	Cae	Rect
Prov		p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
Gizz	p<0.001		p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
Ant	p<0.001	p<0.001		p=1.000	p<0.001	p=1.000	p=0.065	p=1.000	p=1.000	p=0.423
Duo	p<0.001	p<0.001	p=1.000		p<0.001	p=0.185	p<0.001	p<0.001	p=1.000	p=0.616
Pan	p<0.001	p<0.001	p<0.001	p<0.001		p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
Prox.Jej	p<0.001	p<0.001	p=1.000	p=0.185	p<0.001		p<0.001	p=1.000	p=0.981	p=0.001
Dist.Jej	p<0.001	p<0.001	p=0.065	p<0.001	p<0.001	p<0.001		p<0.001	p<0.001	p<0.001
IL	p<0.001	p<0.001	p=1.000	p<0.001	p<0.001	p=1.000	p<0.001		p=0.004	p<0.001
Cae	p<0.001	p<0.001	p=1.000	p=1.000	p<0.001	P=0.981	p<0.001	p=0.004		p<0.001
Rect	p<0.001	p<0.001	p=0.423	p=0.616	p<0.001	p=0.001	p<0.001	p<0.001	p=0.046	

3.3.5.4 INS

Main effect

Insulin gene expression was not different between males and females (females, mean= 4.00 ± 0.16, males, mean= 3.64± 0.15, □2(1) =2.34, p= 0.126). Coal tits had higher INS levels in comparison to blue tits (BT, mean= 3.57± 0.14, CT, mean= 4.07± 0.18, □2 (1) =4.55, p=0.033). The nutritional state of the individuals significantly affected INS gene expression with empty gut birds having higher levels in comparison to full ones (empty, mean= 4.11± 0.20, full, mean= 3.53± 0.10, □2(1) =6.16, p= 0.013). Gut tissues significantly differ in how much INS they express (□2(9) =2323.16, p<0.001) with the pancreas having the highest levels (mean= 7.59± 0.16).

Interactions:

There was a significant sex*tissue interaction (□2(9) =38.91, p<0.001), however, post-hoc Bonferroni-corrected pairwise comparisons did not indicate any sex differences for any of the tissues. There was also a significant tissue*gizzard fullness interaction (□2(9) =48.56, p<0.001) with a difference in INS expression in the antrum, where empty state antrum had higher INS levels than full state antrum (empty, mean= 5.18± 0.36, full, mean= 2.69± 0.32, p<0.001) (Figure 9). However, no other tissue showed INS levels differences in response to gizzard fullness. We detected several significant 3-way interactions. There was an interaction between: sex*species*tissue (□2(9) =33.80, p<0.001), however, Bonferroni-corrected pairwise comparison did not show a significant difference between sexes and species for any tissue. Additionally, there was a significant interaction between sex*tissue*gizzard fullness (□2(9) =29.70, p<0.001) mostly clear in the antrum of females where empty state antrum had higher INS gene expression in comparison to full state antrum (empty, mean= 6.79± 0.18, full, mean= 2.80± 0.60, p<0.001). On the other hand, no such difference in INS levels was detected in the antrum of males (empty, mean= 3.58± 0.70, full, mean= 2.59± 0.22, p=1.000). A significant 3-way interaction was also found between species*tissue*gizzard fullness (□2(9) =22.81, p=0.007) especially in the antrum of blue tits where empty gut antrum had higher INS than when its full (empty, mean= 5.48± 0.62, full, mean= 2.04± 0.12, p<0.001). However, that was not the case for coal tits. Coal tits antrum did not show different INS expression levels in response to gizzard fullness (empty, mean= 4.89± 0.37, full, mean= 3.35± 0.63, p=1.000).

Finally, there was a significant 4- way interaction between sex*tissue*species*gizzard fullness (□2(9) =31.26, p<0.001) where empty gut female blue tit's antrum had higher INS in comparison to full state ones (empty, mean= 6.65± 0.00, full, mean= 1.58± 0.00, p<0.001) but female coal tits antrum did not show a difference in INS levels in response to gizzard fullness (empty, mean= 63.93± 0.37, full, mean= 4.02± 1.20, p=1.000). Similarly, neither male BT antrum nor CT showed a significant difference in INS gene expression

in the different state (BT; empty, mean= 4.31± 1.25, full, mean= 2.50± 0.25, p=1.000; CT; empty, mean= 2.85± 64, full, mean= 2.68± 0.36, p=1.000).

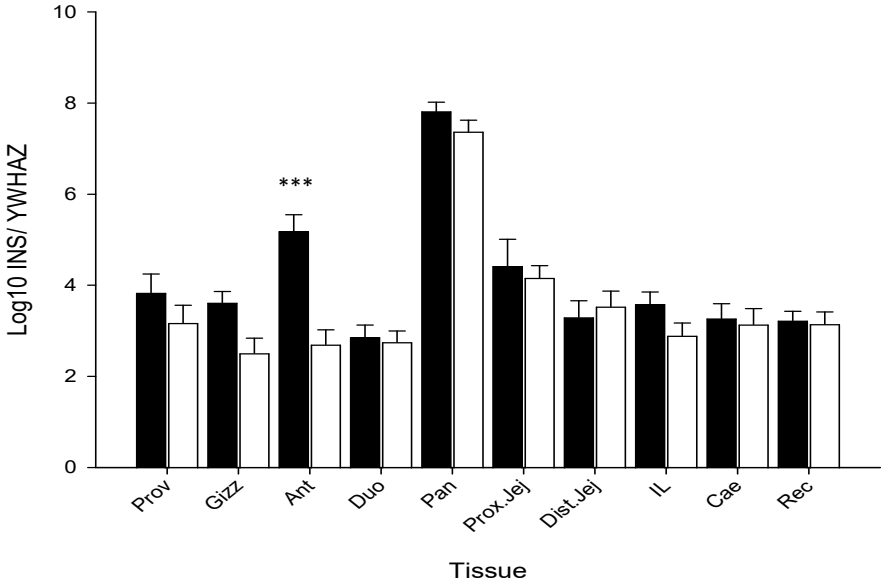


Figure 9: Pairwise comparison of insulin (INS) gene expression across different gut tissues as log10 ratio to YWHAZ using gizzard fullness as a variable. Black bars represent empty gizzard while white ones show full gizzard state. Refer to glossary to see the abbreviation. The asterisk represents significant difference between the two species ((*) p<0.05, (**) p<0.01 (***) p<0.001). Each bar represent mean ± SEM.

Table 5: Pairwise comparison of insulin (INS) gene expression in the different tissues. Tissues are arranged in order of dissection. Refer to glossary to see the abbreviation. Green colour rectangles show non-significant p value.

	Prov	Gizz	Ant	Duo	Pan	Prox.Jej	Dist.Jej	IL	Cae	Rect
Prov		p=1.000	p=1.000	p=1.000	p<0.001	p=1.000	p=1.000	p=1.000	p=1.000	p=1.000
Gizz	p=1.000		p=0.006	p=1.000	p<0.001	p=0.141	p=1.000	p=1.000	p=1.000	p=1.000
Ant	p=1.000	p=0.006		p<0.001	p<0.001	p=1.000	p=1.000	p=0.267	p=0.962	p=0.295
Duo	p=1.000	p=1.000	p<0.001		p<0.001	p<0.001	p=0.599	p=1.000	p=1.000	p=1.000
Pan	p<0.001	p<0.001	p<0.001	p<0.001		p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
Prox.Jej	p=1.000	p=0.141	p=1.000	p<0.001	p<0.001		p=0.111	p=0.047	p=0.005	p=0.023
Dist.Jej	p=1.000	p=1.000	p=1.000	p=0.599	p<0.001	p=0.111		p=1.000	p=1.000	p=1.000
IL	p=1.000	p=1.000	p=0.267	p=1.000	p<0.001	p=0.047	p=1.000		p=1.000	p=1.000
Cae	p=1.000	p=1.000	p=0.262	p=1.000	p<0.001	P=0.005	p=1.000	p=1.000		p=1.000
Rect	p=1.000	p=1.000	p=0.295	p=1.000	p<0.001	p=0.023	p=1.000	p=1.000	p=1.000	

3.3.5.5 GCG/INS ratio in the pancreas only

Main effect

Coal tits had higher GCG/INS ratio in their pancreas in comparison to blue tits (BT, mean= 2.11± 0.22, CT, mean= 3.91± 0.22, $\square^2$ (1) =32.11, p<0.001). GCG/INS ratio was higher in the pancreas of empty state coal tits in comparison to full state ones (empty, mean= 3.38± 0.25, full, mean= 2.65± 0.18, $\square^2$(1) =5.38, p= 0.020). On the other hand, GCG/INS ratio was not different between males and females (females, mean= 3.08 ± 0.23, males, mean= 2.94± 0.20, $\square^2$(1) =0.200, p= 0.654).

Interactions:

There was a significant species*gizzard fullness interaction ($\square^2$(9) =4.88, p=0.027) (Figure 8) mostly seen in coal tits where empty state individuals had slightly higher GCG/INS in their pancreas in comparison to full state ones (empty, mean= 4.63± 0.35, full, mean= 3.20± 0.28, p=0.011). However, no such difference was seen in blue tits (empty, mean= 2.13± 0.35, full, mean= 2.10± 0.25, p=1.000)

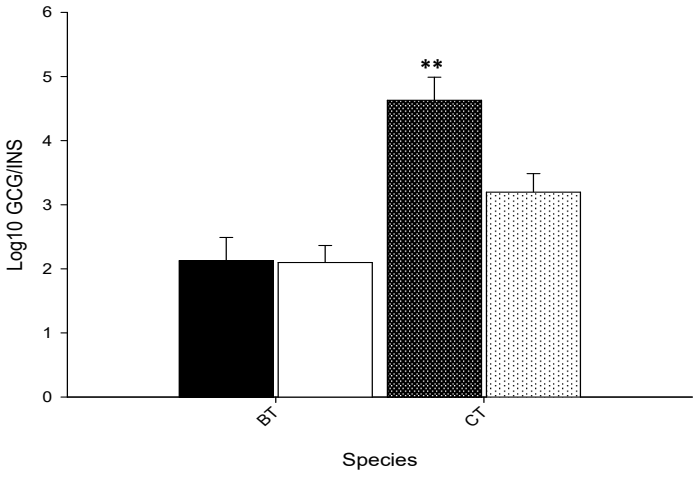


Figure 10: Pairwise comparison showing the glucagon: insulin ratio (GCG/INS) in the pancreas. Blue tit (solid bar), coal tit (dotted bar); empty state (black bar), full state (white bar). The asterisk represents significant difference between the two species ((*) p<0.05, (**) p<0.01 (***) p<0.001). Each bar represent mean ± SEM.

3.4 Discussion

For this part of the study, we aimed to investigate the same group of gut and neuropeptides that were discussed in Chapter 2 but putting an emphasis on the difference in the mode of expression of those hormones between two behaviourally different songbirds, blue tits and coal tits. We aim to gain an insight into specific differences in the way peptide gene expression changes after energetic challenge in a hoarding vs. non-hoarding species as well as the differences in overall expression between species. We hypothesised that the signals gut peptides transduce to the brain are responsible for reporting the nutritional state and the energy expenditure in an individual to the brain leading it to express specific behaviours.

On average, blue tits were heavier than coal tits. However, we did not observe any statistical differences in the fat score. Inboth species full gut individuals had both higher body mass and fat score in comparison to empty individuals. This indicates that our experimental fasting and re-feeding was successful in making a detectable change in the birds' energy stores so that the difference in body mass was not due just to the presence or absence of food in the gizzard.

Several gut and neuropeptides have been found to play major roles in the appetite regulatory system signalling satiety. Here we are interested in comparing the expression pattern of selected peptides between hoarding and non-hoarding species. In the hypothalamus, NPY, AGRP and POMC mRNAs were higher in the blue tits. And even though, neither NPY nor hypothalamic POMC gene expression was affected by the individual nutritional state, AgRP mRNA was significantly higher in empty stomach coal tits in comparison to full ones. Furthermore, the AgRP/POMC ratio in coal tits changed with the nutritional state of the individual where empty state birds had higher levels in comparison to full ones. As for the hindbrain neuropeptides, while coal tits showed higher GCG mRNA in the hindbrain, blue tits had higher POMC levels. As for gut peptides, the only peptide that showed a difference between the two species and the two treatments was insulin, with empty gut female blue tits showing higher insulin mRNA in their antrum. However, for female coal tits the antrum did not show any difference in insulin mRNA levels in either state.

3.4.1 Neuropeptides

Hypothalamus

In our study, the expression of NPY, AGRP and POMC was generally higher in the blue tits in comparison to the coal tits. In particular, POMC gene expression was 2.1× higher compared to only 1.2× for both NPY and AGRP. The AGRP/POMC ratio was also different between the two species. The fact that we did find differences in neuropeptide gene expression between the blue and coal tits might be related to the differences in feeding and hoarding behaviour (which is explored in more details in chapter 4).

As for gizzard fullness, no changes were found in the expression of either NPY or POMC. Our speculation for not detecting changes in gene expression is that 1.5hrs of re-feeding might not be enough to stimulate changes in gene expression levels. Even though we did observe a significant difference in fat score, the difference between the fed and fasted body mass was only about 5%. This difference is less than that observed in other studies (Boswell et al. 2002) where changes in NPY expression were seen after more extended, 24h food deprivation with a 12% loss of body mass compared to the fed state. Having said that, we only detected a significant change in the expression of both AgRP and the AgRP/POMC ratio. For AgRP, empty gut birds showed significantly higher AgRP mRNA in comparison to full individuals which is what we would expect. Studies have shown that AgRP mRNA levels were increased by food deprivation in several bird species. Fang et al. (2014) demonstrated that 48h significantly increased AgRP mRNA in male broiler chickens. Similar results were consistently found in adult Japanese quail fasted for 24h (Phillips-Singh et al. 2003). In both cases, AgRP expression returned to baseline after 24h of re-feeding (Phillips-Singh et al. 2003).

As mentioned, in chapter 2, both AgRP and hypothalamic POMC containing neurons have neural projections to the same brain regions. Those two peptides not only have an antagonising effect on each other, and compete to bind to the same receptor (Boswell and Dunn 2017), but was also shown in rats to be a crucial stimulator for the central melanocortin signalling pathways regulating and controlling the appetite system (Korner et al. 2003). Thus, we examined the AgRP/POMC ratio in relation to the individual nutritional state. We demonstrated that in coal tits, empty state birds had a higher AgRP/POMC ratio in comparison to full ones. This suggests that there may be a species difference in the regulation of melanocortin system signalling and the significance of this in relation to hoarding is explored further in Chapter 4. Korner el al. (2003) found that in both *ad lib* and pair -fed (rats fed only the mean amount of food/ day that the same rat consumed per day as calculated over the 2 days before injection) AgRP injected rats showed a significant elevation in AgRP/POMC ratio that was consistent with the increased stimulation of the central melanocortin signalling pathway. Since AgRP signals for hunger, and POMC terminates a meal (signals satiety), one would expect that a higher ratio would lead the animal to forage more and the decision after finding the food is related to its energetic status at that moment (explored more in chapter 4).

In relation to sex differences, unlike Caughey et al. (2018)'s study in chickens where they were not able to find sex difference in POMC mRNA gene expression, we detected a significant difference between the two sexes in the expression of POMC only specifically in coal tits. Males had higher POMC mRNA in comparison to females. However, no such difference was observed in blue tits. Rancourt et al. (2018) showed that 3-week-old female chickens had higher methylation levels at two CpG sites in the POMC promoter regions than males. DNA methylation is an epigenetic mechanism used by cells to control gene expression. Applying this information to the Rancourt et al. (2018) study, females that had higher methylation in the CpG sites in the POMC promoter had more POMC mRNA expressed. We did not conduct an epigenetic study in our case, so we could not attribute the difference in POMC mRNA between the sexes

to DNA methylation. Having said that, overall, POMC mRNA has always shown contradictory effects in birds. Some studies report no change, while others like our case and Rancourt et al. (2018) observed changes but in the opposite sex. This variability in POMC mRNA results might suggest a very curtailed and fundamental role of POMC in the regulation of appetite in relation to energy homeostasis in animals.

Hindbrain

When it comes to the hindbrain, we only observed species differences in the level of GCG and POMC. While coal tits showed higher GCG mRNA in the hindbrain, blue tits showed higher POMC gene expression. Those differences could be due to behavioural differences between the two species (will be explored in Chapter 4)

For GCG, Larsen et al. (1997) demonstrated that the posttranslational product of the proglucagon in the hindbrain is glucagon like peptide-1 (GLP-1), hence that is likely to be the peptide product of the GCG gene expression we measured. The function of GLP-1 is conserved between mammals and birds (Honda 2016). Thus, we speculate that higher GLP-1 synthesis in coal tits might be linked to satiety and maybe to hoarding too. A study by Williams et al. (2016) demonstrated that in rats, GLP-1 functioned to reduce meal size and prolonged the inter-meal intervals. Thus, we assume that higher levels of GLP-1 in non-hoarding birds are strategically important for individuals to stay satiated for longer periods and reduce the foraging trips in fluctuating conditions.

The higher GCG mRNA levels observed in the coal tits combined with the well-established role of GLP-1 in suppressing food intake made us speculate about the possibility of its involvement in hoarding motivation. Studies have shown that in rats, hindbrain GLP-1 signals the reduction of food intake and the motivation to feed (Alhadeff and Grill 2014). Therefore, it is possible that an elevation of GLP-1 in coal tits while supressing the motivation to eat, might stimulate food hoarding behaviour. On the other hand, we speculate that the higher levels of POMC mRNA gene expression observed in blue tits aid in increasing their foraging tendencies thus increasing food foraging motivation while keeping them satiated. This is explored further by the video analysis in Chapter 4.

The nutritional state of the individual did not cause any changes in the expression of the genes. It is possible that 1.5h re-feeding was not long enough to cause a change in the expression of the neuropeptides. Gelegen et al. (2012) demonstrated that rats fasted for 24h showed a significant reduction of hindbrain neuropeptides gene expression. However, because our birds are very small and fragile, we cannot expose them to a very prolonged fasting period. Additionally, we speculate that since those birds a very metabolically active, they might need a longer re-feeding time frame for satiety peptides to exert their effect on their appetite.

3.4.2 Gut peptides

Pancreatic peptides (Glucagon (GCG), and insulin (INS))

The results presented here demonstrate that both blue and coal tits showed higher GCG mRNA levels in the pancreatic tissues, which is similar to the distribution pattern seen in great tits. GCG expression did not change according to the nutritional state of both blue and coal tits. However, previous studies in chickens fasted for 24-48h demonstrate a strong increase in plasma glucagon peptide which declines with 24h re-feeding (Richards and McMurtry 2008). The contradiction seen between our study and the other study could be related to the differences in body size between our birds and the chicken alongside the differences in their metabolic demands and energetic needs.

Insulin mRNA levels were the highest in the pancreas of both blue and coal tits which again follows the same pattern we observed in great tits. Those observations were also found in ducks (Samols et al. 1969) and chickens (Langslow et al. 1970).

Insulin levels were significantly affected by the nutritional status of the individuals, with empty gut birds having higher insulin mRNA in comparison to fed ones. This result is not what one might expect for insulin to behave if an individual is food deprived. Studies in chickens show that plasma levels of insulin are decreased in fasted individuals (Simon et al. 2011). Studies in healthy humans estimated that within a time frame of 30 min-1h of ingesting a meal, insulin levels rapidly peak, returning to basal levels within 2-4h (Galloway and Chance 1994). On the other hand, in our study we did not measure the peptide, so it is difficult to make conclusions about how insulin transcription is related to the circulating peptide. Especially, since INS mRNA was also found in other gut tissues but in lower magnitude when compared to the pancreas. Therefore, behavioural analysis (Chapter 4) becomes more necessary to pinpoint if the behaviour has any correlation with insulin expression.

The pancreatic GCG/INS ratio was significantly higher in coal tits in comparison to blue tits. Additionally, empty state coal tits expressed more GCG/INS ratio mRNA than fed ones with no difference observed in blue tits in either state. There is very little information about the role of glucagon/insulin ratios and appetite system but given the fact that both hormones had an opposing function to one another, one might expect the regulation of the ratio might have something to do with the original function of those genes separately. However, what we know is that higher GCG/INS would stimulate the mobilization of the nutrient. On the other hand, lower GCG/INS would promote the biosynthesis of proteins and the reduction of blood glucose rise (Kalra and Gupta 2016).

Glucagon related peptides and insulin gene expression outside the pancreas

In the present study, we found higher GCG mRNA gene expression in the rest of the digestive tract in both blue and coal tits. A similar pattern was also found in great tits (Chapter 2) where GCG mRNA levels were the second highest in the rest of the gastrointestinal tract. That tissue distribution in the three tit species is

aligned to what is found in the literature. According to Hiramatsu (2020), the number of GCG producing cells in the small intestine of chicken seems to be higher in more proximal regions, reaching a maximum in the distal ileum.

GCG expression did not seem to respond to the nutritional state of individuals. However, different tissues demonstrated different trends of gene expression in the opposite directions which is typical as each tissue expresses GCG in a unique way. Having said that, overall, the empty state females of the two species expressed more GCG mRNA in comparison to males. While empty state blue tit female showed higher GCG mRNA in their antrum, empty state female coal tits had higher GCG mRNA in their gizzards. Therefore, we speculated the presence of other forms of GCG outside the pancreas. This fact was confirmed because tissue specific action of the enzyme prohormone convertase directs the synthesis of GLP-1 in the L-cells of the gastrointestinal tract which are known to be abundant in the intestine of rodents (Lim and Brubaker 2006). So, in our case we could speculate that what we detected in our study is GLP-1 since we did not measure the actual peptide.

The higher GCG expression we observed in the antrum, may be linked to findings by Rotondo et al. (2011) of a role for GLP-1 into slowing of the gastric emptying process in the mouse antrum, thus indirectly reducing food intake which is in agreement with its anorexigenic action.

Kenan Diler (2008) was able to detect GCG immunoreactive cells in the gizzards of 9-day-old chicks, while Yamaguchi et al. (1987) were able to detect GCG immunoreactive cells in the gizzard of 13-day old quail. However, both studies were also able to detect GCG immunoreactive cells in the adults. Thus, our finding of GCG in the gizzards of adult coal tits is not surprising and agrees with what others have found in other avian species. According to Kokas et al. (1971) the administration of glucagon inhibited the volume of spontaneous gastric juice secretion (in the gizzard) and decreased pepsin output in chickens. Thus, we think that the presence of GCG mRNA in the gizzard might slow/inhibit the gastric emptying process and participate in ileal breakdown by inhibiting small intestine transit. This would prolong the satiation effect. The inhibition function of GCG caused an overall reduction in the absorption of nutrients from the gastrointestinal tract (Perfetti and Merkel 2000). We speculate that higher in the coal tit gizzard might function to maximise the gastric emptying process, thus keeping individuals constantly motivated to forage and store food.

We found higher insulin gene expression in the antrum of empty state male blue tits in comparison to full state ones. Studies have shown that the inhibitory action of insulin might be in part independent of its effect on glucagon secretion (Eisenberg et al. 1963) or its hypoglycaemic effect (Hirschowitz and Robbins 1966). Kemp et al. (1968) showed that in dog's antrum, the presence of insulin causes a re-distribution of potassium within cells (very crucial to the secretory function). Thus, the inhibitory action of insulin on food intake might be in part regulated via the hyperpolarization of the cell, thus decreasing the motility of the antrum, which in turn inhibits the secretion of the hormone gastrin, thus slowing the gastric phase. Why we observed

high insulin mRNA in the empty state of both blue and coal tits rather than the full state might suggest the possibility that insulin have other physiological significance in those tissues.

PYY

In our study PYY gene expression was not different between the blue and coal tits. However, PYY was higher in the duodenum, proximal jejunum, pancreas and the antrum of both species. Similar PYY mRNA distribution was found in the great tits, with higher PYY levels in the antrum, duodenum, pancreas, and proximal jejunum Our results fit the distribution pattern found by Gao et al. (2017). In their study, they showed that chicken PYY is highly expressed in the proventriculus, duodenum, jejunum, ileum and pancreas. Moreover, Reid at al. (2017) using in situ hybridization, found that the major source of gut PYY mRNA is around the distal jejunum, however the duodenum had a relatively lower PYY expression.

On the other hand, although PYY is a known satiety signal, no significant effect of gut fullness was observed in the current study. Moreover, we obtained similar findings in great tits (chapter 2). We were not able to detect any effect of the nutritional state on PYY levels in the great tit experiment. Having said that, studies in chickens have shown a significant elevation of PYY mRNA under *ad lib* conditions in comparison to under 12h fasting (Aoki et al. 2016). Why we were not able to observe an effect of the nutritional state on the expression of PYY mRNA in both studies is still not clear. However, considering how active those small songbirds are, and given that after meal PYY peptide levels increase within 15min and stay elevated 1-2h (Adrian et al. 1985), we speculate that PYY mRNA levels might rapidly return to baseline after the peptide has been secreted because of the high metabolism of titmice.

CCK

In the present study, although both blue and coal tits did not differ in CCK expression, overall, CCK mRNA was higher in the duodenum, proximal and distal jejunum and ileum. In the previous chapter, we also found that great tits showed a wider range of CCK expression in different gut tissues. Studies in chickens have demonstrated that CCK expression levels were highest around the proximal half of the ileum (Reid and Dunn 2018). However, CCK mRNA was lower but detectable in the proventriculus and the boundaries within the antro-duodenal regions in chickens (Reid and Dunn 2018). These observations simply suggest that different species use gut peptides differently according to their physiology, and that usage might largely depend on the way peptide synthesis is distributed within a certain tissue.

Upon investigating the effect of the nutritional state of the individual on CCK expression, we found that CCK was not affected by gizzard fullness. Similar findings were also obtained in the great tit study (chapter 2), where CCK expression was not significantly affected by an individual's nutritional state. Most of the studies in birds looking at CCK and nutritional state have not looked at the CCK expression profile across different gut tissues in response to energetic challenges. For instance, Reid and Dunn (2018) looked at the CCK expression in chickens either fasted for an hour or reintroduced to food after 3h from removing it and given 2.5h to feed to investigate how CCK expression responded to short-term feed restriction. Although

they were not able to detect any significant difference between the two treatments (fasted vs. fed), CCK hybridisation signals between the two groups showed that CCK anticipatory expression might be different if the two groups were under longer nutritional challenges. For that reason, we could speculate that the 1.5h re-feeding time frame in our study might not be long enough to observe a nutritional state effect and that is consistent with the Reid and Dunn (2018) study. CCK is known to stimulate gall bladder contraction and pancreatic exocrine secretion to signal satiety by activating the vagal afferents (Owyang 1996), and those functions are mostly associated with CCK being primarily secreted from tissues of the lower intestine (Fakhry et al. 2017).

Even though the nutritional state of the individuals did not cause any difference in CCK mRNA, gizzard fullness interacted significantly with species, sex and tissue. Empty state female coal tits had higher CCK mRNA in their gizzards in comparison to full ones, while no state difference was seen in blue tits. Having said that, in the great tit study, it was the empty state caecum and the full state proventriculus of males that showed higher CCK mRNA expression. There are not many studies in the literature that have explored the sex differences in songbirds in relation to the nutritional state, however, one study in rats showed that old and young female rats did show higher CCK levels compared to those in males, but they did not do any manipulation with food (Miyasaka et al. 1995). However, we cannot rule out the possibility that CCK peptide levels did not change in either circulation or the gut tissue. It might simply mean that we need to measure circulating CCK peptide levels to have a clear indication in regard to whether CCK gene expression changes in accordance with both the nutritional state as well energy expenditure.

We also observed a significant interaction between sex, species and tissue, but this time, female coal tits showed slightly higher CCK mRNA in their caecum in comparison to female blue tits. But again, since this study is the first to look at CCK expression in different passerine species, we might need to investigate the behavioural videos in order to understand the action of CCK further.

Reasons for a lack in the differences in gut peptides gene expression between fasted and fed birds

Although we found some effects of fasting and refeeding in the neuropeptides, we did not detect several expected changes in gut peptides. This could be due to several factors. One possible factor is stress due to captivity. Housing wild animals in captivity is often very necessary for experimental studies (Dickens and Bentley 2014). And it is well established that captivity induces stress (Owen et al. 2004) which could potentially lead to the dysregulation of behaviour (Wingfield et al. 1998). Song et al. (2012) demonstrated that in laying hens that were exposed to heat and high temperature (stressed), their hypothalamic and peripheral (duodenum and jejunum) CCK mRNA was downregulated causing a reduced intestine mobility. It is therefore possible that stress also prevented the changes in gut peptides that might have happened under non-stressed conditions. That being said, it should be noted that for those small song birds being stressed is a part of their life history since they live in a fluctuating environment where they constantly need to mobilise

their energy sources to meet their high energetic demand. Also, in our case, birds were placed in aviaries for two weeks before moved into cages for our experiment. The aviaries were very well equipped with branches and soft substrate that resembles what the individuals were used to in the wild and they were able to socialize and interact. Pots of both water and food were scattered everywhere so each bird had the opportunity to feed, as well as distributing food on the ground where birds were given a chance to land on the substrate and eat. When moved to the cages, they were given a chance to interact with their cage mate for certain amount of time during the day. Having said all that, till this day, our information regarding the complicated physiological implications of stress and its effect on the appetite system in birds is scarce and not very clear. Moreover, we need to measure corticosterone levels (both baseline and during different time points and before humanely sacrificing the birds) to either rule out or become certain regarding whether not seeing an effect is due to stress or simply because our treatment was not drastic enough.

There are other potential factors as well that could account for our lack of effect of food treatment. First, in the present study the number of individuals was not large enough to pick up subtle statistical differences due to the short nature of the fasting and refeeding (i.e., the statistical power was not large enough). Second, we used Bonferroni- corrections in our statistical model which was so conservative when dealing with our data that it might miss significant effects. Lastly, one important point that should be emphasised here is the fact that in our experiment we did only ever measure the mRNA of the gut peptide which is not a precise representative of the protein that is circulating in the individuals, so we were not able to detect any significant differences in the gene expression given the short re-feeding time frame.

At the end, among the other recognized issues (listed above), comparing only two species might not be enough to make us able to assign the differences to hoarding vs. non-hoarding. To be able to do so, we need more hoarding and more non-hoarding species to have a broader spectrum to look at other factors that might contribute to allocating certain behaviour to a certain individual.

3.5 Conclusion

In conclusion, in the present study we observed few differences between the empty vs full state. The reason might be due to the experimental design, particularly the short period of re-feeding. However, most of the species' differences in peptide expression were observed for neuropeptides rather than gut peptides. Coal tits had higher expression of: AgRP and AgRP/HYP POMC ratio than blue tits. Blue tits did not show any differences in the expression of the neuropeptide as a response to fasting, however, coal tit did. As for hindbrain peptides, while coal tits showed high levels in GCG gene expression, blue tits showed higher POMC levels. Having said that, the hindbrain neuropeptides in neither species responded to fasting. The only gut peptide gene that showed a response to the nutritional state and species difference was insulin in the antrum: the antrum of empty state blue tits showed higher insulin levels in comparison to the full state blue tits, while no such effect was found in the coal tit antrum. Overall, this suggests a possible species difference in the regulation of neurons that may be linked to hoarding behaviour and that possibility is further explored in Chapter 4.

Chapter 4. Different ingestive behaviours are correlated with the expression of both neuro and gut peptides

In the previous chapter, we focused mainly on how gene expression changes according to the nutritional state of coal and blue tits. However, in the current chapter we will mainly highlight how these species respond behaviourally to energetic challenges (e.g., food restriction) and whether there is a correlation between behaviour and changes in gene expression.

The central and peripheral control of both hunger and satiety ensure that the metabolic needs of an individual are met over a wide spectrum of temporal states faced in either the nature or a laboratory set up (Lees et al. 2017). Having said that, it is worth mentioning that a clear distinction between food unpredictability and deprivation/fasting should be made. While the former detailed the likelihood of food sources to change suddenly, uncontrollably and unforeseeably, the latter is more to do with abstaining the individual from all food sources but water for experimental reasons in this case. Avian species commonly experience food deprivation on a normal basis, for example in periods of migration, overwintering and overnight (King 1973). Under those conditions, birds need to exhibit plasticity in feed intake regulation to meet all those challenges. Despite this plasticity, our understanding of avian appetite and hoarding behaviour is poor in comparison to mammals.

As mentioned previously, the typical response of an animal to fasting is to eat more, and birds are not an exception (King 1963). However, birds differ in how motivated they are to eat depending on their metabolic need and their need to engage in other essential survival behaviours. Studies in Japanese quail showed that a 24h fast resulted in a marked increase in food intake when compared to fed individuals (Boswell et al. 2002). Similarly, broiler chickens, although known to have high body weight and limited physical activity, were still motivated to peck and eat when subjected to food restriction despite the well-known notion of them not to be sensitive to food restriction (Bokkers et al. 2004).

Siberian hamsters exhibit physiological and behavioural responses to extreme food deprivation (Wood and Bartness 1996) but not in a manner that we might predict. Developed by 21 days of age, food hoarding in hamsters is considered as an integral part of an innate ingestive behaviour (Etienne et al. 1982). Studies have shown that, unlike rats that increase food intake after a fast (Lawrence and Mason 1955), when food deprived, hamsters do not increase their feed intake, rather they over-hoard (Silverman and Zucker 1976). This is not surprising, since hamsters seem to increase food hoarding when facing any sort of energetically challenging situations such as: pregnancy (Bartness 1997) lactation (Bartness 1997) and food restriction (Bartness and Clein 1994). However, female hamsters shift their behaviour according to the current situation. For instance, females will spend more time with mates if the food is freely available. On the other hand, they will focus on foraging and hoarding following a period of food deprivation (Scheider et al. 2007).

Hoarding in hamsters is a stark contrast to the well-known feeding strategies in several species that seem to compensate for the loss in body mass by increasing food intake when food is available. However, hamsters never increase food intake during the re-feeding period, thus might die due to their failure to compensate for the longer fasting period (Silverman and Zucker 1976). In fact, Syrian hamsters, that are known to hoard the most, eat the least during a re-feeding time frame (Buuckley and Schneider 2003). Therefore, despite never over-eating, hamsters manage to slowly regain the lost body weight seemingly by decreasing their energy expenditure (Bartness and Clein 1994).

Woods et al. (1998) suggested that neuropeptides that stimulate food intake could also stimulate food hoarding. Food deprivation alters the expression of a wide range of central and peripheral peptides including neuropeptides in the central nervous system (CNS) that are involved in energy balance (Bartness et al. 2011).

4.1.1 Hypothalamic NPY/AGRP

As mentioned before, both NPY and AGRP are expressed in the same neural population in the arcuate nucleus (ARC) and are considered as a key mediator for appetitive behaviours (Thomas and Xue 2018). Studies in mice showed that the optogenetic stimulation of those neurons resulted in a robust stimulation of food intake (Krashes et al. 2011). Also, mouse models that allow real-time detection of neural recordings demonstrated that both AGRP and POMC neurons rapidly fire in response to the sensory detection of food (Chen et al. 2015). According to the authors, when mice were food deprived, AGRP neural activity increased, and was then inhibited within a matter of seconds of food being presented. However, when food was removed before being consumed, AGRP firing is stimulated. Those results suggest a regulated mechanism to inhibit foraging and other appetitive related behaviours once food is found (Chen et al. 2015). The activation of AGRP neurons not only induce food consumption but also stimulate other motivational behaviours that drive food obtainment processes such as foraging and the willingness to work for food (Atasoy et al. 2012). Thus, once food is discovered, the appetitive processes will be blocked as a natural transition from foraging mode to feeding (Chen et al. 2015). Having said that, it is in fact the rapid inhibition of the AGRP neurons themselves that provides a direct mechanism to inhibit foraging once food is found

Strikingly, NPY and AGRP appear to mediate ingestive behaviours through discrete mechanisms. Krashes et al. (2013) demonstrated that in mice, NPY stimulation promotes rapid but short-lived increase in food intake, while the firing of AGRP neurons stimulate food intake but in a delayed long-lasting manner.

