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Functional assessment of peripheral mechanisms controlling energy homeostasis in the domestic chicken

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Declaration

I, Angus Reid, declare that I have composed this thesis entirely myself. All work reported herein was completed by me alone, except as otherwise explicitly indicated. This work has never before been submitted for any degree or professional qualifications. All information sources are acknowledged.

Two chapters based on peer-reviewed journal articles are included:

Chapter 4: Peripheral peptide hormones of the PP-fold family
Reid, A. M. A., Wilson, P. W., Caughey, S. D., Dixon, L. M., D'Eath, R. B., Sandilands, V., Boswell, T. & Dunn, I. C. 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.

Chapter 5: Peripheral peptide hormones of the gastrin-cholecystokinin family
Reid, A. M. A. & Dunn, I. C. 2017. Gastrointestinal distribution of chicken gastrin-cholecystokinin family transcript expression and response to short-term nutritive state. *General and Comparative Endocrinology*, submitted 06/09/17, revision requested 28/09/17.

Each of these articles represents original research work to which I made a substantial contribution. Permission from the publisher (Elsevier) is granted as set out in their Authors' and User Rights web document (https://www.elsevier.com/__data/assets/pdf_file/0007/55654/AuthorUserRights.pdf).
My supervisor, Ian Dunn, co-authored both papers, and both chapters include an indication of author contributions.

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Abstract

Heavily-selected livestock production traits rarely come without compromise; altered physiology arising from intensive selection often gives rise to concern of a welfare trade-off. A particularly clear example of welfare challenge caused by genetic selection in chickens is the 'broiler-breeder paradox', wherein breeding populations of broiler-type birds selected for fast growth are feed-restricted in order to reduce growth and maintain reproductive viability at sexual maturity. In order to better-inform management and breeding strategies for alleviating reproductive problems resulting from genetic selection for growth, it is essential to develop a better understanding of the physiological processes underpinning growth. Whereas the molecular mechanisms governing energy balance in mammals have been relatively well-described, analogous avian systems have not received as much research attention and remain somewhat poorly understood. The broad aim of this doctoral project was to contribute to understanding of avian energy balance, particularly in the context of selection for high growth.

Using an advanced broiler-layer intercross chicken line (AIL), high- and low-growth haplotypes at the locus encoding the cholecystokinin A receptor (CCKAR), underlying the most significant QTL for growth in chickens, were characterised. Of over 300 variations detected, a select panel spaced across the CCKAR locus were tested for prediction of bodyweight in a diverse cohort of chicken populations. One intronic SNP was found to be significant ($p<0.05$) and proximal to transcription factor binding sites. The effect of this locus on gross bodyweight remained significant into the 20th AIL generation (~20% at 10wk, $p<0.05$). In this otherwise effectively genetically homogeneous population, several specific physiological traits were predicted by CCKAR haplotype alone, yielding some clues as to the significance of perturbed cholecystokinin (CCK) signalling in broiler strains. While birds with high-growth CCKAR haplotype (HG) did not appear to consume more, feed conversion efficiency (FCE) was improved, at least for males, compared to low-growth (LG) ($p<0.05$). Visceral organ anatomies were morphologically disparate, with HG individuals exhibiting ~1/3 less gallbladder mass ($p<0.01$), and ~10% shorter GI tract ($p<0.01$) and metatarsal bone ($p<0.05$).

Further gaps in knowledge of the expression of peripheral satiety hormones in chicken are addressed in this thesis. Tissue distributions for expression of CCK, gastrin, pancreatic polypeptide (PPY) and peptide YY (PYY), were mapped and their respective dynamic responses to nutritive state examined. CCK was found to be most highly expressed in the brain, whereas PYY, PPY and gastrin were far more abundant in distinct regions of the periphery. Interestingly, peripheral CCK was not responsive to short-term (<10h) satiety in experimental populations where PYY and gastrin were. PYY expression was found to be greatest in the pancreas and consistently upregulated within hours after feeding ($p<0.01$), whereas gastrin expression was confined to the gastric antrum and paradoxically highest in fasting birds ($p<0.01$). PPY expression is strictly limited to the pancreas and appears dependent on longer-term energy state. These results highlight similarities and differences to mammalian systems; notably, the avian pancreas seems to fulfil an exceptional role as a site of signal integration, perhaps unsurprising considering its disproportionate size compared to mammals. Indeed, pancreatic PYY appears to act as a primary peripheral short-term satiety hormone in birds.

This body of work contributes to the understanding of avian energy balance and growth. An invaluable foundation for future research is formed by the identification of the major locations of production, and basic nutrient-responsive trends, for several peripheral avian hormones. Information on the growth role of CCKAR is consolidated and expanded upon, demonstrating a clear genetic contribution to maintenance organ

morphology and overall growth. Such knowledge can be used to reliably assess and advise on selection and management of chickens to stem welfare concerns without compromising production. Comparisons between avian and other vertebrate endocrine systems make for interesting insight into the adaptive role of energy homeostatic mechanisms in divergent evolution of mammals and non-mammalian vertebrates. In some aspects, birds might better represent the ancestral phenotype from which each vertebrate clade arose.

Lay Summary

Feed restriction of breeding meat-type chickens to maintain fertility is considered a welfare challenge and is necessary because of intense selective breeding for high growth. Refining management and breeding strategies for these animals could help reduce the welfare concern, but such an approach requires initial characterisation of the genetic causes of increased growth in meat birds. The aim of this project was to understand how increased growth is brought about by genetic selection and improve knowledge of hormonal control of bodyweight in birds.

Using a genetic hybrid line, the association of DNA sequence with bodyweight was assessed. The DNA region of interest encodes a satiety receptor which is known to be important in achieving increased growth in meat chickens. High- and low-growth individuals are genetically different at many genomic positions in this region, and one particular variation seems to explain a statistically significant difference in bodyweight. The physiological effect of this genetic difference is profound. High-growth birds do not consume more food, yet achieve greater bodyweight than low-growth individuals and have smaller gallbladders, shorter intestines and shorter leg bones.

The roles of satiety hormones in chicken are explored in this thesis; several were mapped to show relative expression in different bodily tissues and their response to feed intake was examined. The results highlight similarities and differences to mammalian hormone systems; for example, the avian pancreas seems to fulfil an exceptional role as a site of signal integration, perhaps unsurprising considering its disproportionate size compared to mammals.

This project forms an invaluable foundation for future research by characterising several avian hormones. Information on genetic selection for growth is consolidated, and a clear genetic contribution to internal organ size and overall growth is demonstrated. This knowledge can be used to reliably advise on management of chickens to improve welfare without compromising production. Comparing avian and mammalian hormonal mechanisms also gives insight into divergent evolution of vertebrate energy signalling.

Research Outputs

Original peer-reviewed articles

Reid, A. M. A., Wilson, P. W., Caughey, S. D., Dixon, L. M., D'Eath, R. B., Sandilands, V., Boswell, T. & Dunn, I. C. (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.

Reid, A. M. A. & Dunn, I. C. (2018) Gastrointestinal distribution of chicken gastrin-cholecystokinin family transcript expression and response to short-term nutritive state. *General and Comparative Endocrinology* **255**, 64-70.

Caughey, S.D., Dunn, I.C., Wilson, P.W., **Reid, A.M.A.**, Boswell, T., Mukhtar, N., Brocklehurst, S. & D'Eath, R.B. (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**, 20-31.

Published abstracts

Reid, A. M. A., Hocking, P. M., Wilson, P. W., Robertson, G. W. & Dunn, I. C. (2015) Understanding genetic selection for growth: the case of satiety in meat chickens. *British Poultry Abstracts* **11**, 10-11.

Reid, A. M. A., Wilson, P. W., Whenham, N. & Dunn, I. C. (2016) A logical approach to understanding physiological mechanisms underpinning growth efficiency in broilers explained by a single selected genetic locus. *British Poultry Abstracts* **12**, 11-12.

Conference papers

Reid, A. M. A., Hocking, P. M., Wilson, P. W., Robertson, G. W. & Dunn, I. C. (2015) Genetic selection for expression of a growth gene in chickens. Proceedings of The Genetics Society themed conference: Breeding for Bacon, Beer and Biofuels, Edinburgh, Scotland.

Reid, A. M. A., Wilson, P. W, Hocking, P. M. & Dunn, I. C. (2016) Decreased expression of the cholecystokinin A receptor (CCKAR) influences bodyweight but not

gross relative feed intake in chickens. Proceedings of the British Society for Neuroendocrinology Annual Meeting, Glasgow, Scotland.

Reid, A. M. A., Wilson, P. W., D'Eath, R. B. & Dunn, I. C. (2016) Is the pancreas a major source of peptide YY? Proceedings of the British Society for Neuroendocrinology Annual Meeting, Glasgow, Scotland.

Reid, A. M. A., Wilson, P. W., Hocking, P. M. & Dunn, I. C. (2016) Is bodyweight set point mediated by the cholecystokinin A-receptor (CCKAR)? Proceedings of the 11th Symposium on Avian Endocrinology (ISAE2016), Niagara-on-the-Lake, Canada.

Reid, A. M. A. & Dunn, I. C. (2017) Pancreatic Peptide YY (PYY) expression is responsive to short-term nutritional state and the pancreas constitutes the major site of PYY mRNA expression in chickens. Proceedings of the World's Poultry Science Association UK Branch Spring Meeting, Chester, England.

Reid, A. M. A. & Dunn, I. C. (2017) Tissue distribution and dynamic regulation of chicken peptide YY (PYY) expression. Proceedings of the 18th International Congress of Comparative Endocrinology (ICCE18), Lake Louise, Canada.

Reid, A. M. A., Wilson, P. W., Hocking, P. M. & Dunn, I. C. (2017) Selection for low cholecystokinin A receptor (CCKAR) expression in fast-growing broiler chickens. Proceedings of the Xth European Symposium on Poultry Genetics, St. Malo, France.

Krause, J. S., **Reid, A. M. A.**, Pérez, J. H., Bishop, V., Cheah, J. C., Wingfield, J. C. & Meddle, S. L. (2018) Hepatic corticosterone binding globulin (CBG) mRNA expression across life history stages in migratory and nonmigratory subspecies of white-crowned sparrow. Proceedings of the Society for Integrative and Comparative Biology Annual Meeting 2018, San Francisco, CA, USA.

Latin species, common names and vertebrate clades

Latin name	**Common name**	**Vertebrate clade**
Ailuropoda melanoleuca	Giant panda	Mammals
Alligator mississippiensis	American alligator	Reptiles
Alligator sinensis	Chinese alligator	Reptiles
Anas platyrhyncos	Mallard	Birds
Anolis carolinensis	Anole (Lizard)	Reptiles
Anser spp	Goose	Birds
Aotus nancymaae	Nancy Ma's night monkey	Mammals
Balaenoptera acutorostrata	Minke whale	Mammals
Bos mutus	Yak	Mammals
Bos Taurus	Cattle	Mammals
Bubalus bubalis	Water buffalo	Mammals
Calidris pugnax	Ruff	Birds
Callithrix jacchus	Common marmoset	Mammals
Calypte anna	Anna's hummingbird	Birds
Camelus bactrianus	Bactrian camel	Mammals
Camelus dromedarius	Dromedary camel	Mammals
Camelus ferus	Wild Bactrian camel	Mammals
Canis lupus	Gray wolf	Mammals
Carassius auratus	Goldfish	Jawed fishes
Castor canadensis	North American beaver	Mammals
Cebus capucinus	White-headed capuchin	Mammals
Cerocebus atys	Sooty mangabey	Mammals
Chelonia mydas	Green sea turtle	Reptiles
Chelonia spp	Turtle	Reptiles
Chlorocebus sabaeus	Green monkey	Mammals
Chrysemys picta	Painted turtle	Reptiles
Chrysochloris asiatica	Cape golden mole	Mammals
Colobus angolensis	Angolan colobus	Mammals
Condylura cristata	Star-nosed mole	Mammals
Coturnix japonica	Japanese quail	Birds
Cricetulus griseus	Chinese hamster	Mammals
Ctenopharyngodon idella	Grass carp	Jawed fishes
Cuculus canorus	Common cuckoo	Birds
Dasypus novemcinctus	Nine-banded armadillo	Mammals
Dipodomys ordii	Ord's kangaroo rat	Mammals
Echinops telfairi	Lesser hedgehog tenrec	Mammals
Elephantus edwardii	North African elephant shrew	Mammals
Equus asinus	Ass	Mammals
Equus caballus	Horse	Mammals
Equus przewalskii	Przewalski's horse	Mammals
Erinaceus europaeus	European hedgehog	Mammals
Falco cherrug	Saker falcon	Birds
Falco peregrinus	Peregrine falcon	Birds
Galeopterus variegatus	Sunda flying lemur	Mammals

Gallus	Red junglefowl (Chicken)	Birds
Gorilla	Western gorilla	Mammals
Heterocephalus glaber	Naked mole-rat	Mammals
Hipposideros armiger	Great roundleaf bat	Mammals
Homo sapiens	Human	Mammals
Ichthyomyzon gagei	Southern brook lamprey	Jawless fishes
Ictidomys tridecemlineatus	Thirteen-lined ground squirrel	Mammals
Jaculus	Lesser Egyptian jerboa	Mammals
Lepidothrix coronata	Blue-crowned manakin	Birds
Leptonychotes weddellii	Weddell seal	Mammals
Leptosomus discolor	Cuckoo Roller	Birds
Lipotes vexillifer	Baiji dolphin	Mammals
Lonchura striata	White-rumped munia	Birds
Loxodonta africana	African elephant	Mammals
Macaca fascicularis	Crab-eating macaque	Mammals
Macaca nemestrina	Southern pig-tailed macaque	Mammals
Manacus vitellinus	Golden-collared manakin	Birds
Mandrillus leucophaeus	Drill	Mammals
Marmota marmota	Alpine marmot	Mammals
Meleagris gallopavo	wild turkey	Birds
Meriones unguiculatus	Mongolian gerbil	Mammals
Mesocricetus auratus	Syrian hamster	Mammals
Microcebus murinus	Gray mouse lemur	Mammals
Microtus ochrogaster	Prarie vole	Mammals
Monodelphis domestica	Gray short-tailed opossum	Mammals
Mus caroli	Ryukyu mouse	Mammals
Mus musculus	House mouse	Mammals
Mus Pahari	Gairdner's shrewmouse	Mammals
Nannorana parkeri	Tibetan frog	Amphibians
Nannospalax galili	UGM blind mole rat	Mammals
Neomonachus schaunslandi	Hawaiian monk seal	Mammals
Nipponia nippon	Crested ibis	Birds
Nomascus leucogenys	Northern white-cheeked gibbon	Mammals
Numida meleagris	Helmeted guineafowl	Birds
Ochotona princeps	American pika	Mammals
Odobenus rosmaris	Walrus	Mammals
Odocoileus virginianus	White-tailed deer	Mammals
Orcinus orca	Killer whale	Mammals
Orycteropus afer	Aardvark	Mammals
Oryctolagus cuniculus	European rabbit	Mammals
Otolemur garnettii	Northern greater galago	Mammals
Pan pansicus	Bonobo	Mammals
Papio Anubis	Olive baboon	Mammals
Parus major	Great tit	Birds
Pavo cristata	Indian peafowl	Birds
Pelodiscus sinensis	Chinese softshell turtle	Reptiles
Peromyscus maniculatus	Deer mouse	Mammals

Petromyzon marinus	Sea lamprey	Jawless fishes
Phascolarctos cinereus	Koala	Mammals
Physeter catodon	Sperm whale	Mammals
Pogona vitticeps	Central bearded dragon	Reptiles
Propithecus coquereli	Coquerel's sifaka	Mammals
Rattus novergicus	Norway rat	Mammals
Rhinolophus sinicus	Chinese horseshoe bat	Mammals
Rhinopithecus bieti	Black snub-nosed monkey	Mammals
Rhinopithecus roxellana	Golden snub-nosed monkey	Mammals
Saimiri boliviensis	Black-capped squirrel monkey	Mammals
Sarcophilus harrisii	Tasmanian devil	Mammals
Schizothorax prenanti	Ya-fish	Jawed fishes
Sorex araneus	Common shrew	Mammals
Sturnus vulgaris	Common starling	Birds
Sus scrofa	Pig	Mammals
Synchiropus splendidus	Mandarinfish	Jawed fishes
Tupaia chinensis	Chinese tree shrew	Mammals
Tursiops truncatus	Bottlenose dolphin	Mammals
Vicugna pacos	Alpaca	Mammals
Xenopus laevis	African clawed frog	Amphibians
Xenopus tropicalis	Western clawed frog	Amphibians

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CHAPTER 1

Introduction

1 Introduction

1.1 Modern poultry farming and food security

By both number of animals killed and gross consumption, the domestic chicken outweighs any other tetrapod species in its contribution to global food production (Fao, 2008; Fao, 2014). Between 2010 and 2017, annual global poultry consumption was 29.4kg per capita (Oecd, 2017), which amounts to over 200 billion kilograms of meat consumed in total worldwide. Chickens account for over 80% of all poultry killed for human food consumption each year (Fao, 2014) and so represent a significant and valuable contribution to global food security, particularly considering the current "shift … to food consumption changes that favour increased proteins from animal sources in diets" (Oecd, 2017). The incredible scale of global poultry agriculture is difficult to appreciate, but might be brought sharply into focus with the objective conclusion that, at this moment in time, the chicken ostensibly constitutes Earth's dominant land vertebrate.

Chickens have a long history as a livestock species, first entering the domestication process over 8000 years ago (West & Zhou, 1989). The success of poultry as an economical source of high-quality nutrition has depended on global dispersal and selective breeding of strains with desirable production traits. Over the past century, highly coordinated artificial selection has resulted in modern commercial chicken strains which can be broadly divided into two categories; egg-type (or 'layers') and meat-type (or 'broilers'). These strains exhibit unprecedented production efficiency compared to their primary ancestral species, the red junglefowl (*Gallus gallus*) (Jackson & Diamond, 1996). Egg-type strains have undergone intensive selection for egg production, with modern lines routinely producing over 300 eggs per bird per year in lay (Hy-Line, 2016a; Hy-Line, 2016b; Lohmann, 2017). Aside from gross production, specific favoured egg traits are also selected, for example: size, shell and yolk colours, breaking strength and chemical composition (Wolc *et al.*, 2011). Meat-type birds have been selected for very fast growth and high bodyweight achievement,

now routinely achieving an as-hatched average market weight of 2kg in less than 35 days (Aviagen, 2015; Cobb, 2015).

1.2 Genetic selection for growth in meat-type chickens

1.2.1 Progress

Meat-type chickens represent an incredibly successful example of selective breeding for growth in a livestock species. Intensive selection for high bodyweight and quick growth has resulted in commercial broiler birds whose growth trajectory, metabolic phenotype and physiological composition scarcely resemble those of the ancestral junglefowl (Jackson & Diamond, 1996; Paxton *et al.*, 2010). Subject to identical rearing conditions with feed provided *ad libitum*, broilers accumulate on average over 400% the body-mass of commercial layer strains and traditional breeds within the first six weeks of life (Sandercock *et al.*, 2009).

1.2.2 Associated welfare problems

Improvement of growth phenotype by means of raw selection for few phenotypic traits does not generally come without compromise. As growth potential of broiler strains has increased, so has the incidence of negative physiological outcomes associated with high growth. Often these manifest as health and welfare issues which challenge the life quality of broiler birds and hence the ethicality of modern poultry meat production.

1.2.2.1 Metabolic complications

Intensively-reared broiler chickens are charged with maintaining hyperactive metabolism to achieve desired weight gain in a short space of time. However, with the necessary genetic selection for very fast growth, and development of modern feed materials to facilitate it, some physiological systems required to support such a change in metabolic phenotype are put under excessive strain, leading to disease. These include cardiovascular and respiratory components, the relatively reduced size

of which leads to hypertension, and long-term hypoxia (Julian, 1998). Cardiomyopathy is also implicated in sudden death syndrome, a common threat to economic production. Broilers are particularly susceptible to heat prostration due to their high metabolic rate, particularly during transport, and this challenges welfare and affects meat quality since affected birds experience heat stress (Mitchell & Kettlewell, 1998). Ascites syndrome – caused by high blood pressure and liver damage, and characterised by fluid accumulation in the abdominal cavity – threatens product quality and economic production, as well as welfare since affected birds suffer tissue damage and struggle to breathe normally (Julian, 1993). Evidence suggests that metabolic disorders are caused by composition of feed or feeding strategy – not necessarily aberrant genetic traits – and it seems possible to alleviate some problems without affecting genetic growth potential (Julian, 1998; De Los Mozos *et al.*, 2017). Understanding anatomical differences causing and resulting from increased growth, as well as endocrine signalling affecting metabolism, might inform alterations in broiler management.

1.2.2.2 Musculoskeletal abnormalities

A widely acknowledged welfare concern in modern meat-type chickens is the disparity between enhanced body mass and relative musculoskeletal integrity, and the resulting inability of heavy birds to support themselves in normal locomotion. Clear problems exist in the locomotive abilities of broilers; abnormal gait and balance problems are regularly reported on (Knowles *et al.*, 2008; Paxton *et al.*, 2013; Duggan *et al.*, 2015; Alves *et al.*, 2016). Part of the problem likely lies in the muscular architecture of broiler chickens, which seems biased toward generating breast muscle mass at the relative expense of supportive pelvic limb musculature (Paxton *et al.*, 2010). Although the reported reduction in pelvic limb muscle mass in broilers compared to junglefowl is small in relative terms (Wall & Anthony, 1995; Paxton *et al.*, 2010), the forward shift of mass resulting from increased breast muscle growth likely exacerbates the situation by contributing to poor balance (Corr *et al.*, 2003; Duggan

et al., 2015) and possibly by changing the direction of strain experienced by the pelvic musculature; hunched stature is widely recognised in broilers and also indirectly contributes to ascites syndrome (Julian, 1998).

Further specific skeletal problems commonly arise in broilers. Tibial dyschondroplasia sees abnormal cartilage growth, causing erratic joint form and abnormal stature (Riddell, 1975). It seems logical that observed bone fractures (Thorp, 1994) occur due to a combination of the aforementioned balance, gait and load problems together with bone deformity, weight-impaired flight and genetic propensity for weaker bones in heavy birds (Duggan *et al.*, 2015). Bone traits generally exhibit high genetic heritability in chickens (Bishop *et al.*, 2000; Whitehead, 2007; Mignon-Grasteau *et al.*, 2016) and so could be included as a selection factor in commercial broiler breeding programmes, however measurement often requires culling and the locomotive problems and some of the health infringements associated with musculoskeletal abnormality do not correlate well with bone strength and so are not simultaneously addressed by this strategy.

1.2.2.3 The broiler breeder paradox

The popularity of chicken meat discussed in section 1.1 demands a global industry which produces in excess of 50 billion birds each year (Fao, 2014). Of course, each of these birds must hatch from an egg produced by parents with the correct genetic makeup to confer desirable growth traits. The problem is that these parent birds must survive and remain healthy into sexual maturity (approximately six months of age), and then remain healthy to produce high-quality offspring. Broiler growth phenotypes are not conducive to good ongoing health in long-lived birds, since fast weight gain continues after normal slaughter age to produce very large individuals with amplified health problems. Broiler breeder males seem to have genetic propensity for aggression, particularly under an *ad libitum* feeding regime, and this interferes with successful copulation (Millman *et al.*, 2000). Females often develop polyfollicular ovaries with perturbed ovarian hierarchy, leading to internal ovulations and production

of inviable eggs (Hocking *et al.*, 1989). Both sexes experience adverse health in overweight condition and complications associated with the aforementioned health problems (and others) increase flock mortality (Julian, 1998; Hocking & Robertson, 2000; Millman *et al.*, 2000). The solution currently employed by broiler breeding operations is to slow growth by restricting feed intake, which restores healthy development (Hocking *et al.*, 1989; Savory *et al.*, 1993; Mench, 2002) but is considered a welfare challenge and generates industry disapproval among welfare charities and pressure groups. The dilemma between allowing birds to eat as much as they choose to, and lowering welfare standard by forcibly manipulating their feed intake is known as the broiler-breeder paradox.

1.3 Energy balance as a selection target

1.3.1 Evolutionary perspective

For members of any eukaryotic species to employ successful survival and propagation strategies, some degree of control of energy resource is required. In multicellular species, not every cell type can liberate (catabolise) or store (anabolise) energy from external sources, yet all cell types require energy to function, and so mechanisms to store and distribute energy are vital for organismal survival. Plants and algae hoard light-derived energy within polysaccharide molecules for future use, and often change their physiology dramatically to suit prevailing seasonal and immediate environmental conditions. Fungi store excess energy as triacylglycerides, and the amount of stored energy is intricately linked to metabolic functions and respiratory strategy (Jain *et al.*, 2016). Animals represent the clade with the most complex energy control needs. Unlike most other eukaryotes, animals most often actively seek and ingest energy sources (food), hence requiring considerable energy even before it has been encountered. In fact, eating is one of many complex behaviours which set animals apart from other eukaryotes. These diverse behaviours include but are not limited to: mating, sociality, locomotion and structure building, and

all require investment of energy in anticipation of strategic reward. Investment of energy in this way demands careful orchestration of energy sensing, storage and expenditure in response to environmental cues. The overall control of anabolic and catabolic cellular processes, choice and consumption of food and energy investment in growth, behaviour and physiological processes is known as energy homeostasis or energy balance. The relative complexity of energy homeostasis in animals compared to other biological clades resonates in the development of complex behaviours and the neural organisation to support those behaviours. There does however exist great intraspecific and considerable interspecific diversity within the animal kingdom in terms of strategy, behaviour and associated physical phenotypes (e.g. growth) and these attributes are dependent to some extent on genetic complement.

Changes in body size, growth potential and energy balance in domestic animal species result from artificial selection for genetic traits which confer desirable phenotypic effects in the organism. In recent decades, advances in the understanding of genetics and selective breeding have prompted efforts to elucidate the underlying genetic loci responsible for selected traits in chickens. Understanding the molecular causes of desirable and undesirable traits allows reduction of genetic impurities in breeding populations and improved production. Several genome-wide association studies have been performed in broiler chickens

1.3.2 Avian energy homeostasis

1.3.2.1 Overview

Vertebrate energy homeostasis depends on a combination of short-term governance of meal size and pattern and longer-term management of stored energy, leading to maintenance of an optimal bodyweight (Boswell, 2005; Speakman *et al.*, 2011; Speakman, 2014). In all vertebrates, acute control of feed intake depends on hormonal signals to convey information about the physiological state of the gut to the brain and appropriately affect behaviour. It is also important that information is fed back from the brain to peripheral effector organs, to prime the gut for efficient digestion

and nutrient uptake (Stanley *et al.*, 2005; Bowen, 2006; Speakman, 2014), and so signalling within the brain directs energy intake and global metabolism (Akieda-Asai *et al.*, 2014; Lopez *et al.*, 2016). Homeostatic energy signalling can be broadly divided into central and peripheral aspects. By virtue of the experimenting species' taxonomy, mammalian systems are generally far better understood than avian counterparts. However, in birds, as in all vertebrate clades, neuropeptides in the brain orchestrate energy homeostasis using the very highly-conserved central melanocortin system to integrate energy signalling from the periphery (Boswell, 2005; Song *et al.*, 2013; Tachibana & Tsutsui, 2016; Honda *et al.*, 2017). Likewise, a variety of hormones are secreted from cells in the gut and peripheral organs in response to feeding and hunger, to relay information about energy availability, with many pathways conserved between mammals and birds, but some key differences (Boswell, 2005; Kaiya *et al.*, 2009; Seroussi *et al.*, 2016; Honda *et al.*, 2017).

1.3.2.2 Peripheral energy signalling

Broadly speaking, endogenous peripheral energy signals can be classified by their functional effect and the energy-responsive mechanism from which they arise. Functionally, orexigens are molecules which stimulate energy intake and anabolism whereas anorexigens produce catabolic effects and curb appetite. Response to nutrient presence or absence produces short-term anorexigens/orexigens, respectively, whereas response to bodyweight and/or composition produces long-term anorexigens. A myriad of peripheral orexigens and anorexigens exist in vertebrates. The following sections give a brief overview of some of these, with focus on the molecules discussed in later chapters and current understanding of their respective actions in birds.

1.3.2.2.1 Peripheral orexigenic factors

The most prominent known peripheral orexigenic factor in mammals is the peptide hormone ghrelin. Ghrelin is released from the mammalian stomach to signal negative energy balance (hunger). Its production is upregulated in absence of gastric contents

(short-term control) and down-regulated by adipostatic leptin and glucostatic insulin, the major long-term anorexigenic signals in mammals (Asakawa *et al.*, 2001). In birds, the action of ghrelin is under debate (Kaiya *et al.*, 2009; Kaiya *et al.*, 2013) and it seems unlikely that endogenous avian ghrelin is a reliable orexigen. It has recently been proposed that the thyroid-derived hormone triiodothyronine (T3) is a better candidate for peripheral orexigenic signalling in avian species, along with a potential role for gastrointestinal distention (Boswell & Dunn, 2017).

1.3.2.2.2 Peripheral anorexigenic factors

Cholecystokinin (CCK) is a vertebrate short-term satiety signal released postprandially from luminal i-cells in the proximal small intestine. In birds, as in mammals, CCK acts at the cholecystokinin A receptor (CCKAR) on afferent vagal fibres, and possibly as an endocrine molecule, signalling energy intake to the brain and inducing digestive activity (Chandra and Liddle, 2007, Song et al., 2013). Release of pancreatic enzymes and bile from the gall bladder which facilitate digestion of feed material is mediated by the direct action of CCK at target organs, and intestinal motility and gastric emptying are also regulated by release of CCK from the gut (Rodriguezmembrilla et al., 1995, Martinez et al., 1993). CCK signalling via CCKAR is known to affect feeding rate across several vertebrate species (Heldsinger et al., 2012, Takiguchi et al., 1997) including chickens (Dunn *et al.*, 2013a). The implications of CCK signalling in energy homeostasis are further discussed in Chapters 3 and 5.

In mammals, CCK acts in synergy with the adipostatic hormone leptin, to inform longer-term feeding behaviour and metabolism, in ambition of optimal bodyweight (Caquineau et al., 2010, Speakman et al., 2011). After decades of uncertainty an avian leptin homolog was recently confirmed to exist (Friedman-Einat *et al.*, 2014; Friedman-Einat & Seroussi, 2014; Prokop *et al.*, 2014), but leptin signalling does not seem to function as a long-term anorexigen in birds as it does in mammals (Friedman-Einat and Seroussi, 2014, Sharp et al., 2008).

Glucagon-like peptide 1 (GLP-1) is another enteroendocrine hormone which postprandially signals short-term energy intake to the pancreas, promoting insulin

production. Similair GLP-1 function is conserved in aves, since metabolic modulation (Tachibana *et al.*, 2007) and feed intake (Furuse *et al.*, 1997) have been evidenced in chickens.

Insulin is known to be a prominent regulator of energy balance across vertebrate taxa. Released from the pancreas in relation to blood glucose concentration, insulin signals to the brain to promote anabolism by downstream increase in glucose absorption and metabolism and also to reduce feed intake (Smit *et al.*, 1998). This function is conserved in birds, as insulin has been shown to affect central regulation of glucose homeostasis and suppress feeding (Honda *et al.*, 2007; Shiraishi *et al.*, 2008b; Shiraishi *et al.*, 2008a).

Pancreatic polypeptide (PP) is a further pancreas-derived hormone which acts to regulate pancreatic endocrine and exocrine secretion when administered centrally in chickens (Denbow *et al.*, 1988). Exogenously-administered PP also acts centrally to reduce food intake and promote catabolism in mammals (Ueno *et al.*, 1999; Batterham *et al.*, 2003), but it is unclear whether this reflects an endogenous role in either clade. The closely-related peptide YY (PYY) is released postprandially from the gut in mammals and acts to shift metabolic balance toward catabolism (Mcgowan & Bloom, 2004; Holzer *et al.*, 2012). The first evidenced avian PYY gene sequences became available very recently (Aoki *et al.*, 2017; Gao *et al.*, 2017; Reid *et al.*, 2017). Early genetic work and structural peptide differences suggest some discordance between the respective roles of avian and mammalian PYY, as discussed in Chapter 4.

1.3.2.3 Integration of energy signals by the central melanocortin system

The role of the central melanocortin system in avian energy homeostasis revolves around integration of incoming energy-signalling factors, namely those discussed in section 4.3.2.2, to instigate appropriate downstream responses. Peripheral orexigenic and anorexigenic signals are transduced to the brain either directly in the circulation (with factors diffusing or being actively transported across the blood-brain

barrier), or by stimulation of vagal afferent fibres innervating the locality of signal production (Boswell, 2005; Dockray, 2009; Zhang & Ritter, 2012; Dockray, 2013). Signals acting at the afferent vagus determine vagal synaptic output at hindbrain neurones in the nucleus of the solitary tract (NTS) which relay (an)orexigenic signals for projection to the hypothalamus (Date *et al.*, 2006; Grill & Hayes, 2012). This explains the results of an earlier mammalian neural experiment which involved disruptive knife-cuts between the hindbrain and hypothalamus (Kirchgessner & Sclafani, 1988).