Similarly, the central administration of both NPY and AGRP in Siberian hamsters markedly elevated food foraging and food hoarding (Teubner et al. 2012). I.c.v administration of NPY robustly increased food intake, food foraging and food hoarding as much as 500%-1000% fold returning to baseline within 24h post injection. Exogenous AGRP increased hoarding to 2000%, which is much higher than for food intake and foraging, however, that increased effect lasted as much as a week post-injection (Day and Bartness 2004). Those results suggested that the long-term increase in ingestive behaviours might be mediated partially by the signalling of AGRP. However, the short-term elevation of ingestive behaviours is mediated via NPY.

4.1.2 Hypothalamic POMC

The melanocortin system is antagonized by the action of AGRP since both AGRP and POMC-derived peptides utilize and compete to bind to the same receptors (Breen et al. 2005). The precursor POMC produces many biologically active peptides that yields a variety of hormones with various functions one of which is appetite regulation (Millington 2007). When the individual is in a calorie deficit state, hypothalamic POMC gene expression declines, and this action is reversed when food is presented to the animal where, in a matter of seconds, POMC neuronal activity is increased (Chen et al. 2015). A study by Zhan et al. (2013) demonstrated that the direct activation of POMC neurons inhibits food intake. There is less known about the action of POMC in relation to food hoarding. However, we could speculate that since POMC and AGRP share the same receptors (melanocortin receptor 3-4 MC3R and MC4R), and AGRP is a long-lasting stimulator of food hoarding, it is only reasonable for POMC to inhibit food hoarding once it binds to its receptor. Having said that, Keen-Rhinehart and Bartness (2007) demonstrated that MTII (a synthetic version of α- melanocortin stimulating hormone (MSH)) was less effective in inhibiting food hoarding, even though it has the ability to block the ability of ghrelin to increase food hoarding.

Since little is known about songbirds' motivation to do certain behaviours when experiencing food deprivation and the correlation of peptide gene expression with those behaviours, the aim of this chapter is to investigate whether there is a correlation between selected food related behaviours and well-known satiety signalling peptides. We predict that when food deprived (fasted), both hoarding (coal tits) and non-hoarding (blue tits) birds will increase their food intake, while hoarding birds would increase their hoarding behaviour. Any decision taken by individuals (whether to eat or hoard) depends on their energetic demands at that moment.

We explored hypothalamic expression of AGRP, NPY and POMC because they correlate with food related behaviours in the mammalian and domestic avian literature. We predict a positive correlation between both NPY and AgRP with an increase in food intake, food grabbing behaviour and hoarding. As for hypothalamic POMC, it should increase with decreasing food intake (increase satiety signalling), food grabbing and hoarding. As for hindbrain neuropeptide, we predict that CCK, GLP-1 and POMC gene expression would correlate negatively with food intake, grabbing and hoarding. Of the gut peptides, we only investigate the pancreatic GCG/INS ratio, as it was the only measurement we found to be affected by the nutritional state of the bird, and we predict it will be higher with increased eating, food grabbing and hoarding.

4.2 Methodology

4.2.1 Animal capture and housing

All details about how the birds were captured and housed, as well as ethical permits under which this was performed, are given in chapter 3.

4.2.2 Sexing the birds.

All details about how blood samples were collected and treated are given in chapter 3.

4.2.3 Gene expression data

Information about the primers used and how RNA was treated and extracted from tissue samples are detailed in chapters 2 and 3.

4.2.4 Experimental design

All the details about how the birds were housed, the type of feed given as well as how they were treated and prepared for the experiment is mentioned in chapter 3.

Note: During the course of the experiment, the initial number of birds in the study were 19 birds, however, during the video recording behaviour days 4 birds were found dead, so we ended up with 15 birds only.

Table 4: A list of all the individuals included in the behavioural study. Note: birds highlighted in red were found dead and have been excluded from the experiment.

Ring identification	Species	Sex	Note
BT61	Blue tit	Male	
BT62	Blue tit	Male	
BT63	Blue tit	Female	
BT64	Blue tit	Female	
BT69	Blue tit	Female	
BT70	Blue tit	Male	
BT71	Blue tit	Male	
BT74	Blue tit	Male	
BT76	Blue tit	Female	Dead
BT77	Blue tit	Male	
BT78	Blue tit	Female	
BT79	Blue tit	Male	
BT80	Blue tit	Female	
BT82	Blue tit	Male	
BT85	Blue tit	Male	
BT86	Blue tit	Male	
BT87	Blue tit	Male	
BT89	Blue tit	Male	Dead
CT10	Coal tit	Male	
CT11	Coal tit	Male	
CT12	Coal tit	Male	
CT13	Coal tit	Male	Dead
CT14	Coal tit	Female	
CT15	Coal tit	Female	
CT2	Coal tit	Male	
CT21	Coal tit	Female	
CT25	Coal tit	Male	
CT27	Coal tit	Male	Returned to aviary

CT28	Coal tit	Male	
CT3	Coal tit	Male	
CT41	Coal tit	Female	
CT44	Coal tit	Female	
CT45	Coal tit	Female	
CT49	Coal tit	Male	
CT50	Coal tit	Female	

4.2.5 Behavioural observation

Videos were recorded for each individual starting from day 4 (the day after having acclimated to the cages) till day 7 (the day before they were humanely sacrificed). Each bird was recorded twice for an hour: when it was fasted and when it was fed. So, for instance, if a bird was recorded fasted in day 4, in day 6 it would be videoed as fed. The treatment was counterbalanced across birds, and the period of fasting/ or re-feeding was either 2h of fasting after lights on, or 1h of fasting after lights on, followed by 1.5h of ad lib access to food before being filmed. Water was always accessible throughout the study.

The behavioural data were quantified separately for the two half hours following introduction of fresh food. We used Behavioral Observation Research Interactive Software (BORIS) to quantify eleven selected behaviours:

a. Eating: duration data measured in seconds and was defined when the bird was observed nibbling on a food item picked from the bowl. The event was considered ended when the individual was seen either dropping the food or doing something else.
b. Flying with food: A duration event identified when the bird was seen picking up food and flying with it. The event ended when the item was dropped.
c. Flying without food: A duration event identified when the bird was seen flying without food.
d. Sitting on a perch: A duration event identified when the bird was observed landing on the perch and sitting there.
e. Sitting on the floor: A duration event identified when the bird was observed sitting on the floor of the cage.
f. Grabbing food: A countable event identified when the bird was seen either grabbing food from the bowl or the floor.
g. Dropping food: A countable event identified when the bird dropped the food item it was carrying.
h. Sitting on the bowl: A duration event identified when the bird was observed landing on the bowl and staying there.
i. Hoarding: A countable event recorded when the bird wasseen carrying a food item and seen hiding it.

j. Retrieving: A countable event recorded when the bird was observed retrieving a previously hoarded food item.

Due to time limitations during the study, three behaviours were chosen: eating, hoarding and food grabbing. Those behaviours were picked because they are considered as a proxy for food foraging behaviour, as well as being the most important variables to test our predictions regarding the correlation between peptide gene expression and ingestive behaviours.

4.2.5 Data analysis

Behaviour analysis

The analysis that follows only focuses on eating, food grabbing and hoarding using the Generalized Linear Models in the SPSS statistical package (IBM 25). Both food grabbing and hoarding (countable events) were treated as Poisson distributed, while eating (duration event) was treated as normally distributed. When analysing the three behaviours, both the first and second half hour were included in the statistical model as a (time of day) variable.

To investigate whether the three behaviours were different between the two species, we used 2×2×2 mixed-design analysis (species (between-subject) × state (within-subject) × time of the day (within-subject)), including all two- and three-way interactions.

Gene expression analysis

To investigate the correlation between gene expression and behaviour we used Generalized Linear Models. Outliers in the gene expression data were removed before analysis. The outliers were identified as such: after doing log (gene/YWHAZ or LBR) for all individuals, values that did not fall within the range of the 95% confidence interval (very high or very low) were identified as outliers and were removed from the data set. For this part of the analysis, we only used the first half hour. However, for food hoarding, since only two coal tits ended up hoarding in the first half hour, both the 1st and 2nd half hour were added up to assess food hoarding behaviour.

When analysing the data to investigate the relationship between neuropeptides and eating and food grabbing behaviour, we used 2×2 analysis (species × state) using the peptides as a covariate. Because peptide gene expression was only measured in one state for any given bird, the state variable was a between-subject variable in this analysis. For gut peptides, selected gut tissues were selected according to both the literature and our observations in Chapters 2 and 3. The only gut peptide that varied its expression according to gizzard fullness in our previous observations was the pancreatic glucagon/insulin ratio. However, when investigating hoarding behaviour, since coal tits hoard and blue tits do not, we ended up analysing coal tits only. So, the food hoarding analysis included the state only as a factor.

All the interactions (both in behavioural analysis and gene expression analysis) were included initially. Non-significant interactions were removed from the model in a stepwise fashion, starting with 3-way interaction

and working our way down. Interactions were removed if $p>0.1$. No 2-way interaction were removed if the 3-way interaction was significant. Therefore, unless mentioned within the results, reader should assume that interactions were not included in the model due to their non-significance.

4.3 Results

4.3.1 Behavioural analysis

Eating

Eating was not significantly different between blue tits (BT) and coal tits (CT) (BT, mean= 193.28s ±13.61, CT, mean= 186.58s±14.59, $\chi 2$ (1) = 0.113, p=0.737). Individuals ate more during the first half hour in comparison to the second half (1st, mean= 228.40s ±14.10, 2nd, mean= 151.46s±14.10, $\chi 2$ (1) = 14.89, p<0.001). Fasted birds spent more time eating in comparison to fed ones (fast, mean= 236.12s ±14.37, fed, mean= 143.75s±13.83, $\chi 2$ (1) = 21.42, p<0.001). There was a significant interaction between times of the day and state ($\chi 2$ (1) = 9.94, p=0.002). While there was no difference in eating duration in fed individuals in both the first and second half hour (1st, mean= 150.82s ±19.56, 2nd, mean= 136.67s±19.56, p=0.609), fasted animals ate more at the first half hour in comparison to the second half (1st, mean= 305.98s ±20.30, 2nd, mean= 166.25s±20.30, p<0.001) (Figure 1).

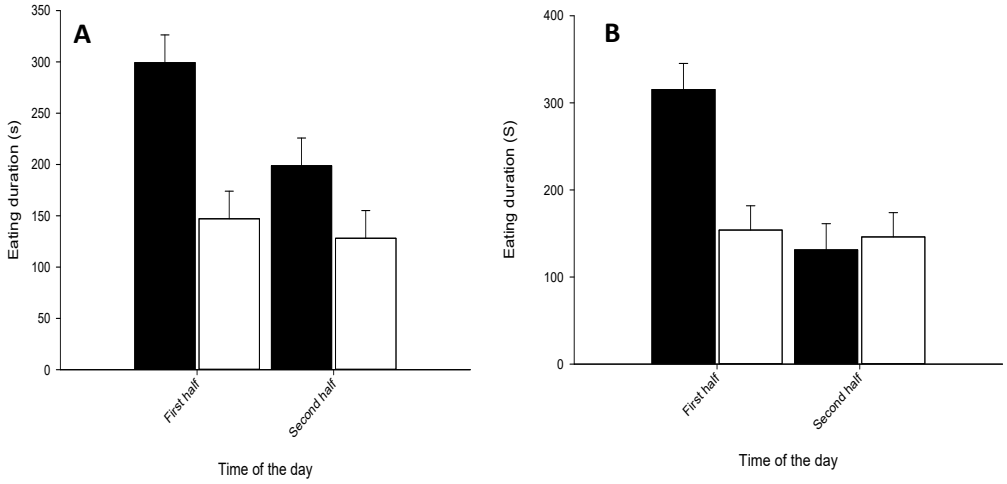


Figure 3: Pairwise comparison illustrating eating behaviour during first and second half hour between blue tits (A) and coal tits (B). Whereas fasted state is represented by black bars and fed state by white bars. Each bar is calculated as mean ± SEM.

Grabbing food

Overall, blue tits grabbed more food items in comparison to coal tits (BT, mean= 11.76 ±0.43, CT, mean= 8.35±0.38, $\chi 2$ (1) = 33.21, p<0.001). Individuals grabbed more food in the first half hour in comparison to the second half (1st, mean= 11.51 ±0.44, 2nd, mean= 8.53±0.38, $\chi 2$ (1) = 25.49, p<0.001) (Figure 2). On the other hand, the nutritional state of the individual did not have a significant effect on the number of food items grabbed (fast, mean= 10.24 ±0.42, fed, mean= 9.59±0.39, $\chi 2$ (1) = 1.27, p=0.259).

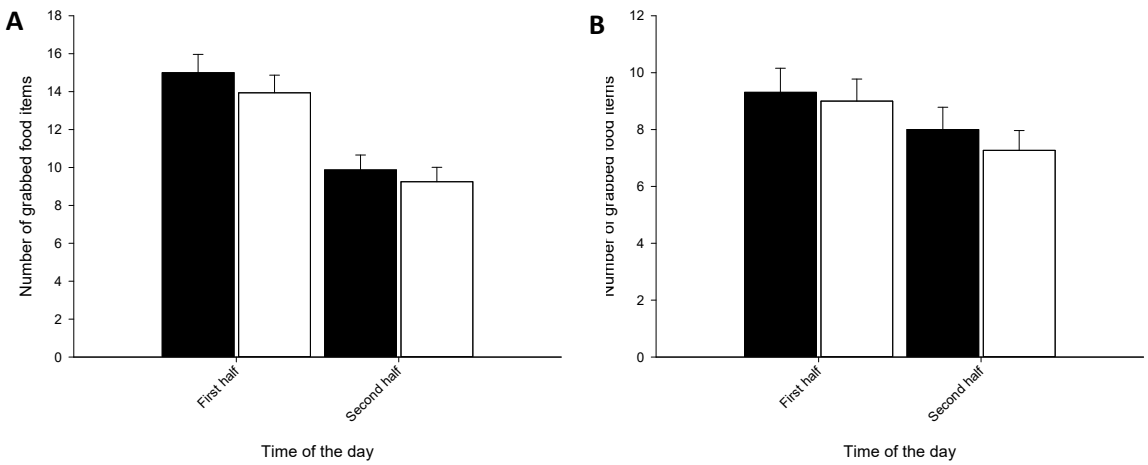


Figure 2: Pairwise comparison illustrating food grabbing behaviour during first and second half hour between blue tits (A) and coal tits (B). Whereas fasted state is represented by black bars and fed state by white bars. Each bar is calculated as mean ± SEM.

Food hoarding

In coal tits, food hoarding was neither affected by the individual nutritional state (fast, mean= 0.88 ±0.19, fed, mean= 0.94±0.18, $\chi 2$ (1) = 0.61, p=0.804), nor the time of the day (1st, mean= 1.06 ±0.19, 2nd, mean= 0.78±0.17, $\chi 2$ (1) = 1.13, p=0.287). State did not interact significantly with time of the day ($\chi 2$ (1) = 1.71, p=0.190).

4.3.2 Correlation between the behaviours

A Spearman rank-order correlation analysis was run to determine the relationship between eating, food grabbing and food hoarding in blue and coal tits when fasted versus when fed across both the first and second half hour. Note: unless mentioned in the results, the reader should assume that non-significant correlations were not included

Blue tits

There was a strong positive correlation between eating and food grabbing behaviour when individuals were fed in the first half hour (rs (14) = 0.789, p<0.001).

Coal tits

There was a strong positive correlation between food grabbing and food hoarding behaviour when individuals were fed in the first half hour (rs (13) = 0.624, p=0.013). Additionally, there was a strong positive correlation between eating and food grabbing behaviour in fed individuals in the second half hour (rs (13) = 0.689, p=0.005).

4.3.3 Relationship between neuropeptides and behaviour

As mentioned in the methods, it should be noted that for the following analysis, we only used the first half hour of the behavioural observation for both eating and food grabbing behaviour, and the first and second half hour combined for food hoarding behaviour (because we only had 2 hoarding coal tits in the first half hour). The state now is a between subject factor, and we also included a co-variate (the different genes that we are interested in). This may change the effect of species and state as previously analysed.

NPY

Eating

There was a significant difference in eating behaviour between the two species with coal tits eating more than blue tits (BT, mean= 118.51s±21.92, CT, mean= 184.28s±20.32, $\chi 2$ (1) = 8.17, p=0.004). Eating behaviour was significantly affected by the state of the individual with fasted birds eating more than fed ones (fast, mean= 218.07s ±18.99, fed, mean= 84.72s±20.24, $\chi 2$ (1) = 27.45, p<0.001).

Eating behaviour was not significantly affected by NPY levels ($\chi 2$ (1) =1.29, p=0.255). However, there was a significant species*NPY interaction ($\chi 2$ (1) =5.93, p=0.015) whereby an increase in blue tit NPY gene expression was strongly associated with increased eating behaviour, while it did not in coal tits (Figure 3).

Food grabbing

There was neither a significant effect of species (BT, mean= 13.52 ±1.48, CT, mean= 8.30±0.94, $\chi 2$ (1) = 0.188, p=0.664) nor nutritional state (fast, mean= 10.41 ±1.07, fed, mean= 10.78±1.28, $\chi 2$ (1) = 1.03, p=0.309) effect on food grabbing behaviour

Food grabbing behaviour was not significantly affected by NPY gene expression ($\chi 2$ (1) =0.903, p=0.342).

Hoarding

Food hoarding was significantly affected by the nutritional status of the individual with fed coal tits hoarding more than fasted ones (fast, mean= 0.33 ±0.16, fed, mean= 1.59±0.34, χ^2 (1) = 8.12, p=0.004).

Food hoarding was not significantly predicted by NPY levels (χ^2 (1) =0.048, p=0.826).

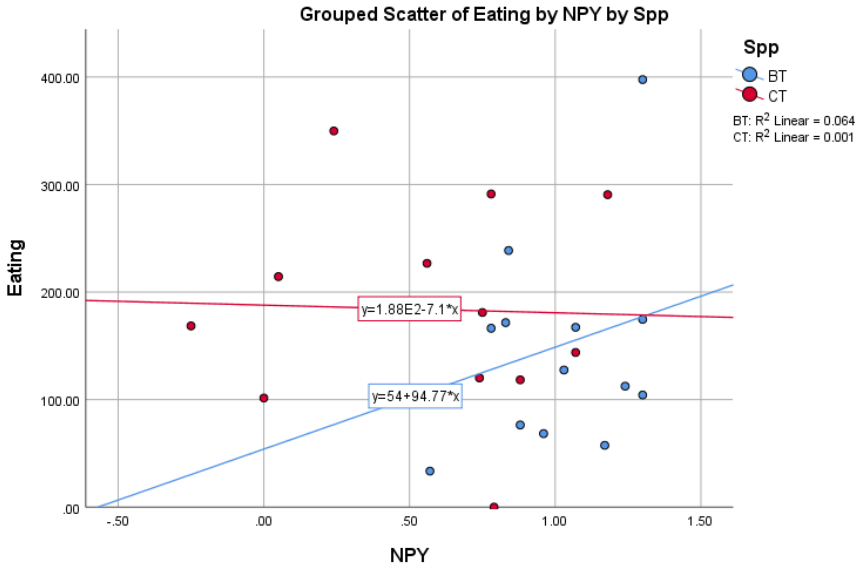


Figure 3: Relationship between NPY and eating duration for coal tits (red) and blue tits (blue).

AGRP

Eating

Eating behaviour was not significantly different between the two species (BT, mean= 153.19s ±19.22, CT, mean= 188.05s±20.94, $\chi 2$ (1) = 1.38, p=0.239). On the other hand, there was a significant effect of the nutritional state on eating behaviour with fasted individuals eating more than fed ones (fast, mean= 230.20s ±20.07, fed, mean= 111.03s±18.53, $\chi 2$ (1) = 18.88, p<0.001).

AGRP gene expression did not predict eating behaviour ($\chi 2$ (1) =0.788, p=0.375).

Grabbing food

Blue tits grabbed more food items in comparison to coal tits (BT, mean= 16.02 ±1.83, CT, mean= 8.56±0.90, $\chi 2$ (1) = 5.96, p=0.015). The nutritional status of the individual significantly affected food grabbing behaviour with fed birds grabbing more food than fasted ones (fast, mean= 10.65 ±1.11, fed, mean= 12.88±1.48, $\chi 2$ (1) 4.25, p=0.039). Although there was a significant interaction between species* state ($\chi 2$ (1) =4.43, p=0.035), pairwise comparison analysis did not detect any significant difference between the different nutritional state within the individuals of either species.

AGRP did not significantly predict food grabbing behaviour ($\chi 2$ (1) =0.064, p=0.801). However, there was a 3-way interaction between state* species and AGRP ($\chi 2$ (1) =10.17, p=0.001) whereby an increase in fed blue tit AGRP was associated with decreased food grabbing behaviour, while an increase in fasted blue tit AGRP was associated with increased food grabbing (Figure 4-A a, b). There was no effect in coal tits.

Food hoarding

Fed coal tits hoarded significantly more than fasted ones (fast, mean= 0.26 ±0.13, fed, mean= 1.58±0.34, $\chi 2$ (1) = 10.86, p=0.001).

Increased AGRP levels was associated with increased food hoarding ($\chi 2$ (1) =5.16, p=0.023) (Figure 4-B).

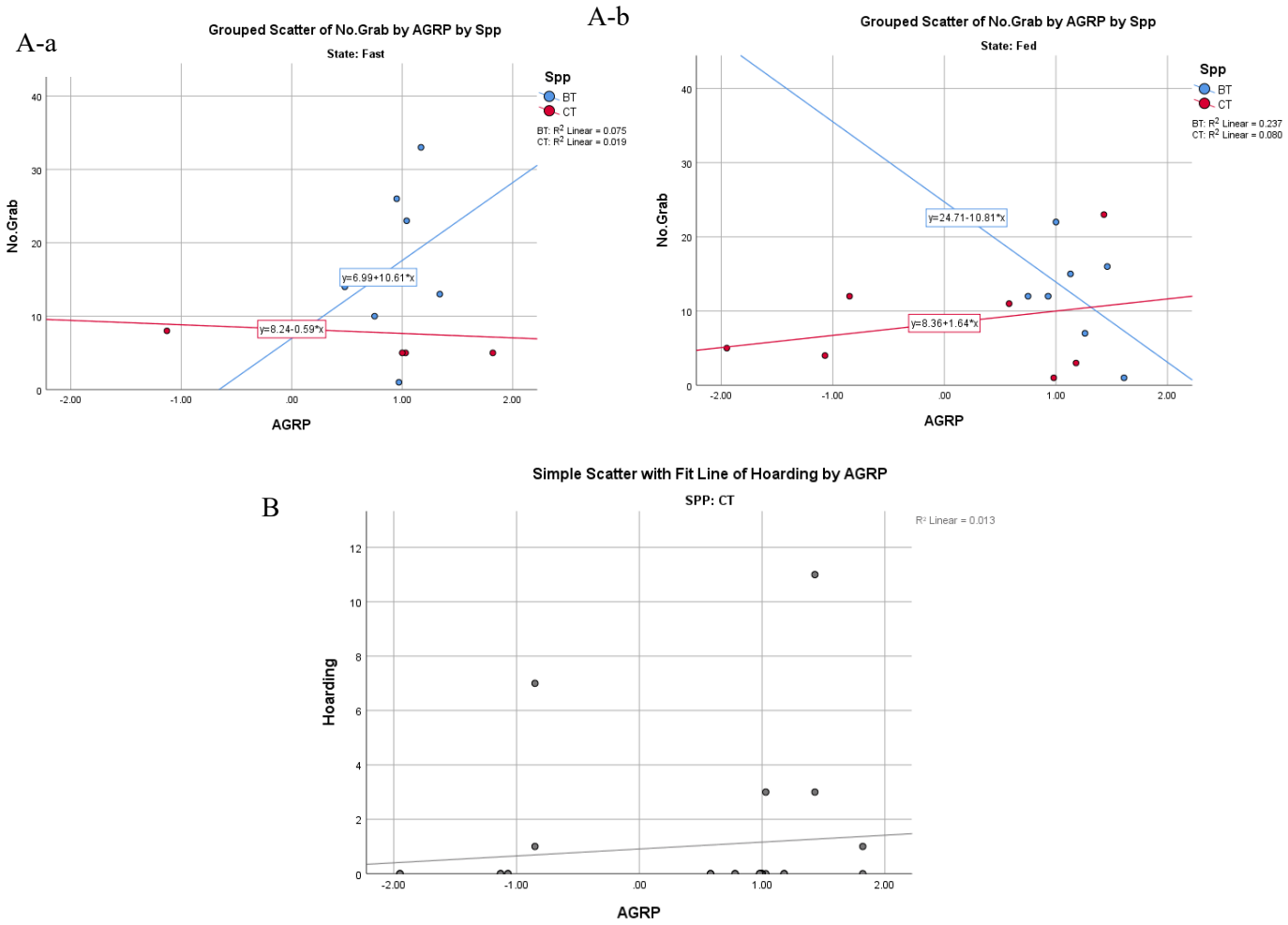


Figure 4: Relationship between AGRP and: the number of grabbed food in blue tits (blue) and coal tits (A- a,b) , and food hoarding behaviour (B).

Hypothalamic POMC (HYP POMC)

Eating

There was a significant effect of species on eating behaviour with coal tits eating more than blue tits (BT, mean= 61.44s ±49.97, CT, mean= 301.72s±58.73, $\chi 2$ (1) = 5.27, p=0.022). There was also a significant effect of the nutritional state of the bird with fasted individuals eating more than fed ones (fast, mean= 248.78s ±21.26, fed, mean= 114.39s±17.37, $\chi 2$ (1) = 18.88, p<0.001).

HYP POMC did not predict eating behaviour ($\chi 2$ (1) =0.788, p=0.375).

Grabbing food

There was neither a species (BT, mean= 4.82 ±1.50, CT, mean= 4.10±1.74, $\chi 2$ (1) = 0.359, p=0.549) nor a state effect (fast, mean= 4.93 ±1.37, fed, mean= 4.01±1.10, $\chi 2$ (1) = 2.50, p=0.113) on food grabbing behaviour.

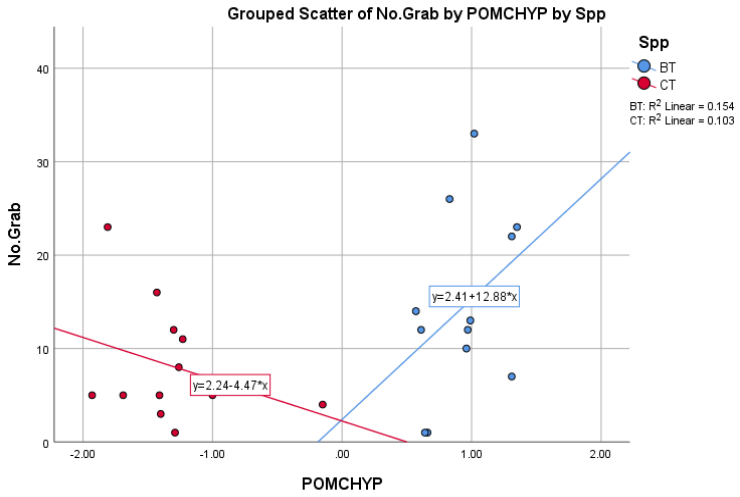
Grabbing behaviour was not significantly affected by HYP POMC ($\chi 2$ (1) =1.37, p=0.241). However, species significantly interacted with HYP POMC ($\chi 2$ (1) =13.43, p<0.001) whereby increased expression of HYP POMC was associated with increased food grabbing behaviour in blue tits, while in coal tits increased levels of HYP POMC was strongly associated with decreased the grabbing behaviour (Figure 5-A).

Food hoarding

The nutritional status of the individuals significantly affected hoarding behaviour with fed coal tits hoarding more than fasted ones (fast, mean= 0.24 ±0.12, fed, mean= 1.41±0.33, $\chi 2$ (1) = 10.43, p=0.001).

Increased HYP POMC gene expression was significantly associated with decreased food hoarding behaviour in coal tits ($\chi 2$ (1) =7.55, p=0.006) (Figure 5-B).

A



B

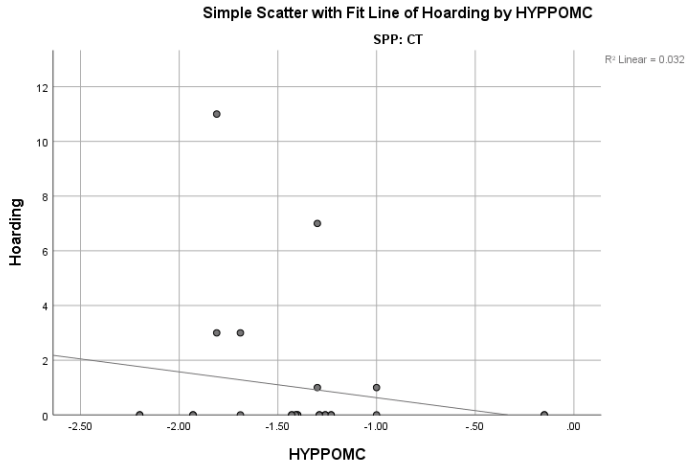


Figure 5: Relationship between hypothalamic POMC and: number of grabbed food in blue tits (blue) and coal tits (red) (A), and food hoarding in coal tits (B).

AGRP/HYP POMC ratio

Eating

There was a significant species difference effect on eating behaviour with coal tits eating more than blue tits (BT, mean= 130.93s ±20.30, CT, mean= 217.61s±23.12, $\chi 2$ (1) = 6.17, p=0.013). Fasted individuals ate more than fed ones (fast, mean= 237.31s ±19.46, fed, mean= 111.23s±17.49, $\chi 2$ (1) = 22.79, p<0.001)

AGRP/HYP POMC ratio did not significantly predict eating behaviour ($\chi 2$ (1) =2.81, p=0.093).

Food grabbing

There was a significant species difference in food grabbing behaviour with coal tits grabbing more food than blue tits (BT, mean= 7.53 ±1.68, CT, mean= 7.67±1.13, $\chi 2$ (1) = 16.81, p<0.001) (Figure 6). There was no nutritional state effect observed on food grabbing behaviour (fast, mean= 7.63 ±2.39 fed, mean= 6.80±1.49, $\chi 2$ (1) = 0.640, p=0.640).

AGRP/HYP POMC ratio did not predict food grabbing behaviour ($\chi 2$ (1) =2.91, p=0.088). However, there was a significant species* AGRP/ HYP POMC interaction ($\chi 2$ (1) =4.64, p=0.031) whereby in blue tits,

increased AGRP/ HYP POMC gene expression was associated with decreased food grabbing behaviour, while there was no obvious effect in coal tits (Figure 6).

Food hoarding

Food hoarding was significantly affected by the nutritional status of the individual with fed coal tits hoarding more than fasted ones (fast, mean= 0.24 ±0.13, fed, mean= 1.52±0.35, $\chi 2$ (1) = 10.93, p=0.001).

AGRP/HYP POMC did not predict food hoarding behaviour in coal tits ($\chi 2$ (1) =3.30, p=0.069).

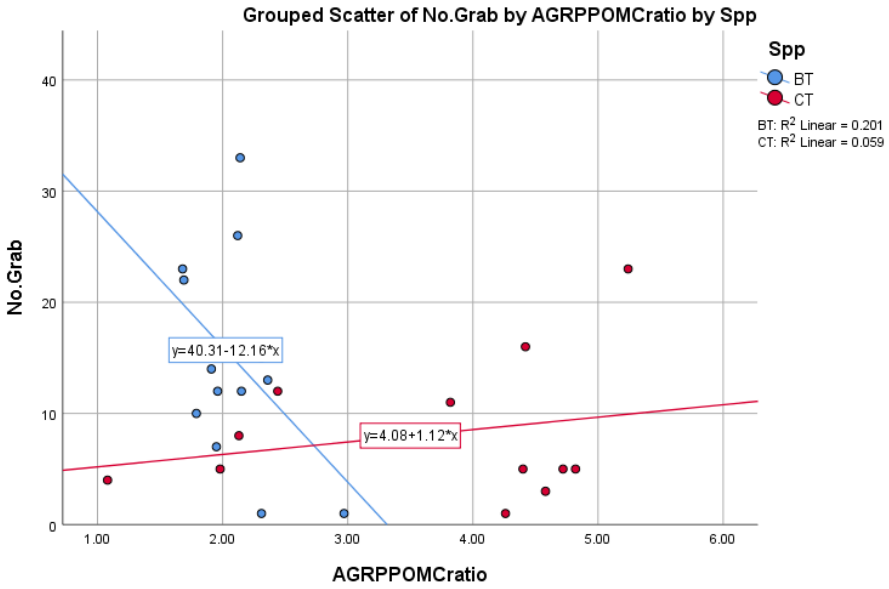


Figure 6: Relationship between AGRP/POMC ratio and the number of grabbed food items in coal tits (red) and blue tits (blue).

Hindbrain CCK

Eating

There was not a significant effect of species on eating behaviour (BT, mean= 151.37s±17.46, CT, mean=191.45s±19.02, $\chi 2$ (1) = 2.40, p=0.121). However, there was a significant effect of the individual nutritional state on eating with fasted birds eating more than fed ones (fast, mean= 228.39s ±19.17, fed, mean= 114.42s±17.61, $\chi 2$ (1) = 18.91, p<0.001).

Increased CCK gene expression was associated with decreased eating behaviour ($\chi 2$ (1) =5.81, p=0.016) (Figure 7-A).

Grabbing food

There was no species difference in food grabbing behaviour (BT, mean= 13.58 ±1.01, CT, mean= 7.51±0.84, $\chi 2$ (1) = 2.51, p=0.113). However, there was a significant effect of nutritional state of individuals on grabbing behaviour with fasted birds grabbing more food than fed ones (fast, mean= 10.98 ±1.07, fed, mean= 9.29±0.85, $\chi 2$ (1) = 26.39, p<0.001). There was a significant interaction between species and state ($\chi 2$ (1)

=7.31, p=0.007) whereby fasted blue tits grabbed more items than fed ones (fast, mean= 15.99 ±1.57, fed, mean= 11.53±1.28, p=0.028), whereas food grabbing behaviour was not significant between fed and fasted coal tits (fast, mean= 7.54 ±1.28, fed, mean= 7.48±1.08, p=0.973)

Hindbrain CCK levels did not significantly predict food grabbing behaviour ($\chi 2$ (1) =3.09, p=0.078). However, CCK interacted significantly with state ($\chi 2$ (1) =27.51, p<0.001), with species ($\chi 2$ (1) =4.21, p=0.040) and 3-way interaction with species*state* HB CCK ($\chi 2$ (1) =6.55, p=0.010) whereby an increase in fasted HB CCK expression in both blue and coal tits was associated with an increased grabbing behaviour, whereas an increase in fed HB CCK expression in both blue and coal tits was associated with decreased grabbing behaviour (Figure 7-B).

Food hoarding

Food hoarding was significantly affected by the nutritional status of the individual with fed birds hoarding more than fasted ones (fast, mean= 0.40 ±016, fed, mean= 1.46±0.33, $\chi 2$ (1) = 6.81, p=0.009).

Food hoarding was not significantly predicted by hindbrain CCK levels ($\chi 2$ (1) =0.494, p=0.482).

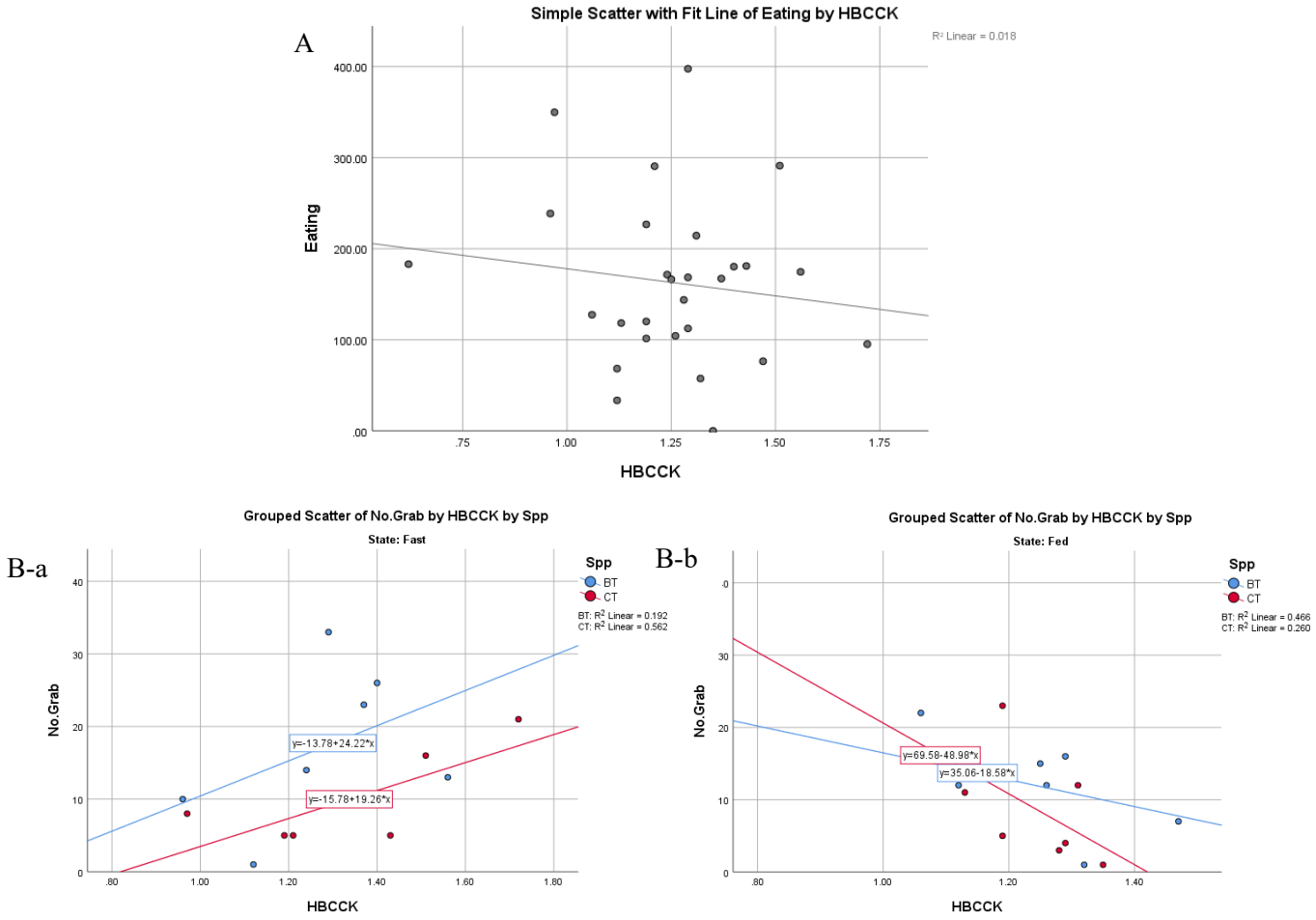


Figure 7: Relationship between hindbrain CCK and: eating duration (A), and the number of grabbed food items in blue tits (blue) and coal tits (red) (B).

Hindbrain GCG

Eating

Eating behaviour was not significantly affected by species (BT, mean= 158.67s±24.59, CT, mean= 193.11±21.94, $\chi 2$ (1) = 1.04, p=0.307) On the other hand, eating was significantly affected by an individual's nutritional state with fasted birds eating significantly more (fast, mean= 240.91s ±20.25, fed, mean= 110.88s±26.0, $\chi 2$ (1) = 5.44, p=0.020). There was a significant species*state interaction ($\chi 2$ (1) =5.40, p=0.020) whereby fasted coal tits ate more than fed ones (fast, mean= 285.87s ±32.18, fed, mean= 100.35s±29.85, p<0.001), whereas blue tit eating was not different when fasted or fed (fast, mean= 195.95s ±24.61, fed, mean=121.40 s±42.58, p=0.130).

Hindbrain GCG did not significantly predict eating behaviour ($\chi 2$ (1) =0.017, p=0.897). However, there was a significant 3-way interaction between species*state*GCG ($\chi 2$ (1) =4.06, p=0.044) whereby an increase in fasted coal tits hindbrain GCG was significantly associated with decreased eating duration, while an increase

in fed coal tit hindbrain GCG gene expression was associated with increased eating behaviour (Figure 8-A a, b). On the other hand, there was no strong relationship for blue tits.

Grabbing food

There was a significant difference between the two species in food grabbing behaviour with blue tits grabbing more food than coal tit (BT, mean= 16.92 ±1.17, CT, mean= 8.32±0.86, $\chi 2$ (1) = 29.74, $p<0.001$). On the other hand, the nutritional state of the individual did not affect food grabbing behaviour (fast, mean= 12.65 ±1.09, fed, mean= 11.13±0.91, $\chi 2$ (1) = 1.14, $p=0.285$).

Increased hindbrain GCG was associated with increased food grabbing behaviour ($\chi 2$ (1) =14.94, $p<0.001$) (Figure 8-B).

Food hoarding

Food hoarding was significantly affected by the nutritional status of the individual with fed birds hoarding more than fasted ones (fast, mean= 0.31 ±0.15, fed, mean= 0.85±0.25, $\chi 2$ (1) = 4.90, $p=0.027$).

Increased hindbrain GCG was significantly associated with increased food hoarding behaviour in coal tits ($\chi 2$ (1) =22.62, $p<0.001$) (Figure 8-C).

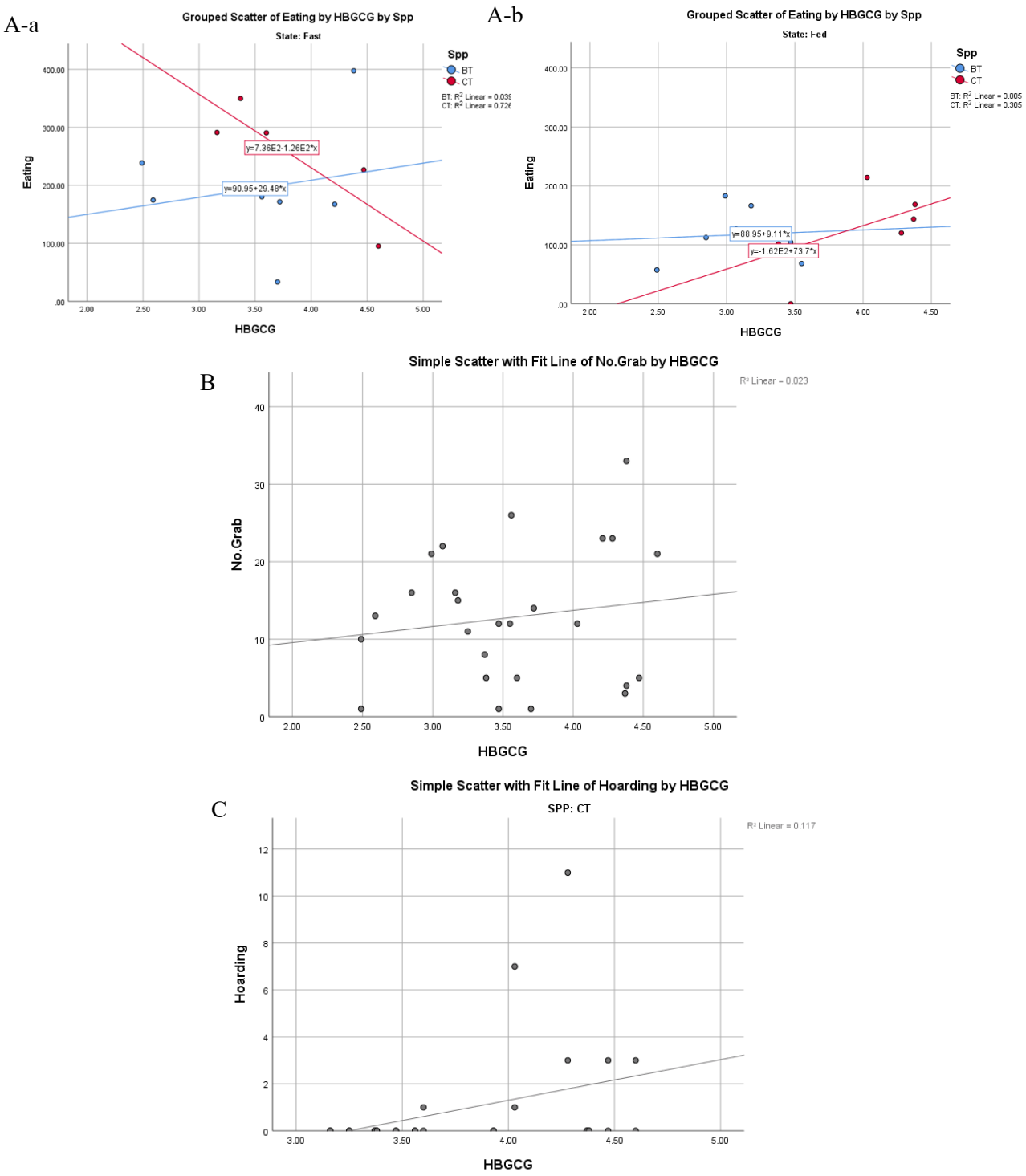


Figure 8: Relationship between hindbrain GCG and: eating duration in in blue tits (blue) and coal tits (red) (A- a, b), food hoarding in coal tits (B).

Hindbrain POMC (HB POMC)

Eating

There was no species difference effect on eating behaviour (BT, mean= 160.30s ±31.04, CT, mean= 173.24s±35.23, $\chi 2$ (1) = 0.047, p=0.829). On the other hand, fasted individuals ate significantly more than fed individuals (fast, mean= 217.98s ±21.22, fed, mean= 118.56s±19.25, $\chi 2$ (1) = 11.26, p=0.001).

HB POMC did not predict eating behaviour ($\chi 2$ (1) =0.070, p=0.792).

Grabbing food

There was neither a significant species (BT, mean= 14.33 ±2.27, CT, mean= 6.42±1.42, $\chi 2$ (1) = 0.581, p=0.446) nor state effect (fast, mean=10.35±1.63, fed, mean= 8.89±1.22, $\chi 2$ (1) = 1.65, p=0.199) on food grabbing behaviour

HB POMC gene expression did not predict food grabbing behaviour ($\chi 2$ (1) =1.28, p=0.257).

Food hoarding

The nutritional state of the individual significantly affected hoarding behaviour, with fed coal tits hoarding more than fasted ones (fast, mean= 0.42 ±0.17, fed, mean= 1.44±0.30, $\chi 2$ (1) = 7.07 p=0.008).

Hindbrain POMC did not predict food hoarding behaviour ($\chi 2$ (1) =0.593, p=0.441). However, there was a significant interaction between state and POMC ($\chi 2$ (1) =5.33, p=0.021) whereby increased in fasted POMC levels was significantly associated with increased hoarding behaviour, while increased fed POMC levels was associated with decreased hoarding, although neither very strongly (Figure 9).

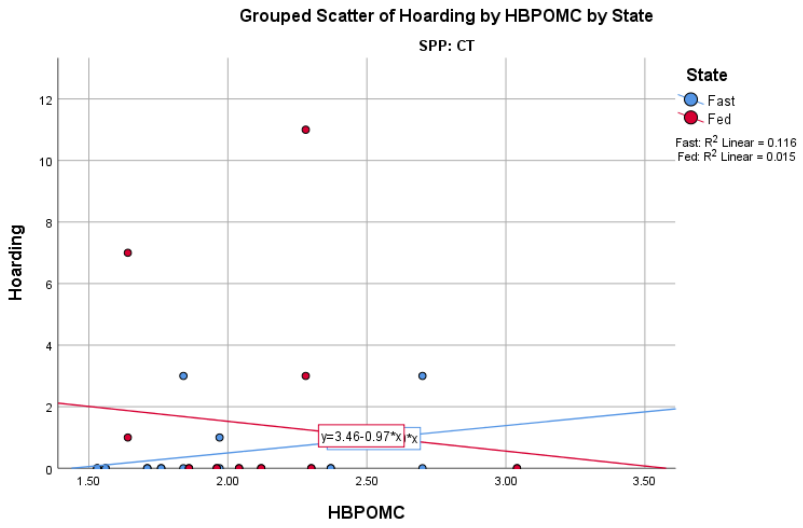


Figure 9: Relationship between hindbrain POMC and food hoarding in coal tits fasted (blue) vs fed (red).

4.3.4 Relationship between gut peptides and behaviour

Pancreatic GCG/INS ratio

Eating

There was no species difference effect on eating behaviour (BT, mean= 150.84s ±18.91, CT, mean= 184.96s±20.43, $\chi 2$ (1) = 1.47, p=0.225). On the other hand, the nutritional status of the individual significantly affected eating duration with fasted birds eating more than when fed (fast, mean= 221.36s ±20.56, fed, mean= 114.44s±19.02, $\chi 2$ (1) = 14.13, p<0.001).

Pancreatic GCG/INS ratio did not predict eating behaviour ($\chi 2$ (1) =1.55, p=0.213).

Food grabbing

Neither species (BT, mean= 13.61 ±1.07, CT, mean= 8.22±0.90 $\chi 2$ (1) = 0.485, p=0.486) nor the nutritional state of individuals (fast, mean= 12.95 ±1.14, fed, mean= 8.64±0.88, $\chi 2$ (1) = 2.47, p=0.116) had a significant effect on food grabbing behaviour. However, there was a significant interaction between species and state ($\chi 2$ (1) =12.78, p<0.001) whereby fasted coal tits grabbed more food in comparison to fed ones (fast, mean= 10.88 ±1.49, fed, mean= 6.20±1.07, p=0.011), whereas there was no significant difference in the number of grabbed food items between fasted and fed blue tits (fast, mean= 15.41 ±1.72, fed, mean= 12.02±1.37, p=0.120).

Pancreatic GCG/INS ratio did not predict grabbing behaviour ($\chi 2$ (1) =0.445, p=0.505). However, there was a significant interaction between state and GCG/INS ratio ($\chi 2$ (1) =4.42, p=0.035). There was also a 3-way interaction between species, state and GCG/INS ratio ($\chi 2$ (1) =13.03, p<0.001) whereby increased fed blue tits GCG/INS ratio was increased food grabbing behaviour, whereas an increase in fed coal tit GCG/INS ratio significantly decreased food grabbing behaviour (Figure 16-A a, b).

Food hoarding

Food hoarding was not significantly affected by the nutritional status of the individuals (fast, mean= 0.44 ±0.16, fed, mean= 0.90±0.26, $\chi 2$ (1) = 3.13, p=0.077).

Pancreatic GCG/INS ratio did not predict food hoarding in coal tits ($\chi 2$ (1) =0.552, p=0.118).

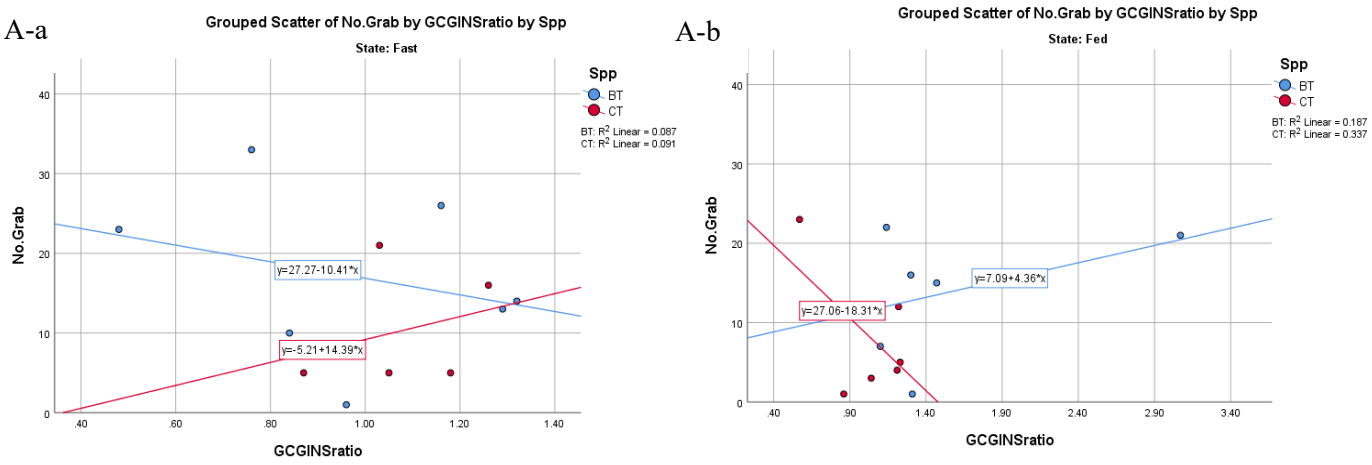


Figure 16: Relationship between pancreatic GCG/INS ratio and food grabbing behaviour in coal tits (red) and blue tits (blue).

4.4 Discussion

This present study aimed to investigate how hoarding (coal tits) vs. non-hoarding (blue tits) titmice behave when subjected to either 18h fasting or 17h fasting followed by 1.5h refeeding and whether there was a relationship between peptide gene expression and different food related behaviours. In this chapter, we are putting more emphasis on the behavioural aspects and whether the gene expression could be explained by those behaviours. Our hypothesis is that the difference in gene expression between the two species that were found in the previous chapter could relate to the behavioural outcomes that were recorded. For simplicity, we divided our analysis into behavioural analysis, correlation between behaviour and neuropeptides and correlation between gut peptides and behaviour.

4.4.1 Behavioural analysis

Overall, we could not detect any difference in eating duration between the two species although we did observe that blue tits were heavier than coal tits. Having said that, fasted individuals spent more time eating in comparison to fed ones, which is expected, because the main outcome of fasting/food restriction is typically increasing the food intake to replenish the energy stores that were depleted.

Why we were not able to detect any difference in eating duration between blue tits and coal tits is not clear. However, blue tits were observed grabbing more food items in comparison to coal tits. This observation indicates that since blue tits are bigger, they might eat faster than the smaller coal tits.

In our study, hoarding behaviour in coal tits was not affected by the nutritional state, even though McNamara et al. (1990) proposed that when the risk of starvation is increased, caching birds tend to increase hoarding behaviour and fat reserves. We could not presume that the 17-18h fasting is not enough, because for those small passerines with a high metabolic rate that period should elicit the behaviour. However, when the state was included as a between-subject variable, we observed that mostly fed individuals hoarded more than fasted ones which could be due to individual differences in motivation rather than a state effect. One of the predictions as to why those coal tits did not hoard might be related to the positioning of the hoarding substrate. In our study, we placed the blocks on the floor, however, field studies have shown that titmice in general prefer to place one item per site scattered over trees (Suhonen and Alatalo 1991). Therefore, we could speculate that if the blocks were hung high in the cage instead of being on the floor, coal tits might hoard. It is logical for songbirds not to consider substrate placed close to the floor as a safe place to store food. That being said, coal tits have been observed storing food items in moss in the ground in the wild. However, in our study, there were not many caching places to store food.

4.4.2 Correlation between neuropeptides and behaviour

For the next two parts, we included different genes as co-variates and changing the effect of species and state as previously analysed. It is worth noting that the peptides were measured on a day when the behaviour did not occur, and individuals were killed at a time when food would have been given.

NPY

We predicted that an increase in NPY gene expression is associated with an increase in food intake and food grabbing behaviour in blue tits. While in coal tits we expected an increase in food intake, grabbing and hoarding behaviour. An increase in blue tit NPY mRNA was associated with an increase in their eating behaviour but this did not apply to the coal tits. We hypothesised that even though coal tits are more active hence needing more energy which can be obtained by eating more, blue tits have bigger brains and thus potentially more neurons to synthesise NPY. Previous studies have demonstrated that food restriction enhances NPY mRNA gene expression in chickens (Boswell et al. 1999) and also stimulates food intake in neonatal chicks (Furuse et al. 1997). Those findings are similar to ours, confirming the orexigenic action of NPY.

As for food hoarding, fasted coal tits hoard more than fed ones, even though NPY gene expression did not predict food hoarding behaviour. In Siberian hamsters, NPY was found to stimulate food hoarding behaviour. The fact that we did not see an effect of NPY on hoarding behaviour, might simply means that NPY might not be involved in food caching behaviour.

AGRP

We predict that an increase in AgRP gene expression is associated with an increase in food intake and food grabbing behaviour in blue tits. While in coal tits we expect an increase in food intake, grabbing and hoarding behaviour.

Both blue and coal tits spent the same duration eating, but fasted birds ate more than fed ones. Moreover, fasted blue tits had higher AGRP mRNA levels (similar to the finding in the previous chapter even though gut fill had no effect on the gene levels) and grabbed more food, while in the fed blue tits, an increase in AGRP levels was associated with a decrease in grabbing behaviour. Experiments in chickens showed that food intake is stimulated by central injection of AGRP, and when subjected to food restriction, hypothalamic AGRP mRNA levels increase, and then decrease by 75% upon 2 days of re-feeding (Lees et al. 2017). Why we only detected an increase in food grabbing behaviour and not eating might be related to the fact that when fasted, the increase in AGRP mRNA signals increased foraging attempts to search for food to eat, and in our case blue tits did have several trips to the feeding bowl to eat but tended to drop food items.

Even though fed coal tits hoarded more than fasted ones, an increase in AGRP gene expression was associated with an increase in food hoarding behaviour. This result might be due to the antagonistic effect of AGRP with hypothalamic POMC. Since an increased hypothalamic POMC decreased hoarding (discussed below). We expect that the ratio between the two genes controls the motivation to hoard.

Hypothalamic POMC

We predicted that an increase in hypothalamic POMC gene expression is associated with a decrease in food intake and food grabbing behaviour in blue tits. While in coal tits we expected a decrease in food intake, grabbing and hoarding behaviour.

An increase in HYP POMC mRNA in blue tits (similar to the molecular finding in the previous chapter) was associated with an increase in grabbing behaviour. While in the case of coal tits, higher HYP POMC gene expression was associated with a decrease in grabbing behaviour. Although the POMC gene product α-MSH is known to decrease food intake in chickens, it also increased energy expenditure (Shojaei et al. 2020) possibly explaining the increase in blue tits activity (grabbing more) keeping them motivated to search for food even if they are satiated.

On the other hand, fed coal tits hoarded more than fasted ones, and the increase in hypothalamic POMC decreased food hoarding behaviour. Given the antagonistic actions of AGRP and POMC, it makes sense that while the increase of AGRP (discussed above) increases food hoarding, the binding of POMC induces the opposite action and decreases food hoarding.

AGRP/HYP POMC ratio

Since both peptides have opposite functions and compete for the same receptor, we predicted that an increase in the ratio is associated with an increase in foraging effort and thus, an increase in food intake and grabbing in blue tits, while coal tits would increase their hoarding efforts on top of increased food intake and grabbing. However, a lower ratio would have an opposite effect (i.e., decreasing food intake, grabbing and hoarding behaviour).

An increase in AGRP/POMC ratio in blue tits was associated with a decrease in grabbing behaviour. It seems that the increase by AGRP and HYP POMC individually increased food grabbing, why the ratio decreased the behaviour might be due to the stronger effect of hypothalamic POMC that might increase the grabbing behaviour.

Fed coal tits hoarded more than fasted ones, however, AGRP/POMC ratio seems not to play a role in the hoarding behaviour. On the other hand, in the previous chapter, the molecular analysis showed that fasted coal tits expressed higher ratios than fed ones. So, it seems generally that when the ratio is increased in the fasted state the bird is more motivated to eat, whereas when fed coal tits are more likely to hoard food even though they are not hungry, they are motivated to forage.

Hindbrain CCK

We predicted that an increase in CCK gene expression is associated with a decrease in food intake and food grabbing behaviour in blue tits. While in coal tits we expected an decrease in food intake, grabbing and hoarding behaviour.

Hindbrain CCK correlated with eating behaviour whereby an increase in CCK gene expression was associated with a decrease in eating behaviour. It has been shown that CCK is more abundant in the brain than the periphery and the most abundant neuropeptide in the CNS. Studies in rats showed a higher CCK immunoreactivity in the cerebral cortex (Innis et al. 1979). The satiation action of CCK is mediated via its receptor CCK-1R which is principally found in the gastrointestinal tract and very restricted brain areas one of which is the brainstem (Zarbin et al. 1983). Another route for the suppressing effect of CCK is through its release into the plasma, where it is transferred into the CCK-1R at the area postrema (AP) in the brainstem. AP has a leaky blood brain barrier (BBB) and monosynaptic connection to NTS where CCK binds to its receptor reducing meal size (Chen et al. 1993). Moreover, studies have shown that hindbrain CCK is intimately involved in mediation of pain stimuli and the opoid system. Thus, we speculate that the actions of CCK might be mediated via the melanocortin system.

Additionally, CCK correlated with food grabbing behaviour. Whereas an increase in fasted CCK levels in the two species was associated with an increase in food grabbing. While an increase in fed CCK expression was associated with a decrease in grabbing behaviour which make sense. Since CCK is mainly involved with reporting satiety, one would expect that when an individual is fed and CCK levels are high, the urge to search for food and eat is depressed because they are satiated, whereas when they are hungry, CCK levels are lower they might be more motivated to forage. However, this is just a speculation, and in is not clear why fasted CCK increased grabbing behaviour. Studies in laboratory mice showed that giving them doses of CCK that can inhibit food intake decreases their exploratory behaviour (Crawley et al. 1981).

Hindbrain CCK did not correlate with food hoarding behaviour in coal tits. Our knowledge regarding the involvement of central CCK in hoarding behaviour is not very clear.

Hindbrain GCG

We predicted that an increase in GCG gene expression is associated with a decrease in food intake and food grabbing behaviour in blue tits. While in coal tits we expected a decrease in food intake, grabbing and hoarding behaviour.

GCG correlated with eating behaviour, whereas an increase in fasted coal tit GCG was associated with a decrease in eating duration, while an increase in fed GCG levels in coal tits was associated with an increase in eating behaviour. Studies in rats showed that the satiation effect of GLP-1 in the hindbrain occurs via its binding to its receptor GLP-1R that is present in both AP and the NTS (Punjabi et al. 2014). However, why increased GCG in fed coal tits caused increased eating is unclear giving the satiation effect of GCG. Additionally, GCG not only correlated with food grabbing behaviour whereby an increase in GCG was associated with an increased grabbing, but it also correlated with food hoarding whereby an increase in GCG levels was associated with increased food hoarding in coal tits. We speculated that the increase in fed GCG gene expression not only made coal tits grab more but also hoard more.

Hindbrain POMC

Similar to hypothalamic POMC, we predicted that an increase in hindbrain POMC gene expression is associated with a decrease in food intake and food grabbing behaviour in blue tits. While in coal tits we expected an decrease in food intake, grabbing and hoarding behaviour

Hindbrain POMC gene expression only correlated with food hoarding behaviour, whereby an increased fasted POMC mRNA was associated with increased food hoarding in coal tits, while increased fed POMC mRNA was associated with decreased hoarding. In the previous chapter, molecular analysis showed that hindbrain POMC gene expression was higher in blue tits and we could not detect an effect of gizzard fullness on the expression of POMC. Moreover, when we compared POMC levels across the three titmice species, it showed a very clear difference in expression specifically in coal tits. Although we have little information regarding the contribution of hindbrain POMC mRNA to food hoarding, what we know is that POMC gene products generally have an anorectic action on appetite, thus we speculate that on one hand higher POMC expression is linked to suppression of food intake, while on the other hand it motivates coal tits to forage and hoard. However, this statement is purely a speculation because the correlation cannot be due to backword causation because we measured the peptides when the birds were sacrificed, and no behaviour was recorded. So, we assume that the peptide expression in the final day is the same if the bird could perform the behaviour.

4.4.3 Correlation between gut peptides and behaviour

Pancreatic peptides

We predicted that an increase in GCG/INS ratio is associated with decreased food intake and grabbing for blue tits, and food hoarding additionally in coal tits. GCG/INS ratio correlated with food grabbing behaviour whereby an increase in fed GCG/INS ratio was associated with increased food grabbing behaviour in blue tits while decreasing it in coal tits. The result observed in coal tits is expected giving that both the increase in GCG and insulin gene expression decreased grabbing behaviour and given the inhibitory effect of both peptides on food intake, it makes sense that coal tits are less motivated to forage for food. However, the increased in grabbing behaviour seen in blue tits is a bit confusing. Given the fact that like coal tits, the individual peptides inhibit grabbing behaviour, you would expect the ratio between them to exert similar effects, but this was not the case. Weston (1996) proposed that the transmission of satiety signals to the brain in response to the digesta is a vital determinant of forage intake regulation. Therefore, it is expected that the intensity of those signals will determine the rate of food intake. According to Newman et al. (1994) in ruminant animals, there is an inverse relation between rumen fill and hunger. Studies in dairy cows showed that insulin concentration increases as rumen fill increases (Gergorini et al. 2009). The authors proposed that as the rumen fill increases during the progression of grazing bouts, individuals decrease their feed intake and instead increased searching time. In the present study, we think that individuals might not have enough time

to re-feed, thus in some cases the report of gut fill might fail to signal to the brain eliciting a certain behaviour that is an appropriate response for the expressed peptides.

We speculated that the transcription processes are not regulated in the short-term rather the long-term. However, the short-term regulation of behaviour is related to the activity of the neurons which then leads to the release of the peptide, not the amount of peptides being made in the neurons themselves.

4.5 Conclusion

Overall, in the present study we observed a fluctuating behavioural response to both food deprivation and re-feeding treatment. Individuals of both species responded as expected to food deprivation most of the time by increasing eating duration and food grabbing behaviour. The AGRP/HYP POMC ratio stood out among the hypothalamic peptide gene expression measurements to correlate with the differences in the behaviour of coal tits. Moreover, GCG and POMC mRNAs in the hindbrain also correlated with hoarding behaviour in coal tits. Thus, we hypothesise that AGRP, HYP POMC, GCG and hindbrain POMC could be used as signals that report the nutritional state of individuals and change according to their motivation to hoard. Overall, hoarding behaviour seems to be induced by re-feeding as it was mostly evident in fed coal tits rather than fasted ones which might imply that being satiated does not suppress the motivation of individuals to hoard food which is expected for those species as they need to be continuously motivated to store food even when not hungry to survive harsh winters.

Chapter 5. Discussion

Appetite regulation is composed of a complex network of signals and information flowing between the central nervous system and the peripheral system. The interactions between those two systems controls and regulate the ingestive behaviours exhibited by individuals. It is the cooperation between the brain and the gut that insures keeping the body in state of energetic homeostasis. The major objectives of this PhD were to: 1. compare the presence and distribution of gene expression of neuropeptides and gut peptides between hoarding and non-hoarding titmice with other avian species. 2. Establish how neuropeptide and gut peptide gene expression is influenced by fasting and re-feeding. 3. Investigate whether there is a correlation between gene expression and different ingestion behaviour.4. Identify candidate peptide gene that could be responsible for regulating hoarding behaviour in hoarding birds.

5.1 Gut peptide tissue distribution

Our study is the first to describe the distribution of gene expression for INS, GCG, CCK and PYY in passerines. Overall, the distribution pattern was generally similar between the three titmice species and the chicken. However, we did also observe some differences.

5.1.1 GCG

Overall, great, blue, and coal tits showed higher GCG mRNA in the pancreas and in different parts of the small intestine. In the case of great tits, while empty state females showed higher GCG mRNA in their duodenum, full state males expressed more GCG in their antrum. As for both blue and coal tits GCG, it exhibited different tissue expression trends. Immunoreactive studies in chickens demonstrate that tissue specific action of the enzyme prohormone convertase directs the synthesis of GLP-1 in the L-cells of the gastrointestinal tract (Monir et al. 2014). Hence, the GCG mRNA we measured in the intestine in our study most likely represents synthesis of GLP-1.

5.1.2 INS

Great, blue, and coal tits all showed higher insulin mRNA gene expression in the pancreas. This observation is not unexpected since insulin is a very well-known pancreatic hormone exclusively expressed in the beta cells of the islets of Langerhans (Fu et al. 2013) and similar localisation was also found in other avian species: ducks (Samols et al. 1969) and chickens (Langslow et al. 1970). According to (Rawdon and Andrew 1999) insulin immunoreactive cells are rare outside the pancreas in vertebrates. Nonetheless, small numbers of insulin positive cells were observed in the proventriculus of rufous collared sparrows (Mendes et al. 2009). The antrum of great tits showed the next lowest insulin expression after the pancreas. Similar finding was also observed in blue tits whereby higher levels of insulin was found in the antrum in comparison to the rest of gut tissues except the pancreas. This unusual presence of insulin outside the pancreas might serve a

physiological function that is yet to be elucidated. So, there is a possibility that insulin may have a physiological significance in those other tissues.

5.1.3 PYY

All three species showed highest PYY expression levels in the antrum, duodenum proximal jejunum and the pancreas. This broad PYY tissue distribution is consistent with that of the chicken. Aoki et al. (2017) found that in chickens, PYY is mainly distributed in the small intestine in comparison to the large intestine. Additionally, PYY-like immunoreactive cells have also been observed in the duodenum and jejunum of chickens (El-Salhy 1982). Moreover, Reid at al. (2017) using in situ hybridization, found that a major site of expression of gut PYY in chickens is around the distal jejunum and that the pancreas had the highest PYY which we have also observed in our study. However, the duodenum had a relatively lower PYY expression which is different than our finding where we were able to detect higher PYY in the duodenum. Wewer Albrechtsen et al. (2016) demonstrated that in mouse, PYY mRNA levels increased along the gastrointestinal tract with the highest levels in the distal colon. Whereas in pigs, PYY levels shifted towards the small intestine. On the other hand, in rats, below detectable levels of PYY were found in the duodenum and proximal jejunum, however, the highest levels were found in the distal ileum. Those difference in PYY distribution might reflect taxonomic and perhaps body size differences in metabolic regulation by PYY, the different energetic demands between different animals and most importantly the variance in feeding modes and patterns among different taxa.

5.1.4 CCK

All three species showed higher CCK gene expression in the proximal and distal jejunum and the ileum. Furthermore, great tits also had higher CCK in the antrum and the caecum, while blue and coal tits expressed more CCK in the duodenum. Nonetheless, CCK levels in both the duodenum and the proximal jejunum in the blue and coal tit were very close to each other, while in great tits those two tissues, had a big difference in CCK expression. Studies in chicken demonstrated that CCK levels were highest around the proximal half of the ileum (Reid and Dunn 2018). However, CCK was lower but detectable in the proventriculus and the boundaries within the antro-duodenal regions in chickens (Reid and Dunn 2018). Previous distribution studies of CCK in mammals showed that CCK was higher in the proximal ileum of rats (Larson and Reheld 1978), while in humans CCK was higher in the duodenum and jejunum (which is similar to our finding) with different forms of CCK localised in certain parts of the gut (Maton et al. 1984). These observations simply suggest that different species use peptides differently according to their physiology, and that usage might largely depend on the way the peptide is distributed within a certain tissue. But also, the well-established role of CCK in digestion is through stimulating gall bladder contraction and pancreatic exocrine secretion to signal satiety by activating the vagal afferents (Owyang 1996). Those functions are closely associated with the fact that CCK is primarily secreted from tissues of the lower small intestine (Fakhry et al. 2017) hence it is expected to see peak expressions within those areas.