Our understanding of vertebrate energy homeostatic signalling has primarily been achieved through study of mammals, however many aspects appear to be echoed in the current looser understanding of avian energy homeostasis (Song et al., 2013; Honda et al., 2017). For example, the central integration of energy signals seems to be maintained across mammals and birds.

Incoming orexigenic and anorexigenic signals have opposite effects on the balance of activity of two first-order neuronal species in the arcuate (or 'infundibular') nucleus (ARC) of the hypothalamus. Here, neurones co-expressing cocaine and amphetamine-regulated transcript (CART) and proopiomelanocortin (POMC) are stimulated by anorexigenic factors (e.g. CCK, GLP-1, PYY, insulin, leptin). This neuronal species exerts a downstream catabolic effect on body-wide energy balance by releasing the anorexigenic POMC gene product alpha melanocyte stimulating hormone (α-MSH) to signal to second-order effector neurones in the paraventricular nucleus (PVN) and lateral hypothalamic area (LHA) (among others), from where onward control of metabolism and innate behaviour is orchestrated. The opposing first-order neuronal species co-expresses agouti-related peptide (AGRP) and neuropeptide Y (NPY). These anabolic neurones are stimulated by orexigenic factors (e.g. ghrelin) and repressed by anorexigenic factors (e.g. CCK, PYY, insulin and, in mammals, leptin), converse to CART/POMC neurones. When stimulated, AGRP/NPY neurones oppose the catabolic signal of CART/POMC neurones in three major ways. Firstly, secreted NPY (acting at PVN/LHA Y_2 receptors) has a

functionally opposite effect to that of MC4R-mediated α-MSH on second order effector neurones. Secondly, secreted AGRP competitively antagonises binding of α-MSH at MC4R in the PVN. And thirdly, to cement their opposition to catabolic first-order neuronal action, AGRP/NPY neurones also synapse directly onto CART/POMC neurones and inhibit their signalling by hyperpolarisation via NPY (at Y_1) and GABA (at $GABA_BR$) (Roseberry *et al.*, 2004). The consequence of stimulation of AGRP/NPY neurones is therefore altered metabolism (toward energy conservation) and behaviour (e.g. increased feed intake). As well as projecting peripheral signals to the ARC, the NTS also relays efferent information from second-order effector neurones in the PVN and LHA, among others, to direct digestive functions and behaviour dependent on nutritive state (Furukawa & Okada, 1992). A schematic summary of the avian central melanocortin system interactions most pertinent to energy homeostasis is shown in Figure 1.1.

The bodyweight achieved when an animal successfully maintains long-term energy homeostasis is referred to as the bodyweight setpoint. Though this is clearly a simplistic view of the function of energy homeostasis, the concept of a bodyweight setpoint is useful when considering responses to positive and negative energy balance. On one hand, behavioural and physiological changes are predicted by relative deviation from the bodyweight setpoint and, reciprocally, alteration of homeostatic control of energy can be explained as a shift in bodyweight setpoint.

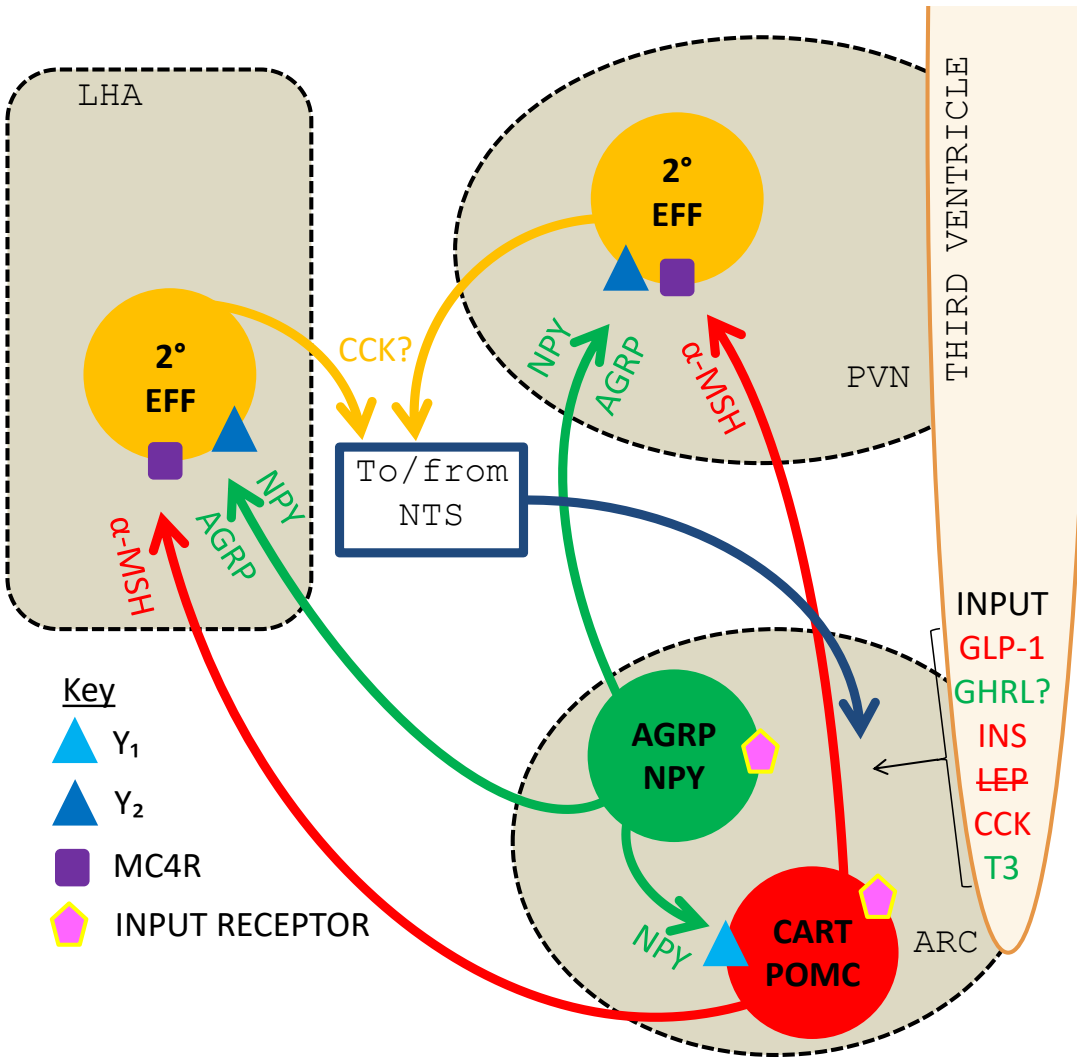


Figure 1.1 – Central melanocortin system dynamics
The major signalling interactions of the central melanocortin system are depicted. Catabolic input molecules are shown in red. Anabolic input molecules are shown in green. Inputs are from circulation (ventricle) or hindbrain (nucleus of the solitary tract; NTS).
When catabolic input outweighs anabolic input at the arcuate nucleus (ARC), catabolic CART/POMC neurones project α-MSH to second-order effector neurones (2°EFF) to stimulate MC4R. 2°EFF project appropriate catabolic signal back to NTS (among other areas), effecting appropriate physiological/behavioural response.
Upon stimulation by anabolic input, AGRP/NPY neurones are activated. Projected AGRP antagonises α-MSH at MC4R. NPY inhibits CART/POMC neuronal activity via Y_1 and stimulates anabolic onward signal from 2°EFF, which is relayed back to the NTS to appropriately alter behaviour/peripheral functions.
The roles of leptin (LEP) and ghrelin (GHRL) in birds are under debate.

1.4 Project hypotheses, design and aims

The artificial genetic selection of broiler chickens for improved growth has shifted their bodyweight setpoint. Such alterations have achieved unprecedented production efficiency but are also associated with negative welfare and economic implications. Delineation of the molecular control of avian energy homeostasis, and how this has been affected by selective breeding, therefore seems pertinent in achieving optimal management strategies for birds used in agricultural commerce. In order to contribute to the understanding of avian energy balance, this project has investigated the regulation and functions of several molecular factors which act to control it. The chapters within this thesis tackle interrelated gaps in the knowledge of avian energy balance and discuss its relevance to the poultry industry.

1.4.1 Consequences of selection at CCKAR (Chapter 3)

One general hypothesis of this project is that such a shift results from inadvertent targeting of genetic loci affecting molecular control of energy balance. Specifically, a candidate gene within the largest chicken genomic QTL for growth, CCKAR, encoding the cholecystokinin A receptor, has previously been identified as a likely historical selection target. Haplotype at this locus explains ~20% difference in bodyweight, and meat-type birds are known to exhibit lowered CCKAR expression, but the genetic basis of this difference is not known, nor are the physiological effects which result in increased growth. It was proposed that finer mapping of the locus would allow identification of the regulatory element(s) affecting CCKAR expression. The first aim of the work in Chapter 3 was therefore to characterise genetic variation at the CCKAR locus and examine association of variants with growth traits. The employed approach involved definition of alternative fixed high and low growth-associated haplotypes for the locus of interest. Identified variations were then genotyped in a diverse population of chicken lines and their association with growth traits analysed to determine candidate causative loci. An additional hypothesis was that it might be possible to infer the physiological mechanism for improved growth by observation of the

anatomical and behavioural phenotypes predicted by each haplotype. Efforts were therefore made to detect major behavioural and physiological differences related to energy intake in an advanced (F_{19}-F_{20}) broiler-layer intercross population, which would enable determination of effects predicted by local markers in an otherwise comparable genetic background.

1.4.2 Peripheral PP-fold hormone characterisation (Chapter 4)

Presented as a published peer-reviewed paper alongside additional subsequent work, Chapter 4 reports pioneering progress in the elucidation and characterisation of the chicken PYY gene and its peptide product, along with that of the closely-related PPY gene (encoding PP). It was first suggested that public databases could be mined using the known chicken PYY peptide sequence in order to determine a putative mRNA sequence, which could then be evidenced and used to characterise expressional regulation. We hypothesised that PYY – a known mammalian satiety factor – would be expressed in the gut, and up-regulated in response to feeding. Several experimental feeding conditions were therefore employed to assess the effect on PYY expression under long- and short-term hunger and satiety. Analysis of pancreatic PP mRNA was also included, so that these closely-related peptide hormones could be compared.

1.4.3 Peripheral gastrin-CCK hormone characterisation (Chapter 5)

Chapter 5 reports the distribution of gastrin-CCK family hormone expression and responsiveness of these genes to short-term hunger and satiety. Because these genes are known to be implicated in energy control, it was hypothesised that their transcripts would have distinct patterns of peripheral expression. Earlier immunological studies of gastrin-CCK family members are uncertain because of potential antibody cross-sensitivity. We therefore set out to map distribution by targeted detection of divergent mRNA regions. It was also hypothesised that

expression would change in response to nutrient intake, so a short-term fed/fasted study was carried out, with expression measurements by qPCR.

CHAPTER 2

Materials and methods

2 Materials and Methods

2.1 Kits and reagents

Appendix 1 contains details for all kits and non-standard reagents.

Details and applications of all oligonucleotide primers and probes used can be found in Appendix 2.

Where possible, reactions for multiple samples were prepared using master mixtures of common reagents before distribution.

All water (H_2O) was type 1 ultrapure (Milli-Q) or type 2 pure (Elix) unless otherwise stated.

2.2 Nucleic acid handling

2.2.1 Genomic DNA (gDNA) preparation

Genomic DNA (gDNA) was prepared from blood. 5µl fresh whole blood was mixed with 300µl DNAzol reagent. After >0.5h incubation at room temperature, 150µl 100% isopropanol was added and mixed by inversion to precipitate the gDNA over a 5-minute room temperature incubation period. DNA was then pelleted in a microfuge at >8,000x*g* before aspiration of the supernatant. The pellet was subsequently washed twice in 70% ethanol, with centrifugation and aspiration after each wash. The resultant gDNA pellet was air-dried in a fume hood for ≥20min before addition of 350µl H_2O and resuspension by gentle agitation at 50°C for ≥1h.

2.2.2 Polymerase chain reaction (PCR)

2.2.2.1 Primer design

All primers were designed using the European Life-sciences Infrastructure for Biological Information (ELIXIR) Primer3 (Koressaar & Remm, 2007; Untergasser *et al.*, 2012) web form (http://primer3.ut.ee/) with appropriate source sequence and default settings except where amplicon size and targeting directions were applied. Selected primer pairs were checked for expected targeting and fidelity using the *in silico* PCR tool of the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgPcr). Details of all primers used in this project can be found in Appendix 2.

2.2.2.2 Reaction conditions

Normal polymerase chain reactions were performed using FastStart Taq polymerase (Roche, Basel, Switzerland) and the supplied buffers as directed by the manufacturer, but with separately prepared 10X dNTP mix (Thermo Fisher Scientific, MA, USA). The standard protocol employed 2µl 10X FastStart buffer with 20mM $MgCl_2$, 2µl 10X dNTP mix, 0.5µl 20µM forward primer, 0.5µl 20µM reverse primer, 0.1µl Taq and 1-2µl template in a final reaction volume of 20µl. Standard thermal conditions were: 95°C for 240s, 40 cycles of (95°C for 30s, 58°C for 30s, 72°C for 30s), 72°C for 420s. Some normal PCR setups were variations on this protocol, as indicated in future sections.

2.2.2.3 Product resolution and visualisation

PCR products to be visualised were <1.5kb and were separated by electrophoresis in 1.5-3% (w/v) agarose in 1X TAE gels containing 0.0001% (v/v) SYBR Safe DNA gel stain (Thermo Fisher Scientific, MA, USA), alongside marker lanes containing Quick-Load 100bp DNA ladder (New England BioLabs Inc., MA, USA). Gels were subsequently visualised by standard blue or UV light transillumination.

2.2.2.4 Product purification

PCR products embedded in agarose gel matrices were individually excised and purified with the QIAquick PCR Purification kit (Qiagen NV, Hilden, Germany) to manufacturer's guidance and eluted in 50µl H_2O.

Prior to sequencing, PCR products were routinely directly purified using a reaction mix containing exonuclease I (ExoI) to degrade single-stranded nucleic acids and shrimp alkaline phosphatase (SAP) to dephosphorylate individual nucleotide residues, rendering them unable to be incorporated into newly-synthesised DNA strands during subsequent sequencing reactions. Both enzymes were procured from New England BioLabs Inc. (MA, USA). To each 10µl PCR product, 0.6µl ExoI, 1.2µl SAP and 1.2µl H_2O were added to give a total reaction volume of 13µl. Preparations were incubated at 37°C for 15min for enzymatic degradation then 80°C for 15min to heat-inactivate both enzymes.

2.2.3 Genotyping

2.2.3.1 CCKAR_MnlI RFLP

Standard restriction fragment length polymorphism (RFLP) SNP genotyping of the CCKAR locus employed MnlI to distinguish between cytosine (MnlI_1) and thymine (MnlI_3) at genomic position galGal5:chr4:73,698,953 (AACCT[C/T]GTTGC), as previously described (Dunn et al., 2013a). Standard PCR (section 2.2.2.2) was employed with primers CCKAR_F3 and CCKAR_altR3 (see Appendix 2) and gDNA template (section 2.2.1) to amplify the genomic region galGal5:chr4:73,698,857-73,699,178 and successful amplification confirmed by standard visualisation (section 2.2.2.3). 10µl crude PCR product was then mixed with 1µl NEBuffer 4 (New England BioLabs Inc., MA, USA), 1µl 0.2% (w/v) bovine serum albumin (BSA), 8ul H_2O and 1.25U MnlI (New England BioLabs Inc., MA, USA) and digested at 37°C for >4h. Digestion products were electrophoresed and visualised (section 2.2.2.3) to determine genotype. MnlI_1 restriction fragment lengths are 43bp, 97bp and 182bp.

MnlI_3 restriction fragment lengths are 140bp and 182bp. Heterozygotes (MnlI_2) exhibit all restriction fragments.

2.2.3.2 DelinvA ALP

Standard amplicon length polymorphism (ALP) genotyping of the CCKAR locus exploited a segregating 136bp genomic deletion at galGal5:chr4:73,708,673-73,708,808. Standard PCR as described in section 2.2.2.2 was employed with primers CCKAR_delinvA_genoF and CCKAR_delinvA_genoR (see Appendix 2) and gDNA template (section 2.2.1), except that FastStart buffer without $MgCl_2$ was used and 2µl 25mM $MgCl_2$ included in the final volume (20µl) and the reaction annealing temperature was adjusted to 68°C. The genomic region galGal5:chr4:73,708,646-73,709,045 was amplified and products were resolved by standard electrophoresis and visualised (section 2.2.2.3). Genotype DelinvA_1 amplicon length is 262bp. Genotype DelinvA_3 amplicon length is 400bp. Heterozygotes (DelinvA_2) exhibit both amplicons.

2.2.3.3 *Molecular sexing*

Sexing was by duplex PCR using gDNA template, and based on a published protocol (Clinton *et al.*, 2001). Each reaction contained 0.4µl 10µM primer W3, 0.4µl 10µM primer W5, 0.5µl 10µM primer R1, 0.5µl 10µM primer R2, 1.5µl 10X dNTP mix, 1.5µl 10X FastStart buffer w/20mM $MgCl_2$, 3µl 5X BB-sucrose solution, 0.75µl DMSO, 0.075µl Faststart taq, 4.375µl H_2O and 2µl gDNA template (see section 2.2.1) in a total volume of 15µl. Thermal conditions were: 94°C for 120s, 30 cycles of (94°C for 10s, 50°C for 15s, 72°C for 20s), 72°C for 300s. PCR products were separated by gel electrophoresis (2% agarose) and visualised as described in section 2.2.2.3. See Appendix 2 for primer details.

2.2.3.4 Outsourced genotyping

Assays for genotyping of the Multistrain (section 2.3.2) for forty variations spread approximately evenly across the sequenced CCKAR region were designed and executed externally by LGC (Middlesex, UK). Chosen variations were identified as segregating in the AIL and associated with the MnlI_1/DelinvA_1 fixed haplotype (i.e. invariant between MnlI_3/DelinvA_3 and galGal4 reference haplotypes). Genomic sequence flanking 50bp either side of each target variation was provided, along with alternative bases for both known alleles. Multistrain gDNA samples were already held by LGC, having been provided by Graeme Robertson (Roslin Institute, Midlothian, Scotland) some months earlier.

2.2.4 Complementary DNA (cDNA) preparation

2.2.4.1 RNA purification

Tissue samples of 40-100mg were homogenised in 1ml Trizol reagent (Life Technologies, Paisley, Scotland) prior to RNA purification and kept chilled throughout. Homogenisation was in 2ml tubes, either by bead beating with 400µl Lysing Matrix D ceramic beads (MP Biomedicals, CA, USA) in the FastPrep-24™ 5G Instrument (MP Biomedicals, CA, USA), or directly with the Ultraturrax T10 homogeniser (IKA-Werke GmbH & Co. KG, Staufen im Breisgau, Germany) with 3x brief H_2O rinses between samples. Total RNA was purified from Trizol homogenate using the Direct-zol RNA MiniPrep kit (Zymo Research Corp., CA, USA) according to the manufacturer's protocol. A standardised 250ul cleared lysate was used for each preparation and remaining Trizol homogenate was stored at ≤-20°C for future use. To remove gDNA contamination, in-column DNase digestion was routinely performed using the supplied DNase treatment materials and protocol. RNA was eluted in 50µl H_2O, quantitated by Nanodrop (Thermo Fisher Scientific, MA, USA) and stored at -70°C until use.

2.2.4.2 Reverse transcription

RNA was reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems Corp., CA, USA) according to the manufacturer's instructions in 20µl reactions. An equal mass of total RNA was used for each sample in any one set; 1µg as standard, though use of a lesser amount was necessary for some sample sets, depending on eluted RNA concentrations. Recovered cDNA products were diluted with 90µl water, to a final volume of 110µl.

2.2.5 Quantitative polymerase chain reaction (qPCR)

2.2.5.1 Primer design

Primer pairs for qPCR assays were designed as described in section 2.2.2.1. The full or known fragment mRNA sequence for the gene of interest was used as the input, and amplicons were limited to 100-300bp where possible, with an acceptable range of 50-500bp. Default settings were used for all other primer chemistry preferences.

Pairs were preferentially designed so that at least one primer spanned an exon-exon boundary to prevent amplification of contaminating gDNA. Where this was not possible, primer pairs were designed so that each primer annealing site corresponded to a different exon, to prevent exponential amplification of gDNA and make substantial gDNA amplification detectable upon scrutiny of the dissociation curve (see section 2.2.5.5).

2.2.5.2 Primer validation

Primer pairs were validated by PCR using the standard FastStart method (section 2.2.2.2) and a mock qPCR reaction based on the standard SYBR green mix method (section 2.2.5.4). Products for each gene were electrophoresed simultaneously using standard agarose gel electrophoresis (section 2.2.2.3) to generate base material for standard curve generation, demonstrate good amplification under qPCR reaction

conditions and ensure good fidelity and matching amplicon size. When several primer pairs were tested for a single gene, all reactions were prepared and products electrophoresed together to help select the best pair by examination of band intensity. All qPCR standard stock products were sequenced to confirm identity (section 2.2.8).

2.2.5.3 Standard curve generation

Selected visualised FastStart amplicon bands indicating good amplification and target specificity were excised and purified as described in section 2.2.2.4. Concentrations were measured by Nanodrop (Thermo Fisher Scientific, MA, USA). Eluted products were diluted 1/500 to give a working stock (stdA), then in a 10-fold series, giving a set of standards (std1-std*n*) for inclusion in qPCR assays to enable extrapolation for unknown sample concentrations.

Standard quantities were calculated using the following equation:

$$x = \frac{v\left({}^{c}/_{d}\right)}{660l}$$

x = standard quantity (nmol.well^{-1})

v = volume of standard used per well (µl)

c = concentration of stock product (ng.µl)

d = stock dilution factor (e.g. 5000 for std1)

l = length of amplicon (bp)

Note the constant 660 is the approximate average molecular weight of DNA in g/mol.

2.2.5.4 Reaction conditions

qPCR reactions were prepared in 96-well plates and employed Brilliant III Ultra-fast SYBR Green qPCR Mastermix (Agilent Technologies, CA, USA). For each sample to be quantitated, 10µl Mastermix, 0.3µl 1/500 diluted ROX reference dye (supplied), 0.4µl 20µM forward primer, 0.4µl 20µM reverse primer and 0.9µl H_2O were combined

in a well before addition of 8µl diluted cDNA sample (see section 2.2.4.2) to give a total reaction volume of 20µl. cDNA sample was substituted for 8µl appropriate standard in sufficient wells to give a standard curve with a minimum of six points in triplicate. Plates were run in the Mx3005p qPCR System (Agilent Technologies, CA, USA).

2.2.5.5 Data output and manual quality control

MxPro software (Agilent Technologies, CA, USA) calculated the number of moles of target cDNA copies per well by extrapolation from the standard curve input values. Several manual routine interpretive checks were carried out for qPCR amplification data within the MxPro program. Standard curve data were validated by scrutiny of amplification plots to ensure even spacing of sequential standards and tight agreement of replicates. Visibly outlying replicates or series were removed. The coefficient of determination (R^2) for standard fluorescence values log-plotted against their known concentrations was assessed to ensure adequacy (≥0.99). Amplification efficiencies of between 90-110%, as calculated by imputation from standard curve data, were deemed acceptable. Dissociation curves were checked to confirm amplification of a single product and lack of gDNA contamination.

2.2.5.6 Reference genes

All qPCR gene-of-interest measurements were normalised to reference gene values from parallel qPCR assays for 1-3 reference genes. Reference genes were *LBR* (encoding lamin B receptor; NM_205342), *YWHAZ* (encoding tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta; NM_001031343) and *NDUFA1* (encoding NADH:ubiquinone oxidoreductase subunit A1; NM_001302115), which are involved in distinct cellular processes and all previously demonstrated to be reliable reference genes in avian species (Dunn *et al.*, 2013a; Olias *et al.*, 2014; Chapman *et al.*, 2016; Reid *et al.*, 2017). Where one reference gene was quantified, its nanomolar value was used as a division factor for

sample-wise normalisation. Where multiple reference genes were quantified, the geometric mean of reference gene values was used as the normalisation factor.

2.2.6 Five-prime rapid amplification of cDNA ends (5'RACE)

The 2nd Generation 5'/3' RACE Kit (Roche, Basel, Switzerland) was used to manufacturer's specifications for all 5' RACE assays. Products were sequenced as described in section 2.2.8.

2.2.7 *In situ* hybridisation

In situ hybridisation method was based on an existing protocol (Meddle *et al.*, 2007).

2.2.7.1 Tissue preparation

Tissues for *in situ* hybridisation were snap-frozen on dry ice at the time of dissection. A cryostat (Leica Biosystems, Wetzlar, Germany) was then used to cut sections of 15µm thickness, and these were adhered to polylysine-coated slides, allowed to air dry and sealed in an airtight box containing desiccant silica gel. Slide boxes were stored at ≤-70°C until use.

2.2.7.2 Oligonucleotide probe design

Oligonucleotide probes for *in situ* hybridisation were manually designed to target a specific region of the mRNA of interest. Target probe parameters were: 48-62% GC content (55% optimal), 43-47mer length (45mer optimal), melting temperature (T_m) at least 20°C greater than the highest predicted tertiary structure T_m predicted by OligoAnalyzer 3.1 online software (Integrated DNA Technologies) and as high as possible with above parameters met. All probes were sourced from Sigma-Aldrich Corp. (MO, USA).

2.2.7.3 Radiolabelling of oligonucleotide probes

Homopolymeric ^{35}S-labelled dATP (PerkinElmer Inc, MA, USA) tails were added to the 3' end of oligonucleotide probes by terminal deoxynucleotidyl transferase (TdT) (Sigma Aldrich, Basel, Switzerland). For each labelling reaction, 2µl 10µM probe was mixed with 26.5µl H_2O, 5µl ^{35}S-labelled dATP, 5µl 2.5mM $CoCl_2$ (supplied with TdT), 10µl Green buffer (supplied with TdT), and 30U (1.5µl) TdT and incubated at 37°C for 1.5h.

Radiolabelled probes were purified using the QIAquick Nucleotide Removal kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and eluted in 50µl EB buffer (supplied). Radioactivity was quantitated by scintillation β-counting of 1µl eluted probe mixed with 3.5ml scintillation fluid.

2.2.7.4 Hybridisation procedure

Tissue slides contained in airtight boxes with desiccant silica gel (see section 2.2.7.1) were removed from the freezer on the day of hybridisation and allowed to warm to room temperature for 2h before opening. Tissues were fixed by room-temperature incubation as follows: 10min in 0.1M PBS with 4% (w/v) PFA, 2x (5min in 0.1M PBS), 10min in TEA-AA solution, brief rinse in H_2O, 3min in 70% ethanol, 3min in 95% ethanol, 3min in 100% ethanol, 3min in 100% $CHCl_3$, 3min in 100% ethanol, 3min in 95% ethanol and then left to air-dry for ≈30min.

Hybridisation solution was prepared by mixing *in situ* hybridisation buffer with 0.02M DTT and sufficient radiolabelled probe to allow 100,000cpm in 25µl total volume per tissue section (approx. 2cm^2 average). 25µl hybridisation solution was spotted onto each tissue section and overlain with a parafilm slip to evenly distribute the solution and maintain humidity. Slides were incubated at 37°C for 16-20h in a humid hybridisation chamber (airtight box lined with moist filter paper).

Following hybridisation, slides were briefly rinsed 3x in 1X SSC solution at room temperature, then 4x 15min in 1X SSC solution at 20°C below probe melting

temperature (see section 2.2.7.2), then 2x 30min in SSC solution at room temperature and finally briefly rinsed in H_2O before allowing to air-dry for 3-20h.

2.2.7.5 Exposure and development

Darkness or safe-light was maintained throughout preparation and exposure. Slides were dipped in K5 Gel Emulsion (Ilford Photo, Knutsford, England) diluted 1:1 (v:v) with H_2O, air-dried for 24h and further exposed in a dark airtight box with desiccant silica gel for 14d at 4°C.

Under safe light conditions, slides were warmed to room temperature (1-2h) before unboxing and incubated in Developer (Ilford Photo, Knutsford, England) diluted 1/5 with H_2O. Following a brief wash in H_2O, slides were incubated 2x in Fixer for 5min then 2x H_2O for 5min.

2.2.7.6 Counterstaining

Automated counterstaining employed the Autostainer XL (Leica Biosystems, Wetzlar, Germany) and the following room temperature incubation process: 30s in Harris haematoxylin, 2x 3min in running tap water, 2min in Scott's tap water substitute (STWS), 5min in running tap water, 2min in 1% (w/v) eosin, 30s in running tap water, 30s in 70% industrial methylated spirit (IMS), 2x 30s in 95% IMS, 2x 2min in 99% IMS, 2min in 99%IMS diluted 1:1 (v:v) with xylene and finally 3x 1min in xylene. Each slide was then sealed with Pertex mounting medium (CellPath Ltd., Powys, Wales) overlain by a coverslip.

2.2.8 Nucleotide sequencing

All sequencing was carried out externally using the LIGHTrun or SUPREMErun Sanger sequencing methods (GATC Biotech AG, Cologne, Germany) with samples provided as directed (http://gatc-biotech.com/).

2.3 Biological resources

2.3.1 Advanced intercross line (AIL)

The advanced intercross line (AIL) is a hybrid population founded by Paul Hocking some years ago from a single broiler-layer mating. Subsequent generations have seen recombination of genetic information from the original broiler and layer haplotypes so that individual genomes are now effectively homogeneous on average. This is particularly useful in dissection of genetic loci controlling phenotypic traits which segregate between broilers and layers (e.g. growth rate) since individual loci can be assessed in an effectively homogeneous genetic background.

2.3.1.1 Population maintenance

AIL individuals used for original live animal experimentation described in this thesis were of the generations F19-F22. Responsibility for organising breeding plans was assumed for each parental generation F18-F23 inclusive. Avoidance of sibling mating was prioritised and achieved for generations F18-F23. Generation F19 individuals were pedigree mated to include progeny from all three extant AIL F18 families, and nine F20 families (each from a different mating) were produced. For generation F20, five egg families were produced. From F21 onward, four egg families were produced per generation and each family was represented in both sexes at the immediately subsequent mating round. By this evolving population maintenance strategy, it is believed that good value was realised in terms of promoting genetic diversity and recombination events as balanced against the financial cost of keeping more birds.

2.3.2 Multistrain

The ‘multistrain’ was a diverse single-generation population comprised of individuals from 12 commercial broiler lines, 12 commercial layer lines and 13 traditional chicken breeds (total n=430). The population was designed to represent the diversity of

modern chicken lines, providing a resource to associate genotypic information with collected phenotypic data.

2.3.3 Tissue panels

2.3.3.1 Broiler panel

A tissue panel comprising samples from basal hypothalamus (BH), breast muscle (BM), liver (Liv), pancreas duodenal end (head; PanH), pancreas splenic end (tail; PanT) crop, proventriculus (ProV), gizzard (Giz), antrum (Ant), antro-duodenal boundary (AD), duodenum (Duo), proximal jejunum (PJ), mid-jejunum (MJ), jejuno-ileal boundary proximal to the vitelline diverticulum (JI), mid-ileum (MI), distal ileum (DI), caecum (Cae) and rectum (Rec) was dissected from Ross 308 broilers culled at six weeks of age (n=4).

2.3.3.2 Layer panel

A tissue panel comprising samples from basal hypothalamus (BH), breast muscle (BM), liver (Liv), pancreas (Pan), crop, proventriculus (ProV), gizzard (Giz), antrum (Ant), antro-duodenal boundary (AD), duodenum (Duo), proximal jejunum (PJ), mid-jejunum (MJ), jejuno-ileal boundary proximal to the vitelline diverticulum (JI), mid-ileum (MI), distal ileum (DI), caecum (Cae) and rectum (Rec) was dissected from Lohmann Brown Classic hens (n=4) culled at peak of lay.

2.3.4 Other biological resources

Alternative chicken populations were used for various experiments as described in future sections.

2.3.5 Tissue dissection

All dissections were completed as quickly as possible after subject death.

2.3.5.1 Samples for RNA purification

Basal hypothalamic samples were excised as blocks of tissue targeted to contain the ARC and PVN. Pancreas and liver samples were taken from the middle region of pancreatic and hepatic lobes respectively, unless otherwise indicated. Gastrointestinal samples were taken in a coronal plane where possible, and otherwise (crop, proventriculus, gizzard) as tissue blocks from a central area of the subject region, to include all tissue strata from the luminal epithelium to serosa.

2.3.5.2 Whole visceral organs

Whole visceral organs and gastrointestinal regions were removed by excision as close as possible to established boundary points. Before weighing, fat and mesenteric tissues were trimmed off and luminal contents removed, except in the case of whole GI tract measurements where fat and luminal contents were left intact.

2.4 Statistical methods

All statistical operations were performed using Genstat 13 (VSN International Ltd., Hemel Hempstead, England). For all tests, probability values (p) of ≤0.05 were considered significant.

2.4.1 Analysis of variance (ANOVA)

Analyses of variance were unbalanced one- or two-way ANOVAs and were used to test for difference between groups. Blocks were included to account for variables not under analysis. Simple residual value plots were examined to ensure approximate normality. If few obvious outliers existed, these were removed before re-examination. If residual values were not normally distributed, data were transformed ($\log_{10}$) and re-examined. Where normality could not be achieved, Kruskall-Wallis test was employed as a non-parametric alternative. Post-hoc calculation of least-significant differences resolved significance of differences between individual groups.

2.4.2 Kruskall-Wallis test

The Kruskall-Wallis test was used only as a non-parametric alternative to ANOVA where data were not normally-distributed. H-statistic (H) and probability of statistically significant difference between groups (p) are reported and pairwise resolution is excluded for analyses involving more than two groups.

2.4.3 Spearman's rank-order correlation

Linear dependence between two variables was tested by Spearman's rank-order correlation. Rho value (r_s) and statistical probability of a correlative relationship (p) are reported.

CHAPTER 3

The cholecystokinin A receptor locus

3 The cholecystokinin A receptor (CCKAR) locus

3.1 Introduction

As discussed in Chapter 1, optimisation of poultry production efficiency and maintenance of acceptable welfare standards demands knowledge of avian energy balance. Specifically, it is of value to identify how selective breeding over recent decades has shaped energy homeostasis phenotypes in modern commercial breeds. Recently, several genome-wide and targeted association studies have identified a region on chicken chromosome 4 as the most significant QTL for growth traits (Ambo *et al.*, 2009; Baron *et al.*, 2011; Rikimaru *et al.*, 2011; Dunn *et al.*, 2013a; Jin *et al.*, 2015; Nassar *et al.*, 2015; Yu-Ping, 2015; Pertille *et al.*, 2017). Further studies of this genomic region have attempted to fine-map causative loci, to generate information that can be included in selection programmes to improve production. A number of positional candidate genes have been identified; FGFBP1 and FGFBP2 (Felicio *et al.*, 2013), PPARGC1A, KLF3 and SLIT2 (Pertille *et al.*, 2015), FAM184B, KCNIP4, MIR15A and GLI3 (Jin *et al.*, 2015), and many others exist at the QTL region, however many targeted studies simply quantify classic trait association and generally conclude with potential marker identification. An approach more proactive and accurate in terms of pinpointing likely loci, and informative in terms of describing the altering effects of intense selection on growth traits, involves identifying candidates with mechanistic as well as positional relevance, and determining the precise genetic basis of the difference caused. It is true that a number of the aforementioned genes could feasibly fulfil significant roles in avian energy homeostasis. For example, FGFBPs (fibroblast growth factor binding proteins) interact with developmental growth factors and predict carcass traits in chickens (Felicio *et al.*, 2013). The PPARGC1A gene product stimulates lipid catabolism (Puigserver *et al.*, 1998) and mitochondrion production (Dorn *et al.*, 2015). However, to date only one gene in the region has been studied in any mechanistic detail, that being CCKAR – the gene encoding the cholecystokinin A receptor (Dunn *et al.*, 2013a; Rikimaru *et al.*, 2013).

3.1.1 CCKAR as a causative candidate

In mammals, the peptide hormone cholecystokinin (CCK) is widely implicated in peripheral digestive function, gut-brain energy signalling and central control of energy homeostasis through its interaction with CCKAR (Crawley *et al.*, 1991). Local peripheral effects originally attributed to CCK are stimulation of the release of bile from the gallbladder and enzymes from the pancreas. Since early characterisation, the involvement of CCK has been recognised in modulating an extensive range of bodily functions including approach behaviours (e.g. feeding, foraging, exploration, sex), nociception and learning/memory (Dockray, 2009; Rehfeld, 2017). As a satiety signal, peripheral CCK acts either directly at the hypothalamic arcuate nucleus by diffusing across the blood-brain barrier, or via a hindbrain relay by local stimulation of vagal afferent fibres, or both (Boswell, 2005; Dockray, 2009; Zhang & Ritter, 2012; Dockray, 2013). Evidence also exists that vagal CCKAR signalling could be as important as local CCKAR in effecting peripheral digestive functions (Furukawa and Okada, 1992), suggesting that these too might be centrally controlled. Dockray (2009) proposes that, complementary to its own satiety signalling role, circulating CCK performs a 'gate-keeping' function, its concentration informing vagal afferents to prime them for appropriate transduction of acute hunger and satiety signals. Behavioural and physiological changes ensue which facilitate digestion and prevent overconsumption (Dockray, 2009).

Both CCK and CCKAR are very highly conserved in vertebrates (see Chapter 5 for information on CCK conservation, CCKAR identity with chicken: *Salmo salar* 61.6%, *Xenopus tropicalis* 70.8%, *Homo sapiens* 75.3%, *Alligator sinensis* 86.5%) and this ancient signalling system seems to have fulfilled important physiological functions since at least the last common ancestor of extant nephrozoans (Janssen *et al.*, 2008). CCKAR specifically binds sulphated CCK molecules whereas a second receptor species (CCKBR) binds both CCK and the related peptide gastrin independent of sulphation, as discussed in Chapter 5.

CCKAR and CCKBR (also known as CCK1R and CCK2R, respectively) exhibit distinct patterns of tissue-specific expression. In chickens, CCKAR is predominantly expressed in the small intestine, pancreas, gallbladder and hypothalamus, consistent with its primary physiological roles. Considerable transcript signal is also seen in other sub-gastric regions of the intestinal tract, adrenal and pituitary glands and testis (Ohkubo *et al.*, 2007). Conversely, chicken CCKBR transcripts are mostly found in the brain – where expression outweighs that of CCKAR in every region studied – but also explicitly in the proventriculus, in keeping with the proposed roles of CCKBR in transduction of peripheral gastrin and central CCK signals (Ohkubo *et al.*, 2007).

Congenital lack of CCKAR (resulting from a naturally-occurring partial gene deletion) is implicated in the obese, hyperglycaemic and hyperinsulinaemic phenotype of the OLETF laboratory rat strain (Takiguchi *et al.*, 1997), however the observed phenotype is not exclusively attributable to one locus in this strain. To further investigate the effects of perturbed CCK signalling on feeding and growth in rodents, a CCKAR-knockout mouse line was generated and found to be refractory to short-term satiating effects of exogenous CCK compared to $CCKAR^{+/+}$ controls, as measured by relative reduction in feed intake (Kopin *et al.*, 1999). These researchers had the foresight to include groups of both $CCKAR^{+/+}/CCKBR^{+/+}$ (wild-type) and previously-generated $CCKAR^{+/+}/CCKBR^{-/-}$ (Nagata *et al.*, 1996) animals, which both exhibited normal response to CCK administration (Kopin *et al.*, 1999), thereby confirming that that the acute appetite-lowering effects of peripheral CCK are mediated by the A-type receptor. Another interesting observation from this study was that neither CCKAR- nor CCKBR-knockout predicted a change in long-term bodyweight, suggesting that bodyweight setpoint is not altered in receptor-deficient mice, although genetic background could not be properly accounted for in the case of CCKBR-knockout. Littermate controls were used in an alternative knockout experiment which targeted the ligand, CCK, and again no effect on bodyweight was detected (Lo *et al.*, 2008). A subsequent mouse study found that brain-specific CCK overexpression in transgenic mice reduced the long-term bodyweight compared to (otherwise genetically identical)

non-transgenic counterparts (Li *et al.*, 2009), which implies that perturbed central CCK signalling might lead to alteration of the bodyweight setpoint in mice, but the mechanism remains unclear. A review of the likely effects of CCKAR loss in OLETF rats notes the species-specificity of CCKAR distribution, even for closely-related species such as rat and mouse (Bi & Moran, 2002). These reviewers conclude that CCKAR in rats is responsible for growth phenotype by both short- and long-term mechanisms. Acute appetite control is perturbed by reduction in peripheral CCK signal transduction, leading to larger meals, and central integration of energy signals is affected by reduced inhibition of hypothalamic NPY expression, leading to a chronic change in energy balance (i.e. revised bodyweight setpoint).

3.1.1.1 Study of CCKAR in livestock species

Interest in CCKAR has been generated by its association with selective breeding for production traits in several livestock species. CCKAR locus effects on growth have been studied in a hybrid pig line founded from two strains divergently selected for growth traits (Houston *et al.*, 2008). This study identified a candidate SNP in the 5' UTR which disrupted binding of the YY1 transcription factor, and the investigators hypothesised that this might lead to reduced CCKAR expression and altered growth trajectory. In goats, the domestication process appears to have favoured a non-synonymous CCKAR gene variant which affects the extracellular ligand-binding domain and ostensibly weakens ligand-receptor interaction (Dong *et al.*, 2015).

3.1.1.2 Previous study of CCKAR in chickens

As discussed in Chapter 1, chickens exhibit some marked differences compared to mammals in control of energy homeostasis. Whilst CCK signalling seems to be conserved, the magnitudes of its many effects might vary appreciably compared to mammals. Genome-wide scanning for loci affecting growth in unrelated populations consistently identify the QTL on chromosome 4 (Rikimaru *et al.*, 2011; Dunn *et al.*, 2013a; Nassar *et al.*, 2015). Some of these studies have been followed up with focussed explorations of the role of CCKAR-mediated CCK signalling. Further

investigation in the Hinai-Dori intercross identified a segregating natural CCKAR variant allele harbouring a novel binding site for YY1 (Rikimaru *et al.*, 2013); the same transcription factor implicated in porcine CCKAR-associated growth phenotype (Houston *et al.*, 2008), although the effect on transcription would need to be opposite in these instances for both to be true. Dunn and colleagues (2013a) studied CCKAR as a candidate to explain segregation of growth phenotype between commercial layer and broiler strains, using the AIL (see section 2.3.1). This work identified relatively reduced expression of CCKAR in high-growth individuals as the causative mechanism, with the associated haplotype explaining a large proportion (~20%) of bodyweight difference. In addition to positive characterisation of this difference at the CCKAR locus, other candidate genes in the chr4 QTL region were tested for expressional differences and found not to vary significantly between haplotypes, within the scope of the sample set examined (tissue choice and environmental factors might have hampered detection, for example). The AIL F16 generation was used, which narrows the explanative region since many more recombination events have taken place on chromosome 4 compared to the F_2 generation used in other studies. Further confidence that a causative variant exists close to the CCKAR gene locus arises from direct correlation of frequency of the most reliable high-growth associated intragenic SNP marker with bodyweight in the Multistrain population, however it might still be several Mb downstream of the CCKAR gene, possibly close to a gene cluster known to affect stature in mammals (Dunn *et al.*, 2013a). Assessment of the organisation of stored energy investment (for example bodyweight relative to stature) might therefore be important in clarifying the likely source of a genetic effect. The expressional effect was also demonstrated to be allele-specific, with an imbalance of expression from each allele in heterozygotes, so the causal element must be cis-regulatory. Taken together, data from the above studies suggest that CCKAR expressional phenotype is a significant contributing factor to growth differences in diverse chicken strains. The nature and precise location of the genetic basis of the expressional difference in the AIL remains unknown however, as does any

physiological explanation for increased bodyweight, aside from the conjectural assumption that CCKAR haplotype determines food intake.

3.2 Aims

Fine-mapping genomic variation to identify potential causative locus/loci explaining the growth effect of the QTL on chicken chromosome 4 was one major objective of the work described in this chapter. It was also hoped that mechanistic explanations for decreased CCKAR expression could be attributed to such variants.

A second broad objective was to describe traits which might be related to the observed effect on bodyweight, for example through altered behaviour or organ morphology, and to elucidate the likely major physiological effects of perturbed CCKAR expression.

3.3 Methods

3.3.1 Genomic assessment

3.3.1.1 Cursory wide-scale association analyses

To improve confidence in targeting the chr4 region responsible for altered CCKAR expression, first a wide-scale analysis of three segregating SNPs was carried out in an unrelated broiler-layer hybrid population. Standard CCKAR_MnlI genotyping (section 2.2.3.1) was performed for the GM8 (a broiler-layer hybrid population unrelated to the AIL, total n=306). Results were analysed together with existing genotypic and phenotypic information for the GM8 (provided by Paul Hocking) to determine association of three segregating SNP markers spaced across the chromosome 4 growth QTL. For long-term bodyweight association, individual birds which lost or gained >5% were removed from the analysis to avoid confounding non-normal effects (e.g. sickness, injury). Analysed SNPs were ch4snp851573063S2 (approx. 1.5Mb upstream of CCKAR), CCKAR_MnlI (within the CCKAR gene) and ch4snp1311324046S2 (approx. 1.5Mb downstream of CCKAR). Genstat was used

to perform ANOVA for each trait between genotypes for each SNP, blocked for all fixed effects. Growth traits for which phenotypic data were available and analysed are listed in Table 3.1.

3.3.1.2 Haplotype definition

The AIL represents a powerful resource with which to investigate genetic causes of differing phenotypes between broiler and layer birds of the founding types, since individual causative loci are represented on an effectively homogeneous genetic background after many generations of interbreeding. In order to fully characterise the CCKAR locus haplotypes associated with high- and low-growth in the AIL, the genomic region surrounding CCKAR (galGal4:chr4:72,810,951-72,831,845) was sequenced for F_{16} birds homozygous each way for the standard genotyping marker CCKAR_MnlI (section 2.2.3.1) (n=2 per haplotype). In total, 27 fragments across the CCKAR locus were amplified by PCR, Exo-SAP purified and sequenced, as described in sections 2.2.2 and 2.2.8. Details of all sequenced fragments can be found in Appendix 3. Fragments were aligned using GAP4 (Staden *et al.*, 2003) and haplotype schematics prepared using SeqBuilder (DNASTAR, Madison, WI, USA).

3.3.1.3 Fine mapping association analyses

Following elucidation of CCKAR haplotypes, 40 variations unique to the high growth-associated haplotype and spread across the CCKAR locus were selected for outsourced genotyping (section 2.2.3.4) of the Multistrain population (section 2.3.2). Genotyping for a deletion downstream of CCKAR (DelinvA) was carried out in-house, as described in section 2.2.3.2. Probability of association with bodyweight was calculated individually for each variant locus by ANOVA blocked for strain and age of weighing.

3.3.1.4 CCKAR 5' RACE

5' RACE was performed for CCKAR as described in section 2.2.6, to determine the transcriptional start site. Input cDNA was prepared from snap-frozen J-line[1] pancreas tissue, as described in section 2.2.4.

3.3.2 Physiological assessments

In order to gather information about morphological composition, visceral organ function and feeding behaviour traits associated with CCKAR high- and low-growth alleles, two separate experimental set-ups were employed as described below. The CCKAR_MnlI marker was used to assign haplotype groups.

3.3.2.1 Experimental set-up 1

AIL F_{19} homozygotes (n=35) were sexed and genotyped as described in sections 2.2.3.1 and 2.2.3.3 and reared in floor pens to 16d, then introduced to randomly-allocated individual cages and allowed to acclimatise for 9 days. Individual bodyweights and feed hopper weights were recorded at 26d and 30d.

3.3.2.2 Experimental set-up 2

AIL F_{20} homozygotes (n=109) were sexed and genotyped as described in sections 2.2.3.1 and 2.2.3.3 and reared with hatchmates in floor pens to 10wk with weekly bodyweight measurements. Homozygotes selected to balance families as much as possible (n=32; 8 per sex per haplotype) were then individually caged at 10.5wk and allowed to acclimatise for 48h. Gross feed intake over the subsequent 5-day period was measured and birds were provided with 50% of their individual average daily intake at 11am, to promote development of a mild hunger state by the time they were killed by cervical dislocation the following day (12wk).

[1] J-line (or simply "J") is an outbred brown leghorn-derived strain available from the National Avian Research Facility, Midlothian, Scotland.

3.3.2.3 Feed intake and conversion ratio

Bodyweight gain (BWG) and gross feed intake (GFI) for the period 26-30d were derived from measurements described in section 3.3.2.1. Feed conversion efficiency (FCE) was calculated for the same period as FCE=BWG/GFI, where BWG and GFI are expressed in equivalent units.

3.3.2.4 Visceral organ capacity

Visceral organs were immediately removed as described in section 2.3.5.2. Spleen, gizzard, proventriculus, pancreas, intact gallbladder (including contents) and emptied gallbladder were each weighed and gallbladder content mass was derived. Whole gastrointestinal tract length was measured as the distance from rostral proventricular boundary to intestinal-cloacal boundary. Right metatarsal bones were disjointed and trimmed to remove cartilage and expose the osteocortical surface before maximal length was measured with a steel caliper.

3.3.2.5 Pancreatic exocrine secretion assay

For birds from experimental set-up 2 (section 3.3.2.2), pancreatic exocrine secretion and its response to exogenous CCK were measured *in vitro* by a two-step process, as described below.

3.3.2.5.1 Pancreatic explants

Whole pancreases were dissected as described in section 2.3.5.2, placed in a covered petri dish and transported on ice to the processing laboratory within 15min of cull by cervical dislocation. For each pancreas, 24 samples from the mid-section of a lobe were sliced 1mm thick using the McIlwain tissue slicer (Stoelting Europe, Dublin, Ireland), of which 12 were dried in an oven at 55°C overnight. Slices immediately adjacent to each dried slice were rinsed individually in fresh minimal essential medium (MEM-α) (Thermo Fisher Scientific, MA, USA) whilst dissection was completed, then individually introduced to wells of a 24-well Nalgene tissue culture dish containing fresh 1.5ml MEM-α at 41°C with shaking, at 30±5min post-cull. Half (six) of the wells

contained CCK at 10nM*. Two sections were immediately (0min) removed from each medium type (CCK+/-), blotted dry, sealed in individual microfuge tubes, snap-frozen on dry ice and stored at -70°C. 1ml medium sample from each containing well was also taken, sealed in a microfuge tube and frozen at -70°C. Further sections and medium samples were recovered in the same manner at 15min and 30min timepoints.

3.3.2.5.2 Colorimetric quantitation of amylase activity

Amylase activity was calculated by photometric determination of starch degradation, based on a previously-published technique (Smith & Roe, 1949). After thawing on ice and in triplicate, 20µl of each recovered medium sample (section 2.4.1) was added to 80µl 2.5X PBS at 41°C with in a 96-well cell culture plate. To begin starch hydrolysis, 100µl 10ng.µl^{-1} starch solution at 41°C was added to each well, the plate lidded and incubated at 41°C with shaking. A standard curve with starch solutions of concentration 10, 7.5, 6.25, 5, 3.75, 2.5, 1.25 and 0 ng.µl^{-1} in place of 10ng.µl starch solution and 20µl MEM-α in place of recovered medium samples was run in triplicate on each plate. After 10min, 40µl 1M HCl was added to each well to halt amylase activity. 10µl from each well was transferred to a fresh plate counterpart well containing 240µl iodine-mix and well mixed. OD_{640} was measured for each well using the Wallac 1420 Victor2 Microplate Reader (PerkinElmer Inc, MA, USA).

3.3.2.6 Intestinal villus morphology

3.3.2.6.1 Tissue mounting for histological examination

A mid-duodenal section of approximately 2-3cm were excised from each bird in experimental set-up 2 (section 3.3.2.2) and fixed in 4% (w/v) PFA in 1X PBS overnight. Sucrose was then added to 10% (w/v) for 4-6h before transfer of tissue to fresh 1X PBS with 30% (w/v) sucrose (cryo-protectant) for >48h before freezing in aluminium foil at -70°C until processing. Fixing and cryo-protection were performed with gentle agitation at 4°C. Short (≈0.5cm) sections were then stored in 70% (v/v) ethanol overnight dehydrated in subusequent 70% (v/v), 95% (v/v) and 3x absolute ethanol washes, cleared by washing twice in xylene and wax-infiltrated by twice

incubating in fresh paraffin at 58°C. All washing, clearing and infiltration steps were 1 hour long. Tissue samples were then embedded in paraffin and sliced 10µm thick on a microtome to yield coronal sections which were mounted on polylysine-coated slides and allowed to air-dry. Slides were then counterstained with haemotoxylin/eosin, as described in section 2.2.7.6.

3.3.2.6.2 Villus morphological measurement

Coronal tissue sections prepared as described in section 3.3.2.6.1 were imaged using the Coolscan V slide scanner (Nikon Corp., Tokyo, Japan). Area measurement employed ImageJ image handling software (Schneider *et al.*, 2012). The sectional area covered by villi was calculated by deduction of the clear luminal space area from the total area within the perimeter of the lamina propria (Figure 3.1). This measurement was used as a proxy for luminal epithelial surface area.

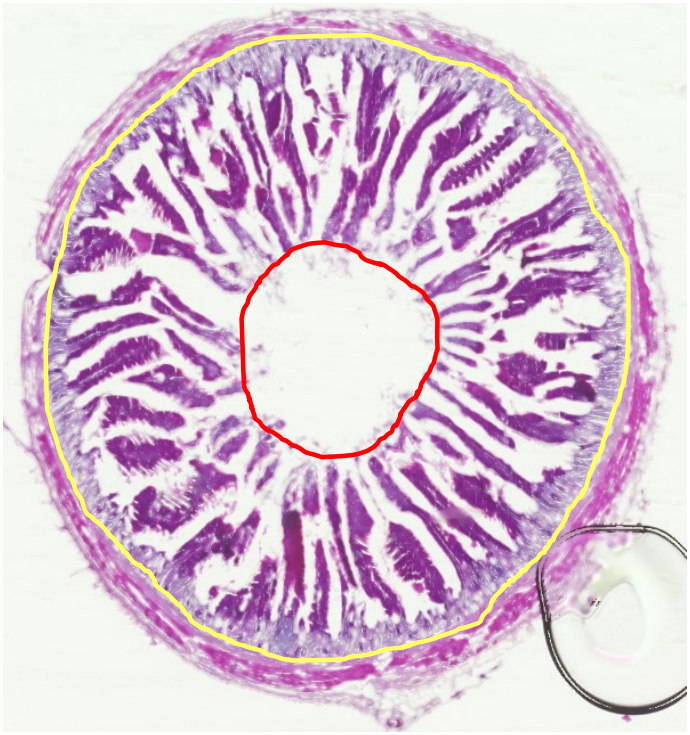


Figure 3.1 – Measurement of villus morphology. The slide area occupied by villar tissue was calculated by deduction of the clear luminal space area (within the indicated red perimeter) from the total area inside the lamina propria (within the indicated yellow perimeter).

3.3.2.7 Whole digestive tract transit duration (WTTD)

To measure whole digestive tract transit duration (WTTD), each bird in experimental set-up 1 (section 3.3.2.1) was administered orally with a gelatin capsule containing 100mg ferric oxide (an inert dye compound) and the time interval until appearance of excreta with distinctive bright red colouring was measured. This approach was based on a previous study (Hughes, 2008). The process was performed in triplicate at 26d,

28d and 30d for each bird, and an average of the three measurements was taken as an individual's WTTD. Order of dye capsule administration was randomised at each replicate.

3.3.2.8 Statistical analysis and interpretation

ANOVAs were performed to assess the significance of experimental factors (CCKAR_MnlI genotype and sex). For the pancreatic exocrine secretion assay (section 3.3.2.5), CCK treatment was also an experimental factor. Nuisance factors were 'hatch' for experimental set-up 1 (section 3.3.2.1) and 'family' for experimental set-up 2 (section 3.3.2.2) and these were used to block ANOVAs.

3.4 Results

3.4.1 Genomic mechanism

3.4.1.1 Cursory wide-scale association analyses

The SNP predicting the most significant effects on growth traits in the GM8 was ch4snp851573063S2 (Table 3.1), located approximately 1.5Mb upstream of the CCKAR gene on chromosome 4. The SNP ch4snp1311324046S2 had previously been identified as the most significant marker for growth traits in the AIL F_8 and F_{16} (Dunn *et al.*, 2013a), and is located approximately 1.5Mb downstream of CCKAR. In both of these populations, the marker CCKAR_MnlI, located within the third intron of the CCKAR gene and therefore between ch4snp851573063S2 and ch4snp1311324046S2, was also found to predict growth traits.

Phenotypic measurement	ch4snp851573063S2	CCKAR_MnlI	ch4snp1311324046S2
Bodyweight 6wk	0.015	0.013	0.625
Bodyweight 12wk	<0.001	<0.001	0.803
Bodyweight 24wk	<0.001	<0.001	0.211
Bodyweight 44wk	<0.001	<0.001	0.091
Bodyweight 48wk	<0.001	0.002	0.057
Bodyweight at sexual mat.	0.004	0.009	0.213
Bodyweight cull	0.005	0.078	0.278
Gain 44-48wk	0.092	0.008	0.674
Gain/MeanBW	0.088	0.014	0.673
Lean breast muscle mass	<0.001	0.003	0.259
Abdominal fat mass	<0.001	<0.001	0.889
Comb mass	0.005	<0.001	0.607
Wattles mass	0.026	0.004	0.302
Right testicle mass	0.005	0.705	0.092
Feed intake 45-46wk	0.002	0.014	0.045
Feed intake 46-47wk	0.021	0.087	0.176
Feed intake 47-48wk	0.001	0.148	0.742
Average food intake 44-48wk	0.002	0.095	0.067
Shank length 6wk	0.007	<0.001	0.601
Shank length 48wk	<0.001	<0.001	0.244

Table 3.1 – Probabilities of association of growth traits with segregating SNP markers ch4snp851573063S2, CCKAR_MnlI and ch4snp1311324046S2 in the GM8 population. Statistically significant associations (p>0.05 ANOVA) are highlighted yellow.

3.4.1.2 Haplotype definition

Haplotypes derived from aligning sequenced reads across the CCKAR locus are detailed in Appendix 3. In total, over 300 novel variations were detected. Haplotypes were compared with the galGal4 reference genome and variations were sorted into three groups: those unique to the broiler-derived, high growth-associated haplotype, those unique to the layer-derived, low growth-associated haplotype and those which were common between AIL haplotypes but different from galGal4.

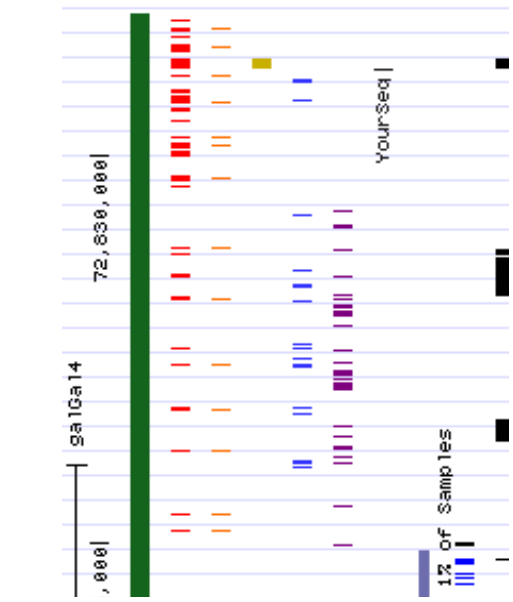
3.4.1.3 Fine mapping association analyses

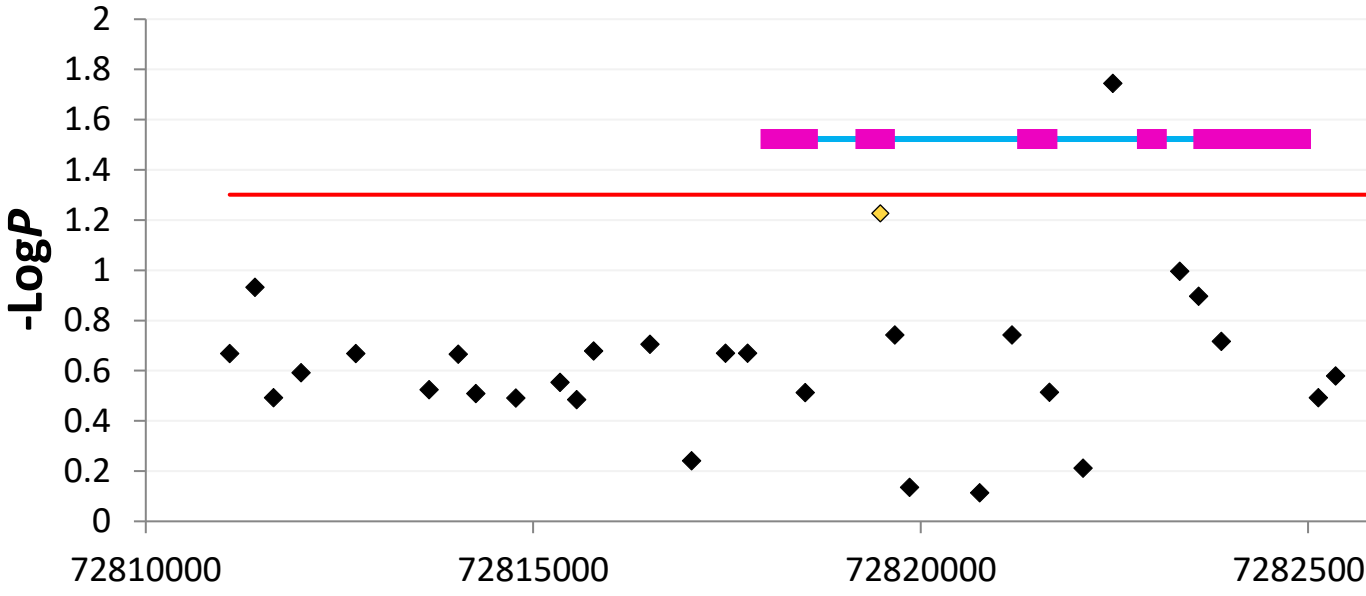
Of the 40 variations for which outsourced genotyping assays were performed, 39 were successful and all of these segregated in the Multistrain. Significance of the association of each SNP with bodyweight is shown in Figure 3.2.

Figure 3.2 (overleaf) – Genotyping and association at the CCKAR locus

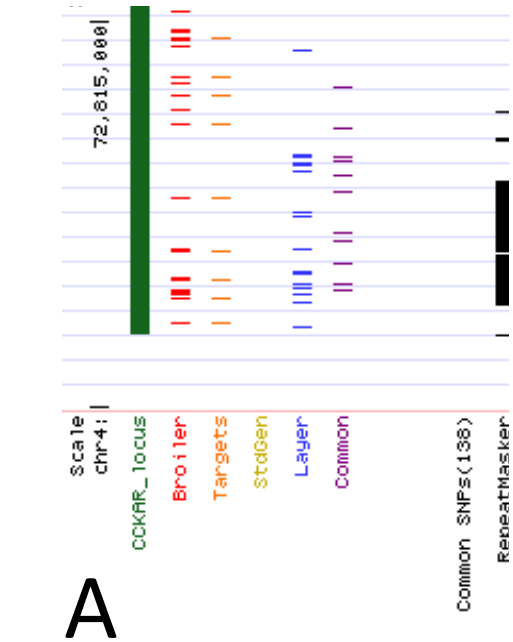
A: The CCKAR genomic locus is shown with tracks to indicate detected variations in the AIL. Variations were unique to high growth-associated haplotype ('Broiler', red), unique to low growth-associated haplotype ('Layer', blue) or common between AIL haplotypes but different from the galGal4 reference genome ('Common', purple). Standard genotyping targets (CCKAR_MnlI and DelinvA) are indicated in yellow ('StdGen'). Genotyping targets for the Multistrain analysis are indicated in orange ('Targets').

B: Results of Multistrain bodyweight association analysis. Each point represents one variation plotted as its genomic position (x-axis) against the inverse log of the probability of its association with bodyweight difference (y-axis). The p=0.05 significance threshold is represented by a red line. Data points corresponding to standard genotyping targets (CCKAR_MnlI and DelinvA) are coloured yellow.





Genomic position galGal4:c



A

B

3.4.1.4 5' RACE result

The CCKAR 5' RACE sequencing product mapped to galGal4:chr4 to evidence a transcriptional start site approximately at position 72,818,171.

3.4.2 Physiological effects

Note: haplotypes are denoted high- (HG) and low- (LG) growth-associated.

3.4.2.1 Bodyweight and stored energy investment

Bodyweight measurements for AIL F_{19} homozygotes from experimental set-up 1 (section 3.3.2.1) are shown in Figure 3.3. No significant differences were detected between genotypes ($F_{1,23}$=0.40, p=0.535) or sexes ($F_{1,23}$=0.04, p=0.840), and there was no significant interaction between these two factors ($F_{1,23}$=1.82, p=0.190).