5.2 Appetite and satiety signalling

Numerous studies have demonstrated the importance of the arcuate nucleus (ARC) in regulating appetite and energy balance in both mammals and birds (Boswell 2005). Our study is the first to show that those same peptide genes are expressed in the hypothalamus of titmice which provides further evidence of the evolutionary conservation of their function. In mammals, both leptin and ghrelin play a key role in regulating the expression of ARC neuropeptides (Klok et al. 2006) but their function is unclear in birds. Even though leptin genome sequences are available for several passerine species this is not yet the case for our titmice species. I attempted to clone leptin using conserved primers and recommended PCR conditions for leptin in our three titmice species but was not successful. Similarly, a ghrelin genome sequence is only available for one passerine species, the rifleman (*Acanthisitta chloris*). However, I was not able to clone ghrelin using the primer sequences conserved between the rifleman and other birds. The difficulty with working with leptin and ghrelin led me to investigate the role of other gut peptides as possible nutritional signals influencing ingestive behaviours including hoarding. Thus, in this PhD, I investigated a wide variety of gut and neuropeptides that have been studied in both mammalian and bird literature and were found to be especially important to regulate and control appetite.

5.2.1 Pancreatic peptides

From the great tit study, although we were not able to detect a nutritional state effect on the expression of appetite controlling peptide gene, we were able to establish a clear pattern of the gut peptide mRNA tissue distribution. Nonetheless, no particular tissue showed a distinct difference in gene expression between the two-treatment groups. However, that was not the case in the blue and coal tit experiment whereby insulin was the only gut peptide that did differ between the two species and the two-treatment groups. Unfortunately, there are not many studies that quantified insulin gene expression in relation to gut fill, however, we know from studies in chickens that plasma levels of insulin are decreased in fasted individuals (Simon et al. 2011). Studies in healthy humans estimated that within a time frame of 30 min-1h of ingesting a meal, insulin levels rapidly peak, returning to basal levels within 2-4h (Galloway and Chance 1994). Having established that, it is worth mentioning that in our study we did not measure the peptide, so it is difficult to draw conclusions about how insulin transcription is related to the circulating peptide.

Those observations, and the difference in insulin mRNA levels between the 3 titmice species made us speculate about the involvement of circadian rhythms (discussed later) in insulin gene expression. Because the experimental design of the great tit study gave individuals a 4h time frame to re-feed, while in the blue and coal tit study, birds were only given 1.5h to re-feed, the longer refeeding time in great tits might have allowed longer processing of insulin satiety signals and reduced mRNA expression in the pancreas.

Numerous studies have indicated a tight relationship between insulin and glucagon in the regulation of appetite and glucose metabolism, althoughwe did not detect such a relationship. It is well-known in birds that glucagon can stimulate insulin secretion, thus causing a rise in insulin levels (Song et al. 2017). However, no

correlation was found between glucagon and insulin. Therefore, we measured the ratio between the two peptide mRNAs (GCG/INS) but only in the pancreas since it is most relevant to investigate it there. The GCG/INS mRNA ratio did not change according to the nutritional state of the great tit; however, the ratio was higher in empty state coal tits but not the fed ones, with no such difference detected in blue tits. There is little information about the role of glucagon/insulin ratios in relation to theappetite system but given the fact that both hormones have an opposing function to one another, one might expect the regulation of the ratio might have something to do with the original function of those genes separately. However, it is likely that a higher GCG/INS mRNA ratio would stimulate the mobilization of glucose. On the other hand, a lower GCG/INS mRNA ratio would promote the biosynthesis of proteins and the reduction of blood glucose rise (Kalra and Gupta 2016).

The GCG/INS mRNA ratio correlated to food grabbing behaviour, whereby an increase in the fed state was associated with a decrease in food grabbing behaviour in coal tits, while an increase in blue tits was conversely associated with increased food grabbing behaviour. Thus, in blue the increased ratio positively correlated with increased foraging behaviour. Since both glucagon and insulin regulate blood glucose, and high glucagon low insulin is associated with hunger, it makes sense that the higher the ratio the more motivated the individual is to search for food to compensate for low energy levels. Having said that, the results seen in coal tits are difficult to interpret.

5.2.2 Glucagon related peptide gene expression outside the pancreas

While female great tits showed higher GCG mRNA in empty state duodenum, full state males showed higher GCG mRNA in their antrum. On the other hand, blue and coal tit GCG mRNA levels did not change according to the nutritional state of the individuals. Having said that, empty state blue tit female showed higher GCG mRNA in their antrum, while empty state female coal tits had higher GCG mRNA in their gizzards. This pattern in GCG tissue distribution reflecting different trends of gene expression is typical as each tissue express GLP-1 in slightly unique way. Moreover, the high expression of GCG in the great tits with empty gizzards is unlike what is expected. According to the well-established role of GLP-1, it is considered as a satiety peptide in chickens (Furuse et al. 1997) and Japanese quail (Shousha et al. 2007). Our ability to detect gizzard fullness effect in great tits but not the other two might be once again related to the longer re-feeding time.

It is not yet clear as to why we observed opposite trends in GCG expression between the two fasted species Our gene expression data came when individuals were humanely killed, and we do not have a video record of that day. Maybe for some reason, on that day individuals ate, grabbed or hoarded more showcasing individual differences. Or possibly individuals respond differently to re-feeding.

5.2.3 PYY

Studies in chickens showed that PYY mRNA levels in the jejunum were significantly higher under *ad libitum* conditions in comparison to when individuals were under 1-2h fasting (Aoki et al. 2017). However,

we were not able to detect any significant change in PYY expression in any of the three tit species studied. Thus, we speculated the involvement of circadian rhythm in great tits (discussed in the next section).

5.2.4 CCK

CCK gene expression was not different between the two-treatment group in either great tit study or the blue and coal tit experiment.

Nevertheless, Reid and Dunn (2018) looked at the CCK expression in chickens either fasted for an hour or had been reintroduced to food after 3h from removing it and given 2.5h to feed before being humanely killed to investigate how CCK expression response to short-term feed restriction. Although they were not able to detect any significant difference between the two treatments (fasted vs. fed), CCK mRNA in situ hybridisation signals between the two groups showed that CCK anticipatory expression might be different if the two groups were under longer nutritional challenges. We speculate that not seeing a nutritional state effect in blue and coal tits case might be related to the short re-feeding period. As for the great tits might be related to circadian rhythm.

5.3 Hindbrain neuropeptides

In all the titmice species studied, we did not detect any difference in GCG mRNA gene expression in response to the nutritional state. However, we did detect that coal tits had significantly higher levels of GCG mRNA in comparison to blue tits. . Larsen et al. (1997) demonstrated that the posttranslational product of the proglucagon in the hindbrain is glucagon like peptide-1 (GLP-1), hence that is likely to be the peptide product of the GCG gene expression we measured our titmice.

As for POMC, in great tit its expression was not changed according to individual state. Similarly, in coal tits and blue tits POMC did not change with the nutritional state. However, blue tits seemed to have higher POMC levels than coal tits. We speculate that the difference we see between the blue and coal tits in hindbrain neuropeptides might be related to behavioural differences.

In the hindbrain, GCG expression was correlated with eating and food grabbing. In fasted coal tits, increased GCG mRNA was related to decreased eating duration, but to increased eating duration in fed birds. Increased GCG expression strongly increased food grabbing in both species. The increase of GCG that decreased eating makes sense, since it is a satiety signal, and this result is consistent with the expression pattern between full and empty great tits. Moreover, this result is consistent with the higher levels of gut GCG found in great tits too, since we speculate that both respond to the same external stimuli.

5.4 Hypothalamic neuropeptides

While no changes in expression were observed in individual peptides in the great, blue and coal tits following fasting and re-feeding, blue tits tended to have a higher hypothalamic neuropeptide than coal tits. An increase in AGRP/POMC mRNA ratio was observed in fasted coal tits but not blue tits. This suggests that there may be species difference in the regulation of melanocortin system signalling and the potential

significance of this in relation to hoarding (see below). Moreover, the lack of effect on POMC is not unexpected as more contradictory results in the literature were demonstrated. Some studies were not able to detect any differences in the POMC mRNA levels even after 24h-48h of food deprivation (Japanese quail, Phillips-Singh et al. 2003; and broiler chicken Song et al. 2012), which was in accordance with similar studies in mammals where no differences in POMC expression was noted (Adam et al. 2002). Others, however, reported significantly decreased POMC expression levels in broiler chicks fasted for either 24-48h (Higgins et al. 2010)

Food grabbing and food hoarding correlated with some of the hypothalamic peptide gene expression. Both AGRP and POMC mRNA levels correlated with food grabbing and hoarding behaviour. An increase in AGRP mRNA in fasted blue tits increased food grabbing behaviour. On the other hand, increased hypothalamic POMC mRNA promoted food grabbing in blue tits but decreased it in coal tits. Generally, the lack of difference in both NPY and AGRP might be related to the short re-feeding time frame, because we have mentioned previously that studies in chicken and Japanese quail found a nutritional state effect on NPY expression levels. However, in those species they had longer food deprivation and refeeding. Moreover, a similar appetite inducing effect was shown in AGRP which is co-expressed with NPY. On the other hand, the decreased AGRP/ POMC ratio response to re-feeding in coal tits might be due to their small size in comparison to both blue and great tits, thus the 1.5h of re-feeding is very convenient for their rapid metabolism. However, the response of AGRP/POMC ratio to the nutritional state of individuals could have potential implications for food hoarding behaviour (discussed in the hoarding section below).

5.5 Circadian rhythms and peptide gene expression

Studies have shown the involvement of both neural and hormonal mechanisms in driving circadian rhythms in hunger and appetite (Scheer et al. 2013). The circadian pacemaker in both mammals and birds influences the hypothalamic nuclei where the neural populations (NPY/AGRP and POMC/CART) reside, and which have been implicated in control and regulation of appetite and weight. There were two peptide genes for which we were able to detect differences in their pattern of expression liniked to a diurnal rhythm. The most important is GCG. In great tits, fasted individuals (sampled at the end of the night) showed higher GCG mRNA in the pancreas, while fed ones (sampled during the day) in the hindbrain and proximal jejunum. However, we were not able to detect an effect of nutritional state on GCG expression in blue tits. Ruiter et al. (2003) demonstrated that in rats,plasma GCG protein concentration was significantly higher in the active phase in comparison to the rest phase in ad lib rats. However, rats fasted for either 18h or 30h showed a peak in GCG protein before the onset of the active phase. Plasma glucagon decreased in the first half of the rest period. However, a steep rise occurred at the end of the light period Thus, it seems that GCG levels are not only under the control of glucose but also under the control of circadian levels. Our result is similar to those observations in rats.

No nutritional state effect was detected in great, blue and coal tits on the expression of CCK mRNA. Xu et al. (2017) investigated the effect of reversing the light: dark cycle for 7 days on the expression of CCK in the duodenum and the pancreas of male Wistar rats. The authors found that those rats showed peak expression of CCK during the light phase (resting phase). This observation is consistent with what we observed in our great tit study. We noticed a significant peak of CCK expression levels in the duodenum, distal jejunum, distal ileum, gizzard and proventriculus during the dark phase which is equivalent to the light phase in rats. Those similar findings suggest that clock genes might be the main driving force behind circadian gene expression through the regulation of the promoter activity of a clock-controlled gene leading to the activation of downstream genes at specific times of the day (Brown and Schibler 1999).

5.6 Control of food hoarding

One of the main aims of this PhD was to identify neural or hormonal signals that not only report and change according to the nutritional status of individuals but could also act as motivators to induce food hoarding behaviour. Overall, we could not detect any difference in the expression of gut peptides as a result of the change in nutritional state of coal tits which could be a motivator for food hoarding, but that does not mean there is no change at all in their encoded peptides as a response to fasting or feeding.

We noted earlier in this thesis the fact that quantifying the mRNA might not be an indication of the peptide expression, and that protein analysis is a more accurate measurement of gene expression as the final product of mRNA translation process. Thus, we could not assume that no gut peptide changed and resulted in hoarding behaviour (recorded on camera).

In blue and coal tits the expression of NPY, AGRP and POMC was generally higher in the blue tits in comparison to the coal tits. In particular, POMC gene expression was 2.1× higher compared to only 1.2× of both NPY and AGRP. As for POMC, we did detect a very clear difference in its expression among the three titmice specifically coal tits. And although we do not have enough information regarding hindbrain POMC contribution to different ingestive behaviours, given its anorexigenic action we speculate that it might have a vital role in inducing food hoarding.

In both studies, we were not able to detect any significant effect of nutritional status on the expression levels of the hypothalamic neuropeptide genes. This made us speculate that the difference in gene expression might not be related to gut fill. AGRP mRNA increased food hoarding in coal tits. Moreover, hypothalamic POMC was linked to decrease food hoarding in coal tits. Additionally, AGRP/ HYP POMC in fasted coal tits was higher than fed birds. Those results indicate that AGRP, POMC and the AGRP/POMC ratio might contribute to food hoarding.

Studies in Siberian hamsters showed that administration of AGRP markedly increase food hoarding behaviour up to 2000% and that effect persist for up to 7 days (Day and Bartness 2004). According to the

authors AGRP induces foraging behaviour not only in Siberian hamster but also in other animals too. And since it is known that AGRP expression increases with fasting, and that Siberian hamsters over-hoard rather than over-eat, it makes sense that for hamsters at least once food is found they rather store it. As for POMC, there is not a lot of information regarding the contribution of POMC to food hoarding behaviour in either mammals or birds. However, our results suggest that POMC decreased food hoarding in coal tits.

In the hindbrain, GCG in coal tits was higher comparison to blue tits and great tits, and increased GCG mRNA increased hoarding. POMC mRNA also correlated with hoarding. An increase in fasted POMC increased hoarding, while an increase in fed POMC decrease it. Moreover, blue tits had higher POMC in comparison to coal tits. When doing a comparison between the three tits we only included the fed state to avoid variation linked to fasting. We found that CCK levels were not different between the three titmice species. However, the comparison between coal and blue tits showed that coal tits the highest GCG expression among the two species, while blue tits showed the highest hindbrain POMC. Our observations of POMC, GCG and CCK gene expression in the hindbrain are the first that we are aware of in birds. This suggests that hindbrain POMC signalling might contribute to the regulation of ingestive behaviours and hoarding.

Ultimately, it is important to note that in our study we have only quantified mRNA from the collected tissues. However, it is generally known that mRNA and protein levels do not always correlate owing to the complexity of the levels of control of transcription, translation and protein degradation. Having said that, in the avian literature few studies have combined measurements of both mRNA and protein (Buccitelli and Selbach 2020). On contrary, studies in mammalian hypothalamic neuropeptide (AgRP and POMC) for instance demonstrated that changes in gene expression are linked directly to peptide secretion and neural firing rates. However, while this might be the case in birds, studies have not been performed to address this (Boswell and Dunn 2017).

This means that we can only assume that the changes we observed in hypothalamic neuropeptide mRNAs were directly correlated with peptide secretion, however this correlation between mRNA and gut peptides is less certain. For example, glucagon mRNA was not correlated with circulating blood glucagon concentrations after fasting and re-feeding in chickens (Richards and McMurtry 2008).

5.7 Future work

For future experimental design, it is necessary to increase the re-feeding time for the titmice. Because those species are very small, and highly active we speculate that digestion and gut emptying process is very fast. Thus, having longer time to feed will insure detecting more satiety signals. Moreover, we could enhance the cage design. We speculated that because coal tits are scatter hoarders, they are more motivated to store food if many hoarding substrates are scattered in their home cage rather than having one place.

Molecularly quantifying leptin and ghrelin would also be informative, since those two peptides are considered the backbone of appetite system in mammals as many gut peptides and neuropeptides rely on their signalling to regulate their activities. |Work by our laboratory demonstrated that systemic administration of mammalian leptin and chicken ghrelin reduced hoarding in coal tits (Henderson et al., 2018). However, the role of these hormones as nutritional feedback signals to the brain in birds has yet to be established (Boswell and Dunn, 2017).

Another important point is that the measurement of mRNA might not relate directly to protein production owing to variation in transcription and translation mechanisms between different genes and tissues (Vogel and Marcotte 2012). Therefore, in the future there is a need to measure the translated peptides in tissues and plasma to help a explain gene expression measurements. There is also limited information on the effect of administering peptides such as GLP-1 on feeding behaviour in passerines such as titmice.

Another possibility is to quantify immediate early gene process via using immunohistochemistry techniques which is a very useful tool of measuring genes that are activated transiently and rapidly in response to a cellular stimulus. This could be particularly useful since we have identified the hindbrain and hypothalamus as potential targets of nutritional signals.

In a longer-term manner, it would be very useful to study the ingestive behaviour in a social context: studies have shown that hierarchy is a very important factor effecting individual daily behaviour. So, it would be interesting to set a study where we could measure the same behaviours and peptides in titmice within social group: where dominant/ subordinate relations are established.

Chapter 6. General conclusion

6.1 Conclusions

In conclusion, the present study overall, have helped us to establish the fact that both the nervous system and the gastrointestinal system shares signalling information that is considered the backbone route for reporting the nutritional state of the individual at different time points contributing in keeping its energy homeostasis at bay. By identifying candidate peptides genes that showed response to the individual nutritional state, we were able to make some distinctions between hoarding and non-hoarding species (figure 1 and 2). Our study suggests that hypothalamic AGRP/POMC primarily could be used as neural signals reporting the nutritional state of titmice. Moreover, hypothalamic AGRP and POMC, and hindbrain GCG and POMC seem to be involved in food hoarding in coal tits.

Having said that, we have showed that Satiety did not suppress hoarding, at least not in cases when the bird has experienced a period of food deprivation, followed by successful foraging. In those conditions, they seem to be more motivated to not only forage more but also store extra food for later consumption. It is not clear that this would also be the case for birds that have continuous access to food sources and therefor are satiated continuously. They might not hoard as much because the food is always there. Thus, those observations further confirm observations from the hamster literature that peptides that are known to control and regulates food intake are also involved in food hoarding.

In this study we compared both gene expression and tissue distribution of several neuropeptides and gut peptides between hoarding and non-hoarding titmice. We were able to establish associations between brain and gut peptide gene expression and ingestive behaviours in response to food deprivation and re-feeding and also to circadian rhythms. By identifying candidate peptide genes that respond to an individual's nutritional state, we were able to make some distinctions between hoarding and non-hoarding species (Figure 1 and 2). Overall, he signalling system between the gut and the central nervous system is an important route for reporting overall nutritional status of the individual. Moreover, we showed that satiety did not suppress hoarding, at least not in cases when the bird has experienced a period of food deprivation, followed by successful foraging. In those conditions, they seem to be more motivated to not only forage more but also store extra food for later consumption. It is not clear that this would also be the case for birds that have a continuous access to food sources and therefore be satiated continuously. They might not hoard as much because the food is always there.

Cyanistes caeruleus

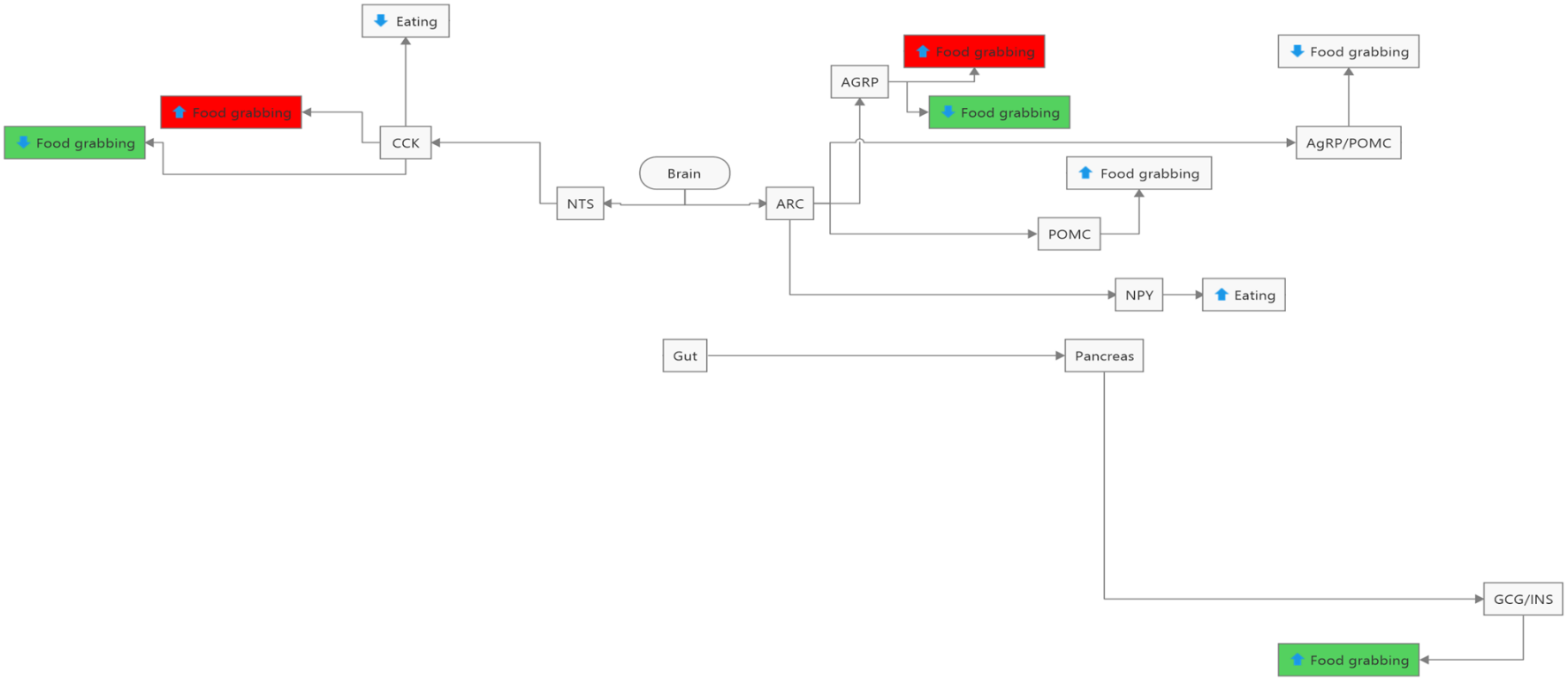


Figure 1. Flow chart illustrating the brain-gut axis of blue tits showing both neuropeptides and gut peptides gene expression and their correlation to eating, food grabbing behaviours. Green boxes are fed state, red boxes are fasted state and white boxes showing an overall effect. The arrows pointing up showcase increase in expression, and the arrows pointing down showcase decrease/ suppression

Pariparus ater

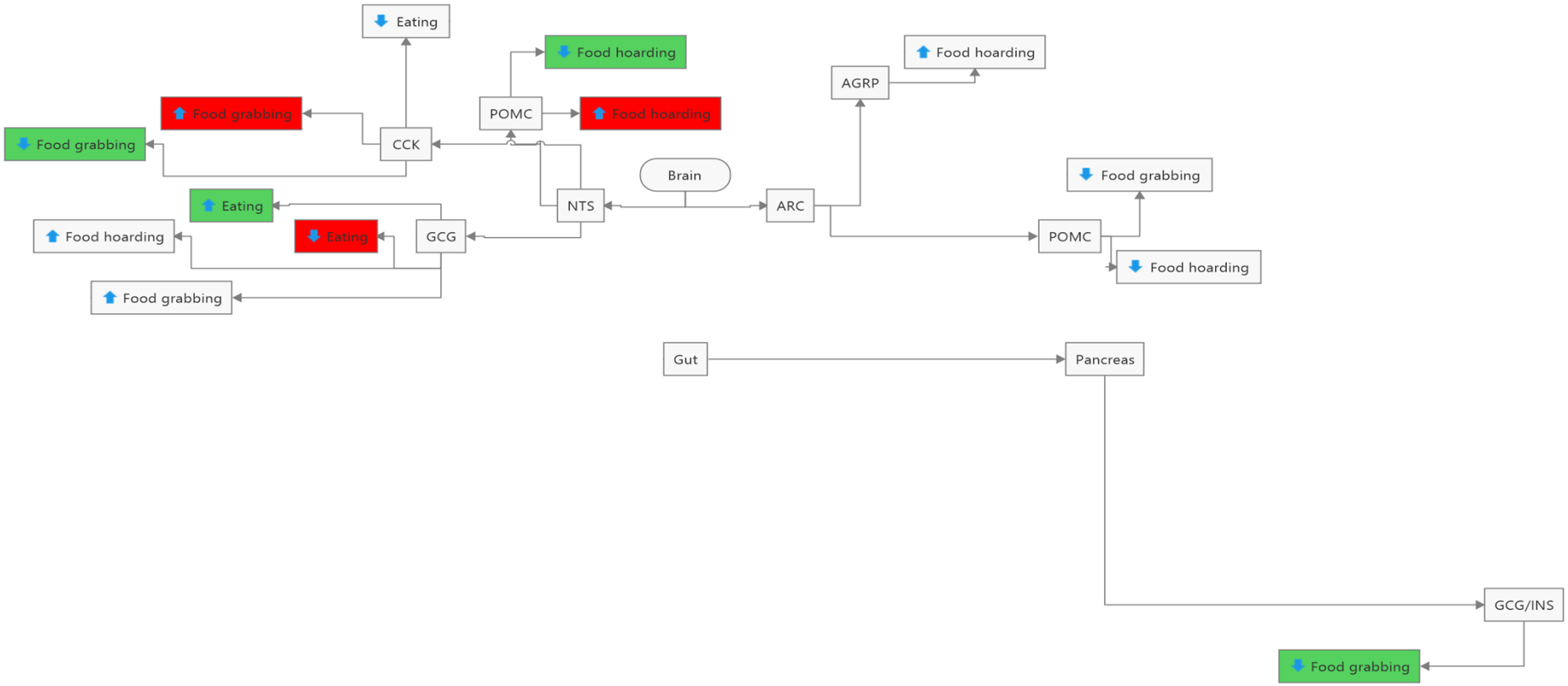


Figure 2. Flow chart illustrating the brain-gut axis of coal tits showing both neuropeptides and gut peptides gene expression and their correlation to eating, food grabbing, and food hoarding behaviours. Green boxes are fed state, red boxes are fasted state and white boxes shows an overall effect. The arrows pointing up showcase increase in expression, and the arrows pointing down showcase decrease/ suppression

Chapter 7. References

7.1 References

Brown J. L. (1964) The evolution of diversity in avian territorial system. Wilson Bulletin 76:160-169.

Etienne A. S, Matathia R, Emmanuelli E, Zinder M, Crapon De Caprona D (1982) Ontogenesis of hoarding behavior in hamster. Journal of Behavioural Biology 1-15.

Wells D. J (1993) Ecological correlates of hovering flight of hummingbirds. Journal of Experimental Biology 178:59-70.

Acosta-Martinez M, Horton T, Levine J (2007) Estrogen receptors in neuropeptide Y neurons: at the crossroads of feeding and reproduction. Trends in Endocrinology & Metabolism 18:48-50.

Acuna-Goycolea C, van den Pol A (2004) Glucagon-Like Peptide 1 Excites Hypocretin/Orexin Neurons by Direct and Indirect Mechanisms: Implications for Viscera-Mediated Arousal. Journal of Neuroscience 24:8141-8152.

Adam C, Archer Z, Findlay P et al. (2002) Hypothalamic Gene Expression in Sheep for Cocaine- and Amphetamine-Regulated Transcript, Pro-Opiomelanocortin, Neuropeptide Y, Agouti-Related Peptide and Leptin Receptor and Responses to Negative Energy Balance. Neuroendocrinology 75:250-256.

Adrian T, Ferri G, Bacarese-Hamilton A et al. (1985) Human distribution and release of a putative new gut hormone, peptide YY. Gastroenterology 89:1070-1077.

Adrian T, Ferri G, Bacarese-Hamilton A et al. (1985) Human distribution and release of a putative new gut hormone, peptide YY. Gastroenterology 89:1070-1077.

Ahima R, Prabakaran D, Mantzoros C et al. (1996) Role of leptin in the neuroendocrine response to fasting. Nature 382:250-252.

Air E, Benoit S, Blake Smith K et al. (2002) Acute third ventricular administration of insulin decreases food intake in two paradigms. Pharmacology Biochemistry and Behavior 72:423-429.

Aldewach H, Wright N, Appleton D, Watson A (1975) The effect of starvation and refeeding on cell population kinetics in the rat small bowel mucosa. Journal of Anatomy 119:105–121.

Alfalah M, Michel M (2004) Neuropeptide Y and related peptides. Springer, Berlin

Alhadeff A, Grill H (2014) Hindbrain nucleus tractus solitarius glucagon-like peptide-1 receptor signaling reduces appetitive and motivational aspects of feeding. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 307: R465-R470.

Al-Massadi O, Fernø J, Diéguez C et al. (2019) Glucagon Control on Food Intake and Energy Balance. International Journal of Molecular Sciences 20:3905.

Altmann G (1972) Influence of starvation and refeeding on mucosal size and epithelial renewal in the rat small intestine. American Journal of Anatomy 133:391-400.

Altschuler S, Bao X, Bieger D et al. (1989) Viscerotopic representation of the upper alimentary tract in the rat: Sensory ganglia and nuclei of the solitary and spinal trigeminal tracts. The Journal of Comparative Neurology 283:248-268.

Amin T, Mercer J (2016) Hunger and Satiety Mechanisms and Their Potential Exploitation in the Regulation of Food Intake. Current Obesity Reports 5:106-112.

Amir S (1986) Central glucagon-induced hyperglycemia is mediated by combined activation of the adrenal medulla and sympathetic nerve endings. Physiology & Behavior 37:563-566.

Andersson M, Krebs J (1978) On the evolution of hoarding behaviour. Animal Behaviour 26:707-711. doi: 10.1016/0003-3472(78)90137-9

Ando R, Kawakami S, Bungo T et al. (2001) Feeding responses to several neuropeptide Y receptor agonists in the neonatal chick. European Journal of Pharmacology 427:53-59.

Anini Y, Brubaker P (2003) Role of Leptin in the Regulation of Glucagon-Like Peptide-1 Secretion. Diabetes 52:252-259.

Anini Y, Fu-Cheng X, Cuber J et al. (1999) Comparison of the postprandial release of peptide YY and proglucagon-derived peptides in the rat. Pfl&#65533;gers Archiv European Journal of Physiology 438:299-306.

Aoki K, Kondo M, Okuda M et al. (2017) Identification, expression analysis, and functional characterization of peptide YY in chickens (Gallus gallus domesticus). General and Comparative Endocrinology 242:11-17.

Aponte G, Taylor I, Soll A (1988) Primary culture of PYY cells from canine colon. American Journal of Physiology-Gastrointestinal and Liver Physiology 254: G829-G836.

Ariyachet C, Tovaglieri A, Xiang G et al. (2016) Reprogrammed Stomach Tissue as a Renewable Source of Functional β Cells for Blood Glucose Regulation. Cell Stem Cell 18:410-421.

Aronsson M, Fuxe K, Dong Y et al. (1988) Localization of glucocorticoid receptor mRNA in the male rat brain by in situ hybridization. Proceedings of the National Academy of Sciences 85:9331-9335.

Artner I, Stein R (1399) Transcriptional regulation of insulin gene expression. In: Pancreatic beta cell in health and disease. 13-30.

Asakawa A, Inui A, Kaga O et al. (2001) Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. Gastroenterology 120:337-345.

Astheimer L, Buttemer W, Wingfield J (1992) Interactions of Corticosterone with Feeding, Activity and Metabolism in Passerine Birds. Ornis Scandinavica 23:355.

Atasoy D, Betley J, Su H, Sternson S (2012) Deconstruction of a neural circuit for hunger. Nature 488:172-177.

Atasoy D, Betley J, Su H, Sternson S (2012) Deconstruction of a neural circuit for hunger. Nature 488:172-177.

Badness T, Powers J, Hastings M et al. (1993) The timed infusion paradigm for melatonin delivery: What has it taught us about the melatonin signal, its reception, and the photoperiodic control of seasonal responses? Journal of Pineal Research 15:161-190.

Bado A, Levasseur S, Attoub S et al. (1998) The stomach is a source of leptin. Nature 790-793.

Bagdade J, Bierman E, Porte D (1967) The Significance of Basal Insulin Levels in the Evaluation of the Insulin Response to Glucose in Diabetic and Nondiabetic Subjects*. Journal of Clinical Investigation 46:1549-1557.

Bagger J, Holst J, Hartmann B et al. (2015) Effect of Oxyntomodulin, Glucagon, GLP-1, and Combined Glucagon +GLP-1 Infusion on Food Intake, Appetite, and Resting Energy Expenditure. The Journal of Clinical Endocrinology & Metabolism 100:4541-4552.

Baggio L, Drucker D (2004) Glucagon-like peptide-1 and glucagon-like peptide-2. Best Practice & Research Clinical Endocrinology & Metabolism 18:531-554.

Baggio L, Huang Q, Brown T, Drucker D (2004) Oxyntomodulin and glucagon-like peptide-1 differentially regulate murine food intake and energy expenditure. Gastroenterology 127:546-558.

Baldwin S, Kendeigh S (1938) Variations in the Weight of Birds. The Auk 55:416-467.

Ballantyne G, Longo W, Savoca P et al. (1989) Deoxycholate-stimulated release of peptide YY from the isolated perfused rabbit left colon. American Journal of Physiology-Gastrointestinal and Liver Physiology 257: G715-G724.

Ballaz S (2017) The unappreciated roles of the cholecystokinin receptor CCK (1) in brain functioning. Reviews in the Neurosciences 28:

Bamberger C, Schulte H, Chrousos G (1996) Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocrine Reviews 17:245-261.

Banks W (2004) The source of cerebral insulin. European Journal of Pharmacology 490:5-12.

Banks W, Kastin A (1985) Peptides and the blood-brain barrier: Lipophilicity as a predictor of permeability. Brain Research Bulletin 15:287-292.

Banks W, Kastin A (1985) Permeability of the blood-brain barrier to neuropeptides: The case for penetration. Psychoneuroendocrinology 10:385-399.

Banks W, Kastin A, Huang W et al. (1996) Leptin enters the brain by a saturable system independent of insulin. Peptides 17:305-311.

Banks W, Tschöp M, Robinson S, Heiman M (2002) Extent and Direction of Ghrelin Transport Across the Blood-Brain Barrier Is Determined by Its Unique Primary Structure. Journal of Pharmacology and Experimental Therapeutics 302:822-827.

Baranowska B, Wolińska-Witort E, Martyńska L et al. (2004) Effects of cocaine–amphetamine regulated transcript (CART) on hormone release. Regulatory Peptides 122:55-59.

Barry W (1976) Environmental Effects on Food Hoarding in Deermice (Peromyscus). Journal of Mammalogy 57:731-746.

Barson J (2020) Orexin/hypocretin and dysregulated eating: Promotion of foraging behavior. Brain Research 1731:145915.

Bartness T, Day D (2002) Food hoarding: a quintessential anticipatory appetitive behavior. Progress in psychobiology and physiological psychology 18:69-100.

Bartness T, Demas G (2003) Comparative studies of food intake: lessons from non-traditionally studied species. In: Food and Fluid Intake, Plenum, New York

Bartness T, Keen-Rhinehart E, Dailey M, Teubner B (2011) Neural and hormonal control of food hoarding. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 301: R641-R655.

Bartness T, Morley J, Levine A (1995) Effects of food deprivation and metabolic fuel utilization on the photoperiodic control of food intake in Siberian hamsters. Physiology & Behavior 57:61-68.

Bartness T, Wade G (1984) Photoperiodic control of body weight and energy metabolism in Syrian Hamsters (Mesocricetus auratus): Role of pineal gland, melatonin, gonads, and diet. Endocrinology 114:492-498.