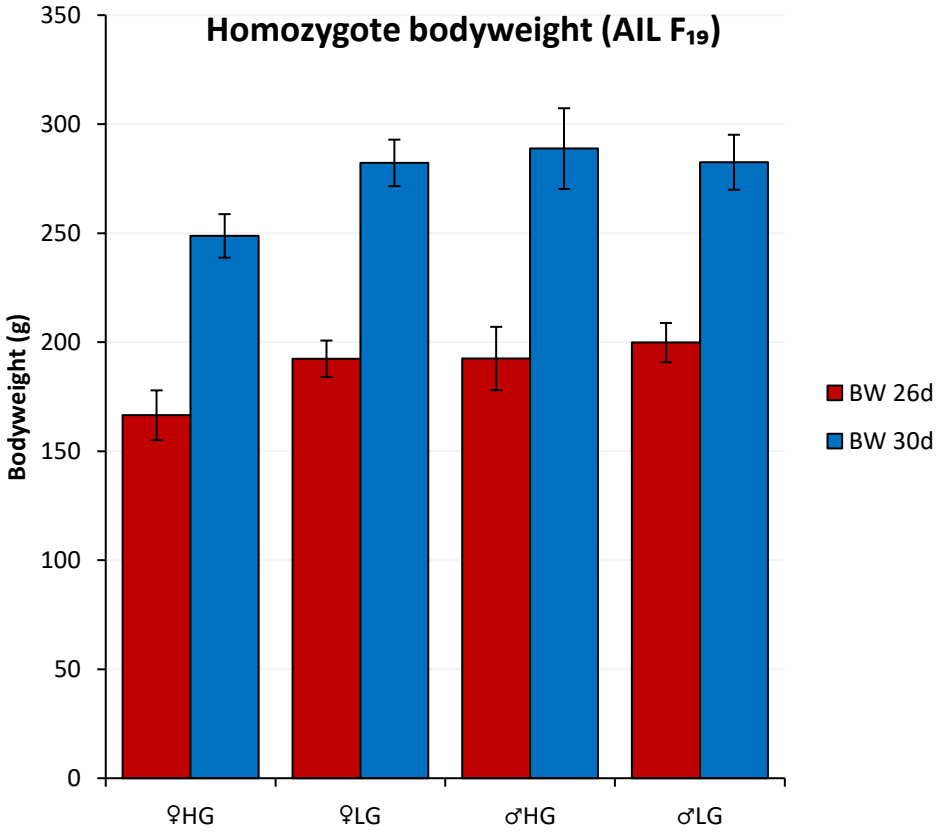


Figure 3.3 – AIL F_{19} bodyweight at 26d and 30d (CCKAR homozygotes)

Bodyweights are plotted for AIL F_{19} birds homozygous for high growth-associated (HG) and low growth-associated (LG) CCKAR haplotypes. No statistically significant differences were detected between CCKAR genotype groups of the same sex and age.

Bodyweight measurements for AIL F_{20} individuals from experimental set-up 2 (section 3.3.2.2) are shown in Figure 3.4. High growth individuals were statistically significantly heavier overall at 5wk, 7wk, 8wk, 9wk and 10wk (10wk $F_{2,75}$=5.29, p=0.007). CCKAR genotype predicted significant difference beteen homozygote males analysed in isolation at 7wk, 8wk, 9wk and 10wk. No significant differences were detected when only female homozygotes were analysed, though the trend was for higher bodyweight in the high growth-associate haplotype.

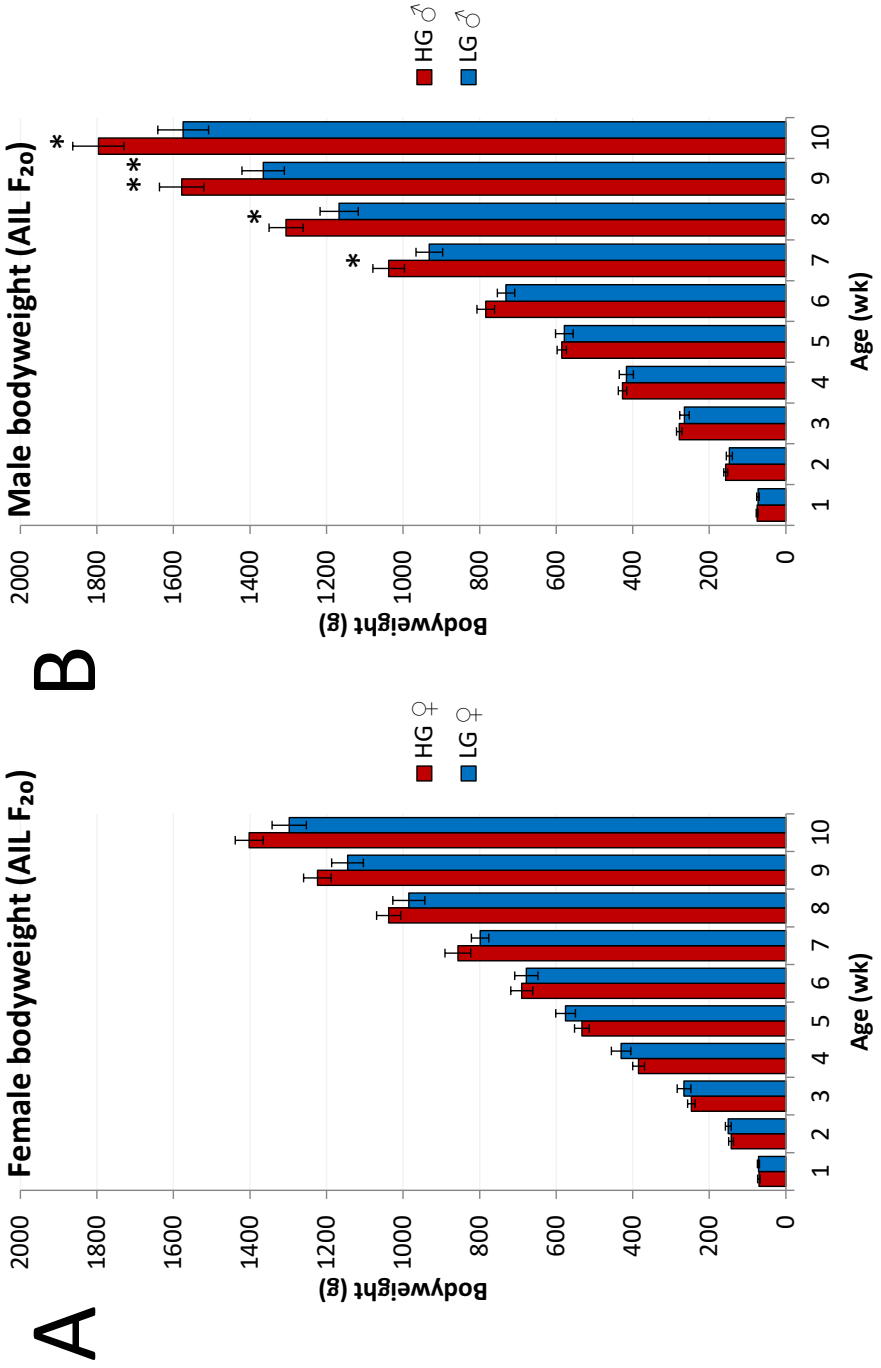


Figure 3.4 – AIL F_{20} homozygote bodyweights. Mean bodyweight ±SEM are plotted for females (A) and males (B). Significant differences between same-sex haplotypes of the same age are indicated (*p≤0.05, **p≤0.01 ANOVA)

Temporal progression of the significance (probability) of the effects of sex and CCKAR genotype are shown in Figure 3.5. Sex verged on significance from hatch or very shortly after, whereas the effect of CCKAR appears to manifest post-hatch.

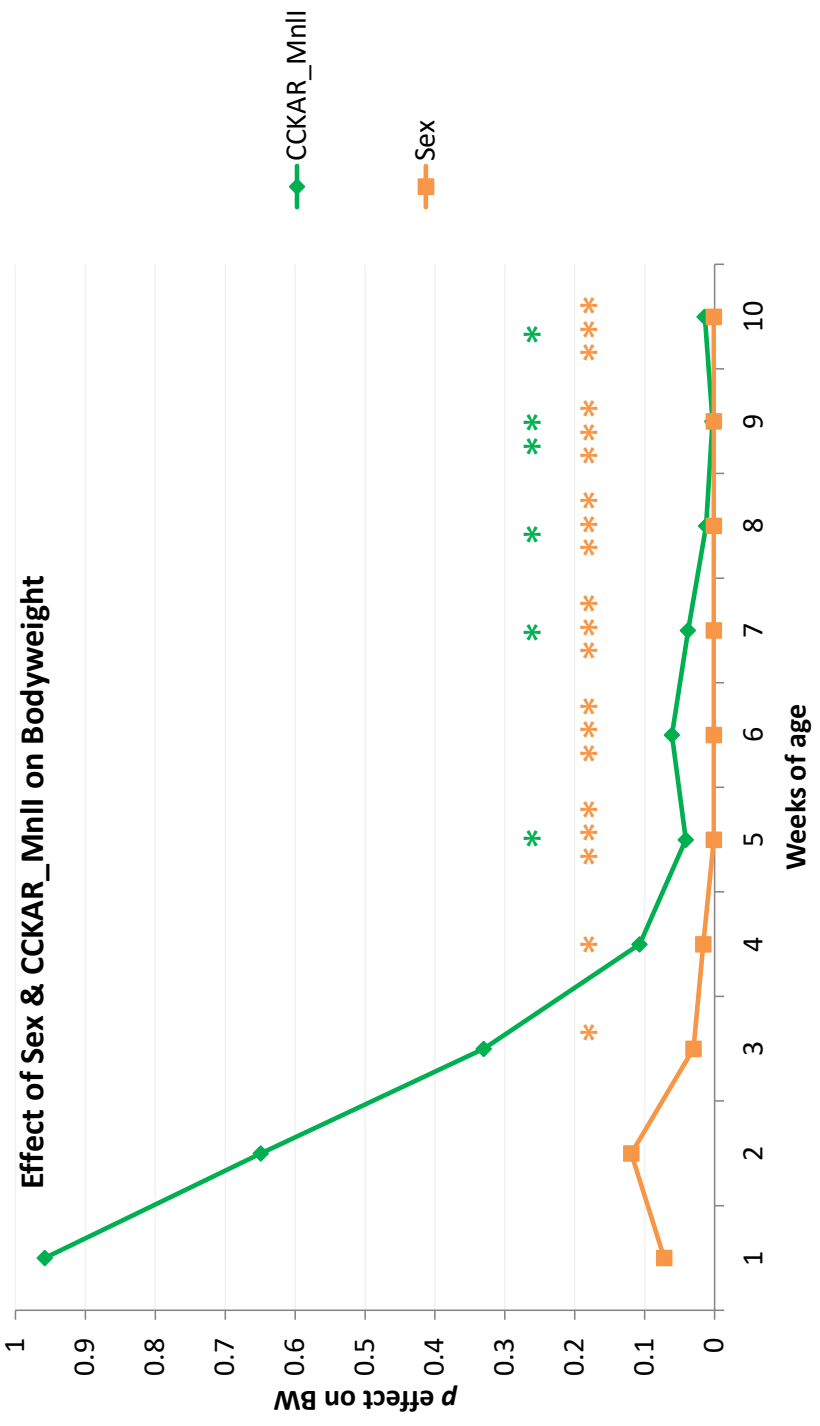


Figure 3.5 – Age-wise significance of the effects of sex and genotype on bodyweight. The significance of the effects of sex and CCKAR genotype are plotted for individuals from 1-10wk. Both sexes and all genotypes including heterozygotes were analysed together and blocked by sex and hatch. Significance of differences between CCKAR genotypes is indicated (*p≤0.05, **p≤0.01, ***p≤0.001 ANOVA).

For initial assessment of stored bodyweight investment, metatarsal bone length was normalised to bodyweight for AIL F_{20} homozygotes (experimental set-up 2, section 3.3.2.2) and these data are presented in Figure 3.6. HG individuals had significantly shorter relative metatarsi overall. Female homozygotes analysed in isolation were found to vary significantly by genotype. A significant difference between homozygote genotypes was not detected in males, however male relative metatarsus length was significantly shorter than their female counterparts.

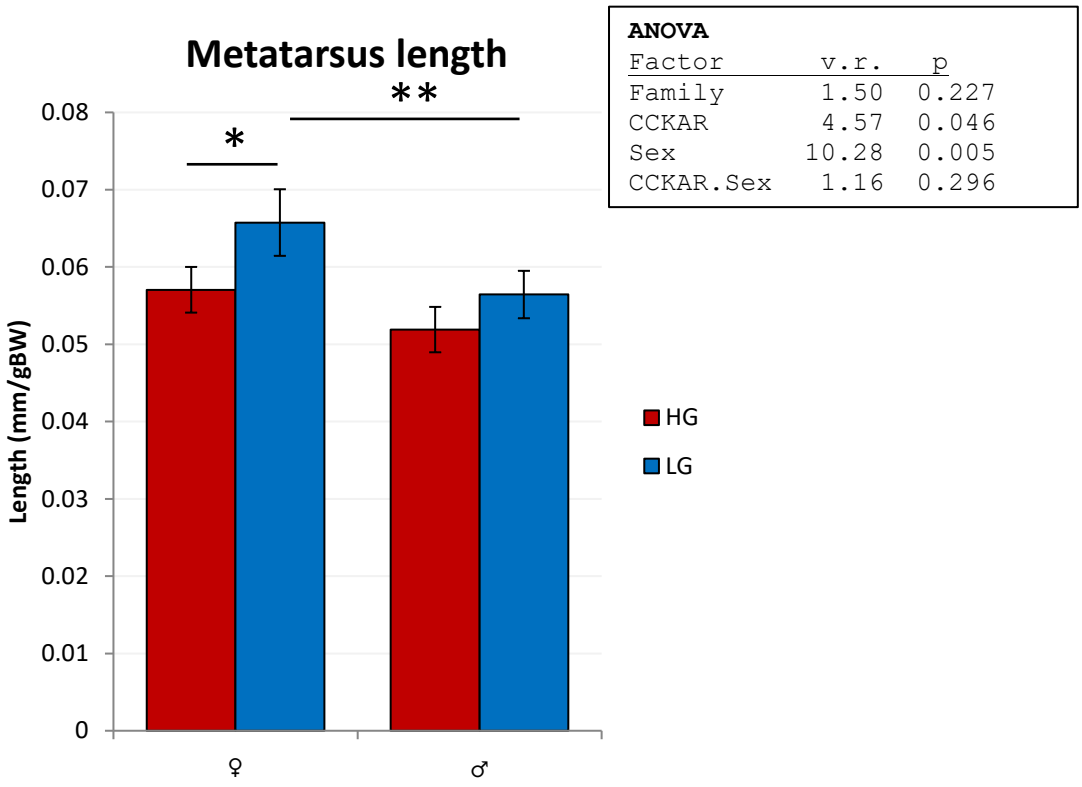


Figure 3.6 – Relative metatarsus length at 12 weeks (CCKAR homozygotes)

Bodyweight-relative metatarsus bone length ±SEM at 12 weeks old is plotted for AIL F_{20} birds homozygous for high growth-associated (HG) and low growth-associated (LG) CCKAR haplotypes. Statistically significant differences between groups are indicated (*p≤0.05, **p≤0.01 ANOVA).

3.4.2.2 Feed intake and conversion ratio

Feed intake data for AIL F_{19} homozygotes (experimental set-up 1, section 3.3.2.1) are shown in Figure 3.7. Aside from hatch, the only significant factor was sex which resolved as significant when all groups were analysed together.

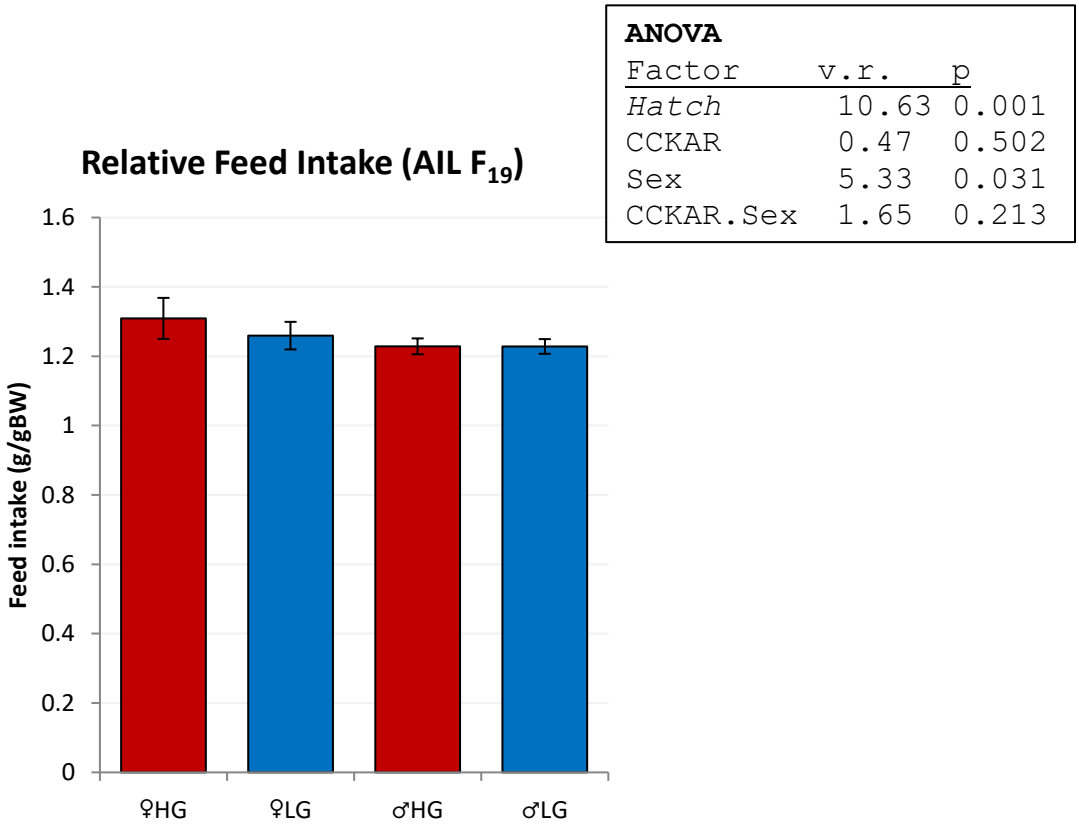
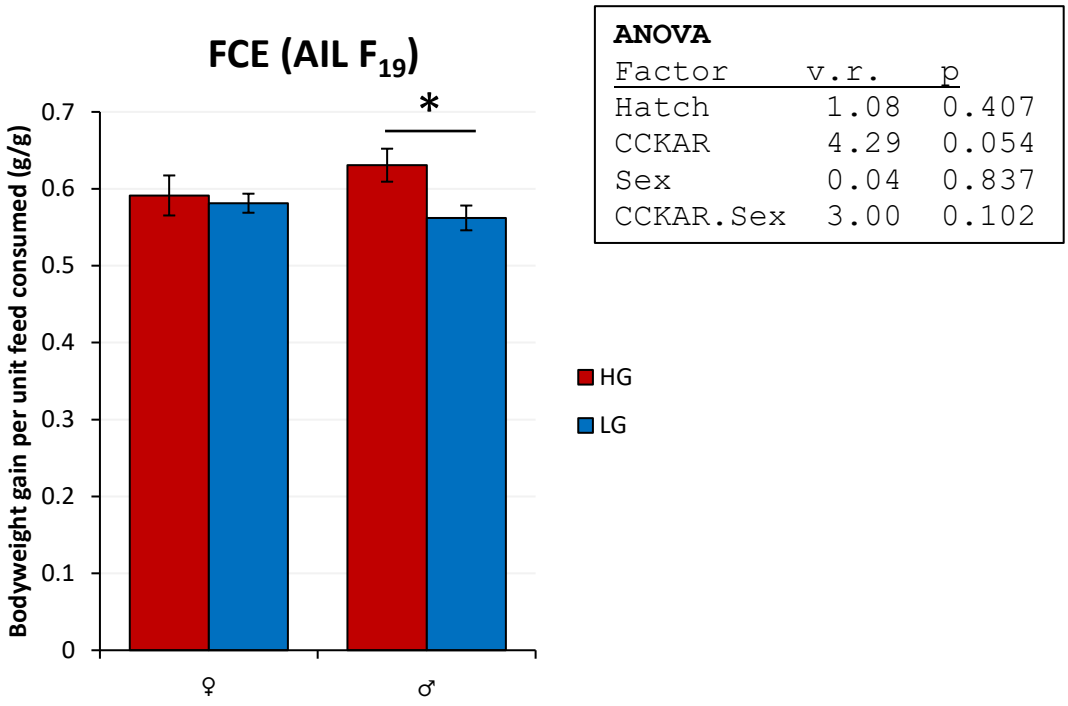


Figure 3.7 – Relative feed intake for AIL F_{19} CCKAR homozygotes

Bodyweight-normalised total feed intake for the period 26-30d is plotted for AIL F_{19} birds homozygous for high growth-associated (HG) and low growth-associated (LG) CCKAR haplotypes. No significant differences between individual groups were detected by two-way ANOVA.

Feed conversion efficiencies (FCEs) for the same experimental population are shown in Figure 3.8. CCKAR genotype predicted a significant difference overall, with HG individuals exhibiting greater feed conversion efficiency. No significant differences were found between CCKAR genotypes when only females were analysed, however males did differ significantly dependent on haplotype.



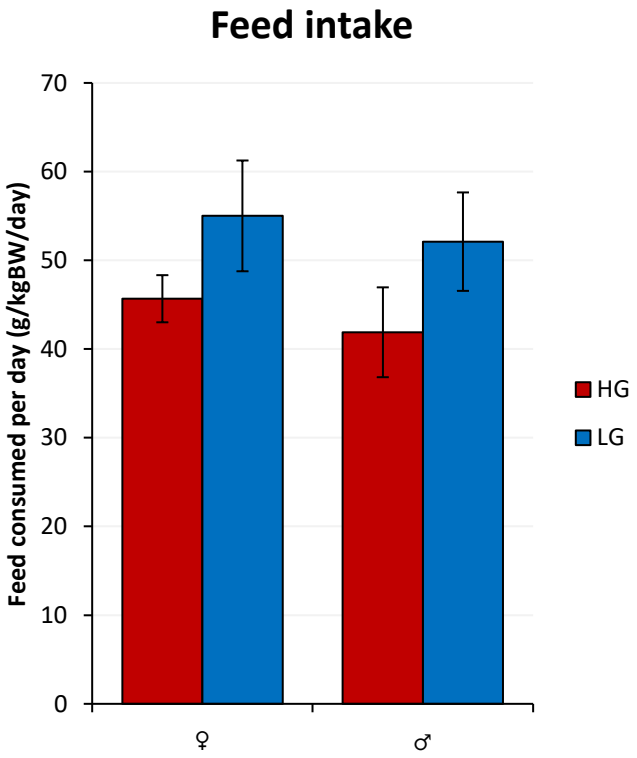
ANOVA

Factor	v.r.	p
Hatch	1.08	0.407
CCKAR	4.29	0.054
Sex	0.04	0.837
CCKAR.Sex	3.00	0.102

Figure 3.8 – Simple feed conversion efficiency (FCE) at 26-30 days (CCKAR homozygotes)

Absolute whole body mass gain per unit feed consumed at 26-30 days old is plotted for AIL F_{19} birds homozygous for high growth-associated (HG) and low growth-associated (LG) CCKAR haplotypes. Statistically significant differences between groups are indicated (*p≤0.05 ANOVA).

Feed intake data for AIL F_{20} homozygotes are shown in Figure 3.9. No significant differences existed for either experimental factor overall, however a trend for HG individuals consuming less feed per unit bodyweight at this age seems apparent.



ANOVA

Factor	v.r.	p
Family	0.37	0.905
CCKAR	2.05	0.170
Sex	0.55	0.468
CCKAR.Sex	0.01	0.920

Figure 3.9 – Daily feed intake at 12 weeks (CCKAR homozygotes)

Bodyweight-relative daily feed intake is plotted for AIL F_{20} birds homozygous for high growth-associated (HG) and low growth-associated (LG) CCKAR haplotypes. No groups differ significantly by two-way ANOVA.

3.4.2.3 Whole digestive tract transit duration (WTTD)

Results from measurement of whole tract transit duration (WTTD) are shown in Figure 3.10. No significant difference was detected between sexes or CCKAR genotypes.

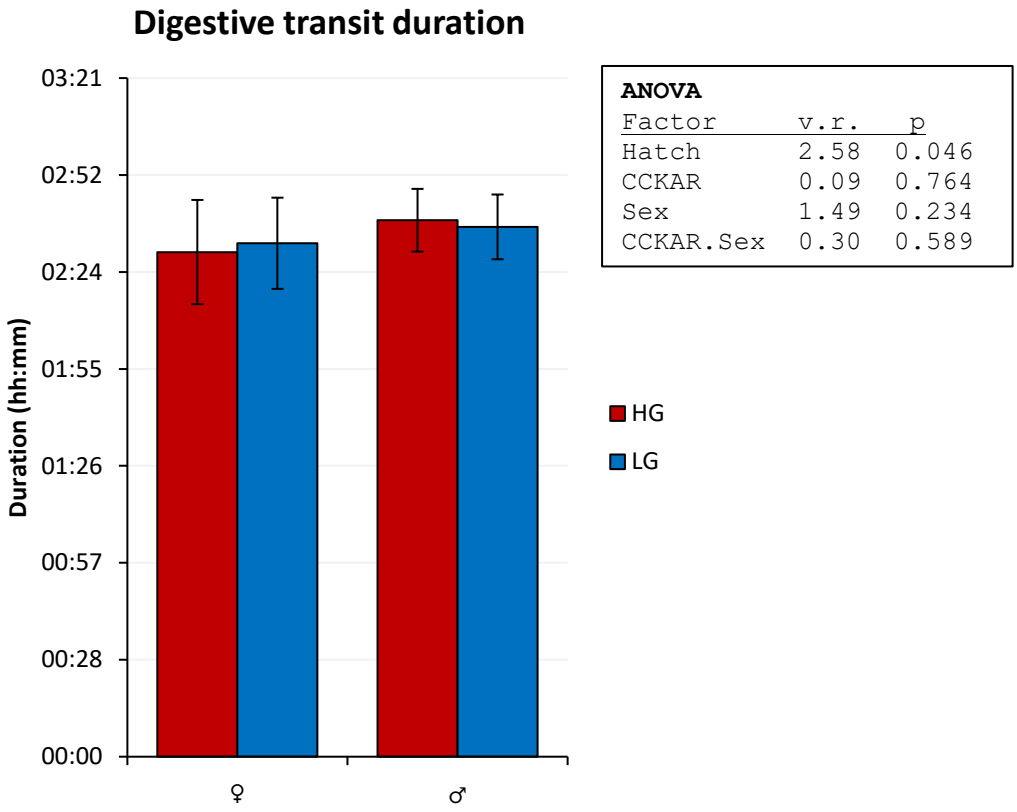


Figure 3.10 – Digestive transit duration at 4 weeks (CCKAR homozygotes)

Absolute digestive transit duration is plotted for AIL F_{19} high growth-associated (HG) and low growth-associated (LG) CCKAR haplotype homozygotes at 4wk. No individual groups differ significantly by two-way ANOVA.

3.4.2.4 Visceral organ capacity

Measurements of bodyweight-relative organ mass for spleen, proventriculus, gizzard, pancreas and gallbladder are shown in Figures 3.11-3.15, respectively. Gallbladder content mass is plotted in Figure 3.16. Gastrointestinal tract length data are shown in Figure 3.17.

Spleen mass was dependent on sex but not family overall, although females did differ significantly when considered in isolation.

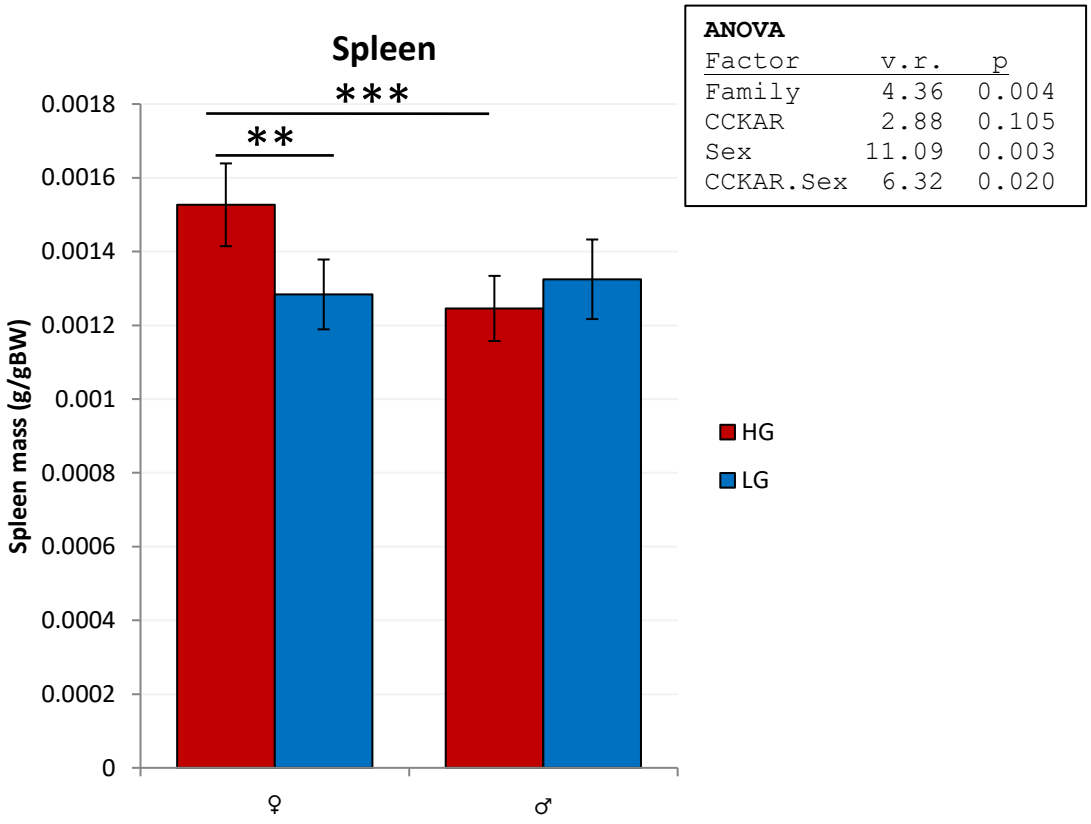


Figure 3.11 – Relative spleen mass at 12 weeks (CCKAR homozygotes)

Bodyweight-relative spleen mass is plotted for AIL F_{20} birds homozygous for high growth-associated (HG) and low growth-associated (LG) CCKAR haplotypes. Significant differences between individual group means are indicated (**p≤0.01, ***p≤0.001).

Normalised to bodyweight, proventricular mass was dependent on CCKAR haplotype but not sex overall, however females were not significantly different dependent on CCKAR haplotype when studied separately. The CCKAR locus predicted a significant effect on relative proventricular mass in males.

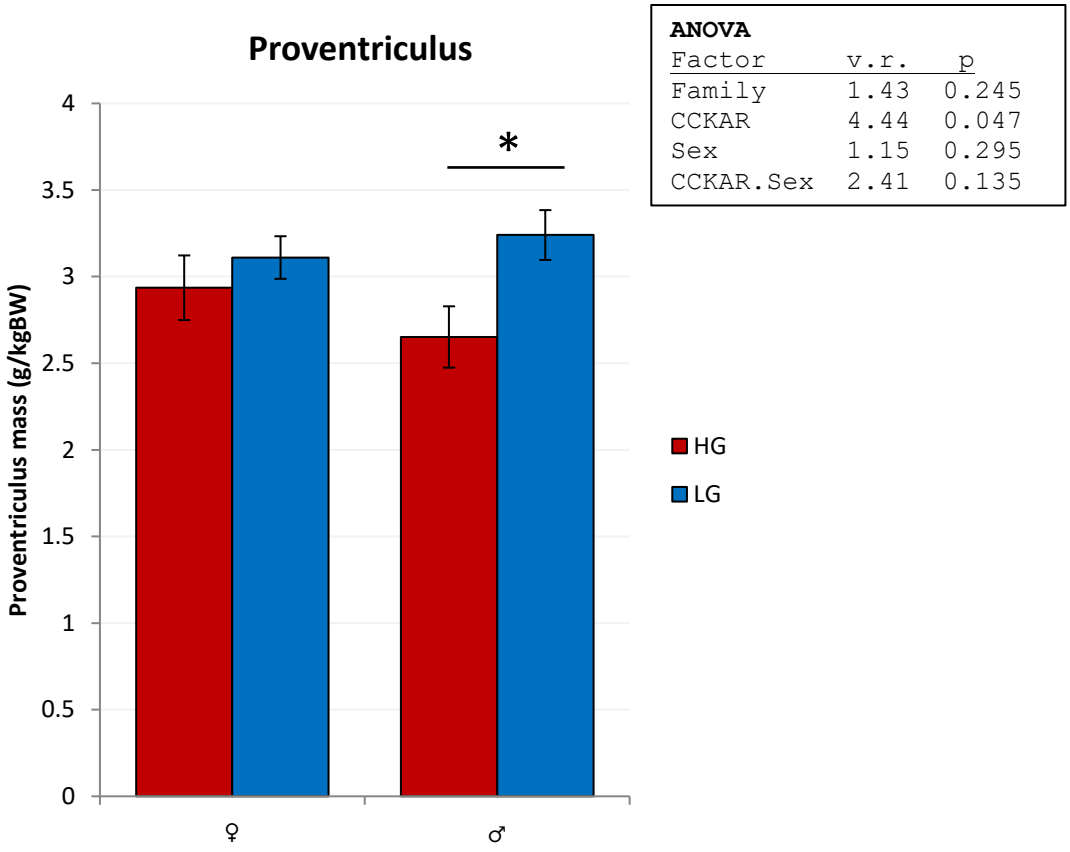


Figure 3.12 – Relative proventriculus mass at 12 weeks (CCKAR homozygotes)

Bodyweight-relative proventriculus mass is plotted for AIL F_{20} birds homozygous for high growth-associated (HG) and low growth-associated (LG) CCKAR haplotypes. Significant differences between individual group means are indicated (*p≤0.05).