Bartness T, Wade G (1985) Photoperiodic control of seasonal body weight cycles in hamsters. Neuroscience & Biobehavioral Reviews 9:599-612.

Baskin D, Breininger J, Schwartz M (1999) Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hypothalamus. Diabetes 48:828-833.

Baskin D, Figlewicz Lattemann D, Seeley R et al. (1999) Insulin and leptin: dual adiposity signals to the brain for the regulation of food intake and body weight. Brain Research 848:114-123.

Batterham R, Bloom S (2003) The gut hormone Peptide YY regulates appetite. Annals of the New York Academy of Sciences 994:162-168.

Batterham R, Cowley M, Small C et al. (2002) Gut hormone PYY3–36 decrease food intake in rodents? Nature 430:3-4.

Baum J, Simons B, Unger R, Madison L (1962) Localization of glucagon in the cells in the pancreatic islet by immunofluorescent techniques. Diabetes 371-374.

Baura G, Foster D, Porte D et al. (1993) Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain. Journal of Clinical Investigation 92:1824-1830.

Beck B, Jhanwar-Uniyal M, Burlet A et al. (1990) Rapid and localized alterations of neuropeptide Y in discrete hypothalamic nuclei with feeding status. Brain Research 528:245-249.

Beer S, Bircham P, Bloom S et al. (1989) The effect of a 72-h fast on plasma levels of pituitary, adrenal, thyroid, pancreatic and gastrointestinal hormones in healthy men and women. Journal of Endocrinology 120:337-350.

Beinfeld M, Meyer D, Eskay R et al. (1981) The distribution of cholecystokinin immunoreactivity in the central nervous system of the rat as determined by radioimmunoassay. Brain Research 212:51-57.

Beinfeld M, Palkovits M (1981) Distribution of cholecystokinin (CCK) in the hypothalamus and limbic system of the rat. Neuropeptides 2:123-129.

Bell G (1986) The glucagon superfamily: Precursor structure and gene organization. Peptides 7:27-36.

Benoit S, Air E, Coolen L et al. (2002) The catabolic action of insulin in the brain is mediated by melanocortins. The Journal of Neuroscience 22:9048-9052.

Berthold P (1976) Animal and vegetable nutrition of omnivorous songbird species: food preference, annual period of nutritional choice, physiological and ecological importance. Journal of Ornithology 117:145-209. doi: 10.1007/bf01643586

Bertile F, Oudart H, Criscuolo F et al. (2003) Hypothalamic gene expression in long-term fasted rats: relationship with body fat. Biochemical and Biophysical Research Communications 303:1106-1113.

Beuving G, Vonder G (1977) Daily rhythm of corticosterone in laying hens and the influence of egg laying. Reproduction 51:169-173.

Biebach H (1975) Seasonal differences in resting metabolic rate ofTurdus merula. Die Naturwissenschaften 62:398-399.

Bielert C, Busse C (1983) Influences of ovarian hormones on the food intake and feeding of captive and wild female chacma baboons (Papio ursinus). Physiology & Behavior 30:103-111.

Bindra D (1948) The nature of motivation for hoarding food. Journal of Comparative and Physiological Psychology 41:211-218. doi: 10.1037/h0055698

Bindra D (1948) What makes rats hoard? Journal of Comparative and Physiological Psychology 41:397-402. doi: 10.1037/h0054521

Bingham E, Hopkins D, Smith D et al. (2002) The Role of Insulin in Human Brain Glucose Metabolism: An 18Fluoro-Deoxyglucose Positron Emission Tomography Study. Diabetes 51:3384-3390.

Bittman E, Dempsey R, Karsch F (1983) pineal melatonin secretion drives the reproductive response to daylength in the ewe. Endocrinology 113:2276-2283.

Bjorbaek C, Kahn B (2004) Leptin Signaling in the Central Nervous System and the Periphery. Recent Progress in Hormone Research 59:305-331.

Boissin J, Assenmacher I (1970) Circadian rhythms in adrenal cortical activity in the quail. Journal of Interdisciplinary Cycle Research 1:251-265.

Bolli G, De Feo P, De Cosmo S et al. (1984) Demonstration of a dawn phenomenon in normal human volunteers. Diabetes 33:1150-1153.

Booth G, Strang J (1936) Changes in temperature of the skin following the ingestion of food. Archives of Internal Medicine 57:533.

Bossema I (1979) Jays and Oaks: An eco-ethological study of a symbiosis. Behaviour 70:1-116.

Boswell T (2005) Regulation of Energy Balance in Birds by the Neuroendocrine Hypothalamus. The Journal of Poultry Science 42:161-181.

Boswell T (2010) Encyclopedia of Animal Behavior. 738-743.

Boswell T, Dunn I (2017) Regulation of agouti-related protein and pro-opiomelanocortin gene Expression in the Avian Arcuate Nucleus. Frontiers in Endocrinology 8:75. 1-11

Boswell T, Dunn I, Corr S (1999) Neuropeptide Y gene expression in the brain is stimulated by fasting and food restriction in chickens. British Poultry Science 40:42-43.

Boswell T, Dunn I, Corr S (1999) Physiology, biochemistry and neurobiology. Neuropeptide Y gene expression in the brain is stimulated by fasting and food restriction in chickens. British Poultry Science 40:42-43.

Boswell T, Lehman T, Ramenofsky M (1997) Effects of plasma glucose manipulations on food intake in white-crowned sparrows. Comparative Biochemistry and Physiology Part A: Physiology 118:721-726.

Boswell T, Li Q (1998) Cholecystokinin Induces Fos Expression in the Brain of the Japanese Quail. Hormones and Behavior 34:56-66.

Boswell T, Li Q, Takeuchi S (2002) Neurons expressing neuropeptide Y mRNA in the infundibular hypothalamus of Japanese quail are activated by fasting and co-express agouti-related protein mRNA. Molecular Brain Research 100:31-42.

Boswell T, Takeuchi S (2005) Recent developments in our understanding of the avian melanocortin system: Its involvement in the regulation of pigmentation and energy homeostasis. Peptides 26:1733-1743.

Brattstrom B (1968) Thermal acclimation in Anuran amphibians as a function of latitude and altitude. Comparative Biochemistry and Physiology 24:93-111.

Briant L, Salehi A, Vergari E et al. (2016) Glucagon secretion from pancreatic a-cells. UPSALA Journal O Medical Sciences 121:113-119.

Brief D, Davis J (1984) Reduction of food intake and body weight by chronic intraventricular insulin infusion. Brain Research Bulletin 12:571-575.

Brobeck J (1945) Effect of variations in activity, food intake and environmental temperature on weight gain in the albino rat. American Journal of Physiology-Legacy Content 143:1-5.

Brobeck J (1960) Food and temperature. In G. Pincus edition. 439-466.

Brobeck J, Tepperman J, Long C (1943) Experimental hypothalamic hyperphagia in the albino rat. Yale Journal of Biology and Medicine 15:831-853.

Broberger C (2005) Brain regulation of food intake and appetite: molecules and networks. Journal of Internal Medicine 258:301-327.

Broberger C, Johansen J, Johansson C et al. (1998) The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice. Proceedings of the National Academy of Sciences 95:15043-15048.

Brodin A (2000) Why do hoarding birds gain fat in winter in the wrong way? Suggestions from a dynamic model. Behavioral Ecology 11:27-39.

Brodin A (2010) The history of scatter hoarding studies. Philosophical Transactions of the Royal Society B: Biological Sciences 365:869-881.

Brodin A, Ekman J (1994) Benefits of food hoarding. Nature 372:510-510.

Brodin A, Nilsson J, Nord A (2017) Adaptive temperature regulation in the little bird in winter: predictions from a stochastic dynamic programming model. Oecologia 185:43-54.

Broggi J, Koivula K, Hohtola E, Orell M (2017) Environmental drivers of roosting body mass variation in boreal Great Tits Parus major. Ibis 159:919-924.

Broggi J, Koivula K, Lahti K, Orell M (2003) Seasonality in daily body mass variation in a hoarding boreal passerine. Oecologia 137:627-633.

Broome M, Hokfelt T, Terenius L (1985) Peptide YY (PYY)-immunoreactive neurons in the lower brain stem and spinal cord of rat. Acta Physiologica Scandinavica 125:349-352.

Brown L, Clegg D, Benoit S, Woods S (2006) Intraventricular insulin and leptin reduce food intake and body weight in C57BL/6J mice. Physiology & Behavior 89:687-691.

Brown S, Schibler U (1999) The ins and outs of circadian timekeeping. Current Opinion in Genetics & Development 9:588-594.

Bruning J, Gautam D, Burks D et al. (2000) Role of Brain Insulin Receptor in Control of Body Weight and Reproduction. Science 289:2122-2125.

Burguera B, Couce M, Long J et al. (2000) The Long Form of the Leptin Receptor (OB-Rb) Is Widely Expressed in the Human Brain. Neuroendocrinology 71:187-195.

Butera P (2010) Estradiol and the control of food intake. Physiology & Behavior 99:175-180.

Buyse M, Ovesjö M, Goïot H et al. (2001) Expression and regulation of leptin receptor proteins in afferent and efferent neurons of the vagus nerve. European Journal of Neuroscience 14:64-72.

Byerly M, Simon J, Lebihan-Duval E et al. (2009) Effects of BDNF, T3, and corticosterone on expression of the hypothalamic obesity gene network in vivo and in vitro. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 296: R1180-R1189.

Caballero B (2007) The Global Epidemic of Obesity: An Overview. Epidemiologic Reviews 29:1-5. doi: 10.1093/epirev/mxm012

Cai G, Mo C, Huang L et al. (2015) Characterization of the Two CART Genes (CART1 and CART2) in Chickens (Gallus gallus). PLOS ONE 10:e0127107.

Campos C, Wright J, Czaja K, Ritter R (2012) CCK-Induced Reduction of Food Intake and Hindbrain MAPK Signaling Are Mediated by NMDA Receptor Activation. Endocrinology 153:2633-2646.

Cardoso T, Pinheiro N, Mota D, Yamada J (1999) Identify-year and localization-of endocrine cells two stogamages of Tangara seledon hrough immunocytochemical study. Rural University Magazine Life Sciences Series 75–83.

Carlessi R, Chen Y, Rowlands J et al. (2017) GLP-1 receptor signalling promotes β-cell glucose metabolism via mTOR-dependent HIF-1α activation. Scientific Reports 7:

Carlini V, Monzón M, Varas M et al. (2002) Ghrelin increases anxiety-like behavior and memory retention in rats. Biochemical and Biophysical Research Communications 299:739-743.

Carlini V, Varas M, Cragnolini A et al. (2004) Differential role of the hippocampus, amygdala, and dorsal raphe nucleus in regulating feeding, memory, and anxiety-like behavioral responses to ghrelin. Biochemical and Biophysical Research Communications 313:635-641.

Carter M, Soden M, Zweifel L, Palmiter R (2013) Genetic identification of a neural circuit that suppresses appetite. Nature 503:111-114.

Carvalheira J, Ribeiro E, Araújo E et al. (2017) Expression of Concern: Selective impairment of insulin signalling in the hypothalamus of obese Zucker rats. Diabetologia

Cassone V, Kumar V (2015) Circadian rhythms. 811–827.

Castaldo L, Lucini C (1991) An immunohistochemical study on the endocrine cells in the gastrointestinal tract of domestic duck. European Journal of Basic and Applied Histochemistry 131-143.

Castillo-Morales A, Monzón-Sandoval J, Urrutia A, Gutiérrez H (2014) Increased brain size in mammals is associated with size variations in gene families with cell signalling, chemotaxis and immune-related functions. Proceedings of the Royal Society B: Biological Sciences 281:20132428.

Caughey S, Wilson P, Mukhtar N et al. (2018) Sex differences in basal hypothalamic anorectic and orexigenic gene expression and the effect of quantitative and qualitative food restriction. Biology of Sex Differences 9:

Caughey S, Wilson P, Mukhtar N et al. (2018) Sex differences in basal hypothalamic anorectic and orexigenic gene expression and the effect of quantitative and qualitative food restriction. Biology of Sex Differences 9:

Caughey S, Wilson P, Mukhtar N et al. (2018) Sex differences in basal hypothalamic anorectic and orexigenic gene expression and the effect of quantitative and qualitative food restriction. Biology of Sex Differences 9:

Chavez M, Riedy C, Van Dijk G, Woods S (1996) Central insulin and macronutrient intake in the rat. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 271: R727-R731.

Chen L, Jiang Q, Kaiya H et al. (2008) Central administration of chicken ghrelin inhibits food intake in adult chickens. 9th International Symposium on Avian Endocrinology 75.

Chen L, Komiya I, Inman L et al. (1989) Effects of hypoglycemia and prolonged fasting on insulin and glucagon gene expression. Studies within situ hybridization. Journal of Clinical Investigation 84:711-714.

Chen Y, Knight Z (2016) Making sense of the sensory regulation of hunger neurons. BioEssays 38:316-324.

Cheung C, Clifton D, Steiner R (1997) Proopiomelanocortin Neurons Are Direct Targets for Leptin in the Hypothalamus. Endocrinology 138:4489-4492.

Christensen K, McMurtry J, Thaxton Y et al. (2012). Metabolic and hormonal responses of modern meat type chickens to fasting. British Poultry Science 54:199-205.

Christensen K, McMurtry J, Thaxton Y et al. (2013) Metabolic and hormonal responses of growing modern meat-type chickens to fasting. British Poultry Science 54:199-205.

Chronwall B (1985) Anatomy and physiology of the neuroendocrine arcuate nucleus. Peptides 6:1-11.

Chrousos G (2009) Stress and disorders of the stress system. Nature Reviews Endocrinology 5:374-381.

Chua S, Koutras I, Han L et al. (1997) Fine Structure of the Murine Leptin Receptor Gene: Splice Site Suppression Is Required to Form Two Alternatively Spliced Transcripts. Genomics 45:264-270.

Cintra A, Fuxe K, Solfrini V et al. (1991) Central peptidergic neurons as targets for glucocorticoid action. Evidence for the presence of glucocorticoid receptor immunoreactivity in various types of classes of peptidergic neurons. The Journal of Steroid Biochemistry and Molecular Biology 40:93-103.

Clark G (1939) The use of the HORSLEY-CLARKE instrument on the rat. Science 90:92-92.

Clark J, Kalra P, Crowley W, Kalra S (1984) Neuropeptide Y and human pancreatic polypeptide stimulate feeding behaviour in rats. Endocrinology 115:427-429.

Colca J, Hazelwood R (1982) Persistence of immunoreactive insulin, glucagon and pancreatic polypeptide in the plasma of depancreatized chickens. Journal of Endocrinology 92:317-326.

Coll A (2007) Effects of pro-opiomelanocortin (POMC) on food intake and body weight: mechanisms and therapeutic potential? Clinical Science 113:171-182.

Cone R (2005) Anatomy and regulation of the central melanocortin system. Nature Neuroscience 8:571-578.

Cone R, Cowley M, Butler A et al. (2001) The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. International Journal of Obesity and Related Metabolic Disorders 25: S63-S67.

Conlon J, O'Harte F (1992) The primary structure of a PYY-related peptide from chicken intestine suggests an anomalous site of cleavage of the signal peptide in preproPYY. FEBS Letters 313:225-228.

Considine R, Sinha M, Heiman M et al. (1996) Serum Immunoreactive-Leptin Concentrations in Normal-Weight and Obese Humans. The New England Journal of Medicine 334:292–295.

Cooper N, Sherry T, Marra P (2015) Experimental reduction of winter food decreases body condition and delays migration in a long-distance migratory bird. Ecology 96:1933-1942.

Cooper S (2007) Daily and seasonal variation in body mass and visible fat in mountain chickadees and Juniper Titmice. The Wilson Journal of Ornithology 119:720-724.

Cordell B, Bell G, Tischer E et al. (1979) Isolation and characterization of a cloned rat insulin gene. Cell 18:533-543.

Corp E, McQuade J, Moran T, Smith G (1993) Characterization of type A and type B CCK receptor binding sites in rat vagus nerve. Brain Research 623:161-166.

Corp E, Woods S, Porte D et al. (1986) Localization of 125I-insulin binding sites in the rat hypothalamus by quantitative autoradiography. Neuroscience Letters 70:17-22.

Covasa M, Forbes J (1994) Exogenous cholecystokinin octapeptide in broiler chickens: Satiety, conditioned colour aversion, and vagal mediation. Physiology & Behavior 56:39-49.

Cowie R, Krebs J, Sherry D (1981) Food storing by marsh tits. Animal Behaviour 29:1252-1259.

Cowley M, Smart J, Rubinstein M et al. (2001) Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature 411:480-484.

Cowley M, Smith R, Diano S et al. (2003) The Distribution and Mechanism of Action of Ghrelin in the CNS Demonstrates a Novel Hypothalamic Circuit Regulating Energy Homeostasis. Neuron 37:649-661.

Craig W (1918) Appetites and Aversions as Constituents of Instincts. Scientific American 85:291-291.

Cummings D (2006) Ghrelin and the short- and long-term regulation of appetite and body weight. Physiology & Behavior 89:71-84.

D'Agostino G, Lyons D, Cristiano C et al. (2016) Appetite controlled by a cholecystokinin nucleus of the solitary tract to hypothalamus neurocircuit. The Journal of eLife e12225.

Dailey M, Bartness T (2009) Appetitive and consummatory ingestive behaviors stimulated by PVH and perifornical area NPY injections. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 296: R877-R892.

Dailey M, Moghadam A, Moran T (2014) Nutrient-specific feeding and endocrine effects of jejunal infusions in obese animals. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 306: R420-R428.

Dailey M, Moran T (2013) Glucagon-like peptide 1 and appetite. Trends endocrinology metabolism 24:85-91.

Dailey M, Stingl K, Moran T (2012) Disassociation between Preprandial Gut Peptide Release and Food-Anticipatory Activity. Endocrinology 153:132-142.

Dallman M, Akana S, StrAck A et al. (1995) The neural network that regulates energy balance is responsive to glucocorticoids and insulin and also regulates HPA axis responsivity at a site proximal to CRF neurons. Annals of the New York Academy of Sciences 771:730-742.

Dallman M, la Fleur S, Pecoraro N et al. (2004) Minireview: Glucocorticoids—Food Intake, Abdominal Obesity, and Wealthy Nations in 2004. Endocrinology 145:2633-2638.

Damiola F (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes & Development 14:2950-2961.

Dark J, Zucker I, Wade G (1983) Photoperiodic regulation of body mass, food intake, and reproduction in meadow voles. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 245: R334-R338.

Date Y, Murakami N, Toshinai K et al. (2002) The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. Gastroenterology 123:1120-1128.

Date Y, Toshinai K, Koda S et al. (2005) Peripheral interaction of ghrelin with cholecystokinin on feeding regulation. Endocrinology 146:3518-3525.

Dawson A, Goldsmith A (1982) Prolactin and gonadotrophin secretion in wild starlings (Sturnus vulgaris) during the annual cycle and in relation to nesting, incubation, and rearing young. General and Comparative Endocrinology 48:213-221.

Day D, Bartness T (2003) Fasting-induced increases in food hoarding are dependent on the foraging-effort level. Physiology & Behavior 78:655-668.

Day D, Bartness T (2004) Agouti-related protein increases food hoarding more than food intake in Siberian hamsters. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 286: R38-R45.

Day D, Keen-Rhinehart E, Bartness T (2005) Role of NPY and its receptor subtypes in foraging, food hoarding, and food intake by Siberian hamsters. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 289: R29-R36.

Day D, Mintz E, Bartness T (2002) Diet choice exaggerates food hoarding, intake and pup survival across reproduction. Physiology & Behavior 75:143-157.

De Araujo I (2009) Gustatory and Homeostatic Functions of the Rodent Parabrachial Nucleus. Annals of the New York Academy of Sciences 1170:383-391.

De Jonghe B, Hayes M, Zimmer D et al. (2012) Food intake reductions and increases in energetic responses by hindbrain leptin and melanotan II are enhanced in mice with POMC-specific PTP1B deficiency. American Journal of Physiology-Endocrinology and Metabolism 303: E644-E651.

De Lecea L, Kilduff T, Peyron C et al. (1998) The hypocretins: Hypothalamus-specific peptides with neuroexcitatory activity. Proceedings of the National Academy of Sciences 95:322-327.

Denbow D (1994) Peripheral Regulation of Food Intake in Poultry. The Journal of Nutrition 124:1349S-1354S.

Denbow D, Duke G, Chaplin S (1988) Food intake, gastric secretion, and motility as affected by avian pancreatic polypeptide administered centrally in chickens. Peptides 9:449-454.

Denbow D, Meade S, Robertson A et al. (2000) Leptin-induced decrease in food intake in chickens. Physiology & Behavior 69:359-362.

Denbow D, Myers R (1982) Eating, drinking and temperature responses to intracerebroventricular cholecystokinin in the chick. Peptides 3:739-743.

Deschodt-Lanckman M, Bui N, Noyer M, Christophe J (1981) Degradation of cholecystokinin-like peptides by a crude rat brain synaptosomal fraction: A study by high pressure liquid chromatography. Regulatory Peptides 2:15-30.

Desjeux J, Amar J, Deschamps I et al. (1979) Duodenal stimulation of insulin secretion. Role of intestinal villi. Archives Francaises de Pediatrie 36:XIX-XXV.

Devaskar S (2001) Neurohumoral regulation of body weight gain. Pediatric Diabetes 2:131-144.

Dhillo W (2007) Appetite Regulation: An Overview. Thyroid 17:433-445.

Dibner C, Schibler U, Albrecht U (2010) The Mammalian Circadian Timing System: Organization and Coordination of Central and Peripheral Clocks. Annual Review of Physiology 72:517-549.

Dickens M, Bentley G (2014) Stress, captivity, and reproduction in a wild bird species. Hormones and Behavior 66:685-693.

Ding B, Lenzi C, Pirone A (2013) Immunohistochemical Study on Glucagon-Like Peptide-1(GLP-1) and Pituitary Adenylate Cyclase Activating Peptide (PACAP) in the Small Intestine of Muscovy Duck during the Prehatching and Posthatching Periods. The Journal of Poultry Science 51:35-41.

Docherty K, Clark A (1994) Nutrient regulation of insulin gene expression. The FASEB Journal 8:20-27.

Druce M, Small C, Bloom S (2004) Minireview: Gut Peptides Regulating Satiety. Endocrinology 145:2660-2665.

Drucker D (1998) Glucagon -like Peptides. Diabetes 159-169.

Drucker D (2001) Minireview: The Glucagon-Like Peptides. Endocrinology 142:521-527.

Drucker D, Mojsov S, Habener J (1986) 6 Cell-specific post-translational processing of preproglucagon expressed from a metallothionine-glucagon fusion gene. The Journal of Biological Chemistry 261:9637-9643.

Dunn I, Wilson P, D'Eath R, Boswell T (2015) Hypothalamic Agouti-Related Peptide mRNA is Elevated During Natural and Stress-Induced Anorexia. Journal of Neuroendocrinology 27:681-691.

Dunn I, Wilson P, Smulders T et al. (2013) Hypothalamic Agouti-Related Protein Expression Is Affected by Both Acute and Chronic Experience of Food Restriction and Re-Feeding in Chickens. Journal of Neuroendocrinology 25:920-928.

Dupont J, Tesseraud S, Derouet M et al. (2008) Insulin immuno-neutralization in chicken: effects on insulin signaling and gene expression in liver and muscle. Journal of Endocrinology 197:531-542.

During M, Cao L, Zuzga D et al. (2003) Glucagon-like peptide-1 receptor is involved in learning and neuroprotection. Nature Medicine 9:1173-1179.

Durkin M, Walker M, Smith K et al. (2000) Expression of a novel neuropeptide Y receptor subtype involved in food intake: an in situ hybridization study of Y5 mRNA distribution in rat brain. Experimental Neurology 165:90-100.

Dutia R, Meece K, Dighe S et al. (2012) β-Endorphin Antagonizes the Effects of α-MSH on Food Intake and Body Weight. Endocrinology 153:4246-4255.

Dylag T, Kotlinska J, Rafalski P et al. (2006) The activity of CART peptide fragments. Peptides 27:1926-1933.

Eastwood C, Maubach K, Kirkup A, Grundy D (1998) The role of endogenous cholecystokinin in the sensory transduction of luminal nutrient signals in the rat jejunum. Neuroscience Letters 254:145-148.

Ebenezer I, de la Riva C, Baldwin B (1990) Effects of the CCK receptor antagonist MK-329 on food intake in pigs. Physiology & Behavior 47:145-148.

Eberlein G, Eysselein V, Schaeffer M et al. (1989) A new molecular form of PYY: Structural characterization of human PYY (3–36) and PYY (1–36). Peptides 10:797-803.

Eisenberg M, Woodward E, Quintana R, Dragstedt L (1963) Insulin inhibition of gastric secretion. Journal of Surgical Research 3:479-484.

Eissele R, Goke R, Willemer S et al. (1992) Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man. European Journal of Clinical Investigation 22:283-291.

Eissele R, Goke R, Willemer.S et al. (1992) Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man. European Journal of Clinical Investigation 22:283-291.

Elias C, Aschkenasi C, Lee C et al. (1999) Leptin Differentially Regulates NPY and POMC Neurons Projecting to the Lateral Hypothalamic Area. Neuron 23:775-786.

Elias C, Lee C, Kelly J et al. (1998) Leptin Activates Hypothalamic CART Neurons Projecting to the Spinal Cord. Neuron 21:1375-1385.

Elias C, Saper C, A Maratos-Flier E et al. (1998) Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area. Journal of Comparative Neurology 402:442–459.

Ellacott K, Cone R (2004) The Central Melanocortin System and the Integration of Short- and Long-term Regulators of Energy Homeostasis. Recent Progress in Hormone Research 59:395-408.

Ellethey H, Jungi T, Hubereicher B (2001) Effects of feeding corticosterone and housing conditions on feather pecking in laying hens (Gallus gallus domesticus). Physiology & Behavior 73:243-251.

Elmquist J, Maratos-Flier E, Saper C, Flier J (1998) Unraveling the central nervous system pathways underlying responses to leptin. Nature Neuroscience 1:445-450.

El-Salhy M, Grimelius L (1983) Immunocytochemical demonstration of polypeptide YY (PYY) in the gastrointestinal tract of the monkey Macaea rhesus. Biomedical Research 4: P289-294.

El-Salhy M, Grimelius L, Lundberg J et al. (1982) Immunocytochemical evidence for the occurrence of PYY, a newly isolated gut polypeptide, in endocrine cells in the gut of amphibians and reptiles. Histochemistry 25-30.

El-Salhy M, Hatlebakk J, Hausken T (2020) Possible role of peptide YY (PYY) in the pathophysiology of irritable bowel syndrome (IBS). Neuropeptides 79:101973.

El-Salhy M, Wilander E, Grimelius L et al. (1982) The distribution of polypeptide YY (PYY)- and pancreatic polypeptide (PP)-immunoreactive cells in the domestic fowl. Histochemistry 75:25-30.

El-Salhy M, Wilander E, Juntti-Berggren L, Grimelius L (1983) The distribution and ontogeny of polypeptide YY (PYY)-and pancreatic polypeptide (PP)-immunoreactive cells in the gastrointestinal tract of rat. Histochemistry 78:53-60.

Emson P, Hunt S, Rehfeld J et al. (1980) Cholecystokinin and vasoactive intestinal polypeptide in the mammalian CNS: distribution and possible physiological roles. Advances in biochemical psychopharmacology 63-74.

Emson P, Rehfeld J, Rossor M (1982) Distribution of Cholecystokinin-Like Peptides in the Human Brain. Journal of Neurochemistry 38:1177-1179.

Ervran A, Blache P, Batailled (1987) Distribution of oxyntomodulin and glucagon in the gastrointestinal tract and the Plasma of the rat. Endocrinology 121:704-713.

Ewer R (1968) Ethology of mammals. 46-62.

F. D, Bent A (1946) Life Histories of North American Jays, Crows, and Titmice. Bird-Banding 18:147.

Fakhry J, Wang J, Martins P et al. (2017) Distribution and characterisation of CCK containing enteroendocrine cells of the mouse small and large intestine. Cell and Tissue Research 369:245-253.

Fan W, Boston B, Kesterson R et al. (1997) Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature 385:165-168.

Fan W, Ellacott K, Halatchev I et al. (2004) Cholecystokinin-mediated suppression of feeding involves the brainstem melanocortin system. Nature Neuroscience 7:335-336.

Fan W, Ellacott K, Halatchev I et al. (2004) Cholecystokinin-mediated suppression of feeding involves the brainstem melanocortin system. Nature Neuroscience 7:335-336.

Fang X, Zhu X, Chen S et al. (2014) Differential gene expression pattern in hypothalamus of chickens during fasting-induced metabolic reprogramming: Functions of glucose and lipid metabolism in the feed intake of chickens. Poultry Science 93:2841-2854.

Fang X, Zhu X, Chen S et al. (2014) Differential gene expression pattern in hypothalamus of chickens during fasting-induced metabolic reprogramming: Functions of glucose and lipid metabolism in the feed intake of chickens. Poultry Science 93:2841-2854.

Fantino M, Cabanac M (1980) Body weight regulation with a proportional hoarding response in the rat. Physiology & Behavior 24:939-942.

Farrell J (1928) Contributions to the physiology of gastric secretion. American Journal of Physiology-Legacy Content 85:685-687.

Fei H, Okano H, Li C et al. (1997) Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. Proceedings of the National Academy of Sciences 94:7001-7005.

Filippi B, Abraham M, Yue J, Lam T (2013) Insulin and glucagon signaling in the central nervous system. Reviews in Endocrine and Metabolic Disorders 14:365-375.

Fleming A (1976) Control of food intake in the lactating rat: Role of suckling and hormones. Physiology & Behavior 17:841-848.

Fleming A (1976) Ovarian influences on food intake and body weight regulation in lactating rats. Physiology & Behavior 17:969-978.

Flier J (2006) AgRP in energy balance: Will the real AgRP please stand up?. Cell Metabolism 3:83-85.

Florant G, Singer L, Scheurink A et al. (1991) Intraventricular insulin reduces food intake and body weight of marmots during the summer feeding period. Physiology & Behavior 49:335-338.

Forbes S, Bui S, Robinson B et al. (2001) Integrated control of appetite and fat metabolism by the leptin-proopiomelanocortin pathway. Proceedings of the National Academy of Sciences 98:4233-4237.

Fretwell S (1972) Populations in a seasonal environment. Princeton U.P., Princeton, N.J.

Fridolf T, Bottcher G, Sundler F, Ahrén B (1991) GLP-1 and GLP- 17-36 amide: influences on basal and stimulated insulin and glucagon secretion in the mouse. Pancreas 6:208-215.

Friedman J, Halaas J (1998) Leptin and the regulation of body weight in mammals. Nature 395:763-770.

Friedman-Einat M, Boswell T, Horev G et al. (1999) The Chicken Leptin Gene: Has It Been Cloned? General and Comparative Endocrinology 115:354-363.

Friedman-Einat M, Cogburn L, Yosefi S et al. (2014) Discovery and Characterization of the First Genuine Avian Leptin Gene in the Rock Dove (Columba livia). Endocrinology 155:3376-3384.

Fryxell J, Sinclair A (1988) Causes and consequences of migration by large herbivores. Trends in Ecology & Evolution 3:237-241.

Fryxell J, Sinclair A (1988) Causes and consequences of migration by large herbivores. Trends in Ecology & Evolution 3:237-241.

Fu Z, R. Gilbert E, Liu D (2013) Regulation of Insulin Synthesis and Secretion and Pancreatic Beta-Cell Dysfunction in Diabetes. Current Diabetes Reviews 9:25-53.

Furuse M (2002) Central regulation of food intake in the neonatal chick. Animal Science Journal 73:83-94.

Furuse M (2007) Central regulation of food intake in the neonatal chick: A review. The Journal of Poultry Science 349–356.

Furuse M, Matsumoto M, Okumura J et al. (1997) Intracerebroventricular injection of mammalian and chicken glucagon-like peptide-1 inhibits food intake of the neonatal chick. Brain Research 755:167-169.

Furuse M, Matsumoto M, Saito N et al. (1997) The central corticotropin-releasing factor and glucagon-like peptide-1 in food intake of the neonatal chick. European Journal of Pharmacology 339:211-213.

Furuse M, Tachibana T, Ohgushi A et al. (2001) Intracerebroventricular injection of ghrelin and growth hormone releasing factor inhibits food intake in neonatal chicks. Neuroscience Letters 301:123-126.

Gagnon J, Anini Y (2013) Glucagon Stimulates Ghrelin Secretion Through the Activation of MAPK and EPAC and Potentiates the Effect of Norepinephrine. Endocrinology 154:666-674.

Galloway J, Chance R (1994) Improving Insulin Therapy: Achievements and Challenges. Hormone and Metabolic Research 26:591-598.

Gao S, Zhang J, He C et al. (2017) Molecular characterization of neuropeptide Y (NPY) receptors (Y1, Y4 and Y6) and investigation of the tissue expression of their ligands (NPY, PYY and PP) in chickens. General and Comparative Endocrinology 240:46-60.

Gardner J, Rothwell N, Luheshi G (1998) Leptin affects food intake via CRF-receptor-mediated pathways. Nature Neuroscience 1:103-103.

Gayle D, Desai M, Casillas E et al. (2006) Gender-specific orexigenic and anorexigenic mechanisms in rats. Life Sciences 79:1531-1536.

Geary N (1990) Pancreatic glucagon signals postprandial satiety. Neuroscience & Biobehavioral Reviews 14:323-338.

Geary N (1998) Satiation. 164-197.

Geary N, Guss J (1995) Glucagon and the control of meal size. Appetite 24:168.

Geary N, Le Sauter J, Noh U (1993) Glucagon acts in the liver to control spontaneous meal size in rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 264:R116-R122.

Geelissen S, Swennen Q, Geyten S et al. (2006) Peripheral ghrelin reduces food intake and respiratory quotient in chicken. Domestic Animal Endocrinology 30:108-116.

Geiser F, Ruf T (1995) Hibernation versus Daily Torpor in Mammals and Birds: Physiological Variables and Classification of Torpor Patterns. Physiological Zoology 68:935-966.

Gelegen C, Chandarana K, Choudhury A et al. (2012) Regulation of hindbrain PYY expression by acute food deprivation, prolonged caloric restriction, and weight loss surgery in mice. American Journal of Physiology-Endocrinology and Metabolism 303: E659-E668.

Greenbaum, D., Colangelo, C., Williams, K., and Grestein, M. (2003) Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biology 4:117.

Genazzani A, Trentini G, Petraglia F et al. (1990) Estrogens Modulate the Circadian Rhythm of Hypothalamic Beta-Endorphin Contents in Female Rats. Neuroendocrinology 52:221-224.

Gerets H, Peeters K, Arckens L et al. (2000) Sequence and distribution of pro-opiomelanocortin in the pituitary and the brain of the chicken (Gallus gallus). The Journal of Comparative Neurology 417:250-262.

Gibbs J, Young R, Smith G (1973) Cholecystokinin decreases food intake in rats. Journal of Comparative and Physiological Psychology 84:488-495.

Giddings S, Chirgwin J, Permutt M (1981) The Effects of Fasting and Feeding on Preproinsulin Messenger RNA in Rats. Journal of Clinical Investigation 67:952-960.

Giduck S, Threatte R, Kare M (1987) Cephalic Reflexes: Their Role in Digestion and Possible Roles in Absorption and Metabolism. The Journal of Nutrition 117:1191-1196.

Göke R, Larsen P, Mikkelsen J, Sheikh S (1995) Distribution of GLP-1 Binding Sites in the Rat Brain: Evidence that Exendin-4 is a Ligand of Brain GLP-1 Binding Sites. European Journal of Neuroscience 7:2294-2300.