Mass of the gizzard relative to bodyweight was significantly affected by CCKAR haplotype. Sex was an insignificant factor.

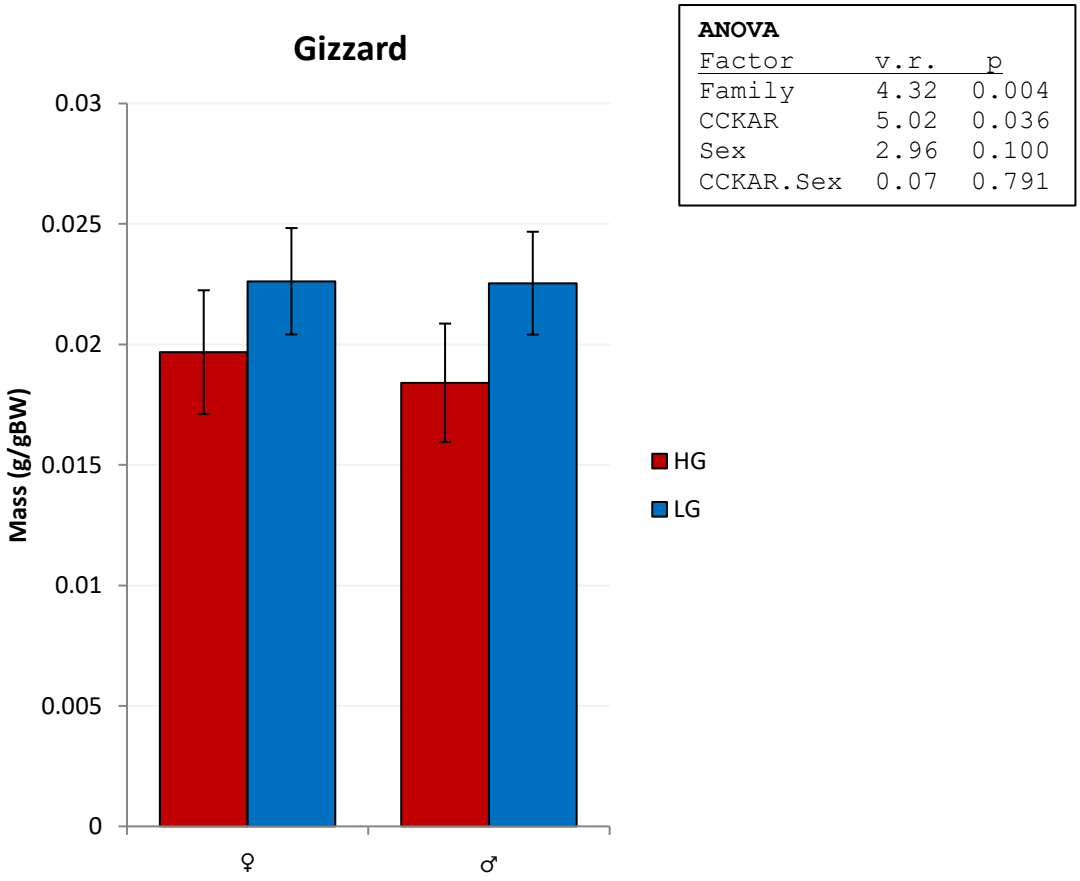


Figure 3.13 – Relative gizzard mass at 12 weeks (CCKAR homozygotes)

Bodyweight-relative gizzard mass is plotted for AIL F_{20} birds homozygous for high growth-associated (HG) and low growth-associated (LG) CCKAR haplotypes. Data were log-transformed for statistical analysis to approximate normality. No individual groups differ significantly.

Relative pancreas mass was not dependent on any factor overall, and there were no significant differences between individual groups.

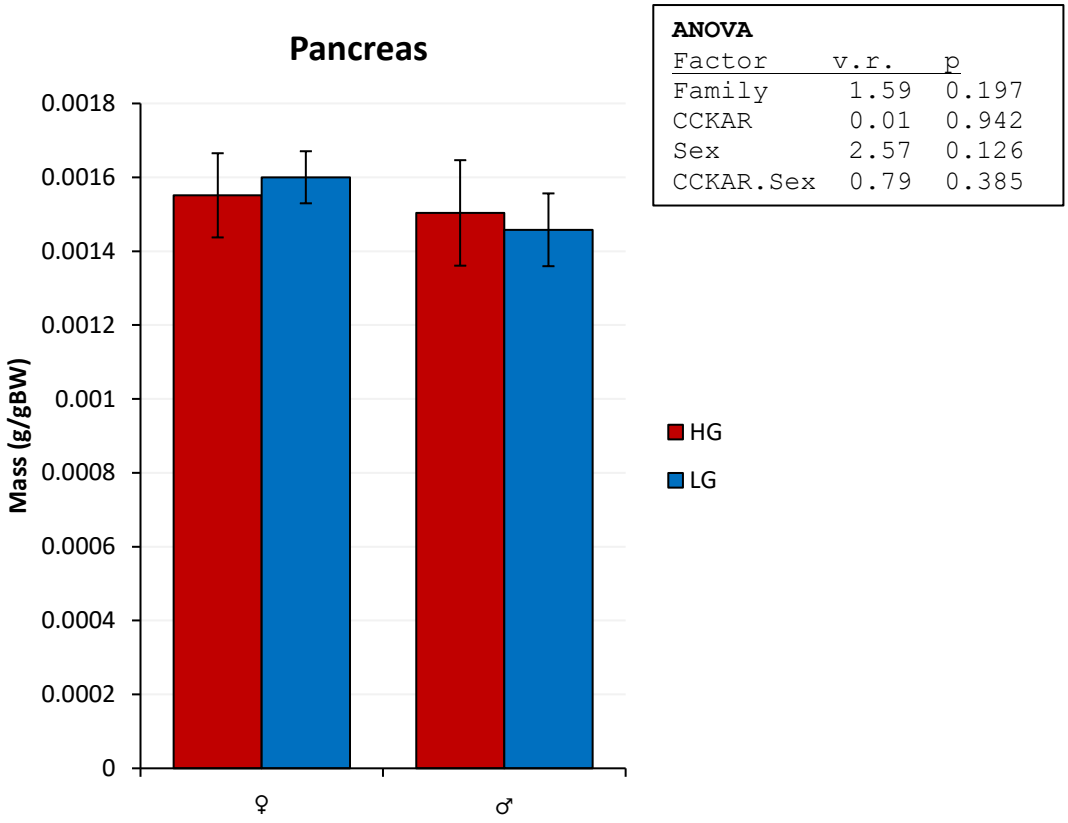


Figure 3.14 – Relative pancreas mass at 12 weeks (CCKAR homozygotes)

Bodyweight-relative pancreas mass is plotted for AIL F_{20} high growth-associated (HG) and low growth-associated (LG) CCKAR haplotype homozygotes at 12wk. No groups differ significantly by two-way ANOVA.

The gallbladder was significantly heavier relative to bodyweight in LG individuals compared to HG individuals, both overall and when each sex was analysed separately.

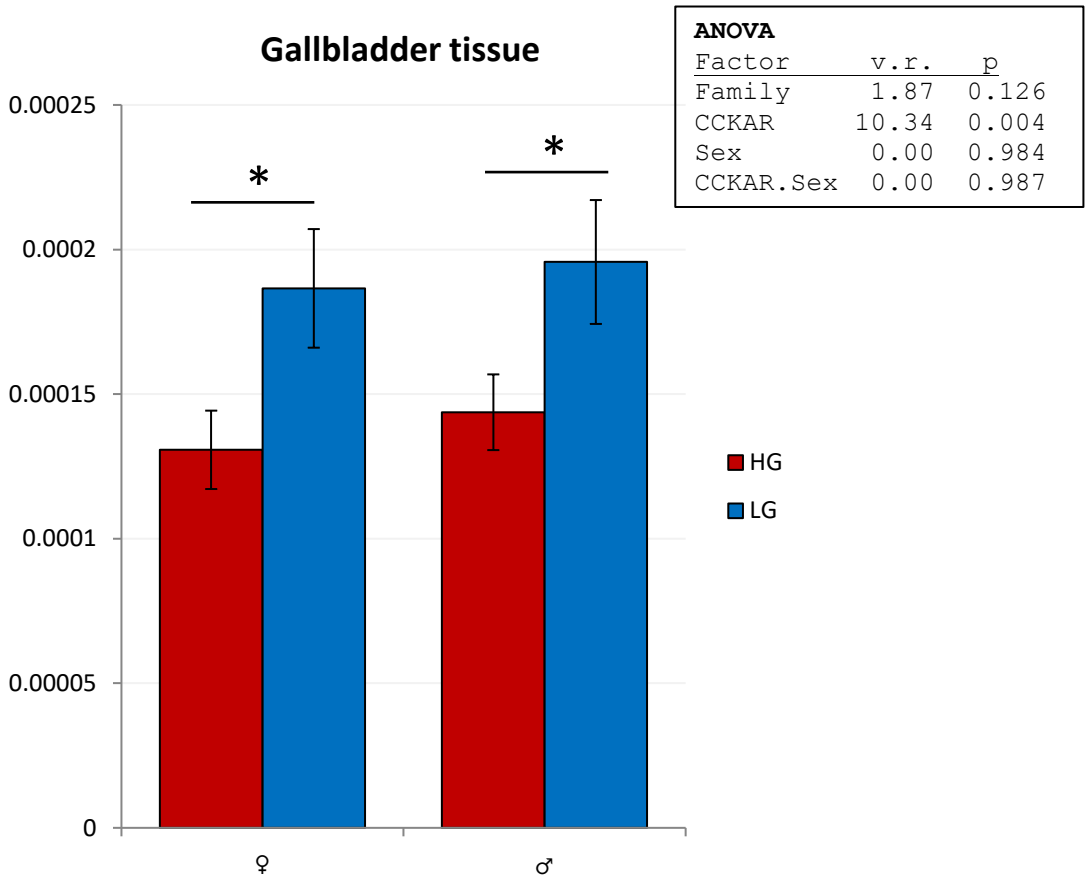


Figure 3.15 – Relative gallbladder tissue mass at 12 weeks (CCKAR homozygotes)

Bodyweight-relative empty gallbladder tissue mass is plotted for AIL F_{20} birds homozygous for high growth-associated (HG) and low growth-associated (LG) CCKAR haplotypes. Significant differences between individual group means are indicated (*p≤0.05).

Gallbladder content mass (relative to bodyweight) was significantly dependent on CCKAR haplotype but no other factors overall. Separate analysis of each sex revealed that female relative gallbladder content volume was highly dependent on CCKAR haplotype, whereas males showed the same trend but not to a level of statistical significance.

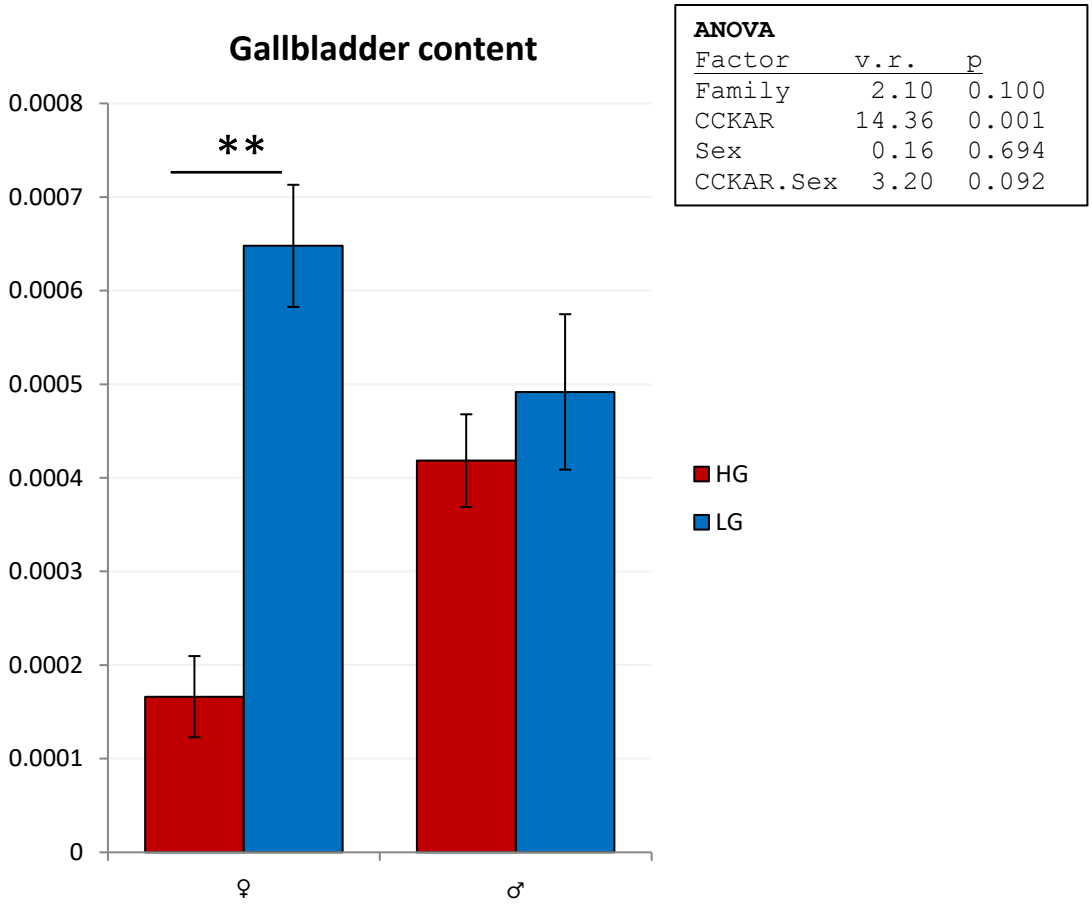


Figure 3.16 – Relative gallbladder content mass at 12 weeks (CCKAR homozygotes)

Bodyweight-relative gallbladder content (bile) mass is plotted for AIL F_{20} birds homozygous for high growth-associated (HG) and low growth-associated (LG) CCKAR haplotypes. Significant differences between individual group means are indicated (**p≤0.01).

CCKAR was the major known factor in determining bodyweight-relative length of the gastrointestinal tract. Sex was also a significant factor explaining a smaller proportion of the difference. A similar trend was found for males and females in terms of the effect of CCKAR haplotype, however statistical significance was only realised in females when sexes were analysed in isolation.

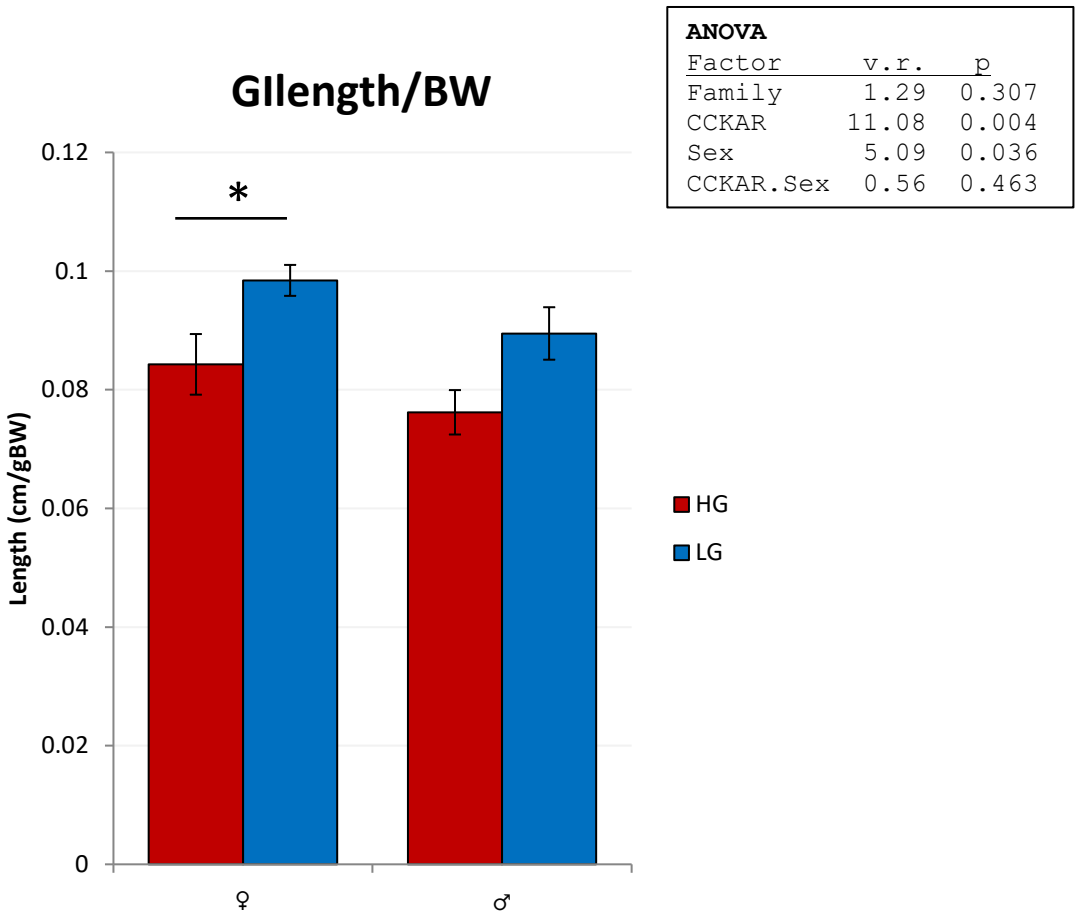
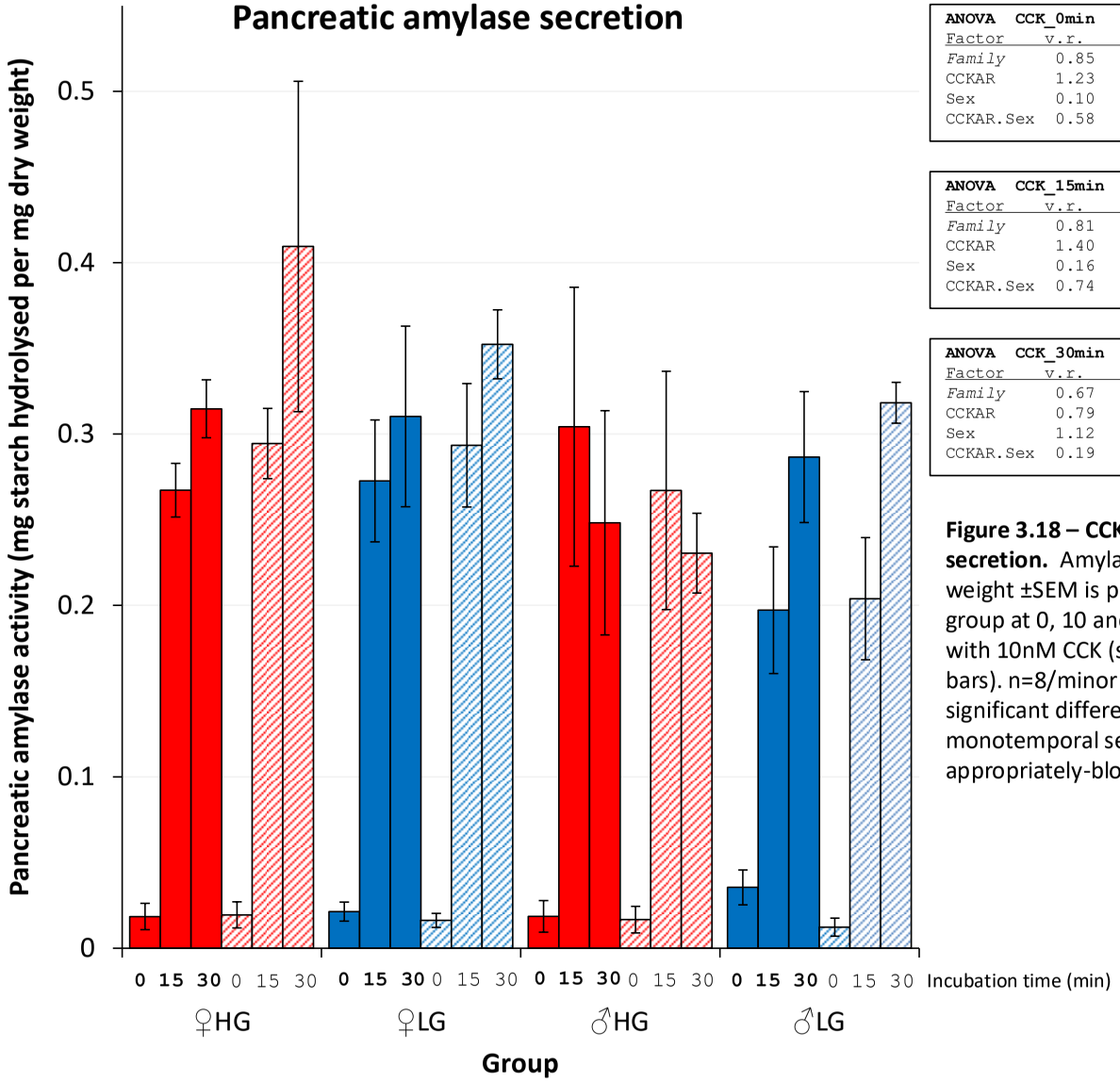


Figure 3.17 – Relative GI tract length at 12 weeks (CCKAR homozygotes)

Bodyweight-relative gastrointestinal tract length is plotted for AIL F_{20} birds homozygous for high growth-associated (HG) and low growth-associated (LG) CCKAR haplotypes. Significant differences between individual group means are indicated (*p≤0.05).

3.4.2.5 Pancreas exocrine function

Figure 3.18 shows the results of the *in vitro* assay for pancreatic exocrine secretion. The obvious increase in amylase activity at the 15min and 30min timepoints confirms that amylase is released from chicken pancreatic explants under the described experimental conditions. CCK treatment did not affect secretion of amylase after 15min or 30min of incubation compared to untreated controls. Neither sex nor CCKAR haplotype had a significant effect on secretion of amylase at any timepoint within CCK treatment or control groups.



ANOVA CCK_0min

Factor	v.r.	p
Family	0.85	0.547
CCKAR	1.23	0.281
Sex	0.10	0.761
CCKAR.Sex	0.58	0.458

ANOVA control_0min

Factor	v.r.	p
Family	0.19	0.976
CCKAR	0.26	0.619
Sex	0.07	0.796
CCKAR.Sex	0.00	0.975

ANOVA CCK_15min

Factor	v.r.	p
Family	0.81	0.575
CCKAR	1.40	0.253
Sex	0.16	0.695
CCKAR.Sex	0.74	0.400

ANOVA control_15min

Factor	v.r.	p
Family	0.99	0.463
CCKAR	0.62	0.440
Sex	1.73	0.204
CCKAR.Sex	0.12	0.731

ANOVA CCK_30min

Factor	v.r.	p
Family	0.67	0.673
CCKAR	0.79	0.387
Sex	1.12	0.304
CCKAR.Sex	0.19	0.666

ANOVA control_30min

Factor	v.r.	p
Family	0.61	0.723
CCKAR	0.14	0.714
Sex	3.84	0.066
CCKAR.Sex	1.68	0.213

Figure 3.18 – CCK-responsive pancreatic amylase secretion. Amylase activity per unit pancreas dry weight ±SEM is plotted for each sex*haplotype group at 0, 10 and 15min incubation in MEM-α with 10nM CCK (solid bars) or without CCK (hashed bars). n=8/minor group. No biologically-relevant significant differences were detected between monotemporal sexes or CCK treatment groups by appropriately-blocked ANOVAs.

3.4.2.6 Intestinal villus morphology

Absolute villar areas are presented in Figure 3.19, and bodyweight-relative villar areas are presented in Figure 3.18. HG individuals tended to have a higher absolute villar area, though no factor was found to predict a significant difference.

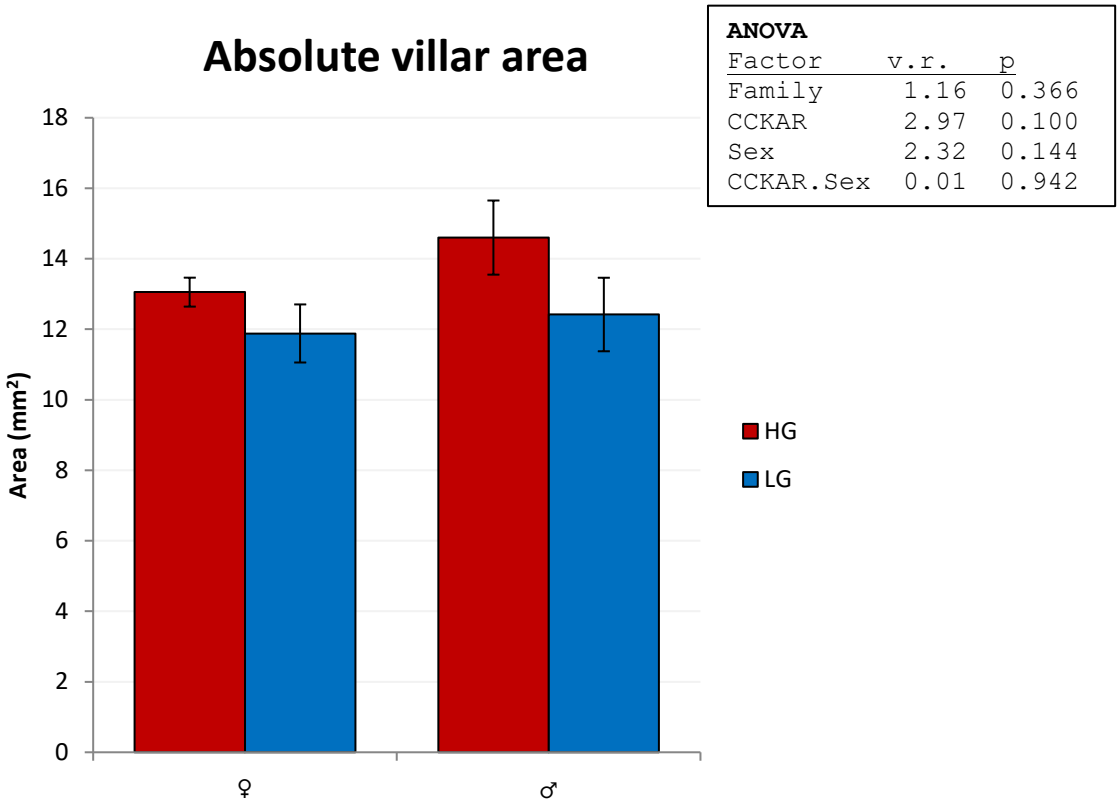


Figure 3.19 – Absolute villar area at 12 weeks (CCKAR homozygotes)

Villar area is plotted for AIL F_{20} high growth-associated (HG) and low growth-associated (LG) CCKAR haplotype homozygotes at 12wk. No individual groups differ significantly by two-way ANOVA.

Bodyweight-relative villar area was independent of CCKAR haplotype, but the trend for males to have relatively smaller villar area relative to their bodyweight approaches significance overall.

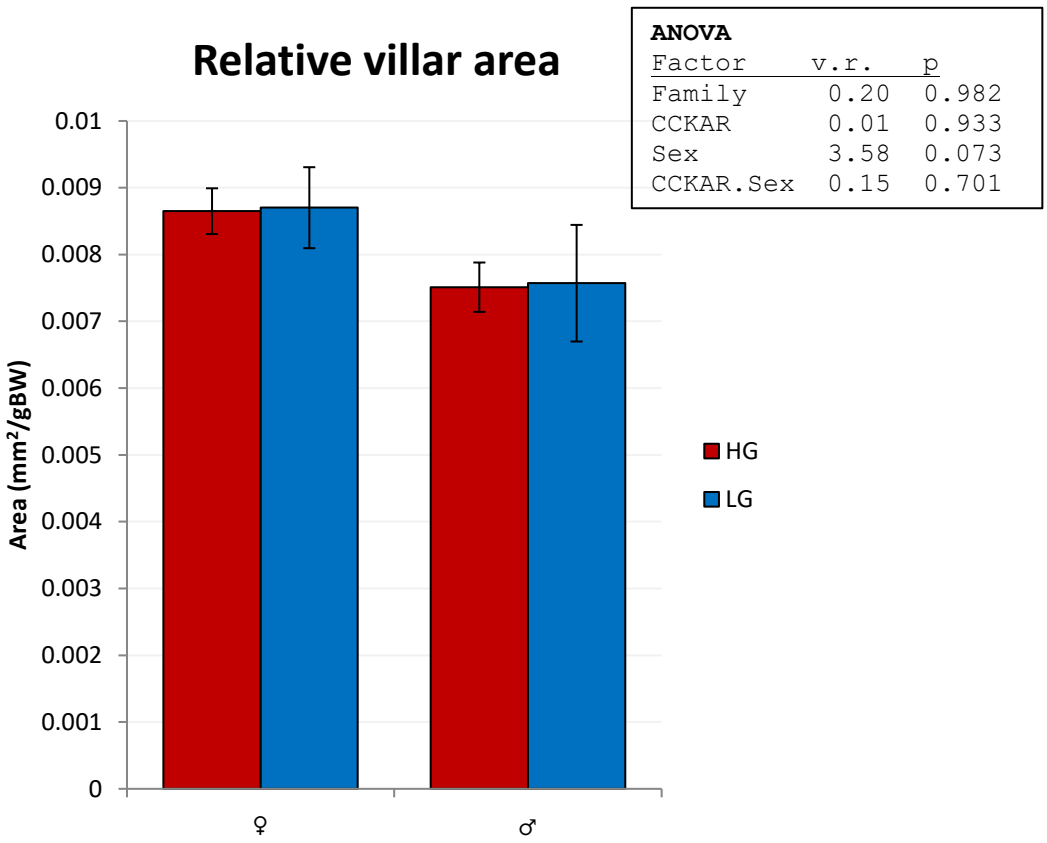


Figure 3.20 – Bodyweight-relative villar area at 12 weeks (CCKAR homozygotes)

Villar area after normalisation to bodyweight is plotted for AIL F_{20} high growth-associated (HG) and low growth-associated (LG) CCKAR haplotype homozygotes at 12wk. No groups differ significantly by two-way ANOVA.

3.5 Discussion and conclusions

3.5.1 Genomic basis of CCKAR-mediated growth phenotypes

The inferred transcriptional start site (section 3.4.1.4; brown leghorn-derived) is discordant with the published transcriptional start site (AB214534.1; white leghorn-derived) (Ohkubo *et al.*, 2007). This is interesting as alternative transcriptional start sites may exist in different chicken lines; however this assay was only carried out for one individual so should be repeated and transcriptional elements examined to confirm authenticity.

3.5.1.1 Association analyses

The probabilities of association of the three wide-range SNP markers tested in the GM8 population (Table 3.1) suggest that the upstream marker ch4snp851573063S2 is the most reliable in predicting growth effects. This was converse to the result obtained in the unrelated AIL, for which the best predictive marker was ch4snp1311324046S2, downstream of CCKAR. Since CCKAR_MnlI, a marker within the CCKAR gene itself, exhibited good reliability for both of these broiler-layer hybrid populations, it was reasoned that these disparate results might be due to divergent linkage disequilibrium or an artefact of the expected multi-factorial nature of the QTL effects. It was reasoned that for an explanatory variant closer to CCKAR not to exist, either two or more recombination events flanking CCKAR_MnlI would be required between ch4snp851573063S2 and ch4snp1311324046S2 in at least one of the AIL and GM8 lines in the period since they diverged. The alternative explanation that a causative variant existed close to CCKAR therefore seemed a more conservative assumption, and it was decided to pursue local haplotype derivation around CCKAR. Since assembly of HG and LG haplotype sequences revealed over 300 novel variations, an abundance of potential genotyping targets for fine mapping existed. The prominent size of the DelinvA deletion immediately made it an obvious candidate for genotyping and also made development of a simple ALP assay possible (Section

2.2.3.2). DelinvA was therefore included alongside CCKAR_MnlI in standard in-house genotyping.

The AIL layer-type founding line exhibits a growth phenotype more reminiscent of the ancestral junglefowl compared to that of the broiler-type founding line, because layers have not been heavily selected for high growth. Genetic loci causing a high-growth effect were hence considered more likely to be unique to the broiler-derived haplotype than to the layer-type or ancestral junglefowl. Only variations unique to the HG haplotype were therefore targeted for genotyping of the Multistrain, alongside the MnlI and DelinvA standard genotyping assays already mentioned.

The Multistrain genotyping and bodyweight association analysis results presented in Figure 3.1B demonstrate that most genotyped variations did not predict a significant effect on bodyweight in this much more diverse population. This is presumably because of the large number of divergent branches and the relatively long timescale over which these strains have diverged. Combined phylogenetic distance of this sort improves the odds that local recombination events are represented in the population overall, thus lowering the likelihood of detection of linkage disequilibrium effects. Only one SNP marker (AR24, located within CCKAR intron 3 at position galGal4:chr4:72,821,650) was found to predict a significant effect on bodyweight.

3.5.1.2 Transcriptional implications

Subsequent *in silico* analysis of this marker did not suggest that it affected any regulatory elements but identified it as the closest marker to another SNP (galGal4:chr4:72,821,636) which disrupts putative binding sites for the transcription factors TGGCA-binding protein, AP-3 and C/EBP-α. Such disruption might contribute to the effect on CCKAR expression, although it must be noted that the junglefowl reference genome agrees with the AIL HG allele at this position. Functional intronic recognition sites for TGGCA-binding protein and AP-3 are not well-documented whereas their effects seem to rely on the recognition site(s) lying upstream of the regulated transcriptional start site. Additionally, the consensus TGGCA-binding

protein recognition site in chicken was determined to be 5'-TGGCANNNTGCCA-3' (Borgmeyer et al., 1984), discordant with the observed sequence. In contrast, there is an abundance of evidence for functional intronic recognition sites for C/EBP-alpha in eukaryotes (Giacopelli et al., 2003, Qiao et al., 2005), including at least one study specifically citing the down-regulating effect of an A>G SNP ablating the recognition site (Murani et al., 2009). The C/EBP (CCAAT/enhancer-binding protein) family of transcription factors seem to be particularly heavily implicated in regulation of genes involved in bodyweight homeostasis such as ADIPOQ, PCK1, LEP, and regulation of these transcription factor is known to be dependent on nutritional status (Ramji and Foka, 2002).

The SNP affecting YY1 binding identified by Rikimaru et al. (2013) was found to segregate in the AIL in the expected phenotype-associated pattern according to their speculation of its effects in chickens, however the concordance of these studies was not recognised at the time of Multistrain genotyping and so by chance this variation was not targeted. Nonetheless, proximal variations flanking this SNP were genotyped and failed to predict a significant effect of bodyweight in the Multistrain. The theory of YY1 binding disruption is tempting however the recognition sequence at the position reported for Hinai-Dori chickens does not conform to the most common mammalian YY1 repressor binding site. The hypotheses of Rikimaru et al. (2013) and Houston et al. (2008) could be substantiated economically by measuring expression of CCKAR mRNA in existing intercrossed animals segregating for the relevant SNP by qPCR.

It might be of value to genotype the YY1-disrupting SNP in the Multistrain population. If it arose fairly recently during the development of heavy chicken breeds, it might be shielded from associative marking (i.e. flanking markers might be common to HG and LG haplotypes even if this SNP does predict a significant effect).

The DelinvA deletion was found to be a missing CR1 regulatory element which improves the prospect of a functional effect on CCKAR expression, however no nearby genotyping targets were found to predict bodyweight.

It would be interesting to determine what proportion of the effect on bodyweight, if any, could be specifically ascribed to DelinvA, YY1 disruption and C/EBP-α, as well as any other arising candidate causative loci, as it seems likely that CCKAR expression is controlled by more than one element in this genomic region. A reverse genetic approach could also be employed to unequivocally link decreased CCKAR expression to increased growth, for example by siRNA knockdown of CCKAR.

3.5.1.3 CCKAR_MnlI-DelinvA recombination

A chance recombination event between CCKAR_MnlI and DelinvA was detected in the AIL F_{19}. The recombination was confirmed as novel by genotyping (section 2.2.3.2) the two AIL founder individuals, which were homozygous in the direction expected (deletion in the broiler). The recombinant allele was only detected in one F_{19} CCKAR_MnlI homozygote (bird ID = ♀4075), and so the experimental populations described in this chapter were not known to be affected, and indeed most individuals were genotyped for DelinvA as described in section 2.2.3.2, so the population-wide influence of this locus independent of the CCKAR_MnlI genotype used for grouping would have been minimal. There must however have been at least one additional recombinant selected which contained both the same recombinant allele as ♀4075, and the reciprocal recombinant product. Such an individual would have presented as a heterozygote at both loci upon genotyping, and must have been selected for breeding since both recombinant alleles have since been detected in generations F_{21}-F_{23} by genotyping in birds whose alternative allele is one of either founding haplotype. Interestingly, the persistence of both alleles suggests that the recombination was germ-line mitotic – not meiotic as would have been conservatively expected – which makes this an exceedingly rare event. It also results in a valuable biological tool with which to further assess association of chr4 regions with growth traits, since the QTL has been split, and each broiler-derived (HG) section can now be isolated alongside the layer-derived (LG) background on the opposite side of the recombination site.

Appropriate breeding strategies were implemented before surrender of control of the AIL population from this project, so these recombinants will be available for use imminently.

3.5.2 Physiological mechanisms explaining altered growth

Persistent reduced expression of CCKAR in HG as compared to LG birds, described in both central and peripheral tissues by Dunn *et al.* (2013a), could conceivably affect growth phenotype by several means. Previous studies have implicated perturbation of appetite control (Dunn *et al.*, 2013a; Rikimaru *et al.*, 2013), generating a neat mechanistic hypothesis for increased growth in the context of central energy homeostasis (see Section 1.3.2). Altered expression of CCKAR might additionally elicit divergent organismal development and/or mature physiology, thereby potentially influencing stored energy investment, digestive capacity and endocrine functions, among other factors. The output of this chapter addresses associations of phenotypic observations with overall growth phenotype, aiming to identify physical differences between HG and LG birds which might facilitate or result from physiological mechanism(s) for increased growth.

3.5.2.1 Note on bodyweight normalisation

Bodyweight normalisation was elected in the handling of physiological data, where it was deemed suitable. This helps to negate the effect of pre-existing gross bodyweight difference causing correlated increase in associated traits (e.g. organ size, feed consumption). This also allowed rapid identification of traits very tightly correlated to bodyweight, which are of interest since defence of relative organ weight implies the necessity of investment of stored energy in that organ to maintain a functional capacity which matches bodyweight. It must however be remembered that great differences in specific heavy parts of the body (e.g. breast muscle) might have a confounding effect on any measurement normalised to bodyweight.

3.5.2.2 Bodyweight and stored energy investment

The difference in growth observed between AIL HG and LG haplotypes only becomes apparent after ~6wk of age (Figures 3.3 and 3.4). The change in statistical significance of the association with growth attributed to CCKAR haplotype suggests that the effect is responsive to the post-hatch environment (whereas sex is predictive from an earlier stage) (Figure 3.5). Of course the egg is a physically limiting environment, so causative mechanisms involving behaviour (e.g. locomotion, feeding) are attractive options for exploration. Concordantly, the CCKAR effect predicted a difference in stored energy investment, since LG metatarsi were longer compared to bodyweight overall (Figure 3.6), but there was no difference in absolute metatarsus length (data not shown) which suggests excess weight is soft tissue.

3.5.2.3 Feed intake

In these studies, improved growth cannot be explained by increased feed consumption, despite the neat hypothesis of Dunn and colleagues (2013a), since feed consumption relative to bodyweight was not increased in HG individuals under normal conditions. In fact, feed intake was comparable between HG and LG groups at 4wk (Figure 3.7) and relatively less in HG by 12wk of age (Figure 3.9). FCE was improved for HG compared to LG individuals (Figure 3.7), suggesting that HG birds are better at extracting nutrients or avoiding energy wastage, or both.

3.5.2.4 Visceral organ capacity and function

Exploration of visceral organ capacities aimed to determine what physiological mechanisms might be at play in achieving greater bodyweight in HG birds.

3.5.2.4.1 Whole digestive tract transit duration (WTTD)

Firstly, it was hypothesised that lowered CCKAR expression might affect CCK-mediated gastrointestinal motility. This could theoretically allow nutrients more time in contact with the gut epithelium and hence improve absorption, however no

difference in WTTD was found between groups in this study (Figure 3.10), so cannot be attributed to simple transit duration.

3.5.2.4.2 Spleen

The spleen was not expected to vary greatly in mass between CCKAR haplotypes, and it did not overall. It was however found to be relatively smaller in LG females compared to HG females, and larger in HG females compared to HG males, each to a level of statistical significance (Figure 3.11). Sex was the most important factor affecting relative spleen mass. The spleen is not classically thought to be related to control of bodyweight, however a similar but sex-independent effect has been reported in turkeys selected for increased bodyweight (Li *et al.*, 2001). It is possible that this effect on spleen mass is unrelated to energy control, since recent evidence suggests a specific role for CCKAR in splenic immune function (El-Kassas *et al.*, 2016).

3.5.2.4.3 Proventriculus and gizzard

The proventriculs and gizzard were prime candidates for altered morphology since altered gastric secretion and mechanical processing of feed might improve nutrient uptake. Both proventriculus and gizzard bodyweight-relative masses exhibited a paradoxical association, being greater in LG birds (Figures 3.12 and 3.13), which suggests that neither gastric exocrine activity nor pre-duodenal mechanical processing of feed are limiting factors in the growth of LG birds. This might be because of the processed nature of the modern feed material these birds were offered; i.e. nutrients are more readily available from pelleted feed than, for example, intact grains.

3.5.2.4.4 Pancreas and gallbladder

The pancreas and gallbladder are both important organs for release of digestive factors (namely enzymes and bile, respectively) into the luminal environment. Alteration of their functional capacity could therefore affect digestive efficiency, and broilers have been shown to exhibit enhanced pancreatic exocrine production

compared to the ancestral junglefowl (Kadhim *et al.*, 2011). Kadhim and colleagues also noted an interesting dynamic in relative pancreas size whereby broilers had a relatively larger pancreas at an early age (<10d) which became relatively smaller at later age (>10d), so disparate relative pancreas size might indicate growth potential, at least at a very young age. The birds studied here were 12wk of age but did not exhibit significant difference in relative pancreas size (Figure 3.14). This is not entirely surprising, since pancreas mass has previously been demonstrated to correlate linearly with bodyweight at later age (9wk) in modern broilers and ancestral fowl (Jackson & Diamond, 1996). This observed fidelity between pancreas mass and bodyweight is interesting in itself, since altered development of the pancreas might be a contributing factor in driving the bodyweight setpoint shift observed in broilers though this remains largely speculative at this time. If true, such a phenomenon is possibly facilitated by the heterocrine nature of pancreatic function, which effects a convincing theoretical double mechanism for increased growth: increased exocrine production improving nutrient absorption on one hand, and elevated endocrine activity acting to lower blood glucose and store energy as body mass on the other. An effect of reduced CCKAR expression on glucose homeostasis in chickens would be in keeping with mechanistic observations made in mammals (section 3.1.1) and seems particularly likely considering the subsequent observation of reduced relative pancreas mass in a congenic rat strain with the OLETF-derived non-functional CCKAR locus (Moralejo *et al.*, 2000). In the present study, no difference in exocrine secretion – using amylase as an index – was detected between sexes, haplotypes or CCK treatment groups (Figure 3.18). It is however conceded that great intra-group variability existed, suggesting that the assay design might not have been conducive to accurate and consistent measurements. The renovation of the protocol used by Hokin and colleagues (1950) was intended to improve reliability by allowing for more replicates, however it might be that the reduction in per-preparation tissue mass made variation between samples more significant. There were also several steps for which timely completion was technically challenging. For example, slicing of the pancreas

was completed within ≈30min of cull, but each tissue slice would experience a unique environment during that time period. Medium samples could only be taken individually, and required removal of all preparations from the incubator, so inconsistencies in timing of sample removal and temperature change were uncontrollable variables. Another limiting step was addition of starch and HCl to begin and halt amylase activity respectively, which could only be completed for 8 wells at a time (of a total of 96 per plate) and so each addition took up to 30s per plate. It is likely that the combined effect of these factors dwarfed any true difference between groups, yet this experimental approach still showed potential since amylase secretion was successfully detected (by comparison of 0min and other timepoints), so if the technically limiting steps could be addressed, repeat of this experiment might be justified.

The gallbladder harboured the most prominent tested difference between HG and LG individuals, with tissue mass (Figure 3.15) and content mass (Figure 3.16) both heavily dependent on CCKAR haplotype overall. The effect on tissue weight was comparable between sex, whereas females exhibited a greater difference in gallbladder content mass. No significant difference in content mass was detected between HG and LG males, however these groups did trend in the same directions as their respective female counterparts and there was no significant interaction between haplotype and sex overall. The gallbladder is the classic target organ for peripheral CCK signalling, so it stands to reason that some functional effect should result from perturbed CCKAR expression. Mechanistically however, the trend for content mass between CCKAR haplotypes is paradoxical. Since CCK is classically implicated in stimulating bile flow, reduced CCK reception would be expected to prevent gallbladder emptying in HG birds, whereas in the current study this group presented with lower gallbladder content mass. This might of course be due to a number of alternative explanations based on possible receptor dynamics, for example reduced expression in the HG sphincter of Oddi might result in sustained CCKAR

hypersensitivity or reduced stimulation-induced internalisation of CCKAR (Cheng *et al.*, 2003).

3.5.2.4.5 Intestinal morphology

Intestinal morphology was of great interest as it has been previously demonstrated both that selection for increased growth was associated with elevated bodyweight-relative intestinal mass (Jackson & Diamond, 1996) and altered villus morphology (Zavarize *et al.*, 2012), and that poultry respond to perception of negative energy balance by increasing luminal epithelial area to improve nutrient uptake (Yamauchi *et al.*, 2010). In the present study, gastrointestinal length was significantly shorter relative to bodyweight in HG birds (Figure 3.16), suggesting that increased intestinal mass results from altered sub-organic structure morphology (e.g. villar shape/size), or that the layer-derived LG allele predicts a relatively increased intestinal length, though this seems less likely. Incidentally, no significant difference in absolute or bodyweight-relative total gastrointestinal mass was detected between haplotypes, however this measurement lacked integrity since gizzard, proventriculus, mesenteric and adipose tissues and gastrointestinal contents were left intact for weighing and so these data are not presented. Villar area (the area of a coronal cross-section of small intestine occupied by villi) was used as a proxy for epithelial surface area due to time constraints. It was reasoned that the dependence of this measurement on intestinal perimeter and villus length made it a suitable proxy, but better resolution might have been achieved by measuring alternative characteristics (e.g. villus length). Nonetheless, a difference in absolute villar area approaching overall significance was detected between CCKAR haplotypes, with the trend of larger area in HG individuals maintained between sexes, in keeping with expectations (Figure 3.19). There was however no difference in relative villar area (Figure 3.20), suggesting that (like pancreas size) villus morphology is intimately tied to total bodyweight. It is not clear whether altered intestinal morphology might result in increased bodyweight or vice-

versa, and in any case a repeated study with a greater number of individuals per group is needed to confirm the putative trend.

3.5.3 General conclusions and future work

The conclusive output of this work is that several hundred novel high- and low- growth-associated variants have been identified. Forty-two markers were assessed for association with bodyweight in diverse chicken lines, with one SNP appearing significant in predicting CCKAR-mediated bodyweight control. Lowered CCKAR expression does not appear to increase feed intake relative to bodyweight at the ages examined, however the HG allele predicted improved FCE, at least in males. Additionally, some morphological traits explained by haplotype at the CCKAR locus have been identified (proventriculus mass, gizzard mass and in particular gallbladder tissue and content mass). Some traits were found to be particularly closely associated with individual whole bodyweight, regardless of CCKAR haplotype (pancreas mass, villar area) and these are of some interest as it is unknown whether bodyweight is caused by or effects these characteristics, and CCKAR may play a role in organic development.

Many of the traits analysed were dependent on sex overall, and some of the significant effects predicted by CCKAR haplotype were only apparent in one sex when sexes were analysed separately. Chickens display obvious sexual dimorphism for bodyweight (e.g. Figure 3.4), as do many avian species. The data described in this chapter implicates CCK signalling via CCKAR in manifestation of dimorphic growth, so it seems likely that sex-linked, trans-acting factors affect either CCK or CCKAR, or both, either directly or indirectly.

The work described in this chapter is provides useful direction for further studies of the genetic basis and physiological mechanism(s) for CCKAR-mediated growth phenotype. Future strategies should make use of existing genomic information and the novel recombinant QTL haplotypes of the AIL to ascribe functional significance to candidate transcription factor binding sites using live birds or transgenic cell lines.

Efforts to identify physiological effects should prioritise collection of data concerning pancreatic, cholecystic and intestinal capacity, since these seem mechanistically plausible and evidence has been generated for their implication in CCKAR-mediated growth phenotype.

Measurement of pancreas capacity at younger ages would enable identification of differences in relative pancreas growth dynamics between HG and LG birds. Refinement of the *in vitro* assay of pancreatic exocrine secretion to reduce variability might resolve any true difference between HG and LG birds. Inclusion of a group treated with a secretion-inhibiting drug (e.g. atropine) could be used to confirm that amylase is actively secreted from explants (and does not merely diffuse). It would also be useful to collect direct evidence by measuring amylase activity in the luminal environment.

Teasing apart the dynamics of CCKAR at the gallbladder for HG and LG haplotypes would require parallel assays for responsiveness to a range of CCK concentrations, but would be of value in explaining the functional effect of reduced receptor expression on release of bile from the gallbladder. *In vitro* preparations of HG and LG sphincter of Oddi with stimulation by exogenous CCK represents a potential reliable and economic option. Being a storage organ, morphology of the gallbladder might be related to bile production as well as stimulation of its secretion, and so examination of genes implicated in bile production, and their dependence on CCKAR haplotype, nutritive state and CCK treatment are additional potential avenues of exploration.

Finally, it should be noted that the observations reported in this chapter do little to describe potential effects of reduced CCKAR signalling in the brain. Although no explanatory difference in feed intake was detected, the effect on bodyweight control might depend on alternative centrally-orchestrated mechanisms of energy balance. Potential examples include conservation of energy by reduced locomotion or thermogenesis, altered respiratory quotient (Lo *et al.*, 2008), or by diversion of energy from other as-yet-unidentified physiological processes, possibly by post-arcuate CCK

signalling at the PVN (Ingram *et al.*, 1989). It would therefore be appropriate for subsequent studies to examine central CCKAR expression more closely, to identify affected brain regions and thus develop a better understanding of how CCKAR haplotype determines central control of energy homeostasis.

CHAPTER 4

Peripheral peptide hormones of the PP-fold family

4 Peripheral peptide hormones of the PP-fold family

4.1 Introduction

Current efforts to investigate genomic effectors of altered growth phenotype in selectively-bred domesticated fowl, such as the characterisation of the molecular basis of selection at the CCKAR locus described in the previous chapter, interrelate with more general interest work geared toward generating a fuller picture of endocrine control of energy homeostasis in birds, and insight into the evolution of (neuro)endocrine mechanisms regulating appetite and energy control in wider vertebrate clades. After all, it is elements of these natural mechanisms that are altered in selection for growth phenotypes in livestock species. Of course the endocrine control of appetite and growth is incredibly complex in vertebrates and birds are no exception, as described in Chapter 1. There exists a myriad of central and peripheral molecular regulators of appetite and energy balance in chickens and, if a global understanding of bodyweight control is to be achieved, the activity and regulation of all these molecules must be well described.

4.1.1 PP-fold hormones

The tetrapod PP-fold family of peptide hormones comprises three members: neuropeptide Y (NPY), pancreatic polypeptide (PP) and peptide YY (PYY). NPY is believed to most closely resemble the common ancestral gene of NPY and its paralogue PYY in all vertebrates, whereas PP is the youngest member and exists in tetrapods only, arising from a more recent duplication of *PYY* (Conlon, 2002). Fish lack PP but have a fish-specific third PP-fold gene more closely related to *PYY*, known as *PYYb* (Volkoff, 2016). All mature vertebrate PP-fold polypeptides share structural homology; at the N-terminus, a loosely structured linear tail most commonly presents three proline residues (*Homo* Pro_2, Pro_5 & Pro_8) which interdigitate with two tyrosine residues (*Homo* Tyr_{20} & Tyr_{27}) which are aligned by inclusion in the subsequent

amphipathic α-helix domain which leads to a disordered C-terminal region (Figure 4.1A).

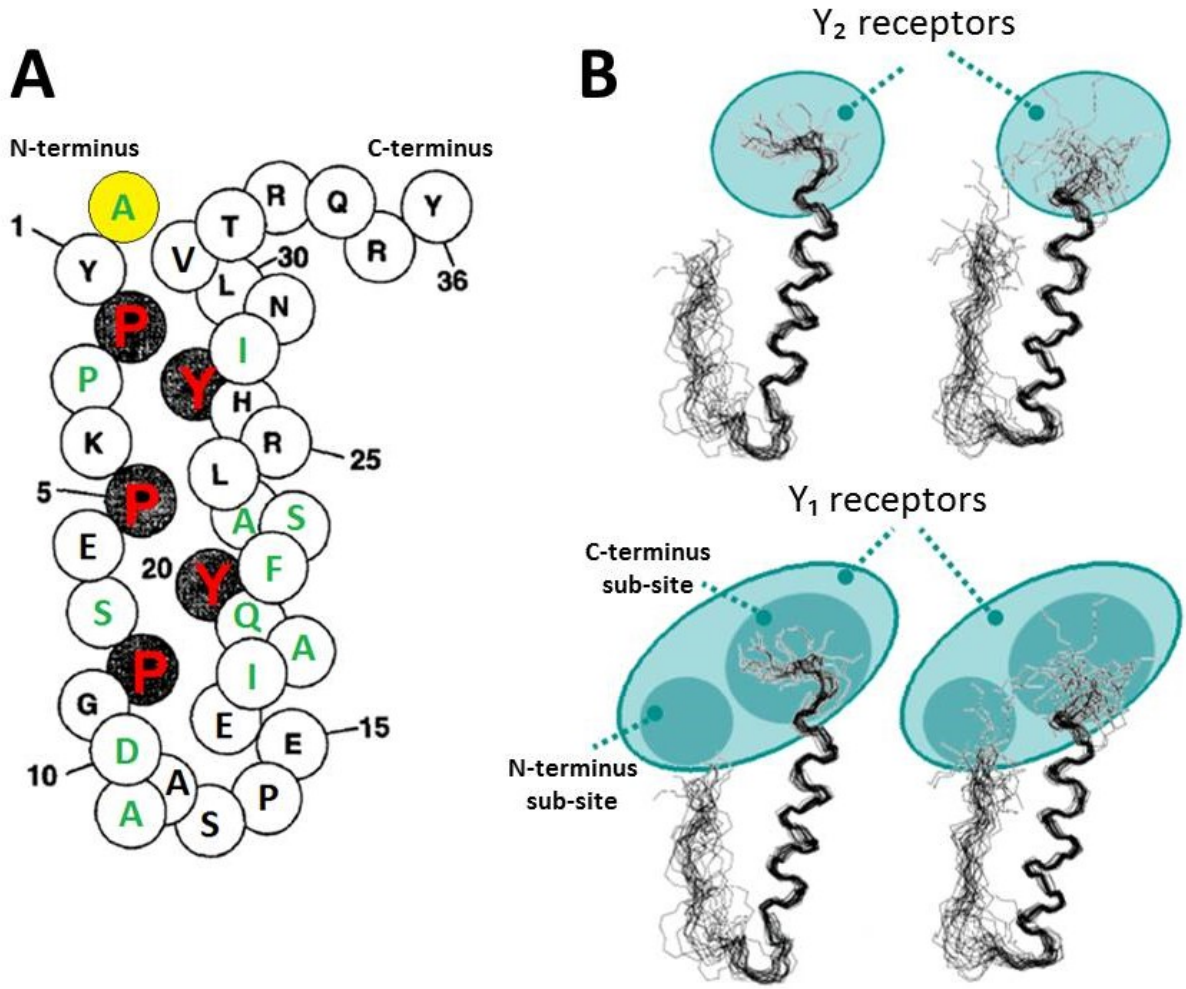


Figure 4.1 – Structural schematics for PYY.
A: The conserved PP-fold motif of chicken PYY is shown. Numbers indicate corresponding mature peptide amino acid positions for human PYY. Green letters indicate differences between human and chicken PYY sequences. The yellow residue does not exist in mature mammalian PYY. Adapted from Larhammar et al. (1996).
B: Conformations of human PYY_{1-36} (hPYY) and PYY_{3-36} (hPYY3-36), and putative receptor interactions are shown. The PYY_{3-36} DPP-IV cleavage product cannot interact at Y_1 receptor N-terminus sub-site but exhibits greater C-terminal structural stability, conferring heightened specificity for Y_2 receptor binding site. Adapted from Nygaard et al. (2006).

4.1.1.1 Neuropeptide Y (NPY)

NPY is mainly implicated in central energy signalling and is co-expressed as an orexigen alongside AGRP in the anabolic first-order neuronal species of the arcuate nucleus (Boswell, 2005), as previously outlined in section 1.3.2.2. Conversely, PP,

PY and PYY are primarily known for their involvement in peripheral energy signalling, although at least PYY (all vertebrates) and PY (fish only) are also detected in the brain (Cerdá-Reverter *et al.*, 2000).

4.1.1.2 Peptide YY (PYY)

PYY is a purported satiety factor expressed in intestinal enteroendocrine cells, increasing in concentration toward the distal end of the mammalian intestinal tract (Ballantyne, 2006). The endogenous activities of PYY are discussed in section 4.1.2, below.

4.1.1.3 Pancreatic polypeptide (PP)

Peripheral PP and PY are almost exclusively found in the pancreas. Tetrapod PP and fish PY have each rapidly and divergently evolved since duplication of the PP/PYY/PY ancestral gene (Cerdá-Reverter *et al.*, 2000; Conlon, 2002), though it remains unclear whether PY and PP arose at a single duplication event (Cerdá-Reverter *et al.*, 2000). For PP at least, structural conservation appears to be of greater importance than precise amino acid sequence in terms of function (Glover *et al.*, 1984) which might explain its accelerated sequence evolution, since the interdigitating proline and tyrosine residues of the PP-fold are conserved (Conlon, 2002).

4.1.2 Endogenous PP-fold roles and receptor diversity

An interesting opposition of energy-regulating roles is apparent for members of the PP-fold family depending on anatomical location, at least in tetrapods, since central NPY and PYY drive energy intake (Kuenzel *et al.*, 1987) and promote anabolism whereas peripheral PP and PYY are satiety hormones (Alumets *et al.*, 1978; Batterham *et al.*, 2002). Physical separation of receptor sites likely facilitates this difference, the response being dependent on the Y receptor type bound and receptive cell species, but this is unlikely to be explained at the blood-brain barrier level, since

all three tetrapod PP-fold family members have been shown to traverse this barrier (Banks *et al.*, 1995; Kastin & Akerstrom, 1999; Nonaka *et al.*, 2003).

PP-fold peptides exert their signal through interaction with several Y receptor types, of which five are known in mammals (Y_1, Y_2, Y_4, Y_5 & Y_6), an additional receptor type (Y_7) has been discovered in fish, amphibians and recently birds (Bromee *et al.*, 2006) and an eighth (Y_8) is lost in all amniotes but persists in teleosts (Larhammar & Bergqvist, 2013). Recent homology studies in holocephalan (Volkoff, 2016) and coelacanth (Larhammar & Bergqvist, 2013) species prove that all the aforementioned Y receptor types existed in the latest common gnathostome ancestor. These ancient Y receptor types are undoubtedly the products of two ancient tetraploidization events which produced at least these seven types (Figure 4.2) and possibly more, depending on losses between and since tetraploidization events (Larhammar & Bergqvist, 2013).

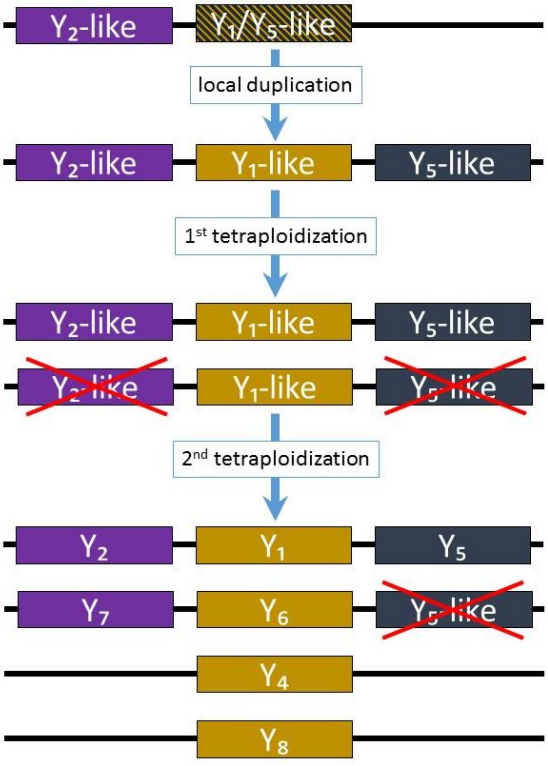


Figure 4.2 – Conjectural evolution of ancient vertebrate Y receptor types.
A local duplication of the ancestral Y_1 /Y_5 -like gene produced 3 syntenic paralogues. The first vertebrate basal tetraploidization event duplicated these three genes, but one syntenic duplicate lost the Y_2 -like and Y_5 -like copies. The subsequent 2nd vertebrate basal tetraploidization produced a further duplicate of each resultant strand, giving a total of eight receptor genes, of which one (Y_5 -like duplicate) was subsequently lost by appearance of the last vertebrate common ancestor. Based on the evolutionary model proposed by Larhammar and Bergqvist (2013).

The less comprehensive Y receptor repertoires of all studied extant gnathostome species must therefore have been achieved by loss of receptor types as gnathostome species diverged. Regardless of the complex evolutionary minutiae of variant Y receptor repertoires, at least one homologue of each of the most ancient duplicates

(Y_1/Y_5-like and Y_2-like) are represented in all studied vertebrate species, so ancient intra-individual receptor diversity is maintained throughout vertebrates. Each ligand and receptor exhibits preferential interactions (Pedragosa-Badia *et al.*, 2013).

Complex mechanistic configurations therefore exist for the interrelated functions of PP-fold hormones in tetrapods. Clarification of the precise effects of each PP-fold molecule requires functional study, which is best informed by knowledge of the anatomical distribution of PP-fold ligands and their Y receptor targets in each species. Two recent studies complementarily describe tissue-specific expression for the full cohort of chicken PP-fold hormones and all 6 Y receptors (He *et al.*, 2016; Gao *et al.*, 2017). These researchers found highest expression of PYY and PPY in the pancreas, with both expressed at lower levels in the brain but only PYY detected in the alimentary tract. NPY transcript abundance was far lower in real terms, and almost exclusively central. Y_1 and Y_7 preferentially bind NPY and are both expressed throughout the brain but also in peripheral tissues, at least for Y_7. PP only activates Y_4 and Y_5, which are predominantly expressed in adipose and pancreatic tissues but also to a lesser extent in some brain regions. PYY interacts appreciably with every Y receptor except Y_6, but is particularly potent at Y_2 which displays the widest distribution of all the Y receptors in chickens. Although this information is incredibly valuable in helping direct further research into PP-fold hormones in chickens, and despite PP first being isolated from chicken pancreas (Kimmel *et al.*, 1975) and the PP-fold structure itself first being described in the same molecule (Blundell *et al.*, 1981), all three tetrapod PP-fold family members remain far better understood in mammals than birds. Insight into this diverse and complex signalling system in chickens might therefore be best understood by study in the context and insight of mammalian studies to date.

4.1.2.1 PYY in glucose homeostasis

PYY is thought to contribute to glycaemic control in the balance of whole-body energy in mammals (Guo *et al.*, 1988; Bertrand *et al.*, 1992; Shi *et al.*, 2015; Ramracheya *et*

al., 2016) by some mechanism involving local regulation of insulin-producing β-cells, but its precise role remains unknown (Batterham & Bloom, 2003). Conflicting evidence exists in the few studies to have investigated its effects directly, since exogenously-administered PYY_{1-36} inhibited insulin release in isolated rat islets *in vitro* and dogs (Guo *et al.*, 1988; Bertrand *et al.*, 1992), but seemingly facilitated glucose-responsive insulin secretion in a separate *in vitro* rat islet preparation (Ramracheya *et al.*, 2016) and PYY overexpression increased insulin-producing islet β-cell proliferation and function in mice (Shi *et al.*, 2015). The latter examples are likely due to inadvertent Y receptor desensitisation and the resulting apparently reversed effects of the ligand, since apparent desensitisation has previously been demonstrated under lower (and ostensibly lower in the case of the work of Shi and colleagues (2015)) PYY concentrations (Bertrand *et al.*, 1992). Acceptance that pancreatic PYY_{1-36} acts locally at β-cell Y_1 receptors to inhibit insulin release (Shi *et al.*, 2015) makes PYY_{1-36} an 'anti-incretin' according to the latest understanding of mammalian glucose homeostasis (Kamvissi *et al.*, 2015). Implication of PYY in regulation of mammalian food intake however depends on cleavage by DPP-IV (see section 4.5) to PYY_{3-36} (Batterham & Bloom, 2003). PYY_{3-36} exhibits significantly lowered interaction with the Y_1 receptor and heightened specificity for the Y_2 receptor since the Y_2 receptor binds only the C-terminal domain (Larhammar, 1996) (4.1B). PYY_{3-36} is therefore presumably able to avoid sequestration by Y_1 receptors distributed ubiquitously in mammalian vascular tissues (Jackerott & Larsson, 1997; Matsuda *et al.*, 2002), travelling unheeded in the bloodstream to traverse the blood-brain barrier and interact with Y_2 receptors in the hypothalamic arcuate nucleus, consistent with the mechanistic paradigm described by Batterham and Bloom (2003).