Goldstone A, Unmehopa U, Bloom S, Swaab D (2002) Hypothalamic NPY and Agouti-Related Protein Are Increased in Human Illness but Not in Prader-Willi Syndrome and Other Obese Subjects. The Journal of Clinical Endocrinology & Metabolism 87:927-937.

Golombek D, Rosenstein R (2010) Physiology of Circadian Entrainment. Physiological Reviews 90:1063-1102.

Goodwin D, Gillmor R (1976) Crows of the world. 354.

Gosler A (2002) Strategy and constraint in the winter fattening response to temperature in the great tit Parus major. Journal of Animal Ecology 71:771-779.

Grandt D, Schimiczek M, Beglinger C et al. (1994) Two molecular forms of Peptide YY (PYY) are abundant in human blood: characterization of a radioimmunoassay recognizing PYY 1–36 and PYY 3–36. Regulatory Peptides 51:151-159.

Graner J, Abraira C (1985) Glucagon in the Cerebrospinal Fluid. New England Journal of Medicine 312:994-995.

Gray T, Morley J (1986) Neuropeptide Y: Anatomical distribution and possible function in mammalian nervous system. Life Sciences 38:389-401.

Greeley G, Hill F, Spannagel A, Thompson J (1987) Distribution of peptide YY in the gastrointestinal tract of the rat, dog, and monkey. Regulatory Peptides 19:365-372.

Green P, Wilkinson C, Woods S (1992) Intraventricular corticosterone increases the rate of body weight gain in underweight adrenalectomized rats. Endocrinology 130:269-275.

GriffIths R, Double M, Orr K, Dawson R (1998) A DNA test to sex most birds. Molecular Ecology 7:1071-1075.

Grill H, Kaplan J (2002) The Neuroanatomical Axis for Control of Energy Balance. Frontiers in Neuroendocrinology 23:2-40.

Guan X, Yu H, Van der Ploeg L (1998) Evidence of altered hypothalamic pro-opiomelanocortin/ neuropeptide Y mRNA expression in tubby mice. Molecular Brain Research 59:273-279.

Gustafson E, Smith K, Durkin M et al. (1997) Distribution of the neuropeptide Y Y2 receptor mRNA in rat central nervous system. Molecular Brain Research 46:223-235.

Gutman R, Choshniak I, Kronfeld-Schor N (2006) Defending body mass during food restriction in Acomys russatus: a desert rodent that does not store food. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 290: R881-R891.

Gwinner E (1989) Photoperiod as a Modifying and Limiting Factor in the Expression of Avian Circannual Rhythms. Journal of Biological Rhythms 4:125-138.

Haftorn S (1989) Seasonal and diurnal body weight variations in tit- mice, based on analyses of individual b. Scandinavian Journal of Ornithology 101:217-235.

Haftorn S (1989) The Diurnal Body Weight Cycle in Titmice Parus spp. Ornis Scandinavica 23:435.

Hagan M, Rushing P, Pritchard L et al. (2000) Long-term orexigenic effects of AgRP-(83—132) involve mechanisms other than melanocortin receptor blockade. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 279: R47-R52.

Hagan M, Rushing P, Schwartz M et al. (1999) Role of the CNS Melanocortin System in the Response to Overfeeding. The Journal of Neuroscience 19:2362-2367.

Håkansson M, Brown H, Ghilardi N et al. (1998) Leptin Receptor Immunoreactivity in Chemically Defined Target Neurons of the Hypothalamus. The Journal of Neuroscience 18:559-572.

Halaas J, Gajiwala K, Maffei M et al. (1995) Weight-reducing effects of the plasma protein encoded by the obese gene. Science 269:543-546.

Hamilton R, Norgren R (1984) Central projections of gustatory nerves in the rat. The Journal of Comparative Neurology 222:560-577.

Han V, Hynes M, Jin C et al. (1986) Cellular localization of proglucagon/glucagon-like peptide I messenger RNAs in rat brain. Journal of Neuroscience Research 16:97-107.

Hara E, Saito M (1980) Diurnal changes in plasma glucose and insulin responses to oral glucose load in rats. American Journal of Physiology-Endocrinology and Metabolism 238: E463-E466.

Harvey S, Scanes C, Brown K (1986) Adrenals, Avian physiology, 4th ed. Springer-Verlag, New York

Harvey S, Scanes C, Brown K (1986) Adrenals, Avian physiology, 4th ed. SpringerVerlag, New York

Hasegawa S, Terazono K, Nata K et al. (1990) Nucleotide sequence determination of chicken glucagon precursor cDNA. FEBS Letters 264:117-120.

Haslam D (2007) Obesity: a medical history. Obesity Reviews 8:31-36.

Hauger R, Dautzenberg F (2000) Regulation of the stress response by corticotropin-releasing factor receptors. In: Conn PM. Neuroendocrinology in physiology and medicine. 261-286.

Havey S, Philips P, Rees A, Hall T (1984) Stress and adrenal functions. Journal of Experimental Zoology 232:633-645.

Havrankova J, Roth J, Brownstein M (1978) Insulin receptors are widely distributed in the central nervous system of the rat. Nature 272:827-829.

Hayes M, Bradley L, Grill H (2009) Endogenous hindbrain glucagon-like peptide-1 receptor activation contributes to the control of food intake by mediating gastric satiation signaling. Endocrinology 150:2654-2659.

Hazelwood R (1965) Avian Physiology, 2nd ed. 313.

Hazelwood R (1973) The avian endocrine pancreas. American Zoologist 13:699-709.

Hazelwood R (1973) The avian endocrine pancreas. American Zoologist 13:699-709.

Hazelwood R, Kimmel J, Pollock H, Barksdale B (1968) Biological characterization of chicken insulin activity in rats and domestic fowl. Endocrinology 83:1331-1336.

Healy S, Dekort S, Clayton N (2005) The hippocampus, spatial memory and food hoarding: a puzzle revisited. Trends in Ecology & Evolution 20:17-22.

Heijboer A, Pijl H, Van den Hoek A et al. (2006) Gut–Brain Axis: Regulation of Glucose Metabolism. Journal of Neuroendocrinology 883–894.

Hell N, Timo-Iaria C (1985) Increase of food intake induced by glucagon in the rat. Physiology & Behavior 34:39-44.

Hellman B, Hellerstrom C (1959) DIURNAL CHANGES IN THE FUNCTION OF THE PANCREATIC ISLETS OF RATS AS INDICATED BY NUCLEAR SIZE IN THE ISLET CELLS. European Journal of Endocrinology XXXI:267-281.

Henderson L, Cockcroft R, Kaiya H et al. (2018) Peripherally injected ghrelin and leptin reduce food hoarding and mass gain in the coal tit (Periparus ater). Proceedings of the Royal Society B: Biological Sciences 285:20180417.

Henry J (1977) The Use of Urine Marking in the Scavenging Behavior of the Red Fox (Vulpes v uLpes). Behaviour 61:82-105. doi: 10.1163/156853977x00496

Herness S, Zhao F (2009) The neuropeptides CCK and NPY and the changing view of cell-to-cell communication in the taste bud. Physiology & Behavior 97:581-591.

Herranz R (2003) Cholecystokinin Antagonists: Pharmacological and Therapeutic Potential. ChemInform 34:

Hetherington A, Ranson S (1942) The relation of various hypothalamic lesions to adiposity in the rat. The Journal of Comparative Neurology 76:475-499. doi: 10.1002/cne.900760308

Higgins S, Ellestad L, Trakooljul N et al. (2010) Transcriptional and pathway analysis in the hypothalamus of newly hatched chicks during fasting and delayed feeding. BMC Genomics 11:162.

Higgins S, Ellestad L, Trakooljul N et al. (2010) Transcriptional and pathway analysis in the hypothalamus of newly hatched chicks during fasting and delayed feeding. BMC Genomics 11:162.

HiguchiSQ H, Yang H, Sabol S (1988) Rat neuropeptide Y Precursor gene expression mRNA structure, tissue distribution, and regulation by glucocorticoids, cyclicAMP, and phorbol ester. The journal of Biological Chemistry 263:6288-6295.

Hill B, De Souza M, Williams N (2011) Characterization of the diurnal rhythm of peptide YY and its association with energy balance parameters in normal-weight premenopausal women. American Journal of Physiology-Endocrinology and Metabolism 301: E409-E415.

Hill J, Elias C, Fukuda M et al. (2010) Direct Insulin and Leptin Action on Pro-opiomelanocortin Neurons Is Required for Normal Glucose Homeostasis and Fertility. Cell Metabolism 11:286-297.

Hiramatsu K (2020) Chicken Intestinal L Cells and Glucagon-like Peptide-1 Secretion. The Journal of Poultry Science 57:1-6.

Hiramatsu K, Yamasaki A, Karasawa Y (2003) Comparative Study on the Distribution of Glucagon-like Peptide-1 (GLP-1)-immunoreactive Cells in the Intestine of Chicken and Ostrich. The Journal of Poultry Science 40:39-44.

Hiramatsu K, Yamasaki A, Shioji T (2005) Immunohistochemical and Morphometrical Studies on the Distribution of Glucagon-like Peptide-1 (GLP-1)-immunoreactive Cells in the Chicken Intestine. The Journal of Poultry Science 42:223-229.

Hirschowitz B, Robbins R (1966) Direct inhibition of gastric electrolyte secretion by insulin, independent of hypoglycemia or the vagus. The American Journal of Digestive Diseases 11:199-212.

Hisadome K, Reimann F, Gribble F, Trapp S (2010) Leptin Directly Depolarizes Preproglucagon Neurons in the Nucleus Tractus Solitarius. Diabetes 59:1890-1898.

Holmberg S, Mikko S, Boswell T et al. (2002) Pharmacological characterization of cloned chicken neuropeptide Y receptors Y1 and Y5. Journal of Neurochemistry 81:462-471.

Holst J (2007) The Physiology of Glucagon-like Peptide 1. Physiological Reviews 87:1409-1439.

Holst J, Wewer Albrechtsen N, Pedersen J, Knop F (2017) Glucagon and Amino Acids Are Linked in a Mutual Feedback Cycle: The Liver–α-Cell Axis. Diabetes 66:235-240.

Holt M, Richards J, Cook D et al. (2018) Preproglucagon Neurons in the Nucleus of the Solitary Tract Are the Main Source of Brain GLP-1, Mediate Stress-Induced Hypophagia, and Limit Unusually Large Intakes of Food. Diabetes 68:21-33.

Holzer P (1992) Peptidergic sensory neurons in the control of vascular functions: mechanisms. Reviews of Physiology, Biochemistry and pharmacology 121:49-146.

Holzer P, Reichmann F, Farzi A (2012) Neuropeptide Y, peptide YY and pancreatic polypeptide in the gut–brain axis. Neuropeptides 46:261-274.

Honda K (2016) Glucagon-related peptides and the regulation of food intake in chickens. Animal Science Journal 87:1090-1098.

Honda K, Kamisoyama H, Saneyasu T et al. (2007) Central administration of insulin suppresses food intake in chicks. Neuroscience Letters 423:153-157.

Honda K, Kamisoyama H, Uemura T et al. (2012) The mechanism underlying the central glucagon-induced hyperglycemia and anorexia in chicks. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 163:260-264.

Honda K, Saneyasu T, Shimatani T et al. (2014) Intracerebroventricular administration of chicken glucagon-like peptide-2 potently suppresses food intake in chicks. Animal Science Journal 86:312-318.

Honda K, Shimatani T, Aoki K et al. (2015) Glucagon-like Peptide-2 Functions as an Anorexigenic Peptide not only in the Central Nervous System but also in the Peripheral Circulation in Broiler Chicks. The Journal of Poultry Science 52:183-187.

Honey R, Weir G (1979) Insulin stimulates somatostatin and inhibits glucagon secretion from the perfused chicken pancreas-duodenum. Life Sciences 24:1747-1750.

Hoosein N, Gurd R (1984) Identification of glucagon receptors in rat brain. Proceedings of the National Academy of Sciences 81:4368-4372.

Horev G, Einat P, Aharoni T et al. (2000) Molecular cloning and properties of the chicken leptin-receptor (CLEPR) gene. Molecular and Cellular Endocrinology 162:95-106.

Horvath T, Naftolin F, Kalra S, Leranth C (1992) Neuropeptide-Y innervation of beta-endorphin-containing cells in the rat mediobasal hypothalamus: a light and electron microscopic double immunostaining analysis. Endocrinology 131:2461-2467.

Hostetter K, Sweazea K, Braun E (2010) The Avian Pancreatic Islets: A Comparison Across Species. Federation of American Societies for Experimental Biology 24:1055.9.

Houston A, McNamara J (1993) A Theoretical Investigation of the Fat Reserves and Mortality Levels of Small Birds in Winter. Ornis Scandinavica 24:205.

Huang G, Li J, Fu H et al. (2012) Characterization of glucagon-like peptide 1 receptor (GLP1R) gene in chickens: functional analysis, tissue distribution, and identification of its transcript variants. Domestic Animal Endocrinology 43:1-15.

Huang G, Li J, Fu H et al. (2012) Characterization of glucagon-like peptide 1 receptor (GLP1R) gene in chickens: functional analysis, tissue distribution, and identification of its transcript variants. Domestic Animal Endocrinology 43:1-15.

Huang G, Li J, Wang H et al. (2014) Discovery of a Novel Functional Leptin Protein (LEP) in Zebra Finches: Evidence for the Existence of an Authentic Avian Leptin Gene Predominantly Expressed in the Brain and Pituitary. Endocrinology 155:3385-3396.

Huda M, Wilding J, Pinkney J (2006) Gut peptides and the regulation of appetite. Obesity Reviews 7:163-182.

Hurley S, Johnson A (2014) The role of the lateral hypothalamus and orexin in ingestive behavior: a model for the translation of past experience and sensed deficits into motivated behaviors. Frontiers in Systems Neuroscience 8:

Hurly T (1992) Energetic reserves of marsh tits (Parus palustris): food and fat storage in response to variable food supply. Behavioral Ecology 3:181-188.

Imakawa K, Day M, Zalesky D et al. (1986) Influence of Dietary-Induced Weight Changes on Serum Luteinizing Hormone, Estrogen and Progesterone in the Bovine Female1. Biology of Reproduction 35:377-384.

Innis R, Snyder S (1980) Cholecystokinin receptor binding in brain and pancreas: Regulation of pancreatic bindign by cyclic and acyclic guanine nucleotides. European Journal of Pharmacology 65:123-124.

Inokuchi A, Oomura Y, Nishimura H (1984) Effect of intracerebroventricularly infused glucagon on feeding behavior. Physiology & Behavior 33:397-400.

Irwin D (2001) Molecular evolution of proglucagon. Regulatory Peptides 98:1-12.

Irwin D, Wong J (1995) Trout and chicken proglucagon: alternative splicing generates mRNA transcripts encoding glucagon-like peptide 2. Molecular Endocrinology 9:267-277.

Jamali A, Tramu G (1997) Daily cycle of fos expression within hypothalamic POMC neurons of the male rat. Brain Research 771:45-54.

James P, Verbeek N (1984) Temporal and energetic aspects of food storage in northwestern crows. Ardea 207-215.

Jarrett R (1979) Rhythms in insulin and glucose. In: Endocrine rhythms. 247-258.

Jarrousse C, Lardeux B, Bourdel G et al. (1980) Portal Insulin and Glucagon in Rats Fed Proteins as a Meal: Immediate Variations and Circadian Modulations. The Journal of Nutrition 110:1764-1773.

Jelinek L, Lok S, Rosenberg G et al. (1993) Expression cloning and signaling properties of the rat glucagon receptor. Science 259:1614-1616.

Jenkins S, Rothstein A, Green W (1995) Food Hoarding by Merriam's Kangaroo Rats: A Test of Alternative Hypotheses. Ecology 76:2470-2481.

Jensen J (2001) Regulatory peptides and control of food intake in non-mammalian vertebrates. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 128:469-477.

Jensen P, Schütz K, Lindqvist C (2002) Red jungle fowl have more contrafreeloading than White Leghorn layers: Effect of food deprivation and consequences for information gain. Behaviour 139:1195-1209.

Jerison H (1973) Evolution of the brain and intelligence. New York

Jhanwar-Uniyal M, Beck B, Burlet C, Leibowitz S (1990) Diurnal rhythm of neuropeptide Y-like immunoreactivity in the suprachiasmatic, arcuate and paraventricular nuclei and other hypothalamic sites. Brain Research 536:331-334.

Jin T (2008) Mechanisms underlying proglucagon gene expression. Journal of Endocrinology 198:17-28.

Johnsen A (1998) Phylogeny of the Cholecystokinin/Gastrin Family. Frontiers in Neuroendocrinology 19:73-99.

Johnsen A (1998) The phylogeny of the cholecystokinin/gastrin family. Regulatory Peptides 40:179.

Jønson L, Schoeman N, Saayman H et al. (2000) Identification of ostrich and chicken cholecystokinin cDNA and intestinal peptides☆. Peptides 21:1337-1344.

Joseph S, Pilcher W, Bennett-Clarke C (1983) Immunocytochemical localization of ACTH perikarya in nucleus tractus solitarius: evidence for a second opiocortin neuronal system. Neuroscience Letters 38:221-225.

Kaiya H van der Geyten S, Kojima M et al. (2002) Chicken Ghrelin: Purification, cDNA Cloning, and Biological Activity. Endocrinology 143:3454-3463.

Kaiya H, Furuse M, Miyazato M, Kangawa K (2009) Current knowledge of the roles of ghrelin in regulating food intake and energy balance in birds. General and Comparative Endocrinology 163:33-38.

Kaiya H, Miyazato M, Kangawa K et al. (2008) Ghrelin: A multifunctional hormone in non-mammalian vertebrates. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 149:109-128.

Kaiya H, Saito E, Tachibana T et al. (2007) Changes in ghrelin levels of plasma and proventriculus and ghrelin mRNA of proventriculus in fasted and refed layer chicks. Domestic Animal Endocrinology 32:247-259.

Kalia M, Sullivan J (1982) Brainstem projections of sensory and motor components of the vagus nerve in the rat. The Journal of Comparative Neurology 211:248-264.

Kalra S, Dube M, Pu S et al. (1999) Interacting Appetite-Regulating Pathways in the Hypothalamic Regulation of Body Weight. Endocrine Reviews 20:68-100.

Kalra S, Gupta Y (2016) The Insulin: Glucagon Ratio and the Choice of Glucose-Lowering Drugs. Diabetes Therapy 1-9.

Kalsbeek A, Strubbe J (1998) Circadian control of insulin secretion is independent of the temporal distribution of feeding. Physiology & Behavior 63:553-560.

Kamatchi G, Rathanaswami P (2012) Inhibition of deprivation-induced food intake by GABAA antagonists: roles of the hypothalamic, endocrine and alimentary mechanisms. Journal of Clinical Biochemistry and Nutrition 51:19-26.

Kamegai J, Tamura H, Shimizu T et al. (2000) Central Effect of Ghrelin, an Endogenous Growth Hormone Secretagogue, on Hypothalamic Peptide Gene Expression. Endocrinology 141:4797-4800.

Kanayama S, Liddle R (1991) Influence of food deprivation on intestinal cholecystokinin and somatostatin. Gastroenterology 100:909-915.

Kastin A, Akerstrom V, Pan W (2002) Interactions of glucagon-like peptide-1 (GLP-1) with the blood-brain barrier. Journal of Molecular Neuroscience 18:07-13.

Kastin A, Pan W (2008) Blood-brain barrier and feeding: regulatory roles of saturable transport systems for ingestive peptides. Current Pharmaceutical Design 14:1615-1619.

Katsurada K, Yada T (2016) Neural effects of gut- and brain-derived glucagon-like peptide-1 and its receptor agonist. Journal of Diabetes Investigation 7:64-69.

Kawakami S, Bungo T, Ando R et al. (2000) Central administration of α-melanocyte stimulating hormone inhibits fasting- and neuropeptide Y-induced feeding in neonatal chicks. European Journal of Pharmacology 398:361-364.

Kawamichi M (1980) Food, food hoarding and seasonal changes of Siberian chipmunks [Entamias sibiricus lineactus]. Japanese Journal of Ecology 30:211-220.

Keen-Rhinehart E, Bartness T (2005) Peripheral ghrelin injections stimulate food intake, foraging, and food hoarding in Siberian hamsters. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 288: R716-R722.

Keen-Rhinehart E, Bartness T (2008) Leptin inhibits food-deprivation-induced increases in food intake and food hoarding. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 295: R1737-R1746.

Keen-Rhinehart E, Ondek K, Schneider J (2013) Neuroendocrine regulation of appetitive ingestive behavior. Frontiers in Neuroscience 7: 213.

Kemnitz J, Gibber J, Lindsay K, Eisele S (1989) Effects of ovarian hormones on eating behaviors, body weight, and glucoregulation in rhesus monkeys. Hormones and Behavior 23:235-250.

Kemp D, Herrera F, Tsukamoto M, Eisenberg M (1968) Insulin-Potassium Effect on Gastric Acid Secretion and Antral Motility in Dogs. Gastroenterology 54:190-196.

Kenan ; DİLER Ç (2008) Localizations of cells that secrete bombesin, glucagon, somatostatin-14 in stomach (proventriculus and gizzard) in chicken (Gallus gallus domestica L.) during the prenatal and postnatal periods. Ankara Üniversitesi Veteriner Fakültesi Dergisi 50:1-1.

Kendeigh S, Kontogian J, Mazac A, Roth R (1969) Environmental regulation of food intake by birds. Journal of Comparative Biochemistry and Physiology 941-957.

Kennedy G (1953) The role of depot fat in the hypothalamic control of food intake in the rat. Proceedings of the Royal Society of London Series B - Biological Sciences 140:578-592.

Kerdelhue B, Karteszi M, Pasqualini C et al. (1983) Circadian variations in β-endorphin concentrations in pituitary and in some brain nuclei of the adult male rat. Brain Research 261:243-248.

Khorooshi R, Helwig M, Werckenthin A et al. (2008) Seasonal regulation of cocaine- and amphetamine-regulated transcript in the arcuate nucleus of Djungarian hamster (Phodopus sungorus). General and Comparative Endocrinology 157:142-147.

Kieffer T, Habener J (1999) The glucagon-like peptides. Endocrine Reviews 20:876–913.

King J (1972) Adaptive periodic fat storage by birds. Proceedings of the 15th International Ornithlogical Congress, the Hague, the Netherlands 30 August-5 September 1970. 200-217.

Klee W (1979) Peptides of the central nervous system.

Kleiber M (1961) The Fire of Life. Wiley, New York

Kobelt P, Tebbe J, Tjandra I et al. (2005) CCK inhibits the orexigenic effect of peripheral ghrelin. The American Journal of Physiology, Regulatory, Integrative and Comparative Physiology 288: R751–R758. doi: 10.1136/gut.2004.061010

Koenig W (1991) The effects of tannins and lipids on digestion of acorns by acorn Woodpeckers. The Auk 108:79–88.

Kojima M, Hosoda H, Date Y et al. (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656-660.

Kojima S, Tohei A, Ikeda M, Anzai N (2015) An endogenous tachykinergic NK2/NK3 receptor cascade system controlling the release of serotonin from colonic mucosa. Current Neuropharmacology 13:830-835.

Kokas E, Kaufman S, Long J (1971) Effect of glucagon on gastric and duodenal secretion in chickens. Zeitschrift f�r Vergleichende Physiologie 74:315-325.

Kontogiannis J (1967) Day and Night Changes in Body Weight of the White-Throated Sparrow, Zonotrichia Albicollis. The Auk 84:390-395.

Konturek S, Konturek J (2000) Cephalic phase of pancreatic secretion. Appetite 34:197-205.

Konturek S, Konturek J, Pawlik T, Brzozowki T (2004) Brain-gut axis and its role in the control of food intake. Journal of Physiology and Pharmacology 55:137-154.

Korner J, Savontaus E, Chua S et al. (2001) Leptin Regulation of AgRP and Npy mRNA in the Rat Hypothalamus. Journal of Neuroendocrinology 13:959-966.

Korner J, Wissig S, Kim A et al. (2003) Effects of Agouti-Related Protein on Metabolism and Hypothalamic Neuropeptide Gene Expression. Journal of Neuroendocrinology 15:1116-1121.

Korner J, Wissig S, Kim A et al. (2003) Effects of Agouti-Related Protein on Metabolism and Hypothalamic Neuropeptide Gene Expression. Journal of Neuroendocrinology 15:1116-1121.

Krams I, Cirule D, Suraka V et al. (2010) Fattening strategies of wintering great tits support the optimal body mass hypothesis under conditions of extremely low ambient temperature. Functional Ecology 24:172-177.

Krashes M, Koda S, Ye C et al. (2011) Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. Journal of Clinical Investigation 121:1424-1428.

Krebs J, McCleery R (1982) Optimization in Behavioural Ecology. Behavioural Ecology: An Evolutionary Approach, 2nd ed. 91-121.

Kristensen P, Judge M, Thim L et al. (1998) Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature 393:72-76.

Kruuk H (1964) Predators and anti-predator behaviour of the black-headed gull (Larus ridibundus L.). Behaviour Supplement 1-129. doi: 10.2307/4511169

Kuenzel W (1982) Central neural structures affecting food intake in birds: the lateral and ventral hypothalamic areas. In: Aspects of Avian Endocrinology. 211-216.

Kuenzel W (1989) Neuroanatomical substrates involved in the control of food intake. Poultry Science 68:926-937.

Kuenzel W, Douglass L, Davison B (1987) Robust feeding following central administration of neuropeptide Y or peptide YY in chicks, Gallus domesticus. Peptides 8:823-828.

Kuenzel W, McMurtry J (1988) Neuropeptide Y: Brain localization and central effects on plasma insulin levels in chicks. Physiology & Behavior 44:669-678.

Kuhre R, Albrechtsen N, Windeløv J et al. (2014) GLP-1 amidation efficiency along the length of the intestine in mice, rats and pigs and in GLP-1 secreting cell lines. Peptides 55:52-57.

Kumar V, Singh B, Rani S (2004) The bird clock: A complex, multi-oscillatory and highly diversified system. Biological Rhythm Research 35:121-144.

Kuurk H (1972) The spotted hyena a study of predation and social behaviour. 335.

La Fleur S, Kalsbeek A, Wortel J et al. (2001) A Daily Rhythm in Glucose Tolerance. Diabetes 50:1237-1243.

Lage R, Diéguez C, Vidal-Puig A, López M (2008) AMPK: a metabolic gauge regulating whole-body energy homeostasis. Trends in Molecular Medicine 14:539-549.

Lambert P (1997) A role for novel CART peptide fragments in the central control of food intake. Neuropepides 620-621.

Landgraf D, Neumann A, Oster H (2017) Circadian clock-gastrointestinal peptide interaction in peripheral tissues and the brain. Best Practice & Research Clinical Endocrinology & Metabolism 31:561-571.

Langley K (1994) The nueroendocrine concept today. Annals of the New York Academy of Sciences 1-17.

Langlois I (2003) The anatomy, physiology, and diseases of the avian proventriculus and ventriculus. Veterinary Clinics of North America: Exotic Animal Practice 6:85-111.

Langslo D, Butler E, Hales C, Pearson A (1970). The response of plasma insulin, glucose and non-esterified fatty acids to various hormones, nutrients and drugs in the domestic fowl. Journal of Endocrinology 46:243-260.

Langslow D (1978) Gluconeogenesis in Birds. Biochemical Society Transactions 6:1148-1152.

Langslow D, Butler E, Hales C, Pearson A (1970) The response of plasma insulin, glucose and non-esterified fatty acids to various hormones , nutrients and drugs in the domestic fowl. Journal of Endocrinology 46:243-260.

Langslow D, Freeman B (1972) Partial pancreatectomy and the role of insulin in carbohydrate metabolism ingallus domesticus. Diabetologia 8:206-210.

Larsen P, Tang-Christensen M, Holst J, Ørskov C (1997) Distribution of glucagon-like peptide-1 and other preproglucagon-derived peptides in the rat hypothalamus and brainstem. Neuroscience 77:257-270.

Larsen P, Tang-Christensen M, Jessop D (1997) Central Administration of Glucagon-Like Peptide-1 Activates Hypothalamic Neuroendocrine Neurons in the Rat. Endocrinology 138:4445-4455.

Larsson L, Holst J, Hkanson R, Sundler F (1975) Distribution and properties of glucagon immunoreactivity in the digestive tract of various mammals: An immunohistochemical and immunochemical study. Histochemistry 44:281-290.

Larsson L, Rehfeld J (1978) Distribution of gastrin and CCK cells in the rat gastrointestinal tract. Histochemistry 58:23-31.

Lattin C, Bauer C, de Bruijn R, Michael Romero L (2012) Hypothalamus–pituitary–adrenal axis activity and the subsequent response to chronic stress differ depending upon life history stage. General and Comparative Endocrinology 178:494-501.

Lavin J, Wittert G, Sun W et al. (1996) Appetite regulation by carbohydrate: role of blood glucose and gastrointestinal hormones. American Journal of Physiology-Endocrinology and Metabolism 271: E209-E214.

Layer P, Holst J (1993) GLP-1: a humoral mediator of the ileal brake in humans? Digestion 385–386.

Lea S, Tarpy R (1986) Hamsters' demand for food to eat and hoard as a function of deprivation and cost. Animal Behaviour 34:1759-1768.

Lee G, Proenca R, Montez J et al. (1996) Abnormal splicing of the leptin receptor in diabetic mice. Nature 379:632-635.

Lee S, Zabolotny J, Huang H et al. (2016) Insulin in the nervous system and the mind: Functions in metabolism, memory, and mood. Molecular Metabolism 5:589-601.

Lees J, Lindholm C, Batakis P et al. (2017) The physiological and neuroendocrine correlates of hunger in the Red Junglefowl (Gallus gallus). Scientific Reports 7:

Lembart P, Wilding J, Ghatei M, Bloom S (1994) A role for GLP-l-(7-36) NH2 in the central control of feeding behavior. Digestion 54:360-361.

Lepkovsky S (2020) Hypothalamic-adipose tissue interrelationship. federation proceedings 32:1705-1708.

Lepkovsky S, Snapir N, Furuta F (1968) Temperature regulation and appetitive behavior in chickens with hypothalamic lesions. Physiology & Behavior 3:911-915.

Leslie R, Gwyn D, Hopkins D (1982) The central distribution of the cervical vagus nerve and gastric afferent and efferent projections in the rat. Brain Research Bulletin 8:37-43.

Levine A, Morley J (1984) Neuropeptide Y: A potent inducer of consummatory behavior in rats. Peptides 5:1025-1029.

Li J, Finniss S, Yang Y et al. (2000) Agouti-Related Protein-Like Immunoreactivity: Characterization of Release from Hypothalamic Tissue and Presence in Serum1. Endocrinology 141:1942-1950.

Liddle R, Goldfine I, Rosen M et al. (1985) Cholecystokinin bioactivity in human plasma. Molecular forms, responses to feeding, and relationship to gallbladder contraction. Journal of Clinical Investigation 75:1144-1152.

Lilliendahl K (2002) Daily patterns of body mass gain in four species of small wintering birds. Journal of Avian Biology 33:212-218.

Lim G, Brubaker P (2006) Glucagon-Like Peptide 1 Secretion by the L-Cell: The View from Within. Diabetes 55: S70-S77.

Lima S (1986) Predation Risk and Unpredictable Feeding Conditions: Determinants of Body Mass in Birds. Ecology 67:377-385.

Lin H, Chey W, Zhao X (2000) Release of distal gut peptide YY (PYY) by fat in proximal gut depends on CCK☆. Peptides 21:1561-1563.

Linden A, Carlquist M, Hansen S, Uvnas-Moberg K (1989) Plasma concentrations of cholecystokinin, CCK-8, and CCK-33, 39 in rats, determined by a method based on enzyme digestion of gastrin before HPLC and RIA detection of CCK. Gut 30:213-222.

Ling M, Hotta E, Kilianova Z et al. (2004) The melanocortin receptor subtypes in chicken have high preference to ACTH-derived peptides. British Journal of Pharmacology 143:626-637.

Liu L, Song Z, Sheikhahmadi A et al. (2012) Effect of corticosterone on gene expression of feed intake regulatory peptides in laying hens. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 162:81-87.

Liu L, Zhu L (2012) Effect of 24 h Fasting on Gene Expression of AMPK, Appetite Regulation Peptides and Lipometabolism Related Factors in the Hypothalamus of Broiler Chicks. Asian-Australasian Journal of Animal Sciences 25:1300-1308.

Liu X, Shi J, Du W et al. (2011) Glucocorticoids decrease body weight and food intake and inhibit appetite regulatory peptide expression in the hypothalamus of rats. Experimental and Therapeutic Medicine 2:977-984.

Loh K, Zhang L, Brandon A et al. (2017) Corrigendum to “Insulin controls food intake and energy balance via NPY neurons” [Mol Metabol 6 (2017) 574–584]. Molecular Metabolism 6:1562.

Lõhmus M, Fredrik Sundström L, El Halawani M, Silverin B (2003) Leptin depresses food intake in great tits (Parus major). General and Comparative Endocrinology 131:57-61.

Lopaschuk G, Ussher J, Jaswal J (2010) Targeting Intermediary Metabolism in the Hypothalamus as a Mechanism to Regulate Appetite. Pharmacological Reviews 62:237-264.

Loudon A (1994) Photoperiod and the regulation of annual and circannual cycles of food intake. Proceedings of the Nutrition Society 53:495-507.

Lu X, Shieh K, Kabbaj M et al. (2002) Diurnal rhythm of agouti-related protein and its relation to corticosterone and food intake. Endocrinology 143:3905-3915.

Lucas J (1994) Regulation of cache stores and body mass in Carolina chickadees (Panes carolinensis). Behavioral Ecology 5:171-181.

Lyman C (1954) Activity, Food Consumption and Hoarding in Hibernators. Journal of Mammalogy 35:545-552. doi: 10.2307/1375580

MacDonald P, El-kholy W, Riedel M et al. (2002) The Multiple Actions of GLP-1 on the Process of Glucose-Stimulated Insulin Secretion. Diabetes 51: S434-S442.

MacDougald O, Mandrup S (2002) Adipogenesis: forces that tip the scales. Trends in Endocrinology & Metabolism 13:5-11.

MacDougall-Shackleton S, Sherry D, Clark A et al. (2003) Photoperiodic regulation of food storing and hippocampus volume in black-capped chickadees, Poecile atricapillus. Animal Behaviour 65:805-812.

Macroberts M, Macroberts B (1976) Social Organization and Behavior of the Acorn Woodpecker in Central Coastal California. Ornithological Monographs iii-115.

Maffei M, Halaas J, Ravussin E et al. (1995) Leptin levels in human and rodent: Measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nature Medicine 1:1155-1161.

Maniatis G, Demiris N, Kranis A et al. (2012) Genetic analysis of sexual dimorphism of body weight in broilers. Journal of Applied Genetics 54:61-70.

Margolis R, Altszuler N (1967) Insulin in the cerebrospinal fluid. Nature 1375–1376.

Marín Bivens C, Thomas W, Stanley B (1998) Similar feeding patterns are induced by perifornical neuropeptide Y injection and by food deprivation. Brain Research 782:271-280.

Markman S, Tadmor-Melamed H, Arieli A, Izhaki I (2006) Sex differences in food intake and digestive constraints in a nectarivorous bird. Journal of Experimental Biology 209:1058-1063.

Marks J, Porte D, Stahl W, Baskin D (1990) Localization of insulin receptor mRNA in rat brain by in situ hybridization. Endocrinology 127:3234-3236.

Martin J, Novin D (1977) Decreased feeding in rats following hepatic-portal infusion of glucagon. Physiology & Behavior 19:461-466.

Martínez del Rio C, Schondube J, McWhorter T, Herrera L (2001) Intake responses in nectar feeding birds: Digestive and metabolic causes, osmoregulatory consequences, and coevolutionary effects. American Zoologist 41:902-915.