4.1.2.2 PYY in food intake

Peripheral PYY is understood to act as a satiety factor in the gut-brain axis, released from gut enteroendocrine cells after meals to relay its signal to the hypothalamus for integration by the central melanocortin system by acting at Y_2 receptors both at vagal

afferent inputs (paracrine) and directly at the arcuate nucleus (endocrine) (Batterham *et al.*, 2002; Mcgowan & Bloom, 2004; Ueno *et al.*, 2008). In keeping with this model, peripherally-administered exogenous PYY does curb appetite in mammals (Batterham *et al.*, 2003; Neary *et al.*, 2008); however centrally-injected PYY stimulates appetite in rats (Alhadeff *et al.*, 2015) and chickens (Kuenzel *et al.*, 1987), converse to its role as a satiety factor. Although endogenous PYY expression has been evidenced in the brain of vertebrates (Cerda-Reverter *et al.*, 2000; Gelegen *et al.*, 2012; Alhadeff *et al.*, 2015; Reid *et al.*, 2017), the primary appetite-regulating central PP-fold ligand is recognised as NPY. With that in mind, whilst exogenously-administered central PYY might represent a higher-than-physiological concentration (and so desensitisation cannot be ruled out), it more likely mimics the actions of NPY which is co-expressed with AGRP by anabolic first-order neurones to conserve energy and stimulate food intake. Specificity for the Y_2 receptor is conferred by proteolytic processing of mammalian PYY_{1-36} to PYY_{3-36} (Nygaard *et al.*, 2006) by DPP-IV (see section 4.5); a cleavage which activates the satiety role of peripheral PYY (Batterham & Bloom, 2003). The actions of PYY in regulation of food intake seem therefore dependent on both molecular form and anatomical location.

4.2 Aims

Pursual of the anatomical distribution and dynamic regulation of chicken PYY and PP expression represented an opportunity for significant contribution to the field of avian endocrinology. This objective was hampered by lack of an avian PYY gene sequence on which to base expression assays. The primary aims of the work described in the published article forming the basis of this chapter (section 4.4) were therefore to determine the previously unknown gene sequence for chicken peptide YY (PYY) and characterise the endogenous role of chicken PYY and PPY by means of plotting the anatomical distribution and nutrition-dependent regulation of their expression. A further objective was to investigate the evolution of susceptibility to DPP-IV proteolysis in vertebrates, to give a better handle on how this aspect of energy

homeostasis differs between different the vertebrate clades, as addressed in section 4.5.

4.3 Methods

4.3.1 *In silico* sequence derivation

To derive putative sequences for unknown PYY mRNAs, publicly-available RNA-seq short reads in the sequence read archive (Leinonen *et al.*, 2011b) were mined. The European Nucleotide Archive (Leinonen *et al.*, 2011a) was first employed to identify relevant experimental datasets by entering appropriate search terms (e.g. 'gallus' and 'brain or intestine'). The tblastn alignment search tool (NCBI) was then used to search the target datasets using the known chicken PYY peptide sequence (Conlon & Oharte, 1992) as a query sequence. Returned short reads were downloaded and aligned using GAP (Guo *et al.*, 1988). Contiguous sequence alignments were interrogated using ExPASy Translate (Gasteiger *et al.*, 2003) to identify which consensus sequence(s) translated to correctly resemble the PYY peptide sequence. Agreeable mRNAs were then used as query sequences in nucleotide BLAST (NCBI) to mine the same search set, until no further sequence extensions were achieved. The resultant sequence was the putative PYY mRNA for the species of interest.

4.3.2 Standard methods used for the published article

Development of chicken PYY, PPY (PP), YWHAZ and NDUFA qPCR assays was as described in 2.2.5. 5'RACE was performed for chicken PYY as described in section 2.2.6. Existence of the theoretical chicken PYY mRNA (section 4.3.1) was successfully evidenced by sequencing (section 2.2.8) of the 5'RACE and qPCR amplicons, and these sequence fragments were uploaded to Genbank (accession MF455302 & MF455303, respectively). Animal experimentation is described in the published article (section 4.4).

4.4 Journal Article

4.4.1 Author contributions

The work reported within this article materialised in coordination with a wider grant-funded project and was carried out in collaboration with researchers from Scotland's Rural College (SRUC) and Newcastle University. Other members of the authorship team for this paper (ID, TB, RD & VS) were responsible for attracting funding for some of the animal resource and all listed authors contributed to practical animal work including husbandry and dissection of animals. ID derived chicken PYY mRNA sequence by aligning SRA reads (article section 2.1). PW quantified reference gene expression for the long-term nutritional state experiment (article section 2.2.3). SC developed the qPCR assay for PPY (PP). Conceptualisation and execution of all other molecular work, interpretation of results and manuscript preparation were carried out by AR independently, with minor administrative input from co-authors.

4.4.2 Article as published

Pages 88-97 contain the article in published PDF format.

Notes: Sectional and figure/table citations within the manuscript are native.

Figures within the published paper are prefixed 'P1-' when cited elsewhere in this thesis.

References within the article are not replicated in the thesis reference list unless cited elsewhere in this thesis.

4.4.3 Article conclusion

At the time this research was carried out, no avian PYY sequence was available and thus no knowledge of the anatomical distribution or dynamic regulation of PYY gene expression had been achieved in birds. The work described in this article was therefore pioneering in the field of avian endocrinology, although two separate accounts of the chicken PYY gene sequence were published whilst the manuscript was in preparation (Aoki *et al.*, 2017; Gao *et al.*, 2017). Our independent elucidation of PYY mRNA sequence information, particularly the 5' end and proposed transcriptional start site, was important in reconciling the disagreement between these other studies, since Gao and colleagues (2017) seem to have inadvertently included an erroneous segment; presumably an artefact of mispriming during sequencing. We also evidenced the sequence of a second galliforme PYY mRNA (*Coturnix japonica*), demonstrating the conservation of the additional N-terminal alanine residue of the mature peptide by signal peptide cleavage site detection with SignalP (Petersen *et al.*, 2011). Both of the above articles describe select distribution of peripheral PYY mRNA, however neither match the intestinal and pancreatic resolution offered by the paper in hand (representing the two major anatomical sites of PYY expression). The level of resolution seems particularly important in this case, since both Aoki *et al.* (2017) and Gao *et al.* (2017) concluded that the jejunum is the major site of intestinal PYY expression, whereas we were able to determine that the highest expression level is found at the jejuno-ileal boundary (article section 3.2) but the studies agree that avian PYY distribution differs markedly from mammals. The data between studies do not disagree; simply the conclusions arising from disparate levels of resolution. We were also able to demonstrate that regional PP-fold expressional distribution varies within the pancreas (article section 3.3.1), with both PYY and PP more highly expressed in the splenic tail end compared to the duodenal head end, and that this distributional gradient arises ontogenically by 12 weeks of age and is apparent at least as early as six weeks of age for broilers of the Ross 308 strain (article Figure 2). The existing studies on regional pancreatic distribution of avian PP-fold hormones

(Alumets *et al.*, 1978; Tomita *et al.*, 1985) deal only with PP, neglecting to mention PYY. This potentiates problems concerning immunological specificity, however the previous finding that PP peptide concentration was greater in the duodenal head end in pre-adolescent chicks (8-10wk) remain unaccountable in the context of our findings. This suggests either that gradients of regional chicken pancreatic PYY distribution are very plastic, or that concentration of the translated product is not strictly dependent on mRNA level.

The work of Aoki et al. demonstrated a simple effect of different feeding conditions on the expression of PYY, in an experiment similar to that described in section 2.2.2 of our article – namely short-term fed vs. fasted groups – however these researchers measured mRNA expression in the small intestine (jejeunum). They did not include pancreas material when plotting anatomical distribution and so likely considered the jejunum to be the major source of peripheral PYY. Both the results of Gao et al. and our own study identify the pancreas as the major site of PYY expression in chickens; however ours is the only study to date to measure the response of pancreatic PYY expression to nutritive state. The findings that pancreatic PYY responds to short-term energy state whereas pancreatic PP changes over longer periods represent significant steps in developing an understanding of PP-fold hormone dynamics in birds, and how these differ to mammals. It was also deduced that this response is dependent on nutrient uptake – as opposed to physical distention alone – since birds fed a diet with soluble fibre inclusion did not exhibit elevated pancreatic PYY expression (article section 3.3.3).

4.5 Dipeptidyl peptidase IV (DPP-IV) susceptibility

As mentioned in the article discussion (article section 4), the shared proteolytic insusceptibility between goldfish and chicken mature PYY originally suggested that DPP-IV processing might be a relatively recent development in mammalian evolution and inapplicable to non-mammalian clades. Since publication of this paper, further work has been completed *in silico* which substantiates this conclusion and

undermines the alternative explanation that DPP-IV susceptibility has been lost in some birds (e.g. galliforme lineage).

DPP-IV is a serine protease which selectively cleaves the N-terminal dipeptides Xaa-Pro and Xaa-Ala from peptide molecules (Hopsu-Havu & Glenner, 1966; Mentlein, 1999; Rawlings & Salvesen, 2013). Peptides susceptible to DPP-IV cleavage must have proline or alanine as the penultimate N-terminal residue (P1), and must not have proline in the third position (P1') (Mcdonald & Schwabe, 1977). Although these criteria for DPP-IV cleavage are strict, their simplicity allows broad substrate diversity; for example, mammalian PP_{1-36} molecules are presumably susceptible to cleavage regardless of identity of the N-terminal amino acid residue (Conlon, 2002; Kamvissi *et al.*, 2015). The additional N-terminal residue of chicken PYY is remnant of altered signal peptide cleavage (Conlon & Oharte, 1992; Conlon, 1995) and completely ablates sensitivity to DPP-IV since the N-terminal sequence Ala-Tyr-Pro does not conform to the substrate criteria for the exopeptidase activity of DPP-IV. Conlon and colleagues (1992) recognised that chicken PYY was not a substrate for DPP-IV but incompletely reasoned that the N-terminal sequence Xaa-Pro-Pro confers insusceptibility to DPP-IV cleavage (when in fact the chicken N-terminal sequence Ala-Tyr-Pro does not resemble the DPP-IV recognition motif whatsoever). The idea that a proline residue at position P1' might confer resistance to DPP-IV cleavage in non-mammalian PYY molecules is however significant, since several other species have proline in the third position, with no 37^{th} N-terminal residue (Conlon, 2002). Investigating DPP-IV susceptibility and resistance in vertebrate species could therefore yield interesting information about the evolution of the hormone and its functional reliance on DPP-IV activity.

4.5.1 Comparative investigation

4.5.1.1 Principle and method

Determining with confidence the evolutionary history of chicken PYY structure, and phylogeny across vertebrate clades, was not possible with so few sequences available from species closely related to chicken. The previously-described (section 4.3.1) SRA-mining process was therefore employed to derive the mRNA and translated peptide sequences for several species for which RNA-seq data was available but no assembled entries were available in the NCBI database: Helmeted guineafowl (*Numida meleagris*), Indian peafowl (*Pavo cristata*), Goose (*Anser spp.*) and Mallard (*Anas platyrhynchos*), in addition to the chicken (*Gallus gallus*) and Japanese quail (*Coturnix japonica*) sequences already derived (section 4.4). Putative mRNA sequences were translated using ExPASy Translate (Gasteiger *et al.*, 2003) to identify the correct open reading frame and obtain the translated amino acid sequence which was then assessed to locate the peptide signal cleavage site using SignalP (Petersen *et al.*, 2011). The derived peptide sequences were then aligned with several known vertebrate PYY peptide sequences harvested from the NCBI database using MUSCLE (Edgar, 2004).

4.5.1.2 Results

Figure 4.3 shows alignment of vertebrate PYY pro-peptide amino acid sequences. A cladogram and phylogenetic tree were also generated to demonstrate the evolutionary relationship between preproPYY molecules, and mature PYY peptide susceptibility to DPP-IV is indicated Figure 4.4.

Figure 4.3 (overleaf) – Alignment of vertebrate preproPYY molecules. Vertebrate preproPYY molecules are shown aligned by sequence. Identity to grass carp NPY (row 1) is noted. Amino acid residue positions are coloured blue depending on conservation between preproPYY molecules, with darker blue signifying greater conservation as per the indicated key. The region analogous to chicken mature PYY_{1-37} is boxed in red. Substitutions of interdigitating residues of the PP-fold motif are highlighted yellow. Species whose sequences derived from SRA data are highlighted pink.

From Figure 4.3, it is clear that the mature PYY_{1-36} peptide is the most highly-conserved region of preproPYY amino acid sequences in vertebrates. The interdigitating residues of the PP-fold motif (Figure 4.1) are incredibly highly conserved: Pro_2 is conserved in all but 1 studied species (*Echinops telfairi*), Pro_5 and Pro_8 are completely conserved, Tyr_{20} is completely conserved and Tyr_{27} is conserved in all but three closely-related species (*Lipotes vexillifer, Tursiops truncates and Orcinus orca*). A further highly-conserved segment is found at mature peptide residues 12-16, corresponding to the turn motif which forms the fold. The C-terminal octapeptide of PYY_{1-36} is also very highly conserved (allowing for leucine-isoleucine substitutions at position 28).

Figure 4.4 (overleaf) – Phylogeny of vertebrate preproPYY molecules. A cladogram (grey) and phylogenetic tree (brown) are shown for vertebrate preproPYY molecules.

DPP-IV susceptibility/resistance is indicated as follows:

- * Susceptible to DPP-IV cleavage
- * Susceptible to sequential DPP-IV cleavage
- * Resistant (additional N-terminal residue)
- XPP Resistant (proline at position 3)
- XXP Resistant (proline at position 3 and no proline at position 2)
- ? No signal peptide cleavage site detected

Every molecule segregated exactly with other molecules from the same major vertebrate clade (agnatha, aves, fishes, reptilia, amphibia or mammalia) in the PYY_{1-36} phylogenetic analysis (Figure 4.4). Susceptibility to DPP-IV cleavage of mature PYY was found to exist primarily in mammalian peptides.

4.6 Discussion and conclusions

Elucidation of the first avian PYY mRNA sequences facilitates discovery in further avian species and opens the door to further study of their expression in birds. The concentration of PYY mRNA at the pancreas is interesting, since pancreatic PYY is thought to be an intrinsic mediator of glucose homeostasis in mammals, as discussed in section 4.1.2.1. Compared to mammals, birds maintain distinct glycaemic control and glucose storage and metabolism strategies (Braun & Sweazea, 2008) and so might require tighter PYY-mediated control of incretin release. For the first time in chickens, pancreatic PYY expression has been shown to respond to short-term nutritional state, implicating PYY as a short-term regulator of avian energy homeostasis dependent on chemical (not physical) gut fill. Pancreatic PP is demonstrated to respond to longer-term energy state and may be an important regulator of long-term energy homeostasis.

In all, the work described in the article of section 1.4 represents significant contribution to the field of avian endocrinology. Clearly there is much still to learn about PP-fold hormone dynamics in birds, as in all vertebrates; however the knowledge accumulated in this paper, together with the recent publications mentioned above (He *et al.*, 2016; Aoki *et al.*, 2017; Gao *et al.*, 2017), forms a good base from which to pursue further characterisation.

From the alignment of vertebrate PYY sequences (Figure 4.3) and phylogenetic analysis of the same (Figure 4.4), it is obvious that PYY structure is highly conserved. Residues forming the PP-fold motif and C-terminal octapeptide are particularly conserved, presumably because overall tertiary structure and C-terminal amino acid

sequence are important for receptor interaction. From examination of the information on DPP-IV susceptibility (Figure 4.4), it could be postulated that proline occupied the third amino acid position in the ancient PYY structure, and sensitivity to DPP-IV must have developed by substitution of this proline residue at the time of mammalian divergence. Since all tested galliforme sequences exhibit the altered signal peptide cleavage initially observed in the chicken, this likely arose at a single event in the galliforme lineage. Absence of pressure to maintain a DPP-IV-sensitive sequence would facilitate such a divergence, though the precise molecular change responsible is unknown, as are the exact effects of the additional residue on the ligand chemistry, but it might conceivably affect receptor specificity. The species notably inconsistent in PP-fold motif residue conservation were *Echinops telfairi* – which was also found to resist DPP-IV cleavage (Figure 4.4), suggesting a possible altered or redundant role for PYY. The PYY of dolphin species *Lipotes vexillifer*, *Tursiops truncatus* and *Orcinus orca* were also found to be insusceptible and segregated with two whale species (*Physeter catodon* and *Balaenoptera acutorostrata*) exhibiting DPP-IV insusceptibility in the phylogenetic analysis (Figure 4.4). It seems likely that the evolutionary distance from land mammals and unique environment of these species would demand specialised energy homeostatic mechanisms. Reciprocally, some avian (*Calypte anna* and *Nipponia nippon*) PYY peptides appear to have developed novel DPP-IV susceptibility (Figure 4.4) which is not entirely surprising since the mutation conferring the necessary amino acid substitution (Pro_3>Ala) can be achieved with a single nucleotide mutation at the genomic DNA level. It would be interesting to probe further for potential reasons and effects of anomalous PYY characteristics, but such investigation is outwith the scope of this thesis. All vertebrate PYY molecules studied exhibit reasonable identity with the grass carp NPY outgroup and human and chicken NPY controls (Figure 4.3). Mature NPY peptide is susceptible to DPP-IV cleavage (Kos *et al.*, 2009) but central NPY is presumably protected by its anatomical location, since DPP-IV is found in the periphery.

From the current study, in the context of the roles of mammalian PYY in glucose homeostasis and food intake and considering the insusceptibility of non-mammalian PYY to DPP-IV cleavage, it could be proposed that chicken pancreatic PYY is not directly involved in feed intake and might instead act primarily to lower insulin levels, hence affecting central melanocortin system signal integration indirectly. It is notable that PYY expression took several hours to increase (article section 3.3.2), since regulation of an appetite-regulating satiety factor might have been expected to increase more acutely.

The complexity and ubiquity of the vertebrate PP-fold signalling pathways makes this system a daunting research subject. Berglund (2005) hints that the elusiveness of PP-fold peptide roles might be due to "a large degree of redundancy," but the obvious careful temporal and geographical orchestration of expression of ligands, receptors and probably targeting proteases suggests that such an explanation is too simplistic and instead tempts the conclusion that this complex ancient hormonal signalling system fulfils diverse roles in modern vertebrates, as yet poorly understood because of insufficient experimentation. In order to resolve PP-fold dynamics in each species, further targeted study is required but a wider appreciation of conserved roles in diverse vertebrates might be key in identifying the most fundamental mechanisms since these are likely conserved.

As in all animals, future study of PP-fold molecules in aves should aim toward a full understanding of ligand and receptor distribution, receptor specificity and how this is affected (if at all) by proteolytic processing of ligands. Measurements of endogenous ligand expression, such as those described in section 1.4, are critical to determine which environmental cues PP-fold peptides respond to. Exogenous administration of PP-fold molecules is of course illuminating and useful in confirmatory mechanistic studies, but special care should be taken to avoid overstimulation (desensitisation) as this can lead to confounding results.

For the chicken, distributions of PP & PYY have now been described in some detail (Aoki *et al.*, 2017; Gao *et al.*, 2017; Reid *et al.*, 2017), but room still exists for

improvement in the resolution of peripheral and central structures. Likewise, anatomical distributions of chicken Y_2 & Y_5 (He *et al.*, 2016), Y_6 (Bromee *et al.*, 2006) and Y_7 (Bromee *et al.*, 2006; He *et al.*, 2016) receptors have been described. Distributional mapping of NPY and receptors Y_1 and Y_4 are lacking in chickens and these might prove pivotal in inferring the function of PP-fold ligands. This is especially true for Y_1 in determining the role of PYY in regulation of feeding, since the ability of PYY_{1-37} to reach the arcuate nucleus and interact with Y_2 receptors might depend on lack of vascular Y_1 receptors. The physiological distribution of chicken DPP-IV has not been studied. In terms of exogenous administration, central and peripheral injections of PYY have caused significant behavioural effects but no studies have employed exogenous NPY. The extant descriptions of responsive endogenous PP-fold hormone expression (Aoki *et al.*, 2017; Reid *et al.*, 2017) are valuable but barely scratch the surface in terms of interrogation of possible endogenous response dynamics. Finally, it would be interesting to pursue further knowledge of the dynamics and significance of pancreatic PYY/PP ontogeny since these molecules might play important roles in embryonic development and subsequent growth. Characterisation of such roles might be possible with use of targeted gene knockouts and exogenously-applied selective receptor (ant)agonists.

CHAPTER 5

Peripheral hormones of the gastrin-cholecystokinin family

5 Peripheral hormones of the gastrin-cholecystokinin family

5.1 Introduction

It is clear from the basis and findings of work described in Chapter 3 that the peptide hormone cholecystokinin (CCK) is an important regulator of energy homeostasis in birds. Understanding the regulation and functions of endogenous CCK is therefore of interest in describing hormonal control of avian energy balance, which will in turn inform strategies to alleviate welfare and production problems in poultry farming, as discussed in Chapter 1. Primary characterisation of the function of a gene commonly involves mapping distribution of its expression in the species of interest. This allows general inference of the likely physiological role(s) fulfilled by the gene product, and paves the way for experimentation to delineate transcriptional and post-transcriptional dynamics *in vivo*. Chicken CCK has received little attention in this respect. Mapping of the gene products themselves has been attempted (Martinez *et al.*, 1993b). The problem with the strategy employed by these researchers is that it depends on immunological specificity, which is difficult to satisfactorily demonstrate. In the case of CCK, conserved structure with gastrin (GAST) gene products (the only other known member of the gastrin-cholecystokinin gene family) increases the likelihood of cross-reactivity. CCK and gastrin exhibit structural homology in their C-terminal receptor binding motif, common to all peptide isoforms. It is therefore not possible to reliably distinguish between isoforms, or even source gene, without exhaustive testing of cross-reactive potential for all known gene products. The obvious alternative to immunohistochemistry in plotting expressional activity is detection of transcripts. This requires knowledge of the mRNA sequence for the gene(s) of interest, but transcripts of different genes – whose products might be structurally similar – can generally be differentiated by targeting regions of low sequence identity. A gastrointestinal mRNA distribution profile was produced by RT-qPCR as part of a recent review of avian gut

hormones (Honda *et al.*, 2017). But these researchers do not mention the likelihood of non-specific amplification from non-target genes with similar sequence (i.e. gastrin), and their primer selection suggests that they might not have taken this into account. In any case, the distribution plot achieved is rather low-resolution, with only gastrointestinal samples measured, and only 7 regions sampled in total. It was therefore decided that, ahead of measuring expressional response to energy state, a higher-resolution distribution of CCK and gastrin expression should be determined by RT-qPCR, with primers designed to exclude amplification of the alternative gene family mRNA. This information was then used to investigate the response of endogenous CCK and gastrin to short-term hunger and satiety induced by short-term feeding regimes.

5.2 Journal article

5.2.1 Author contributions

AR designed and carried out all animal experimentation, completed molecular laboratory work, performed statistical analyses, interpreted results and prepared the manuscript. ID contributed to manuscript revision.

5.2.2 Article as submitted

Pages 112-124 contain the article manuscript as submitted to *General and Comparative Endocrinology* on 06 Sep 2017. Reviewers' comments have been received and the article is now under minor revision before publication.

Notes: Sectional and figure/table citations within the manuscript are native.

Figures have been included proximal to relevant text and prefixed '5.' when cited elsewhere in this thesis.

References cited in this paper are included in the thesis reference list.

Article published 01 Jan 2018 (Appendix 4).

Title:

Gastrointestinal distribution of chicken gastrin-cholecystokinin family transcript expression and response to short-term nutritive state

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Abstract:

The related peptide hormones Cholecystokinin (CCK) and gastrin (GAST) are conserved throughout vertebrate clades and implicated in energy homeostasis. CCK is generally accepted as a satiety hormone in poultry, but the role of gastrin remains poorly studied. Functional dissection of these ligands is required to characterise the molecular control of growth & satiety in the domestic chicken, for which there is an increasingly pressing mandate. There are limited descriptions of physiological distributions for the two genes in birds, and these are mostly reliant on immunohistochemistry which can prove problematic due to the shared structure of the targets. Therefore, we have defined the tissue distributions of CCK and gastrin in the chicken, focussing on the gastrointestinal tract, by using transcript-dependent techniques to improve reliability by increasing specificity. Though considerably more highly expressed in the brain, gastrointestinal CCK transcripts were dispersed throughout the small intestine and particularly around the proximal ileum. Gastrin expression was strictly limited to the gastric antrum region of the intestinal tract, albeit very highly expressed. We demonstrate that CCK mRNA expression does not respond as expected for a short-term satiety hormone, and that the short-term response of gastrin expression is paradoxical compared to its role in mammals. These results partially corroborate previous peptide distribution studies and initiate exploration of the nutrient-responsive roles of these hormones in avian energy balance.

Keywords:

Satiety; avian; hormone; feeding; poultry

1. Introduction

Recent years have seen increasing interest in the characterisation of avian energy homeostasis, both in order to optimise poultry production and welfare and to better understand endocrine regulation of vertebrate energy balance and evolution of the mechanisms which underlie it. The 'broiler-breeder paradox' – restriction of feed intake to maintain reproductive health in broiler parent flocks – is a prominent example of welfare concern arising from intense selective breeding in chickens for meat production. This might be solved or ameliorated if hormonal response to nutrition was better understood and breeding or husbandry managed to prevent aberrant follicular development (Decuypere *et al.*, 2006). Further concerns surround force-feeding in the production of foie gras, and the need for development of alternatives are currently under debate (Guemene & Guy, 2004; Rochlitz & Broom, 2017). Some steps have been taken to describe how endocrine and neuroendocrine signalling is affected under such atypical feeding conditions in poultry (Boswell *et al.*, 1999; Davail *et al.*, 2003; De Jong *et al.*, 2003; Dunn *et al.*, 2012; Dunn *et al.*, 2013b), however much work is yet required to fully understand the molecular control of avian growth and its significance to modern agricultural practice, particularly considering the contrasting characteristics of energy balance mechanisms in birds compared to other vertebrates (Honda *et al.*, 2017).

The gastrin-cholecystokinin peptide family comprises the variably processed and modified products of two genes; gastrin (GAST) and cholecystokinin (CCK) and represents one set of hormones relatively well-described in mammals but neglected in birds. Both genes are conserved across vertebrate species, likely arising from a duplication event early in the vertebrate lineage (Johnsen, 1998), and descend from an ancient peptide class conserved throughout metazoans (Dupré & Tostivint, 2014; Yu & Smagghe, 2014). Gastrin and CCK have related physiological roles in vertebrates, being heavily implicated in peripheral signalling to regulate appetite and digestive organ activity, as well as in emotion and behaviour (Ballaz, 2017). Products

of both genes are variably processed to an impressive spectrum of molecules, relative abundances of which are dependent on species, tissue dietary composition, and specific degradation rates among other factors, as comprehensively summarised by Guilloteau *et al*. (2006). All CCK and gastrin molecules have similar C-terminal structures and bind a common receptor (CCKBR) with similar efficacy dependent on sulphation at the C-terminus-proximal tyrosyl residue whereas CCKAR is only practically bound by tyrosyl-sulphated CCK (Huang *et al.*, 1989; Guilloteau *et al.*, 2006). This posttranslational complexity undermines the validity of immunological studies employing antibodies raised against certain molecular forms. Common physiological effects seem to be conferred by all functional products of each gene (Guilloteau *et al.*, 2006), so studies on the gene transcript may be more reliable and will complement the interpretation of existing studies which used immunological tools.

The basic gastrointestinal distributions of CCK and gastrin transcript and peptides have been described in chickens (Martinez *et al.*, 1993b; Honda *et al.*, 2017), however these studies either lack resolution or are dependent on antibodies as discussed . Likewise, although some work has been carried out to assess the function of CCK as a regulator of appetite (Tachibana *et al.*, 2012), stimulation of acid secretion by gastrin (Campbell *et al.*, 1991; Furuse & Dockray, 1995) and CCK and gastrin as modulators of gastrointestinal motility (Martinez *et al.*, 1993a), the response of native gastrin and CCK expression to disparate nutritive states in birds has not been addressed. We therefore set out to better describe the anatomical distribution of CCK and gastrin production, and how their expression is affected by short-term hunger and satiety states in the domestic chicken.

2. Materials and Methods

2.1. Animal Material

Use of animals was approved by the Roslin Institute Animal Welfare and Ethical Review Body and experiments were carried out under the Animals (Scientific Procedures) Act 1986, project licence 70/7909.

2.1.1 Distribution of gastrin and CCK expression

In order to assess the distribution of expression of gastrin and CCK in chicken tissues by qPCR, four Lohmann Classic hens reared in standard conditions were killed by barbiturate overdose at peak of lay and a range of tissue samples was collected from intestine, visceral organs, brain and musculo-skeletal tissue. Material for *in situ* hybridisation was harvested from broiler breeders reared in standard conditions with commercial food restriction to achieve the breeding company's target growth rate (Aviagen, 2013) until 11 weeks of age when birds were moved to individual cages. Following a 5-day cage acclimatisation period, birds were fed either *ad libitum* or continued commercial restriction for a further 2.5d before cull by barbiturate overdose. The antrum was dissected to include part of the gizzard and duodenum at either side. A section of proximal ileum just posterior to the vitelline diverticulum was also dissected. All samples were snap-frozen on dry ice.

2.1.2 Response to short-term nutritive state

To characterise the responses of gastrin and CCK to short-term hunger and satiety, 50 NOVOgen brown birds were sexed by genotyping (Clinton *et al.*, 2001) at 2d and reared to 6d in a single floor pen before being split into four floor pens; two containing males (n=14/pen), and two containing females (n=11/pen), balanced by bodyweight for each sex. *Ad libitum* feeding was provided until 16d, temperature was 26°C, light was 14L:10D with lights-on at 0700h, and all birds were handled daily for 4 days prior to cull at 17d. Feed was removed from all pens at 05:00 on the day of cull, and reintroduced to one pen of each sex after 3h (08:00). The remaining pens maintained

fast for the remainder of the experiment. 2.5±0.5h of feed after reintroduction of feed or maintenance of fast (10:00-11:00), five females and seven males from each treatment were culled. All remaining birds were culled 7.5±0.5h after reintroduction of feed or maintenance of fast (15:00-16:00). All birds were killed by cervical dislocation and immediately dissected to harvest 40-100mg samples of gastric antrum and proximal ileum, which were snap-frozen on dry ice. All samples were taken in a coronal plane to include all intestinal tissue strata.

2.2. Design of oligonucleotide primers and probes

Details of all primers and probes used in this study are summarised in Table 1. Novel primers to amplify chicken preprogastrin (GI:45382320) and chicken CCK (*GI*:48976040) mature mRNA sequences were designed using Primer3 (Rozen & Skaletsky, 2000; Untergasser *et al.*, 2012). Oligonucleotide probes for *in situ* hybridisation were designed manually to conform to the following parameters: ~55% GC content (48-62%), ~45mer length (43-47mer) and melting temperature (T_m) as high as possible within those parameters and at least 20°C greater than the highest predicted tertiary structure T_m predicted by OligoAnalyzer 3.1 online software (Integrated DNA Technologies). Chicken CCK and gastrin preprohormone mRNA sequences were aligned using MUSCLE (Edgar, 2004) to identify regions that were divergent and to avoid selecting regions of similarity between the two transcripts for targeting oligonucleotide primer and probe annealing (Figure 1). Similarity was calculated for each probe against the unintended target mRNA reverse-complement by the Smith-Waterman algorithm using EMBOSS Water (Smith & Waterman, 1981; Rice *et al.*, 2000) and found to be 48.9% for AR_GAST_ISH1 and 60.9% for AR_GAST_ISH1. BLASTN (NCBI) returned no unintended chicken targets for either probe. Primers for quantification of LBR, YWHAZ and NDUFA1 as reference genes were described previously (Reid *et al.*, 2017). Sigma-Aldrich UK supplied all oligonucleotide primers and probes.