Marubashi S, Tominaga M, Katagiri T et al. (1985) Hyperglycaemic effect of glucagon administered intracerebroventricularly in the rat. Acta Endocrinologica 108:6-10.

Masuzaki H, Ogawa Y, Sagawa N et al. (1998) Non-adipose Tissue Production of Leptin. Obstetrical & Gynecological Survey 53:156-158.

Maton P, Selden A, Chadwick V (1984) Differential distribution of molecular forms of cholecystokinin in human and porcine small intestinal mucosa. Regulatory Peptides 8:9-19.

Mayer J, Thomas D (1967) Regulation of Food Intake and Obesity. Science 156:328-337.

McConn B, Gilbert E, Cline M (2018) Appetite-associated responses to central neuropeptide Y injection in quail. Neuropeptides 69:9-18.

McConn B, Gilbert E, Cline M (2019) Fasting, and refeeding induce differential changes in hypothalamic mRNA abundance of appetite-associated factors in 7-day-old Japanese quail, Coturnix japonica. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 227:60-67.

McElroy J, Wade G (1986) Short photoperiod stimulates brown adipose tissue growth and thermogenesis but not norepinephrine turnover in Syrian hamsters. Physiology & Behavior 37:307-311.

McGhee N, Jefferson L, Kimball S (2009) Elevated corticosterone associated with food deprivation upregulates expression in rat skeletal muscle of the mTORC1 repressor, REDD1. The Journal of Nutrition 139:828-834.

McGowan M, Andrews K, Kelly J, Grossman S (1990) Effects of chronic intrahypothalamic infusion of insulin on food intake and diurnal meal patterning in the rat. Behavioral Neuroscience 104:373-385.

McNamara J, Houston A (1990) The value of fat reserves and the trade-off between starvation and predation. Acta Biotheoretica 38:37-61.

McNamara J, Houston A, Krebs J (1990) Why hoard? The economics of food storing in tits, Parus spp. Behavioral Ecology 1:12-23.

Meena H, Nakhate K, Kokare D, Subhedar N (2009) GABAA receptors in nucleus accumbens shell mediate the hyperphagia and weight gain following haloperidol treatment in rats. Life Sciences 84:156-163.

Meguid M, Fetissov S, Varma M et al. (2000) Hypothalamic dopamine and serotonin in the regulation of food intake. Nutrition 16:843-857.

Meier A, Fivizzani A, Ottenweller J (1978) Daily rhythm of plasma corticosterone binding activity in the white-throated sparrow, Zonotrichiaalbicollis. Life Sciences 22:401-405.

Meijer T, Mohring F, Trillmich F (1994) Annual and Daily Variation in Body Mass and Fat of Starlings Sturnus vulgaris. Journal of Avian Biology 25:98.

Mendes R do Nascimento A, Dos Santos C et al. (2009) An immunocytochemical study of the endocrine cells in the stomach and duodenum of Zonotrichia capensis subtorquata (Passeriformes, Emberizidae). Acta Histochemica 111:83-92.

Mercer A, Hentges S, Meshul C, Low M (2013) Unraveling the Central Proopiomelanocortin Neural Circuits. Frontiers in Neuroscience 7:

Mercer J, Hoggard N, Moar K (1998) Localization of Leptin Receptor (Ob-R) Messenger Ribonucleic Acid in the Rodent Hindbrain. Endocrinology 139:29-34.

Mercer J, Hoggard N, Williams L et al. (1996) Co-expression of Leptin Receptor and Preproneuropeptide Y mRNA in Arcuate Nucleus of Mouse Hypothalamus. Journal of Neuroendocrinology 8:733-735.

Mercer J, Hoggard N, Williams L et al. (1996) Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. FEBS Letters 387:113-116.

Mercer J, Moar K, Ross A et al. (2000) Photoperiod regulates arcuate nucleus POMC, AGRP, and leptin receptor mRNA in Siberian hamster hypothalamus. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 278: R271-R281.

Mercer J, Moar K, Ross A, Morgan P (2000) Regulation of leptin receptor, POMC and AGRP gene expression by photoperiod and food deprivation in the hypothalamic arcuate nucleus of the male Siberian hamster (Phodopus sungorus). Appetite 34:109-111.

Mercer R, Chee M, Colmers W (2011) The role of NPY in hypothalamic mediated food intake. Frontiers in Neuroendocrinology 32:398-415.

Merchenthaler I, Lane M, Shughrue P (1999) Distribution of pre-pro-glucagon and glucagon-like peptide-1 receptor messenger RNAs in the rat central nervous system. The Journal of Comparative Neurology 403:261-280.

Mialhe P (1958) Glucagon, insulin and endocrine regulation of blood sugar in ducks. Journal of Endocrinology 28:1-134.

Mialhe P, Karmann H, Laurnet F, Gross R (1997) Pancreatic hormones other than insulin in birds. In: Prespectives in avian endocrinology. 245-252.

Miller G, Viek P (1944) An analysis of the rat's response to unfamiliar aspects of the hoarding situation. Journal of Comparative Psychology 37:221-231.

Millington G (2007) The role of proopiomelanocortin (POMC) neurones in feeding behaviour. Nutrition & Metabolism 4:18.

Mishra I, Singh D, Kumar V (2016) Daily expression of genes coding for neurotransmitters in central and peripheral tissues of redheaded bunting: Implication for circadian regulation of physiology in songbirds. Chronobiology International 33:280-292.

Mistlberger R (1994) Circadian food-anticipatory activity: Formal models and physiological mechanisms. Neuroscience & Biobehavioral Reviews 18:171-195.

Miyachi Y, Jitsuishi W, Miyoshi A et al. (1986) The distribution of polypeptide YY-Like immunoreactivity in rat tissues. Endocrinology 118:2163-2167.

Miyachi Y, Jitsuishi W, Miyoshi A et al. (1986) The Distribution of Polypeptide YY-Like Immunoreactivity in Rat Tissues. Endocrinology 118:2163-2167.

Miyasaka K, Funakoshi A (2003) Cholecystokinin and cholecystokinin receptors. Journal of Gastroenterology 38:1-13.

Miyasaka K, Ohta M, Masuda M et al. (1995) Sex difference in gene expressions of cholecystokinin (CCK) and CCK receptor in young and old rats. Archives of Gerontology and Geriatrics 21:157-165.

Mizuno T, Kleopoulos S, Bergen H et al. (1998) Hypothalamic pro-opiomelanocortin mRNA is reduced by fasting and [corrected] in ob/ob and db/db mice but is stimulated by leptin. Diabetes 47:294-297.

Mo C, Zhong Y, Wang Y et al. (2014) Characterization of glucagon-like peptide 2 receptor (GLP2R) gene in chickens: functional analysis, tissue distribution, and developmental expression profile of GLP2R in embryonic intestine. Domestic Animal Endocrinology 48:1-6.

Mogenson G, Wu M (1988) Disruption of food hoarding by injections of procaine into mediodorsal thalamus, GABA into subpallidal region and haloperidol into accumbens. Brain Research Bulletin 20:247-251.

Moghadam A, Moran T, Dailey M (2017) Alterations in circadian and meal-induced gut peptide levels in lean and obese rats. Experimental Biology and Medicine 242:1786-1794.

Moiron M, Mathot K, Dingemanse N (2018) To eat and not be eaten: diurnal mass gain and foraging strategies in wintering great tits. Proceedings of the Royal Society B: Biological Sciences 285:20172868.

Mojsov S (2000) Glucagon-like Peptide-1 (GLP-1) and the Control of Glucose Metabolism in Mammals and Teleost Fish. American Zoologist 40:246-258.

Mojsov S, Kopczynski M, Habener J (1990) Both amidated and nonamidates forms of glucagon-like peptide I are synthesized in the rat intestine and the pancreas. Journal of Biological Chemistry 8001-8008.

Mondal M, Date Y, Yamaguchi H et al. (2005) Identification of ghrelin and its receptor in neurons of the rat arcuate nucleus. Regulatory Peptides 126:55-59.

Monir M, Hiramatsu K, Yamasaki A et al. (2013) The Influence of restricted feeding on glucagon-like peptide-1 (GLP-1) containing cells in the chicken small intestine. Anatomia, Histologia, Embryologia 43:153-158.

Moran T, Dailey M (2011) Intestinal feedback signalling and satiety. Physiology & Behavior 105:77-81.

Moran T, Robinson P, Goldrich M, McHugh P (1986) Two brain cholecystokinin receptors: implications for behavioral actions. Brain Research 362:175-179.

Moran T, Robinson P, McHugh P (1985) The pyloric cholecystokinin receptor a site of mediation for satiety? Annals of the New York Academy of Sciences 448:621-623.

Morgan C, Stellar E, Johnson O (1943) Food-deprivation and hoarding in rats. Journal of Comparative Psychology 35:275-295.

Morley J, Hernandez E, Flood J (1987) Neuropeptide Y increases food intake in mice. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 253: R516-R522.

Morley J, Levine A, Grace M, Kneip J (1985) Peptide YY (PYY), a potent orexigenic agent. Brain Research 341:200-203.

Morse D (1980) Behavioral mechanisms in ecology. 383.

Morton G, Schwartz M (2001) The NPY/AgRP neuron and energy homeostasis. International Journal of Obesity S56-S62.

Morton M (1967) Diurnal feeding patterns in white-crowned sparrows, Zonotrichia leucophrys gambelii. The Condor 69:491-512.

Mountjoy K, Robbins L, Mortrud M, Cone R (1992) The cloning of a family of genes that encode the melanocortin receptors. Science 257:1248-1251.

Müller T, Finan B, Clemmensen C et al. (2017) The New Biology and Pharmacology of Glucagon. Physiological Reviews 97:721–766.

Munn A, Kern P, McAllan B (2010) Coping with chaos: unpredictable food supplies intensify torpor use in an arid-zone marsupial, the fat-tailed dunnart (Sminthopsis crassicaudata). Naturwissenschaften 97:601-605.

Murphy B, Fioramonti X, Jochnowitz N et al. (2009) Fasting enhances the response of arcuate neuropeptide Y-glucose-inhibited neurons to decreased extracellular glucose. American Journal of Physiology-Cell Physiology 296:C746-C756.

Murphy K, Bloom S (2004) Gut hormones in the control of appetite. Experimental Physiology 89:507-516.

Mustonen A, Pyykönen T, Nieminen P (2005) Adiponectin and peptide YY in the fasting blue fox (Alopex lagopus). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 140:251-256.

Nakazato M, Murakami N, Date Y et al. (2001) A role for ghrelin in the central regulation of feeding. Nature 409:194-198.

Näslund E, Hellström P (2007) Appetite signalling: From gut peptides and enteric nerves to brain. Physiology & Behavior 92:256-262.

Nauck M, B�sing M, �rskov C et al. (1993) Preserved incretin effect in type 1 diabetic patients with end-stage nephropathy treated by combined heterotopic pancreas and kidney transplantation. Acta Diabetologica 30:39-45.

Neary N, Goldstone A, Bloom S (2004) Appetite regulation: from the gut to the hypothalamus. Clinical Endocrinology 60:153-160.

Nef S, Verma-Kurvari S, Merenmies J et al. (2003) Testis determination requires insulin receptor family function in mice. Nature 426:291-295.

Nishimura K, Hiramatsu K, Monir M et al. (2013) Ultrastructural study on colocalization of glucagon-like peptide (GLP)-1 with GLP-2 in chicken intestinal L-cells. Journal of Veterinary Medical Science 75:1335-1339.

Nizielski S, Levine A, Gosnell B et al. (1985) The effect of neuropeptide Y (NPY) and norepinephrine (NE) on feeding in hypophagic ground squirrels. Federation Proceedings 545.

Nonaka N, Shioda S, Niehoff M, Banks W (2003) Characterization of Blood-Brain Barrier Permeability to PYY3-36 in the Mouse. Journal of Pharmacology and Experimental Therapeutics 306:948-953.

Norgren R, Smith G (1988) Central distribution of subdiaphragmatic vagal branches in the rat. The Journal of Comparative Neurology 273:207-223. doi: 10.1002/cne.902730206

Nyby J, Wallace P, Owen K, Thiessen D (1973) An influence of hormones on hoarding behavior in the Mongolian gerbil (Meriones unguiculatus). Hormones and Behavior 4:283-288.

Ollmann M, Wilson B, Yang Y et al. (1997) Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. Science 278:135-138.

Olszewski P, Bomberg E, Grace M, Levine A (2007) α-Melanocyte stimulating hormone and ghrelin: Central interaction in feeding control. Peptides 28:2084-2089.

Orbetzova M (2012) Appetite Regulatory Peptides and Insulin Resistance. INTECH Open Access Publisher

Ornithology B (2020) Resources for data collection. In: BTO - British Trust for Ornithology. https://www.bto.org/our-science/projects/ringing/taking-part/resources-ringers/resources-data-collection. Accessed 13 Aug 2020

Orth D (1992) Corticotropin-releasing hormone in humans. Endocrine Reviews 13:164-191.

Ortiz-Huidobro R, Velasco M, Larqué C et al. (2021) Molecular Insulin Actions Are Sexually Dimorphic in Lipid Metabolism. Frontiers in Endocrinology 12:

Owen D (1954) The winter weights of titmice. Ibis 96:299-309.

Owen M, Swaisgood R, Czekala N et al. (2004) Monitoring stress in captive giant pandas (Ailuropoda melanoleuca): behavioral and hormonal responses to ambient noise. Zoo Biology 23:147-164.

Owyang C (1996) Physiological mechanisms of cholecystokinin action on pancreatic secretion. American Journal of Physiology-Gastrointestinal and Liver Physiology 271: G1-G7.

Palkovits M, Eskay R (1987) Distribution and possible origin of β-endorphin and ACTH in discrete brainstem nuclei of rats. Neuropeptides 9:123-137.

Pan W, Tu H, Kastin A (2006) Differential BBB interactions of three ingestive peptides: Obestatin, ghrelin, and adiponectin. Peptides 27:911-916.

Parker J, Bloom S (2012) Hypothalamic neuropeptides and the regulation of appetite. Neuropharmacology 63:18-30.

Passaro E, Debas H, Oldendorf W, Yamada T (1982) Rapid appearance of intraventricularly administered neuropeptides in the peripheral circulation. Brain Research 241:338-340.

Paternoster S, Falasca M (2018) Dissecting the Physiology and Pathophysiology of Glucagon-Like Peptide-1. Frontiers in Endocrinology 9:

Pavlov I (1910) The work of the digestive glands. C. Griffin, London

Pelletier G, Li S, Luu-The V, Labrie F (2007) Oestrogenic Regulation of Pro-Opiomelanocortin, Neuropeptide Y and Corticotrophin-Releasing Hormone mRNAs in Mouse Hypothalamus. Journal of Neuroendocrinology 19:426-431.

Perello M, Stuart R, Nillni E (2007) Differential effects of fasting and leptin on proopiomelanocortin peptides in the arcuate nucleus and in the nucleus of the solitary tract. American Journal of Physiology-Endocrinology and Metabolism 292: E1348-E1357.

Perfetti R, Merkel P (2000) Glucagon-like peptide-1: a major regulator of pancreatic beta-cell function. European Journal of Endocrinology 717-725. doi: 10.1530/eje.0.1430717

Perley M, Kipnis D (1966) Effect of Glucocorticoids on Plasma Insulin. New England Journal of Medicine 274:1237-1241.

Perrins C (1979) British tits. Collins, London

Perry B, Wang Y (2012) Appetite regulation and weight control: the role of gut hormones. Nutrition & Diabetes 2: e26-e26.

Phillips-Singh D, Li Q, Takeuchi S et al. (2003) Fasting differentially regulates expression of agouti-related peptide, pro-opiomelanocortin, prepro-orexin, and vasoactive intestinal polypeptide mRNAs in the hypothalamus of Japanese quail. Cell and Tissue Research 313:217-225.

Phoenix C, Goy R, Gerall A, Young W (1959) Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. Endocrinology 65:369-382.

Piersma T, Lindström Å (1997) Rapid reversible changes in organ size as a component of adaptive behaviour. Trends in Ecology & Evolution 12:134-138.

Pirone A, Ding B, Giannessi E et al. (2012) Glucagon-like peptide 1 (GLP-1) in the gastrointestinal tract of the pheasant (Phasianus colchicus). Acta Histochemica 114:535-539.

Plata-Salamán C, Oomura Y, Shimizu N (1986) Dependence of food intake on acute and chronic ventricular administration of insulin. Physiology & Behavior 37:717-734.

Plavnik I, Hurwitz S (1990) Performance of broiler chickens and turkey poults subjected to feed restriction or to feeding of low-protein or low-sodium diets at an early age. Poultry Science 69:945-952.

Pliquett R, Führer D, Falk S et al. (2006) The Effects of insulin on the central nervous system - Focus on appetite regulation. Hormone and Metabolic Research 38:442-446.

Poitout V, Hagman D, Stein R et al. (2006) Regulation of the insulin Gene by glucose and fatty acids. The Journal of Nutrition 136:873-876.

Polak J, Bloom S, Rayford P et al. (1975) Identification of cholecystokinin-secreting cells. The Lancet 306:1016-1018.

Pollock J, Rowland N (1981) Peripherally administered serotonin decreases food intake in rats. Pharmacology Biochemistry and Behaviour 15:179-183.

Porte D, Baskin D, Schwartz M (2002) Leptin and Insulin Action in the Central Nervous System. Nutrition Reviews 60: S20-S29.

Porter D, Faivre E, Flatt P et al. (2012) Actions of incretin metabolites on locomotor activity, cognitive function and in vivo hippocampal synaptic plasticity in high fat fed mice. Peptides 35:1-8.

Powley T (2000) Vagal circuitry mediating cephalic-phase responses to food. Appetite 34:184-188.

Powley T, Berthoud H, Fox E, Laughton W (1992) the dorsal vagal complex forms of sensory motor lattice: the circuitry of gastrointestinal reflexes. In Ritter. Neuroanatomy and physiology of Abdominal Vagal Afferents. 55-79.

Pravosudov V (1985) Search for and storage of food by Parus cinctus lapponicus and P. montanus borealis. Zoology 1036–1043.

Pravosudov V (2003) Long-term moderate elevation of corticosterone facilitates avian food-caching behaviour and enhances spatial memory. Proceedings of the Royal Society of London Series B: Biological Sciences 270:2599-2604.

Pravosudov V, Grubb T (1997) Energy management in passerine birds during the non-breeding Season. Current Ornithology 14:189-234.

Pravosudov V, Grubb T (1997) Management of fat reserves and food caches in tufted titmice (Parus bicolor) in relation to unpredictable food supply. Behavioral Ecology 8:332-339.

Pravosudov V, Kitaysky A, Wingfield J, Clayton N (2001) Long-term unpredictable foraging conditions and physiological stress response in mountain chickadees (Poecile gambeli). General and Comparative Endocrinology 123:324-331.

Pravosudov V, Lucas J (2001) A dynamic model of short-term energy management in small food-caching and non-caching birds. Behavioral Ecology 12:207-218.

Prentice A (2005) Early influences on human energy regulation: Thrifty genotypes and thrifty phenotypes. Physiology & Behavior 86:640-645. doi: 10.1016/j.physbeh.2005.08.055

Proietto J, Rohner-Jeanrenaud F, Ionescu E, Jeanrenaud B (1987) Role of the oropharynx in regulation of glycemia. Diabetes 36:791-795.

Proszkowiec-Weglarz M, Dupont J, Rideau N et al. (2017) Insulin immuno-neutralization decreases food intake in chickens without altering hypothalamic transcripts involved in food intake and metabolism. Poultry Science 96:4409-4418.

Qian J, Scheer F (2016) Circadian System and Glucose Metabolism: Implications for Physiology and Disease. Trends in Endocrinology & Metabolism 27:282-293.

Quiñones M, Al-Massadi O, Gallego R et al. (2015) Hypothalamic CaMKKβ mediates glucagon anorectic effect and its diet-induced resistance. Molecular Metabolism 4:961-970.

Rancourt R, Schellong K, Tzschentke B et al. (2018) DNA methylation and expression of proopiomelanocortin(POMC) gene in the hypothalamus of three-week-old chickens show sex-specific differences. FEBS Open Bio 8:932-939.

Rawdon B, Andrew A (1999) Gut Endocrine Cells in Birds: An Overview, with Particular Reference to the Chemistry of Gut Peptides and the Distribution, Ontogeny, Embryonic Origin and Differentiation of the Endocrine Cells. Progress in Histochemistry and Cytochemistry 34:3-79.

Raybould H (2007) Mechanisms of CCK signaling from gut to brain. Current Opinion in Pharmacology 7:570-574.

Reddy A, Cronin A, Ford H, Ebling F (1999) Seasonal regulation of food intake and body weight in the male Siberian hamster: studies of hypothalamic orexin (hypocretin), neuropeptide Y (NPY) andpro-opiomelanocortin (POMC). European Journal of Neuroscience 11:3255-3264.

Reeve J, Green G, Chew P et al. (2003) CCK-58 is the only detectable endocrine form of cholecystokinin in rat. American Journal of Physiology-Gastrointestinal and Liver Physiology 285: G255-G265.

Rehfeld J (1978) Immunochemical studies on cholecystokinin: Distribution and molecular heterogeneity in the central nervous system and small intestine of man and dog. The Journal of Biological Chemistry 253:4022-4030.

Rehfeld J (2017) Cholecystokinin—From Local Gut Hormone to Ubiquitous Messenger. Frontiers in Endocrinology 8:

Reid A, Dunn I (2018) Gastrointestinal distribution of chicken gastrin-cholecystokinin family transcript expression and response to short-term nutritive state. General and Comparative Endocrinology 255:64-70.

Reid A, Wilson P, Caughey S et al. (2017) Pancreatic PYY but not PPY expression is responsive to short-term nutritional state and the pancreas constitutes the major site of PYY mRNA expression in chickens. General and Comparative Endocrinology 252:226-235.

Remage-Healey L, Romero L (2001) Corticosterone and insulin interact to regulate glucose and triglyceride levels during stress in a bird. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 281: R994-R1003.

Rexford S, Ahima M, Antwi D (2008) Brain regulation of appetite and satiety. The Journal of Endocrinology & Metabolism Clinics of North America 37:811–823.

Richards M, McMurtry J (2008) Expression of proglucagon and proglucagon-derived peptide hormone receptor genes in the chicken. General and Comparative Endocrinology 156:323-338.

Richards M, McMurtry J (2009) The avian proglucagon system. General and Comparative Endocrinology 163:39-46.

Richards M, Rosebrough R, Coon C, McMurtry J (2010) Feed intake regulation for the female broiler breeder: In theory and in practice,. Journal of Applied Poultry Research 19:182-193.

Richards P, Parker H, Adriaenssens A et al. (2013) Identification and Characterization of GLP-1 Receptor-Expressing Cells Using a New Transgenic Mouse Model. Diabetes 63:1224-1233.

Richards W, Hillsley K, Eastwood C, Grundy D (1996) Sensitivity of vagal mucosal afferents to cholecystokinin and its role in afferent signal transduction in the rat. The Journal of Physiology 497:473-481.

Richardson R, Boswell T, Raffety B et al. (1995) NPY increases food intake in white-crowned sparrows: effect in short and long photoperiods. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 268: R1418-R1422.

Richardson R, Boswell T, Raffety B et al. (1995) NPY increases food intake in white-crowned sparrows: effect in short and long photoperiods. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 268: R1418-R1422.

Richardson R, Boswell T, Weatherford S et al. (1993) Cholecystokinin octapeptide decreases food intake in white-crowned sparrows. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 264: R852-R856.

Rijnsdorp A, Daan S, Dijkstra C (1981) Hunting in the kestrel, Falco tinnunculus, and the adaptive significance of daily habits. Oecologia 50:391-406.

Ritter R (2004) Gastrointestinal mechanisms of satiation for food. Physiology & Behavior 81:249-273.

Ritter R, Covasa M, Matson C (1999) Cholecystokinin: proofs and prospects for involvement in control of food intake and body weight. Neuropeptides 33:387-399.

Ritter S, Weatherford S, Stone S (1986) Glucagon-induced inhibition of feeding is impaired by hepatic portal alloxan injection. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 250: R682-R690.

Ritter W (1938) The California woodpecker and I. University of California Press, Berkeley

Rix I, Nexøe-Larsen C, Bergmann N et al. (2021) Glucagon Physiology. In: Ncbi.nlm.nih.gov. https://www.ncbi.nlm.nih.gov/books/NBK279127/. Accessed 15 Aug 2021

Roberts R (1979) The Evolution of Avian Food-Storing Behavior. The American Naturalist 114:418-438.

Rodriguez-Sinovas A, Fernandez E, Manteca X et al. (1997) CCK is involved in both peripheral and central mechanisms controlling food intake in chickens. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 272: R334-R340.

Rogers C (1987) Predation Risk and Fasting Capacity: Do Wintering Birds Maintain Optimal Body Mass? Ecology 68:1051-1061.

Rogge G, Jones D, Hubert G et al. (2010) Erratum: CART peptides: regulators of body weight, reward and other functions. Nature Reviews Neuroscience 11:218-218.

Roman C, Derkach V, Palmiter R (2016) Genetically and functionally defined NTS to PBN brain circuits mediating anorexia. Nature Communications 7:

Roman C, Sloat S, Palmiter R (2017) A tale of two circuits: CCK NTS neuron stimulation controls appetite and induces opposing motivational states by projections to distinct brain regions. Neuroscience 358:316-324.

Ronveaux C, de Lartigue G, Raybould H (2014) Ability of GLP-1 to decrease food intake is dependent on nutritional status. Physiology & Behavior 135:222-229.

Rorig G (1905) Studies on the economic importance of insectivorous Bird. Agricultural and Forestry 4:1-50.

Rossi M, Kim M, Morgan D et al. (1998) A C-terminal fragment of Agouti-related Protein increases feeding and antagonizes the effect of Alpha-melanocyte stimulating hormone in vivo. Endocrinology 139:4428-4431.

Rotondo A, Amato A, Lentini L et al. (2011) Glucagon-like peptide-1 relaxes gastric antrum through nitric oxide in mice. Peptides 32:60-64.

Rui L (2013) Brain regulation of energy balance and body weight. Reviews in Endocrine and Metabolic Disorders 14:387-407.

Ruiter M, La Fleur S, van Heijningen C et al. (2003) The daily rhythm in plasma glucagon concentrations in the rat is modulated by the biological clock and by feeding behaviour. Diabetes 52:1709-1715.

Safi K, Seid M, Dechmann D (2005) Bigger is not always better: when brains get smaller. Biology Letters 1:283-286.

Sahu A, Kalra P, Kalra S (1988) Food deprivation and ingestion induce reciprocal changes in neuropeptide Y concentrations in the paraventricular nucleus. Peptides 9:83-86.

Sainsbury A, Cusin I, Rohner-Jeanrenaud F, Jeanrenaud B (1997) Adrenalectomy prevents the obesity syndrome produced by chronic central neuropeptide Y infusion in normal rats. Diabetes 46:209-214.

Saito E, Kaiya H, Tachibana T et al. (2005) Inhibitory effect of ghrelin on food intake is mediated by the corticotropin-releasing factor system in neonatal chicks. Regulatory Peptides 125:201-208.

Saito E, Kaiya H, Takagi T et al. (2002) Chicken ghrelin and growth hormone-releasing peptide-2 inhibit food intake of neonatal chicks. European Journal of Pharmacology 453:75-79.

Saito E, Takagi T, Nakanishi T et al. (2002) Ghrelin Activates Behavior of Neonatal Chicks in a Short Period of Post-Intracerebroventricular Injection. Journal of Applied Animal Research 22:33-41.

Saladin R, De Vos P, Guerre-Millot M et al. (1995) Transient increase in obese gene expression after food intake or insulin administration. Nature 377:527-528.

Salaneck E, Holmberg S, Berglund M et al. (2000) Chicken neuropeptide Y receptor Y2: structural and pharmacological differences to mammalian Y21. FEBS Letters 484:229-234.

Sam A, Troke R, Tan T, Bewick G (2012) The role of the gut/brain axis in modulating food intake. Neuropharmacology 63:46-56.

Samola E, Tyler M, Marks V, Mialhe P (1969) The physiological role of glucagon in different species. Proceedings 206-219.

Samols E (1983) Glucagon and insulin secretion. In: Lefebvre, P. J., ed. Handbook of experimental pharmacology, 485-518.

Samols E, Marri G, Marks V (1966) Interrelationship of Glucagon, Insulin and Glucose: The Insulinogenic Effect of Glucagon. Diabetes 15:855-866.

Sanacora G, Kershaw M, Finkelstein J, White J (1990) Increased Hypothalamic Content of Preproneuropeptide Y Messenger Ribonucleic Acid in Genetically Obese Zucker Rats and Its Regulation by Food Deprivation*. Endocrinology 127:730-737.

Sander L, Porter J (1988) Influence of bombesin, CCK, secretin and CRF on corticosterone concentration in the rat. Peptides 9:113-117.

Saneyasu T, Honda K, Kamisoyama H et al. (2011) Neuropeptide Y effect on food intake in broiler and layer chicks. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 159:422-426.

Saper C, Chou T, Elmquist J (2002) The need to feed: homeostatic and hedonic control of eating. Neuron 199-211.

Sapolsky R (1996) Why stress is bad for your brain. Science 273:749-750.

Savory C, Gentle M (1983) Effects of food deprivation, strain, diet, and age on feeding responses of fowls to intravenous injections of cholecystokinin. Appetite 4:165-176.

Sawchenko P (1998) Toward a new neurobiology of energy balance, appetite, and obesity: The anatomists weigh in. The Journal of Comparative Neurology 402:435-441.

Sayegh A, Washington M, Raboin S et al. (2014) CCK-58 prolongs the intermeal interval, whereas CCK-8 reduces this interval: Not all forms of cholecystokinin have equal bioactivity. Peptides 55:120-125.

Scanes C, Braun E (2012) Avian metabolism: its control and evolution. Frontiers in Biology 8:134-159.

Scanes C, Merrill G, Ford R et al. (1980) Effects of stress (hypoglycaemia, endotoxin, and ether) on the peripheral circulating concentration of corticosterone in the domestic fowl (Gallus domesticus). Comparative Biochemistry and Physiology Part C: Comparative Pharmacology 66:183-186.

Schaffhauser A, Stricker-Krongrad A, Brunner L et al. (1997) Inhibition of Food Intake by Neuropeptide Y Y5 Receptor Antisense Oligodeoxynucleotides. Diabetes 46:1792-1798.

Scheer F, Morris C, Shea S (2013) The internal circadian clock increases hunger and appetite in the evening independent of food intake and other behaviors. Obesity 21:421-423.

Schick R, Zimmerman P, Walde T et al. (1992) Glucagon-like peptide (GLP)-l-(7-36) amide: a central suppressor of food intake in fasted rats. Gastroenterology 102:A756-.

Schmidt-Nielsen K (1997) Animal Physiology Adaptation and Environment. Cambridge University Press, Cambridge

Schneeberger M, Gomis R, Claret M (2014) Hypothalamic and brainstem neuronal circuits controlling homeostatic energy balance. Journal of Endocrinology 220:T25-T46.

Schneeman B (2002) Gastrointestinal physiology and functions. British Journal of Nutrition 2, S159–S163.

Schneider J (2006) Metabolic and hormonal control of the desire for food and sex: Implications for obesity and eating disorders. Hormones and Behavior 50:562-571.

Schneider J, Casper J, Barisich A et al. (2007) Food deprivation and leptin prioritize ingestive and sex behaviour without affecting estrous cycles in Syrian hamsters. Hormones and Behavior 51:413-427.

Schwartz G, McHugh P, Moran T (1993) Gastric loads and cholecystokinin synergistically stimulate rat gastric vagal afferents. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 265: R872-R876.

Schwartz M, Bergman R, Kahn S et al. (1991) Evidence for entry of plasma insulin into cerebrospinal fluid through an intermediate compartment in dogs. Quantitative aspects and implications for transport. Journal of Clinical Investigation 88:1272-1281.

Schwartz M, Dallman M, Woods S (1995) Hypothalamic response to starvation: implications for the study of wasting disorders. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 269: R949-R957.

Schwartz M, Figlewicz D, Baskin D et al. (1992) Insulin in the brain: a hormonal regulator of energy balance. Endocrine Reviews 13:387-414.

Schwartz M, Seeley R, Campfield L et al. (1996) Identification of targets of leptin action in rat hypothalamus. Journal of Clinical Investigation 98:1101-1106.

Schwartz M, Seeley R, Woods S et al. (1997) Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. Diabetes 46:2119-2123.

Schwartz M, Sipols A, Grubin C, Baskin D (1995) Differential effect of fasting on hypothalamic expression of genes encoding neuropeptide Y, galanin, and glutamic acid decarboxylase. Brain Research Bulletin 31:361-367.

Schwartz M, Sipols A, Marks J et al. (1992) Inhibition of hypothalamic neuropeptide Y gene expression by insulin. Endocrinology 130:3608-3616.

Schwartz M, Woods S, Porte D et al. (2000) Central nervous system regulation of food intake. Nature 661-671.

Secchi A, Caldara R, Caumo A et al. (1995) Cephalic-phase insulin and glucagon release in normal subjects and in patients receiving pancreas transplantation. Metabolism 1153–1158.

Seeley R, Yagaloff K, Fisher S et al. (1997) Melanocortin receptors in leptin effects. Nature 390:349-349.

Seibert H (1949) Differences between Migrant and Non-Migrant Birds in Food and Water Intake at Various Temperatures and Photoperiods. The Auk 66:128-153.

Seoane L, López M, Tovar S et al. (2003) Agouti-Related Peptide, Neuropeptide Y, and Somatostatin-Producing Neurons Are Targets for Ghrelin Actions in the Rat Hypothalamus. Endocrinology 144:544-551.

Seroussi E, Cinnamon Y, Yosefi S et al. (2016) Identification of the Long-Sought Leptin in Chicken and Duck: Expression Pattern of the Highly GC-Rich Avian leptin fits an autocrine/paracrine rather than endocrine function. Endocrinology 157:737-751.

Serrano J, Bevins C, Young S, de Pablo F (1989) Insulin gene expression in chicken ontogeny: Pancreatic, extrapancreatic, and prepancreatic. Developmental Biology 132:410-418. doi: 10.1016/0012-1606(89)90237-6

Shcherbina L, Lindqvist A, Thoren Fischer A et al. (2018) CART is a regulator of GIP and GLP-1 secretion and expression. The Journal of Molecular and Cellular Endocrinology 476:8-16.

Sherry D (1985) Food storage in birds and mammals. Advances in the Study of Animal Behaviour 153-188.

Sherry D (1989) Food Storing in the Paridae. Wilson Ornithological Society 101:289-304.

Shettleworth S, Hampton R, Westwood R (1995) Effects of season and photoperiod on food storing by black-capped chickadees,Parus atricapillus. Animal Behaviour 49:989-998. doi: 10.1006/anbe.1995.0128

Shimizu H, Bray G (1993) Effects of castration, estrogen replacement and estrus cycle on monoamine metabolism in the nucleus accumbens, measured by microdialysis. Brain Research 621:200-206.

Shintani M, Ogawa Y, Ebihara K et al. (2001) Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 Receptor Pathway. Diabetes 50:227-232.

Shiraishi J, Tanizawa H, Fujita M et al. (2011) Localization of hypothalamic insulin receptor in neonatal chicks: Evidence for insulinergic system control of feeding behaviour. Neuroscience Letters 491:177-180.

Shiraishi J, Yanagita K, Fujita M, Bungo T (2008) Central insulin suppresses feeding behaviour via melanocortins in chicks. Domestic Animal Endocrinology 34:223-228.

Shirakawa T, Honma S, Honma K (2001) Multiple oscillator in the suprachiasmatic nucleus. Chronobiology International 18:371-387.

Shousha S, Nakahara K, Kojima M et al. (2005) Different effects of peripheral and central ghrelin on regulation of food intake in the Japanese quail. General and Comparative Endocrinology 141:178-183.

Shousha S, Nakahara K, Nasu T et al. (2007) Effect of glucagon-like peptide-1 and -2 on regulation of food intake, body temperature and locomotor activity in the Japanese quail. Neuroscience Letters 415:102-107.

Shutter J, Graham M, Kinsey A et al. (1997) Hypothalamic expression of ART, a novel gene related to agouti, is up regulated in obese and diabetic mutant mice. Genes & Development 11:593-602.

Siegel P, Dunnington E (1990) Behavioural genetics. In: Poultry Breeding and Genetics. 877-896.

Siegel P, Haberfeld A, Mukherjee T et al. (1992) Jungle fowl–domestic fowl relationships: a use of DNA fingerprinting. World's Poultry Science Journal 48:147-155.

Silva A, Bloom S (2012) Gut Hormones and Appetite Control: A Focus on PYY and GLP-1 as Therapeutic Targets in Obesity. Gut and Liver 6:10-20.

Silverin B (1998) Stress response in birds. Poultry and Avian Biology Reviews 9:153-168.

Silverman H, Zucker I (1976) Absence of post-fast food compensation in the golden hamster (Mesocricetus auratus). Physiology & Behavior 17:271-285.

Simon J (1989) Chicken as a useful species for the comprehension of insulin action. Critical Reviews in Poultry Biology 121-148.

Simon J, Derouet M, Gespach C (2000) An Anti-Insulin Serum, But Not a Glucagon Antagonist, Alters Glycemia in Fed Chickens. Hormone and Metabolic Research 32:139-141.

Simon J, Freychet P, Rosselin G (1977) A study of insulin binding sites in the chicken tissues. Diabetologia 13:219-228.

Simon J, LeRoith D (1986) Insulin receptors of chicken liver and brain Characterization of α and β subunit properties. European Journal of Biochemistry 158:125-132.

Simon J, Leroith D (1986) Insulin receptors of chicken liver and brain. Characterization of alpha and beta subunit properties. European Journal of Biochemistry 158:125-132.

Simon J, Rideau N, Taouis M, Dupont J (2011) Plasma insulin levels are rather similar in chicken and rat. General and Comparative Endocrinology 171:267-268.

Simon J, Rosselin G (1978) Effect of fasting, glucose, amino acids and food intake on in vivo insulin release in the chicken. Hormone and Metabolic Research 10:93-98. doi: 10.1055/s-0028-1093450

Sinha M, Ohannesian J, Heiman M et al. (1996) Nocturnal rise of leptin in lean, obese, and non-insulin-dependent diabetes mellitus subjects. Journal of Clinical Investigation 97:1344-1347.

Sipols A, Baskin D, Schwartz M (1995) Effect of intracerebroventricular insulin infusion on diabetic hyperphagia and hypothalamic neuropeptide gene expression. Diabetes 44:147-151.

Smith C, Bright-Taylor B (1974) Does a glucostatic mechanism for food intake Control Exist in Chickens? Poultry Science 53:1720-1724.

Smith C, Follmer D (1972) Food preferences of squirrels. Ecology 53:82-91.

Smith C, Reichman O (1984) The evolution of food caching by birds and mammals. Annual Review of Ecology and Systematics 15:329-351.

Smith G (1988) Humoral mechanisms in the control of eating and body weight. 59-65.

Smith G, Gibbs J (1994) Satiating effect of cholecystokinin. Annals of the New York Academy of Sciences 713:236-241.

Smith G, Jerome C, Cushin B et al. (1981) Abdominal vagotomy blocks the satiety effect of cholecystokinin in the rat. Science 213:1036-1037.

Smits M, Tonneijck L, Muskiet M et al. (2016) Gastrointestinal actions of glucagon-like peptide-1-based therapies: glycaemic control beyond the pancreas. Diabetes, Obesity and Metabolism 18:224-235.

Sobhani I, Buyse M, Goïot H et al. (2002) Vagal stimulation rapidly increases leptin secretion in human stomach. Gastroenterology 122:259-263.

Solomon T (1985) Pancreatic polypeptide, peptide YY, and neuropeptide Y family of regulatory peptides. Gastroenterology 88:838-841.

Song G, Pacini G, Ahrén B, D'Argenio D (2017) Glucagon increases insulin levels by stimulating insulin secretion without effect on insulin clearance in mice. Peptides 88:74-79.

Song Z, Liu L, Sheikhahmadi A et al. (2012) Effect of Heat Exposure on Gene Expression of Feed Intake Regulatory Peptides in Laying Hens. Journal of Biomedicine and Biotechnology 2012:1-8.

Song Z, Liu L, Yue Y et al. (2012) Fasting alters protein expression of AMP-activated protein kinase in the hypothalamus of broiler chicks (Gallus gallus domesticus). General and Comparative Endocrinology 178:546-555.

Speakman J (2018) The evolution of body fatness: trading off disease and predation risk. The Journal of Experimental Biology 221: jeb167254.

Stadlbauer U, Woods S, Langhans W, Meyer U (2015) PYY3–36: Beyond food intake. Frontiers in Neuroendocrinology 38:1-11.

Stanley S, Wynne K, Bloom S (2004) Gastrointestinal Satiety Signals III. Glucagon-like peptide 1, oxyntomodulin, peptide YY, and pancreatic polypeptide. American Journal of Physiology-Gastrointestinal and Liver Physiology 286: G693-G697.

Steiner R, Kabigting E, Lent K, Clifton D (1994) Diurnal Rhythm in Proopiomelanocortin mRNA in the Arcuate Nucleus of the Male Rat. Journal of Neuroendocrinology 6:603-608.

Stellar E (1994) The physiology of motivation. Psychological Review 61:5-22.

Stephens T, Basinski M, Bristow P et al. (1995) The role of neuropeptide Y in the antiobesity action of the obese gene product. Nature 377:530-532.

Strader A, Buntin J (2001) Neuropeptide-Y: A possible mediator of prolactin-induced feeding and regulator of energy balance in the ring dove (Streptopelia risoria). Journal of Neuroendocrinology 13:386-392.

Strader A, Buntin J (2003) changes in agouti-related peptide during the ring dove breeding cycle in relation to prolactin and parental hyperphagia. Journal of Neuroendocrinology 15:1046-1053.

Strader A, Schiöth H, Buntin J (2003) The role of the melanocortin system and the melanocortin-4 receptor in ring dove (Streptopelia risoria) feeding behaviour. Brain Research 960:112-121.

Strubbe J, Mein C (1977) Increased feeding in response to bilateral injection of insulin antibodies in the VMH. Physiology & Behavior 19:309-313.

Strubbe J, Steffens A (1993) Neural control of insulin secretion. Hormone and Metabolic Research 25:507-512.

Strubbe J, Van Dijk G (2001) Involvement of central GLP-1 circuitry in gastrointestinal motility. European Winter Conference on Brain Research 51:403.

Stunkard A (1975) Satiety Is a Conditioned Reflex. Psychosomatic Medicine 37:383-387.

Suominen A, Glimm D, Tedesco D et al. (1998) Intestinal nutrient-gene interaction: the effect of feed deprivation and refeeding on cholecystokinin and proglucagon gene expression. Journal of Animal Science 76:3104.

Suzuki K, Simpson K, Minnion J et al. (2010) The role of gut hormones and the hypothalamus in appetite regulation. Endocrine Journal 57:359-372.

Swart I, Jahng J, Overton J, Houpt T (2002) Hypothalamic NPY, AGRP, and POMC mRNA responses to leptin and refeeding in mice. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 283: R1020-R1026.

Tachibana T, Hiramatsu K, Furuse M et al. (2005) Distribution of proglucagon mRNA and GLP-1 in the brainstem of chicks. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 140:203-207.

Tachibana T, Hirofuji K, Matsumoto M et al. (2004) The hypothalamus is involved in the anorexic effect of glucagon-like peptide-1 in chicks. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 137:183-188.

Tachibana T, Matsumoto M, Furuse M et al. (2003) Central, but not peripheral, glucagon-like peptide-1 inhibits crop emptying in chicks. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 134:777-781.

Tachibana T, Sugahara K, Ohgushi A et al. (2001) Intracerebroventricular injection of agouti-related protein attenuates the anorexigenic effect of alpha-melanocyte stimulating hormone in neonatal chicks. Neuroscience Letters 305:131-134.

Tachibana T, Tsutsui K (2016) Neuropeptide Control of Feeding Behaviour in Birds and Its Difference with Mammals. Frontiers in Neuroscience 10:

Takahashi K, Cone R (2005) Fasting induces a large, leptin-dependent increase in the intrinsic action potential frequency of orexigenic arcuate nucleus neuropeptide Y/ agouti-related protein neurons. Endocrinology 146:1043-1047.

Takeuchi S, Takahashi S (1999) A possible involvement of melanocortin 3 receptor in the regulation of adrenal gland function in the chicken. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1448:512-518.

Tang-Christensen M, Larsen P, Goke R et al. (1996) Brain GLP-1 (7–36) amide receptors play a major role in regulation of food and water intake. American Journal of Physiology R848-R856.

Tang-Christensen M, Larsen P, Goke R et al. (1996) Central administration of GLP-1-(7-36) amide inhibits food and water intake in rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 271: R848-R856.

Tang-Christensen M, Larsen P, Thulesen J et al. (2000) The proglucagon-derived peptide, glucagon-like peptide-2, is a neurotransmitter involved in the regulation of food intake. Nature Medicine 6:802-807.

Taouis M, Chen J, Daviaud C et al. (1998) Cloning the chicken leptin gene. Gene 208:239-242.

Tartaglia L, Dembski M, Weng X et al. (1995) Identification and expression cloning of a leptin receptor. Cell 1263–1271.

Tatemoto K (2004) Neuropeptide Y: History and overview. In Michel MC, ed. Neuropeptide Y and related peptides. 1–21.

Taylor I, Feldman M, Richardson C, Walsh J (1978) Gastric and cephalic stimulation of human pancreatic polypeptide release. Gastroenterology 75:432-437.

Tempel D, Leibowitz S (1994) Adrenal steroid receptors: interactions with brain neuropeptide systems in relation to nutrient intake and metabolism. Journal of Neuroendocrinology 6:479-501.

Teshigawara K, Takeuchi S, Takahashi S (2000) Widespread expression of Agouti-related protein (AGRP) in the chicken: a possible involvement of AGRP in regulating peripheral melanocortin systems in the chicken. Biochimica et Biophysica Acta 261-269.

Teubner B, Bartness T (2010) Cholecystokinin-33 acutely attenuates food foraging, hoarding and intake in Siberian hamsters. Peptides 31:618-624.

Teubner B, Keen-Rhinehart E, Bartness T (2012) Third ventricular co-injection of subthreshold doses of NPY and AgRP stimulate food hoarding and intake and neural activation. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 302: R37-R48.

Thim L, Clausen J, Kristensen P et al. (1999) Tissue-specific processing of cocaine- and amphetamine-regulated transcript peptides in the rat. Regulatory Peptides 94:75.

Thomas M, Xue B (2018) Mechanisms for AgRP neuron-mediated regulation of appetitive behaviours in rodents. Physiology & Behavior 190:34-42.

Thornton J, Cheung C, Clifton D, Steiner R (1997) Regulation of Hypothalamic Proopiomelanocortin mRNA by Leptin in ob/ob Mice. Endocrinology 138:5063-5066.

Toates F (1978) A circadian rhythm of hoarding in the hamster. Animal Behaviour 26:631.

Torekov S, Madsbad S, Holst J (2011) Obesity - an indication for GLP-1 treatment? Obesity pathophysiology and GLP-1 treatment potential. Obesity Reviews 12:593-601.

Toshinai K, Mondal M, Nakazato M et al. (2001) Upregulation of ghrelin expression in the stomach upon fasting, insulin-induced hypoglycemia, and leptin administration. Biochemical and Biophysical Research Communications 281:1220-1225.

Totzke U, Hübinger A, Dittami J, Bairlein F (2000) The autumnal fattening of the long-distance migratory garden warbler (Sylvia borin) is stimulated by intermittent fasting. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 170:627-631.

Tseng H, Yang S, Yang S, Shieh K (2015) Hepatic circadian-clock system altered by insulin resistance, diabetes and insulin sensitizer in mice. PLOS ONE 10: e0120380.

Tsigos C, Chrousos G (2002) Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. Journal of Psychosomatic Research 53:865-871.

Tung Y, Hewson A, Dickson S (2004) Glucocorticoid-dependent stimulation of adiposity and appetite by a ghrelin mimetic in the rat. European Journal of Endocrinology 905-911.

Turcek F, Kelso L (1968) Ecological aspects of food transportation and storage in corvidae. Communications in Behavioural Biology Part A 1:277-297.

Ueno H, Nakazato M (2016) Mechanistic relationship between the vagal afferent pathway, central nervous system and peripheral organs in appetite regulation. Journal of Diabetes Investigation 7:812-818.

Unger R, Orci L (1976) Physiology and pathophysiology of glucagon. Physiological Reviews 56:778-826.

Valassi E, Scacchi M, Cavagnini F (2008) Neuroendocrine control of food intake. Nutrition, Metabolism and Cardiovascular Diseases 18:158-168.

Van Cauter E, Blackman J, Roland D et al. (1991) Modulation of glucose regulation and insulin secretion by circadian rhythmicity and sleep. Journal of Clinical Investigation 88:934-942.

Van Cauter E, Polonsky K, Scheen A (1997) Roles of Circadian Rhythmicity and Sleep in Human Glucose Regulation. Endocrine Reviews 18:716-738.

Van Dijk G, De Groote C, Chavez M et al. (1997) Insulin in the arcuate nucleus reduces fat consumption in rats. Brain Research 147-152.

Vander Wall S (1990) Food hoarding in animals. University of Chicago Press, Chicago

Vander Wall S, Jenkins S (2003) Reciprocal pilferage and the evolution of food-hoarding behavior. Behavioral Ecology 14:656-667.

Vanderhaeghen J, Signeau J, Gepts W (1975) New peptide in the vertebrate CNS reacting with antigastrin antibodies. Nature 257:604-605.

Vanderweele D, Geiselman P, Novin D (1979) Pancreatic glucagon, food deprivation and feeding in intact and vagotomized rabbits. Physiology & Behavior 23:155-158.

Vestlund J, Bergquist F, Licheri V et al. (2020) Activation of glucagon-like peptide-1 receptors and skilled reach foraging. Addiction Biology

Von Pernau J (1720) from the titmouse. 226.

Vrang N (2006) Anatomy of hypothalamic CART neurons. Peptides 27:1970-1980.

Wade G, Bartness T (1984) Effects of photoperiod and gonadectomy on food intake, body weight, and body composition in Siberian hamsters. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 246: R26-R30.

Wager-Srdar S, Gannon M, Levine A (1987) The effect of cholecystokinin on food intake in gonadectomized and intact rats: The influence of sex hormones. Physiology & Behavior 40:25-28.

Wakita M, Kawamura H, Watanabe S (1994) Hoarding behavior in the pigeon (Columbia livia): performance under the restriction of food availability. Behavioural Processes 31:167-176.

Wall S, Balda R (2010) Ecology and Evolution of Food-storage Behavior in Conifer-seed-caching Corvids. Zeitschrift für Tierpsychologie 56:217-242. doi: 10.1111/j.1439-0310.1981.tb01298.x

Walther C, Mörl K, Beck-Sickinger A (2011) Neuropeptide Y receptors: ligand binding and trafficking suggest novel approaches in drug development. Journal of Peptide Science 17:233-246.

Wang J, Wang Y, Li X et al. (2008) Cloning, Tissue Distribution, and Functional Characterization of Chicken Glucagon Receptor. Poultry Science 87:2678-2688. doi: 10.3382/ps.2008-00260

Wang T, Hung C, Randall D (2006) THE COMPARATIVE PHYSIOLOGY OF FOOD DEPRIVATION: From Feast to Famine. Annual Review of Physiology 68:223-251.

Watanabe M, Hayasaki H, Tamayama T, Shimada M (1998) Histologic distribution of insulin and glucagon receptors. Brazilian Journal of Medical and Biological Research 31:243-256.

Watanabe T, Hiramatsu K, Nishimura K, Ono T (2018) Glucagon-like peptide-1 receptor expression in the pancreatic D cells of three avian species; white leghorn chickens, northern bobwhites, and common ostriches. The Journal of Poultry Science 55:199-203.

Webster A (2003) Physiology and behaviour of the hen during induced molt. Poultry Science 82:992-1002.

Weigle D (1987) Pulsatile Secretion of Fuel-Regulatory Hormones. Diabetes 36:764-775.

Wettergren A, Schjoldager B, Mortensen P et al. (1993) Truncated GLP-1 (proglucagon 78?107-amide) inhibits gastric and pancreatic functions in man. Digestive Diseases and Sciences 38:665-673.

Wewer Albrechtsen N, Kuhre R, Toräng S, Holst J (2016) The intestinal distribution pattern of appetite- and glucose regulatory peptides in mice, rats and pigs. BMC Research Notes 9:

Wiener P, Wilkinson S (2011) Deciphering the genetic basis of animal domestication. Proceedings of the Royal Society B: Biological Sciences 278:3161-3170.

Wilding J (2002) Neuropeptides and appetite control. Diabetic Medicine 19:619-627.

Willesen M, Kristensen P, Rømer J (1999) Co-Localization of Growth Hormone Secretagogue Receptor and NPY mRNA in the Arcuate Nucleus of the Rat. Neuroendocrinology 70:306-316.

Williams D, Baskin D, Schwartz M (2009) Evidence that Intestinal Glucagon-Like Peptide-1 Plays a Physiological Role in Satiety. Endocrinology 150:1680-1687.

Williams D, Cummings D, Grill H, Kaplan J (2003) Meal-related ghrelin suppression requires postgastric feedback. Endocrinology 144:2765-2767.

Williams E, Chang R, Strochlic D et al. (2016) Sensory Neurons that Detect Stretch and Nutrients in the Digestive System. Cell 166:209-221.

Williams K, Washington M, Johnson-Rouse T et al. (2016) Exogenous glucagon-like peptide-1 acts in sites supplied by the cranial mesenteric artery to reduce meal size and prolong the intermeal interval in rats. Appetite 96:254-259.

Wingate D, Ewart W (1981) The brain-gut axis. In Textbook: Gastroennterology. 50-69.

Wingfield J, Breuner C, Jacobs J (1997) corticosterone and behavioural responses to unpredictable events. Journal of Endocrinology Press 267-278.

Wingfield J, Maney D, Breuner C et al. (1998) Ecological Bases of Hormone—Behavior Interactions: The “Emergency Life History Stage”. American Zoologist 38:191-206.

Wirth M, Giraudo S (2000) Agouti-related protein in the hypothalamic paraventricular nucleus: effect on feeding. Peptides 21:1369-1375.

Witter M, Cuthill I (1993) The ecological costs of avian fat storage. Philosophical Transactions of the Royal Society of London Series B: Biological Sciences 340:73-92.

Witter M, Cuthill I (1993) The ecological costs of avian fat storage. Philosophical Transactions of the Royal Society of London Series B: Biological Sciences 340:73-92.

Wójcik-Gładysz A, Szlis M (2016) Hypothalamo-gastrointestinal axis – role in food intake regulation. Journal of Animal and Feed Sciences 25:97-108.

Wolfe J (1939) An exploratory study of food-storing in rats. Journal of Comparative Psychology 28:97-108.

Wood A, Bartness T (1996) Food deprivation-induced increases in hoarding by Siberian hamsters are not photoperiod-dependent. Physiology & Behavior 60:1137-1145.

Woods S (2004) Gastrointestinal Satiety Signals I. An overview of gastrointestinal signals that influence food intake. American Journal of Physiology-Gastrointestinal and Liver Physiology 286: G7-G13.

Woods S (2009) The Control of Food Intake: Behavioral versus Molecular Perspectives. Cell Metabolism 9:489-498.

Woods S (2013) Metabolic signals and food intake. Forty years of progress. Appetite 71:440-444.

Woods S, Lotter E, McKay L, Porte D (1979) Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. Nature 282:503-505.

Woods S, Lutz T, Geary N, Langhans W (2006) Pancreatic signals controlling food intake; insulin, glucagon and amylin. Philosophical Transactions of the Royal Society B: Biological Sciences 361:1219-1235.

Woods S, Porte D (1977) Relationship between plasma and cerebrospinal fluid insulin levels of dogs. American Journal of Physiology-Endocrinology and Metabolism 233: E331.

Woods S, Seeley R (2000) Adiposity signals and the control of energy homeostasis. Nutrition 16:894-902.

Woods S, Seeley R, Porte D, Schwartz M (1998) Signals That Regulate Food Intake and Energy Homeostasis. Science 280:1378-1383.

Wren A, Small C, Abbot C et al. (2001) Ghrelin causes hyperphagia and obesity in rats. Diabetes 2540–2547.

Wren A, Small C, Ward H et al. (2000) The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology 141:4325-4328.

Wynne K, Stanley S, McGowan B, Bloom S (2005) Appetite control. Journal of Endocrinology 184:291-318.

Xu B, Kalra P, Farmerie W, Kalra S (1999) Daily changes in hypothalamic gene expression of neuropeptide Y, galanin, proopiomelanocortin, and adipocyte leptin gene expression and secretion: effects of food restriction1. Endocrinology 140:2868-2875.

Xu L, Wu T, Li H et al. (2017) An individual 12-h shift of the light–dark cycle alters the pancreatic and duodenal circadian rhythm and digestive function. Acta Biochimica et Biophysica Sinica 49:954-961.

Xu Y, Nedungadi T, Zhu L et al. (2011) Distinct Hypothalamic Neurons Mediate Estrogenic Effects on Energy Homeostasis and Reproduction. Cell Metabolism 14:453-465.

Yamaguchi S, Yamada J, Kitamura N, Yamashita T (1987) Histological and immunohistochemical study on ontogeny of the endocrine cells in the quail gizzard. Gegenbaur's morphological yearbook 133:71-78.

Yang H, Wang Q, Wang D (2014) Food hoarding, but not food intake, is attenuated by acute diazepam treatment in female Mongolian gerbils (Meriones unguiculatus). Hormones and Behavior 66:186-195.

Yaswen L, Diehl N, Brennan M, Hochgeschwender U (1999) Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. Nature Medicine 5:1066-1070.

Yuan L, Lin H, Jiang K et al. (2008) Corticosterone administration and high-energy feed results in enhanced fat accumulation and insulin resistance in broiler chickens. British Poultry Science 49:487-495.

Yue S, Irwin D (2005) Structure and expression of the chicken proglucagon gene. Molecular and Cellular Endocrinology 230:69-76.

Yvonne H, Potenza M (2013) Stress and Eating Behaviors. Minerva Endocrinologica 38:255–267.

Zafra M, Molina F, Puerto A (2006) The neural/cephalic phase reflexes in the physiology of nutrition. Neuroscience & Biobehavioral Reviews 30:1032-1044.

Zhan C, Zhou J, Feng Q et al. (2013) Acute and long-term suppression of feeding Behaviour by POMC neurons in the brainstem and hypothalamus, respectively. Journal of Neuroscience 33:3624-3632.

Zhang D, Bula W, Stellar E (1986) Brain cholecystokinin as a satiety peptide. Physiology & Behaviour 36:1183-1186.

Zhang R, Nakanishi T, Ohgushi A et al. (2001) Suppression of food intake induced by corticotropin-releasing factor family in neonatal chicks. European Journal of Pharmacology 427:37-41.

Zhang Y, Proenca R, Maffei M et al. (1994) Positional cloning of the mouse obese gene and its human homologue. Nature 372:425-432.

Zheng H, Patterson L, Berthoud H (2002) CART in the dorsal vagal complex: sources of immunoreactivity and effects on Fos expression and food intake. Brain Research 957:298-310.

Zhou J, Hegsted M, McCutcheon K et al. (2006) Peptide YY and Proglucagon mRNA Expression Patterns and Regulation in the Gut*. Obesity 14:683-689.

Chapter 8. Appendices

8.1 Great tit stepwise deletion tables of the final model in addition to the terms (factors) removed with their p-values and X2 values

A. Gut cholecystokinin (CCK)

CCK Final model			
Factors	**Df**	**X^2**	**p-value**
Sex	**1**	**0.434**	**0.510**
Gizzard full	**1**	**1.31**	**0.252**
Tissue	**9**	**2.55E+10**	**<0.001**
Sex*Gizzard full	**1**	**1.22**	**0.269**
Sex*Tissue	**9**	**7779371258**	**<0.001**
Gizzard full*Tissue	**9**	**78.24**	**<0.001**
Sex*Gizzard full*Tissue	**9**	**111.04**	**<0.001**

B. Gut glucagon (GCG)

GCG Final model			
Factors	**Df**	**X2**	**p-value**
Sex	**1**	**0.22**	**0.639**
Gizzard full	**1**	**0.644**	**0.422**
Tissue	**9**	**5.76E+06**	**0**
Sex*Gizzard full	**1**	**<0.0019**	**0.924**
Sex*Tissue	**9**	**11977.55**	**<0.001**
Gizzard full*Tissue	**9**	**3.29E+11**	**<0.001**
Sex*Gizzard full*Tissue	**9**	**2.05E+11**	**<0.001**

C. Gut peptide YY (PYY)

PYY final model			
Factors	**Df**	**X2**	**p-value**
Sex	**1**	**49.86**	**<0.001**
Gizzard full	**1**	**0.721**	**0.396**
Tissue	**9**	**1.21E+04**	**<0.001**
Sex*Gizzard full	**1**	**5.12E-01**	**0.474**
Sex*Tissue	**9**	**1.45E+12**	**<0.001**
Gizzard full*Tissue	**9**	**3.86E+12**	**<0.001**
Sex*Gizzard full*Tissue	**9**	**3.47E+01**	**<0.001**

D. Gut insulin (INS)

Term removed	**Order of removal**	**X2**	**p-value**
Species*Sex*Gizzard full	**1**	**0.07**	**0.792**
Sex*Gizzard full	**2**	**0.344**	**0.558**
Species*Gizzard full	**3**	**0.491**	**0.483**
Species*Sex	**4**	**0.394**	**0.530**

INS final model			
Factors	**Df**	**X2**	**p-value**
Sex	**1**	**0.077**	**0.781**
Gizzard full	**1**	**0.115**	**0.735**
Tissue	**9**	**2.04E+13**	**<0.001**
Sex*Gizzard full	**1**	**0.02**	**0.888**
Sex*Tissue	**9**	**2.80E+11**	**<0.001**
Gizzard full*Tissue	**9**	**2.72E+11**	**<0.001**
Sex*Gizzard full*Tissue	**9**	**4.49E+12**	**<0.001**

E. Pancreas glucagon/ insulin ratio (GCG/INS)

Term removed	Order of removal	X2	p-value
Sex*gizzard fullness	1	0.708	0.4

GCG/ INS final model			
Factors	Df	X2	p-value
Sex	1	0.066	0.797
Gizzard full	1	0.039	0.834

8.2 Blue and coal tit stepwise deletion tables of the final model in addition to the terms (factors) removed with their p-values and X2 values

A. Neuropeptide Y (NPY)

NPY Final model			
Factors	Df	p-value	X2
Species	1	0.017	5.68
Sex	1	0.16	1.97
Gizzard full	1	0.813	0.056

B. Agouti related peptide (AgRP)

Term removed	Order of removal	X2	p-value
Species*Sex*Gizzard full	1	0.817	0.366
Sex*Gizzard full	2	1.05	0.304

AgRP final model			
Factors	**Df**	**X2**	**p-value**
Species	**1**	**4.65**	**0.031**
Sex	**1**	**1.43**	**0.231**
Gizzard full	**1**	**4.57**	**0.032**
Species*Sex	**1**	**1.93**	**0.165**
Species*Gizzard full	**1**	**5.12**	**0.024**

C. Hypothalamic Proopiomelanocortin (POMC)

Term removed	Order of removal	X2	p-value
Species*Sex*Gizzard full	**1**	**0.198**	**0.656**
Species*Gizzard full	**2**	**0.023**	**0.880**

Hypothalamic POMC final model			
Factors	**Df**	**X2**	**p-value**
Species	**1**	**184.48**	**<0.001**
Sex	**1**	**4.95**	**0.026**
Gizzard full	**1**	**0.497**	**0.481**
Species*sex	**1**	**5.39**	**0.200**
Sex*Gizzard full	**1**	**3.69**	**0.055**

D. Hypothalamic agouti related protein/ Proopiomelanocortin ratio (AgRP/POMC)

Term removed	Order of removal	X2	p-value
Species*Sex*Gizzard full	**1**	**1.06**	**0.301**
Sex*Gizzard full	**2**	**1.44**	**0.229**
Species*Sex	**3**	**1.83**	**0.1752**

AgRP. Hypothalamic POMC final model			
Factors	**Df**	**X2**	**p-value**
Species	**1**	**13.31**	**<0.001**
Sex	**1**	**1.84**	**0.174**
Gizzard full	**1**	**6.09**	**0.014**
Species*Gizzard full	**1**	**3.62**	**0.057**

A. Hindbrain cholecystokinin (CCK)

Hindbrain CCK final model			
Factors	**Df**	**X2**	**p-value**
Species	**1**	**1.42**	**0.233**
Sex	**1**	**<0.0012**	**0.967**
Gizzard full	**1**	**3.67**	**0.055**
Species*Gizzard full	**1**	**1.85**	**0.173**

B. Hindbrain glucagon (GCG)

Term removed	Order of removal	X2	p-value
Species*Sex*Gizzard full	**1**	**0.316**	**0.574**
Species*Sex	**2**	**0.052**	**0.819**
Sex*Gizzard full	**3**	**1.03**	**0.309**

Hindbrain GCG final model			
Factors	**Df**	**X2**	**p-value**
Species	**1**	**16.26**	**<0.001**
Sex	**1**	**1.51**	**0.218**
Gizard full	**1**	**1.32**	**0.251**
Species*Gizzard full	**1**	**4.87**	**0.027**

C. Hindbrain Proopiomelanocortin (POMC)

Term removed	Order of removal	X2	p-value
Species*Sex*Gizzard full	**1**	**0.288**	**0.592**
Sex*Gizzard full	**2**	**0.011**	**0.916**
Species*Sex*Gizzard full	**3**	**1.08**	**0.298**

Hindbrain POMC final model			
Factors	**Df**	**X2**	**p-value**
Species	**1**	**111.94**	**<0.001**
Sex	**1**	**0.877**	**0.349**
Gizzard full	**1**	**0.072**	**0.788**
Species*sex	**1**	**0**	**0.990**
Species*Gizzard full	**1**	**0.114**	**0.736**
Sex*Gizzard full	**1**	**0.834**	**0.361**
Species*Sex*Gizzard full	**1**	**3.05**	**0.081**

D. Gut cholecystokinin (CCK)

Gut CCK final model			
Factors	**Df**	**X2**	**p-value**
Sex	**1**	**0.188**	**0.665**
Species	**1**	**0.606**	**0.436**
Tissue	**9**	**1518.83**	**<0.001**
Gizzard full	**1**	**0.899**	**0.343**
Species*Sex	**1**	**2.55**	**0.11**
Sex*Tissue	**9**	**21.9**	**<0.001**
Sex*Gizzard full	**1**	**0.016**	**0.899**
Species*Tissue	**9**	**25.29**	**<0.001**
Species*Gizzard full	**1**	**3.19**	**0.074**
Tissue*Gizzard full	**9**	**19.38**	**0.022**
Sex*Species*Tissue	**9**	**22.24**	**<0.001**
Sex*Species*Gizzard full	**1**	**0.102**	**0.75**
Sex*Tissue*Gizzard full	**9**	**11.52**	**0.242**
Species*Tissue*Gizzard full	**9**	**18.5**	**0.03**
Sex*Species*Tissue*Gizzard full	**9**	**18.6**	**0.029**

E. Gut glucagon (GCG)

Gut GCG final model			
Factors	**Df**	**X2**	**p-value**
Sex	**1**	**0.023**	**0.88**
Species	**1**	**1.77**	**0.183**
Tissue	**9**	**2100.13**	**<0.001**
Gizzard full	**1**	**0.096**	**0.757**
Species*Sex	**1**	**1.43**	**0.23**
Sex*Tissue	**9**	**6.56**	**0.682**
Sex*Gizzard full	**1**	**2.33**	**0.127**
Species*Tissue	**9**	**105.12**	**<0.001**
Species*Gizzard full	**1**	**0.731**	**0.393**
Tissue*Gizzard full	**9**	**46.54**	**<0.001**
Sex*Species*Tissue	**9**	**14.62**	**0.102**
Sex*Species*Gizzard full	**1**	**43.98**	**0.026**
Sex*Tissue*Gizzard full	**9**	**43.79**	**<0.001**
Species*Tissue*Gizzard full	**9**	**35.61**	**<0.001**
Sex*Species*Tissue*Gizzard full	**9**	**27.28**	**<0.001**

F. Gut peptide YY (PYY)

Term removed	Order of removal	X2	p-value
Species*Sex*Tissue*Gizzard full	**1**	**5.99**	**0.74**
Sex*Species*Tissue	**2**	**6.36**	**0.703**
Sex*Tissue*Gizzard full	**3**	**13.11**	**0.158**
Species*Tissue*Gizzard full	**4**	**12.3**	**0.197**

PYY final model			
Factors	**Df**	**X2**	**p-value**
Sex	**1**	**1.52**	**0.217**
Species	**1**	**0.187**	**0.666**
Tissue	**9**	**247.003**	**<0.001**
Gizzard full	**1**	**0.334**	**0.564**
Species*Sex	**1**	**0.155**	**0.94**
Sex*Tissue	**9**	**9.5**	**0.392**
Sex*Gizzard full	**1**	**1.98**	**0.159**
Species*Tissue	**9**	**26.66**	**<0.001**
Species*Gizzard full	**1**	**0.695**	**0.404**
Tissue*Gizzard full	**9**	**12.07**	**0.209**
Sex*Species*Gizzard full	**1**	**2.9**	**0.088**

G. **Gut insulin (INS)**

INS Final model			
Factors	**Df**	**X2**	**p-value**
Sex	**1**	**2.34**	**0.126**
Species	**1**	**4.55**	**0.033**
Tissue	**9**	**2323.16**	**<0.001**
Gizzard full	**1**	**6.16**	**0.013**
Species*Sex	**1**	**1.32**	**0.249**
Sex*Tissue	**9**	**38.91**	**<0.001**
Sex*Gizzard full	**1**	**0.874**	**0.35**
Species*Tissue	**9**	**3.52**	**0.94**
Species*Gizzard full	**1**	**0.098**	**0.755**
Tissue*Gizzard full	**9**	**48.56**	**<0.001**
Sex*Species*Tissue	**9**	**33.8**	**<0.001**
Sex*Species*Gizzard full	**1**	**1.64**	**0.199**
Sex*Tissue*Gizzard full	**9**	**29.7**	**<0.001**
Species*Tissue*Gizzard full	**9**	**22.81**	**<0.001**
Sex*Species*Tissue*Gizzard full	**9**	**31.26**	**<0.001**

H. Pancreatic glucagon/ insulin ratio (GCG/INS)

Term removed	Order of removal	X2	p-value
Species*Sex*Gizzard full	**1**	**1.06**	**0.301**
Sex*Gizzard full	**2**	**0.38**	**0.538**
Species*Sex	**3**	**1.65**	**0.198**

GCG.INS ratio final model			
	Df	**X2**	**p-value**
Species	**1**	**32.11**	**<0.001**
Sex	**1**	**0.2**	**0.654**
Gizzard full	**1**	**5.38**	**0.02**
Species *Gizzard full	**1**	**4.88**	**0.027**