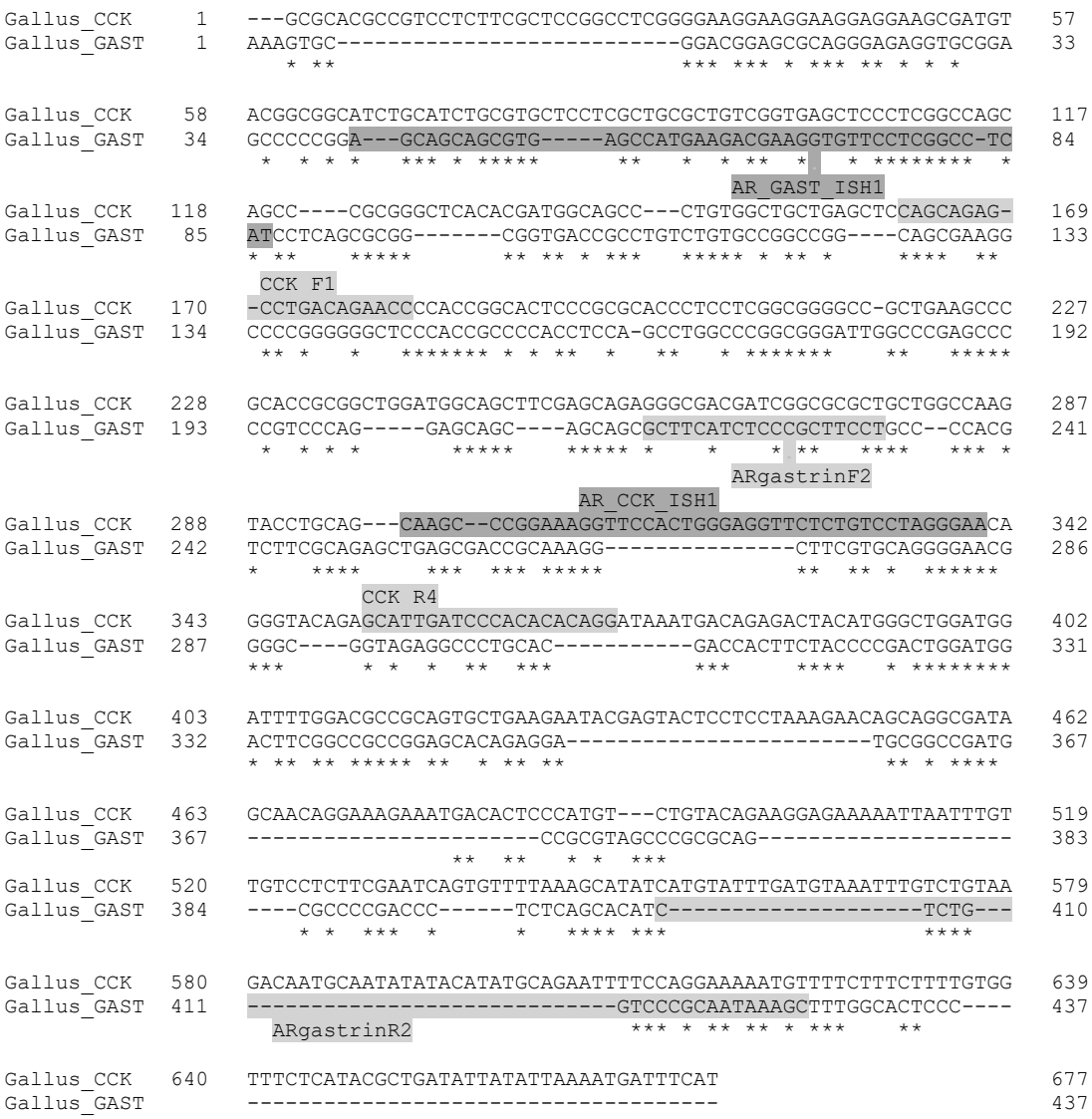

Figure 1. Alignment of CCK and gastrin mRNA sequences. Oligonucleotide primer (light grey) and probe (dark grey) annealing positions are indicated to show targeted areas of low shared identity. Further details of primers and probes used in this study can be found in table 1.

2.3. Preparation of cDNA

Total RNA was isolated from tissue homogenised in TRIzol reagent (Invitrogen) using the Direct-zol RNA Kit (Zymo Research) to manufacturer's specifications, with in-column DNase treatment. 1µg total RNA per sample was reverse transcribed using the High Capacity Reverse Transcription Kit (Applied Biosystems) in 20µl reactions according to manufacturer's guidelines and the product diluted to 110µl total volume per sample with water.

2.4. Quantitative polymerase chain reaction (qPCR)

Brilliant III Ultra-fast SYBR Green qPCR Mastermix and the Mx3005p qPCR System with MxPro software (Agilent Technologies) were employed according to the manufacturers' guidelines and as described previously (Whenham *et al.*, 2015). Briefly, 10µl SYBR mix, 8µl cDNA product, 0.4µl 20µM forward primer, 0.4µl 20µM reverse primer, 0.3µl 1/500 ROX reference dye solution and 0.9µl H_2O were mixed for each 20µl reaction. Thermal conditions were consistent for all assays: 50°C; 120s, 95°C; 120s, (40 cycles of 95°C; 15s, 60°C; 30s), then 95°C; 60s, 60°C; 30s, 95°C; 15s. Apparent reaction efficiencies were between 96-99%, as determined by analysis of the standard dilution curve. Amplicons were bidirectionally sequenced using LightRUN Sanger sequencing (GATC Biotech) to confirm identity. LBR, NDUFA1 and YWHAZ were chosen as reference genes due to their reliability in previous avian studies (Mcderment *et al.*, 2012; Olias *et al.*, 2014) and quantified as above. Normalisation was achieved by dividing the raw expression value for the gene of interest by the geometric mean of the LBR and YWHAZ raw expression values.

2.5. *In situ* hybridisation

In situ hybridisation employed reagents and protocol as described previously (Meddle *et al.*, 2007). Briefly, oligonucleotide probes specific to mRNAs of interest (see Table 1) were radiolabelled with ^{35}S dATP and incubated overnight with fixed 15µm tissue sections on polysine slides. Slides were exposed for 14 days in autoradiographic emulsion before development, fixation and haemotoxylin/eosin counterstaining.

3. Results

3.1 Distribution of gastrin and CCK

Figure 2 shows distribution of gastrin and CCK mRNA expression levels as assessed by qPCR across a panel of chicken tissues. CCK was found to be primarily expressed in the basal hypothalamus (Figure 2a), whereas gastrin was exclusively expressed in the gastric antrum region (Figure 2b). Peripheral CCK exhibited peak expression in

the small intestine, particularly around the proximal half of the ileum, with low but detectable expression in other visceral regions, particularly the proventriculus and antro-duodenal boundary regions of the gastrointestinal tract.

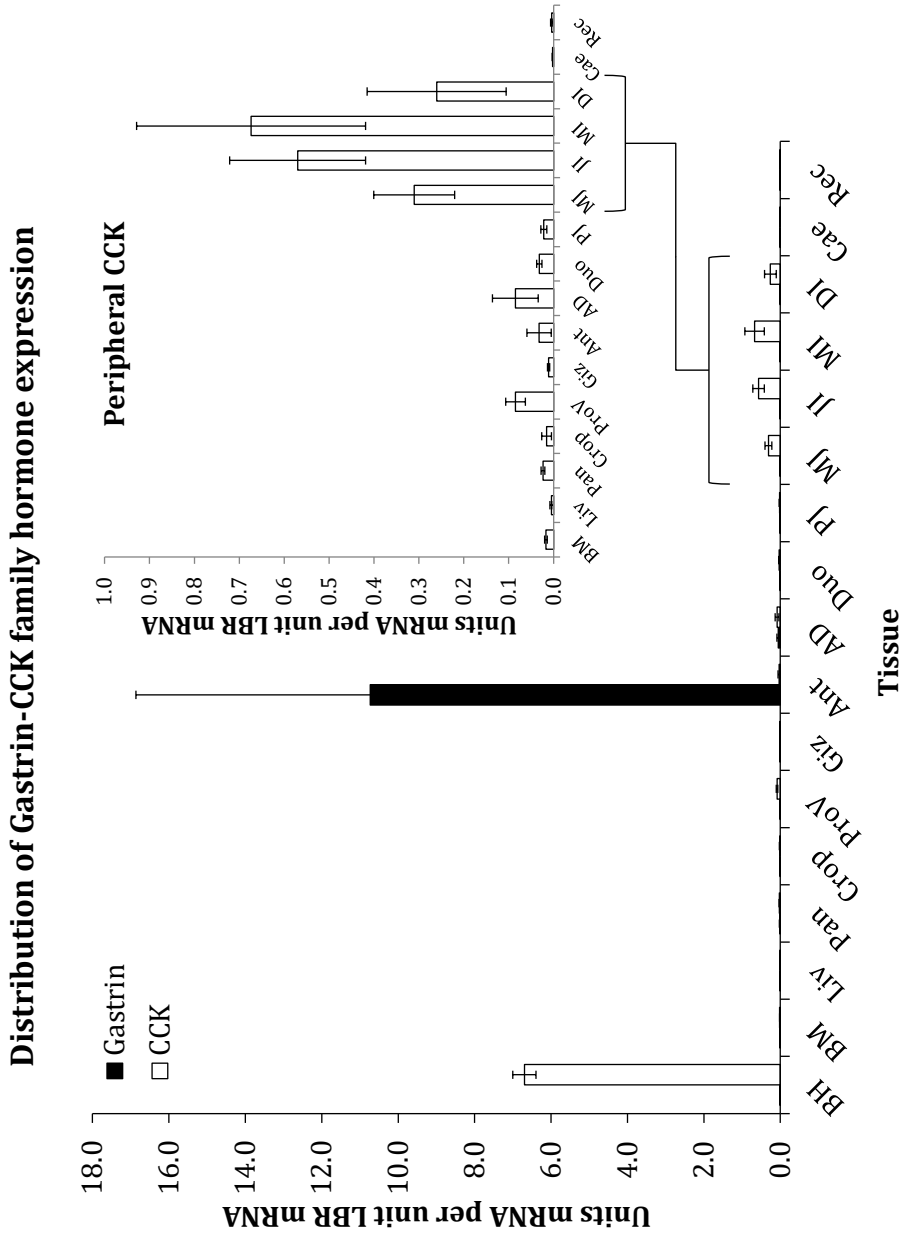

Figure 2. Tissue distribution of chicken Gastrin-CCK family hormone expression. Normalised relative mean (±SEM) gastrin (filled bars) and CCK (open bars) mRNA expression for 17 tissue types in Lohmann Classic brown laying hens (n=4): basal hypothalamus (BH), breast muscle (BM), liver (Liv), pancreas (Pan), crop, proventriculus (ProV), gizzard (Giz), antrum (Ant), antro-duodenal boundary (AD), duodenum (Duo), proximal jejunum (PJ), mid-jejunum (MJ), jejuno-ileal boundary just distal to the vitelline diverticulum (JI), mid-ileum (MI), distal ileum (DI), caecum (Cae) and rectum (Rec).

Peripheral observations were corroborated by *in situ* hybridisation results which clearly showed a distinct region of high gastrin expression in the antral epithelium (Figure 3a) but no detectable gastrin in the ileum (Figure 3b). Discrete high CCK expression was detected in luminal villus cells of the proximal ileum (Figure 3b) and lower but detectable CCK expression at the proximal duodenum, but not the antrum (Figure 3a). Notably, both assays agree that antral gastrin mRNA concentration is far greater than ileal CCK mRNA concentration (Figures 2 & 3). The intensity of ileal CCK hybridisation signal was observed to differ considerably between *ad libitum*-fed and restricted birds (Figure 3b), but no quantitative analyses were performed for this assay.

Oligo name	Type	Sequence (5'-3')	Target acc. no. & amplicon length
CCK_F1	Primer	CAGCAGAGCCTGACAGAACC	NM_001001741.1 210bp
CCK_R4	Primer	CCTGTGTGTGGGATCAATGC	
ARgastrinF2	Primer	GCTTCATCTCCCGCTTCCT	NM_205400.1 212bp
ARgastrinR2	Primer	GCTTTATTGCGGGACCAGAG	
YWHAZ_F	Primer	GTGGAGCAATCACAACAGGC	NM_001031343.1 223bp
YWHAZ_R	Primer	GCGTGCGTCTTTGTATGACTC	
LBR-F	Primer	GGTGTGGGTTCCATTTGTCTACA	NM_205342.1 80bp
LBR-R	Primer	CTGCAACCGGCCAAGAAA	
NDUFA1-F1	Primer	ATGTGGTACGAGATCCTGCC	NM_001302115.1 203bp
NDUFA1-R1	Primer	TTCTCCAGACCCTTGGACAC	
AR_CCK_ISH1	Probe	TTCCCTAGGACAGAGAACCTCC-CAGTGGAACCTTTCCGGGCTTG	NM_001001741.1 -
AR_GAST_ISH1	Probe	ATGAGGCCGAGGAACACCTTCG-TCTTCATGGCTCACGCTGCTGCT	NM_205400.1 -

Table 1. Details of oligonucleotide primers and probes

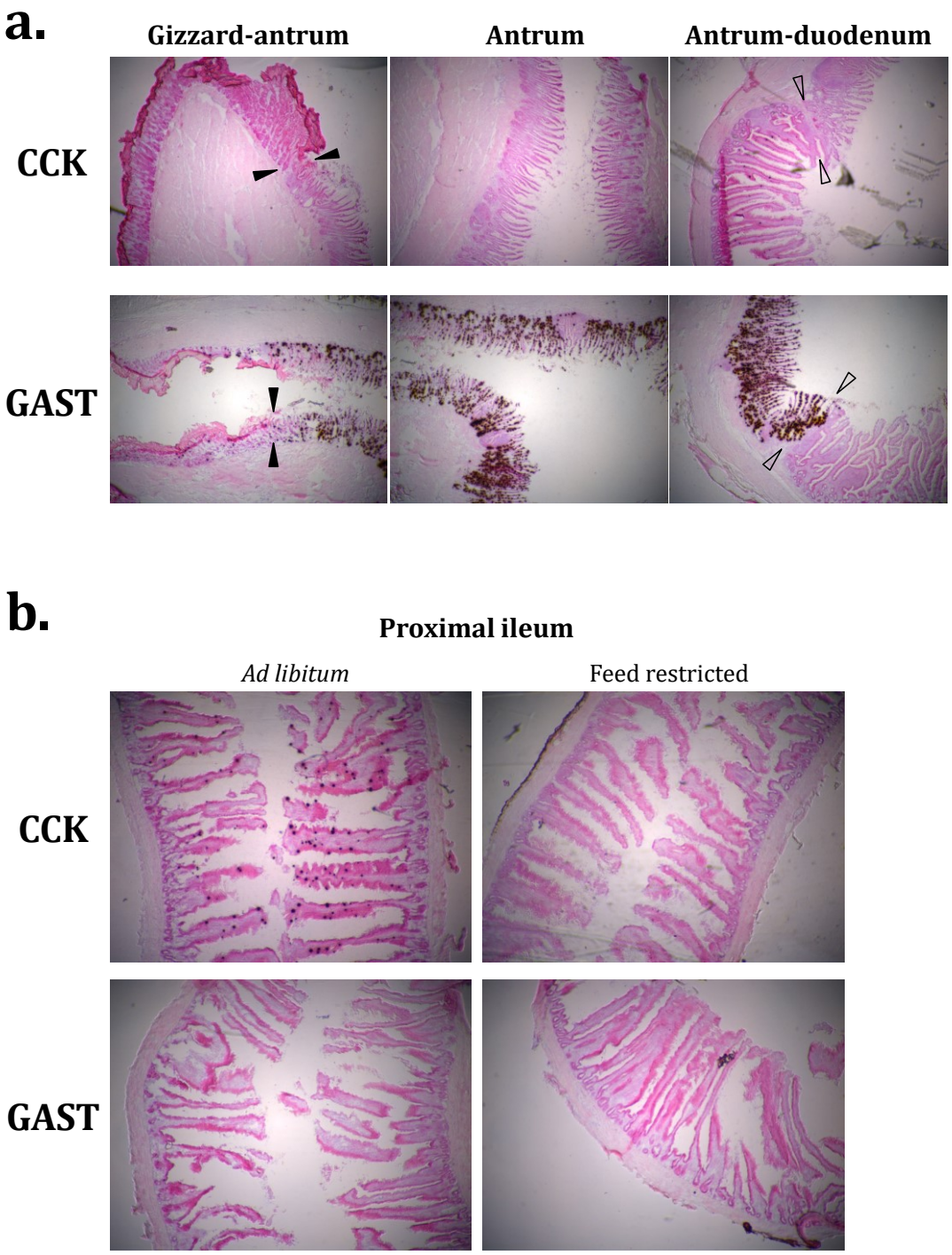

Figure 3. *In situ* hybridisation around the gastric antrum and proximal ileum. 15µm tissue sections are shown for the gastric antrum in *ad lib*-fed birds (a). Hybridisation signal for CCK (top row) or GAST (bottom row) transcripts. Arrows signify transition from gizzard to antrum (filled) and antrum to duodenum (open). Further 15µm sections are shown for the proximal ileum in *ad lib*-fed and feed restricted birds (b). Hybridisation signal for CCK (top row) or GAST (bottom row).

3.2 Response to short-term nutritive state

Sex was not found to be a significant factor in any analysis, so data from both sexes are presented together. No significant difference in CCK expression was detected between treatments ($F_{1,42}$=0.99, P=0.324) or sampling times ($F_{1,42}$=1.32, P=0.257), and there was no treatment by sampling time interaction ($F_{1,42}$=0.96, P=0.332) (Figure 4a). Gastrin expression was higher in the fasted groups compared to the *ad libitum*-fed groups across both sampling times ($F_{1,42}$=8.6, P=0.005), and lower at the later sampling time compared to the earlier sampling time across both treatments ($F_{1,42}$=13.52, P<0.001), but there was no interaction between treatment and sampling time ($F_{1,42}$=0.00, P=0.990) (Figure 4b).

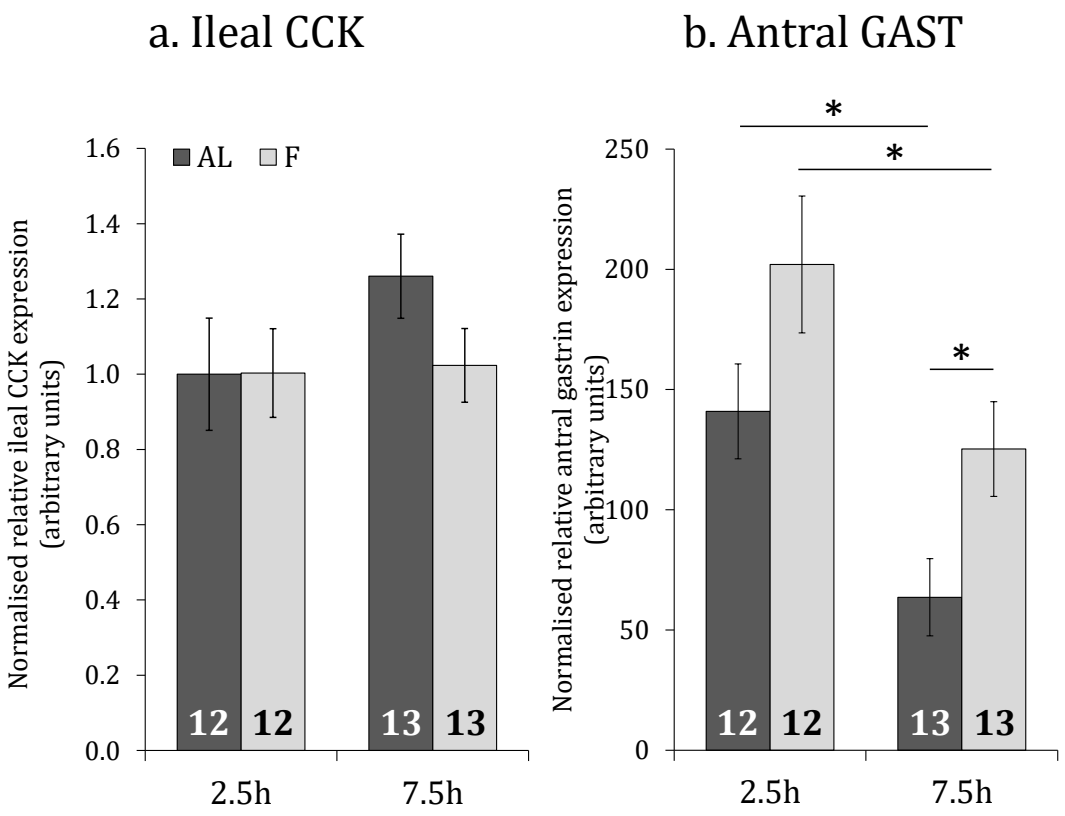

Figure 4. Response of ileal CCK and antral gastrin to short-term satiety state. Normalised relative mean (±SEM) ileal CCK (a) and antral gastrin (b) mRNA expression for birds fed *ad libitum* or fasted for 2.5h and 7.5h. Number of birds in each group are shown within each bar. Asterisks (*) represent statistical significance at p<0.05.

Notably, across both the distribution assay and the feeding experiment, gastrin expression was found to be far higher than CCK expression in their sites of highest expression (antrum and proximal ileum, respectively) in real terms (i.e., moles of transcript per mg tissue).

4. Discussion

Using qPCR and *in situ* hybridisation, we have corroborated and further resolved the results of previous studies of distribution of native gastrin-cholecystokinin peptide family expression in the domestic chicken (Martinez *et al.*, 1993b; Honda *et al.*, 2017). Whereas Martinez et al. (1993b) employed an immunohistochemical approach (and therefore antibodies which might have been cross-reactive or insensitive to some processed peptide forms), our methods targeted the common mRNA transcript for each gene which allowed greater control of specificity as target regions of low shared identity could be prioritised (Figure 1). This allowed information on the aggregate expression of the numerous variably processed peptide products of each gene to be inferred, since neither GAST nor CCK are thought to routinely produce splice variants (Håkanson & Rehfeld, 2002). Chicken gastrin expression is strictly limited to the gastric antrum (Figure 2), suggesting a specific role in responding to the luminal environment at the transition from gizzard to small intestine. This is in keeping with the gastric acid secretion-regulating function of vertebrate gastrin, as originally demonstrated in the chicken (Campbell *et al.*, 1991). CCK was far more highly expressed in the brain than any peripheral region sampled (Figure 2), which reinforces the role of CCK as an important neuropeptide in birds and is consistent with broad distribution of active CCK peptides (Rehfeld, 2017). This skewed distribution is particularly noteworthy in the context of the recent report that mammalian brain CCK exists almost exclusively in the sulphated form, potentiating activity at the A-type receptor (Agersnap *et al.*, 2016). Of course heightened central expression of CCK does not negate its importance in peripheral regulation of gastrointestinal function, especially since vagal transduction of peripheral CCK feeds into central energy

balance, although it should be noted that in mammals this transduction is mediated to some extent by leptin (Dockray, 2013), which seems unlikely in birds (Seroussi *et al.*, 2016). CCK in the periphery was most highly expressed in the proximal ileum, consistent with intestinal CCK expression in mice (Fakhry *et al.*, 2017), but its absolute expression is remarkably low compared to that of gastrin in the gastric antrum. This is interesting as it suggests that the magnitude of paracrine gastrin binding at B-type receptors local to the antrum must be profound in comparison to CCK binding, assuming expression of the transcript translates to peptide release. This difference in expression has to be taken in context however, since the total gastrin-expressing intestinal region (the gastric antrum) is very short compared to the tissue expressing CCK, which is effectively most of the small intestine (Figure 2). Gastrin and CCK seem to have functionally opposite effects on regulation of gastric acid (Guilloteau *et al.*, 2006), however the inhibitory effect of CCK is dependent on signalling via CCKAR (Chen *et al.*, 2004), whereas gastrin acts only at CCKBR, so disparate threshold ligand concentrations for each of these signalling routes might explain this apparent paradox. More work in defining the distribution and relative functions of receptor distribution in the chicken is required to further tease apart the significance and implications of these regional expressional differences of avian CCK and gastrin.

Although birds are considered 'monogastric,' their gastric lumen is compartmentalised into the proventriculus (glandular stomach) and ventriculus or 'gizzard' (muscular stomach). The proventriculus best resembles the mammalian monogastric stomach in form and function, and so is sometimes referred to as the 'true stomach' (Mussehl *et al.*, 1933; Zaher *et al.*, 2012). The strict delineation of avian gastrin within the 'antrum' region observed here resembles primary mammalian gastrin production at the pyloric antrum which suggests homology of these gastrointestinal structures between birds and mammals. This provides evidence that the mammalian monogastric stomach can be considered homologous to the entire gastric region in birds (i.e. the gizzard is a specialised compartment of the whole 'true stomach' and

not for example an adaptated region of intestinal tissue), in approximate keeping with extant belief (Smith *et al.*, 2000; Nielsen *et al.*, 2001). Its strength and fidelity of expression make gastrin a candidate marker for evolutionary comparisons of vertebrate digestive tract physiology.

CCK did not alter significantly in response to short-term satiety state within the scope of the fed/fasted experiment (section 2.1.2.) (Figure 4a). This was unexpected since CCK is heavily implicated in the short-term satiety response in vertebrate species (Havel, 2001; Murashita *et al.*, 2007; Moran, 2009; Gibbons *et al.*, 2016; Honda *et al.*, 2017; Volkoff *et al.*, 2017), however the feed/fast durations tested here might belie the true short-term expression response if this is considerably more immediate than 2.5h, as demonstrated in murine cell culture (Hand *et al.*, 2010) and has recently been described for pacu fish (Volkoff *et al.*, 2017) but not yellowtail fish (Murashita *et al.*, 2007). Indeed the circulating peptide longevity is known to be very short (Liddle *et al.*, 1985), although a delay in transcriptional response might have been expected, as observed for the satiety factor peptide YY in chickens (Reid *et al.*, 2017). Furthermore, differences in the rate of mRNA translation remain unknown and activity may depend on differential post-translational processing, rather than differential expression (Sayegh *et al.*, 2014). In all, the results herein suggest that CCK expression is not significantly affected by short-term nutrient availability in the chicken, however anticipatory expression might differ between groups under longer-term nutritional challenge, particularly considering the difference in CCK hybridisation signal between *ad libitum*-fed and feed-restricted birds (Figure 3b). In addition, very short-term expressional response to feeding might have been missed by virtue of sampling times in this design.

Gastrin expression differed significantly between treatments, with fasted individuals exhibiting greater expression compared to their fed counterparts at both sampling timepoints (Figure 4b). This suggests that the short-term nutrient-responsive

regulation of gastrin expression in chickens manifests within 2.5h and is maintained for at least 7.5h. The observed trend seems paradoxical; why is gastrin, an accepted vertebrate satiety factor, upregulated under fasting conditions in the chicken? Longer-term conditioning to food availability and heightened expression in anticipation of meal consumption might explain this phenomenon, since these birds were fed *ad libitum* for the entire rearing period before induction to experimental treatment. If this is the case, it might be sensible to consider heightened gastrin expression a means to maintain peptide stocks for secretion upon anticipated detection of nutrients at the gastric antrum. The idea that gastrin expression might be regulated by conditioning is mimicked in the observation that a strong diurnal pattern is apparently maintained regardless of treatment, with gastrin expression decreasing across the experimental timescale for both treatments (Figure 4b). Attenuation of gastrin expression throughout the waking day makes inherent sense for the diurnal chicken, since it would be ineffective for an animal to produce much gastric acid during, or shortly before, inactive hours. Considering the regulatory interplay between gastrin and gastric acid production (Campbell *et al.*, 1991), relatively lowered postprandial expression of gastrin might simply be due to the inhibitory effect of gastrin-stimulated gastric acid on production of gastrin itself.

In conclusion, we have demonstrated tissue distribution of the gastrin/cholecystokinin family of hormones in chicken to a previously unattained resolution. CCK expression does not seem to respond to short-term satiety, contrary to some antecedent vertebrate studies. Gastrin expression did alter between fed and fasted treatments, however its expression was paradoxically lower in acute satiety and higher in acute hunger, which might be an artefact of conditioning to *ad libitum* feeding conditions. Higher resolution studies of the expressional response of these hormones to nutritive state will undoubtedly clarify similarities and differences to mammals and other vertebrate clades. Future investigators should consider disparate nutrient availability for longer time periods and periprandial sampling.

5. Acknowledgements

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5.3 Discussion and conclusions

The distributional data for CCK and gastrin expression reported in this paper represent the highest-resolution determination of endogenous CCK and gastrin production mapping available to date, and the most reliable in terms of specificity. The results from subsequent experimental induction of short-term hunger and satiety leave understanding of the endogenous roles of chicken CCK and gastrin in conundrum. CCK and gastrin were each measured at their respective sites of highest peripheral expression, namely the proximal ileum and gastric antrum, respectively. Sampling timepoints were 2.5h and 7.5h after reintroduction of feed, following a 3h fast. The alternative treatment group, sampled at the same timepoints, were fasted throughout. The intestinal expression of CCK at the proximal ileum did not change dependent on disparate short-term satiety state between these groups. This was highly unexpected, since CCK is classically considered a short-term satiety factor in vertebrates. Antral gastrin expression did differ significantly between these groups, but only 7.5h after feed reintroduction, and in the opposite direction to that expected. As discussed in the article conclusion, this might be due to the dilution of gastric acid (which stimulates gastrin production) in the fed group, compared to resultant lower pH at the antrum of the fasted group. It seems likely that longer-term nutrient deprivation would cause cessation of elevated gastrin expression until after feed was consumed. Similarly, perhaps CCK is not expressionally responsive over such a short time period, or perhaps its upregulation is dampened at the 2wk age examined. As shown in article figure 3(b), a considerable difference in CCK expression is apparent in older birds fed differently (*ad libitum* or commercial restriction) for 2.5 days.

Certainly further investigation of the endogenous responses of peripheral CCK and gastrin are required if their roles in hormonal control of energy balance in birds is to be understood. The confirmation of gastrin and CCK gene expression detailed in this paper provides a reliable source of distribution information for future studies.

The relatively high hypothalamic expression of CCK should not go undiscussed, since it seems that central-derived CCK dwarfs that produced in the periphery. It might therefore be that a CCK-mediated response to short-term satiety involves brain-derived CCK.

Further studies of endogenous CCK and gastrin should incorporate samples from birds at a range of ages and with sampling timepoints spaced over longer periods of disparate feeding. It would also be interesting to examine regional expression of CCK in the brain, and how this is affected by short- and long-term manipulation of energy state.

CHAPTER 6

Final discussion

6 Final discussion

In order to optimise health, welfare and production of chickens as the most-produced livestock, and thus protect the security of this important source of human nutrition, it is necessary to understand how birds achieve their growth potential. Energy balance is integral to all biological processes, yet relatively little is known from studies in birds and our understanding of avian energy homeostasis is largely built on the foundation of mammalian investigations. The available evidence supports broad functional conservation of energy homeostatic mechanisms across vertebrate clades, invariably orchestrated by the central melanocortin system. This overall conservation in this diverse taxon is not surprising considering the complexity of the system and its vital role in maintenance of energy balance. Some notable differences nonetheless clearly exist between mammals and birds, including the debated function of avian ghrelin in contrast to its orexigenic mammalian counterpart could conceivably affect balance of the central melanocortin system activity. More pertinent to the content of this thesis is the discordance in posttranslational processing of PP-fold hormones (discussed in chapter 4), which plausibly results in drastically altered overall receptor specificity of the hormonal milieu accessible to the hypothalamic feeding centre. Furthermore, lack of a highly-conserved leptin structure certainly implies altered function between the two clades, and birds probably do not rely on synergistic leptin-CCK signalling to maintain long-term energy homeostasis.

6.1 Balance of energy via a neuroendocrine switch

Section 1.3.2.3 describes how orchestration of energy homeostasis depends on balancing activities of anabolic AGRP/NPY and catabolic POMC/CART neurones of the central melanocortin system. An interesting asymmetry to this oppositional fundament of central energy control exists. Anabolic first-order neurones increase AGRP and NPY expression in response to orexigenic input, and downregulate these genes in response to anorexigenic input. CART/POMC neurones do not exhibit such

bidirectional plasticity in their transcriptional response to (neuro)endocrine factors. Whilst POMC transcription responds positively to stimulation by anorexigenic factors, it is not dependent on incoming anorexigenic signals (Phillips-Singh *et al.*, 2003). Additionally, whilst stimulated AGRP/NPY neurones actively inhibit secretory activity of CART/POMC counterparts, a reciprocal inhibition is not believed to be true. This means that, in the absence of any extraphysiological input at all (i.e. nutrient starvation), CART/POMC neurones will express POMC in direct relation to endogenous anorexigenic signals (e.g. insulin). Conversely, AGRP/NPY neurone activity might be dampened by these endogenous anorexigens, but in the absence of satiety signalling (e.g. CCK, GLP-1), and presence of stimulation by endogenous orexigens (e.g. ghrelin), AGRP/NPY neurones become highly active. The result is that the balance of signals reaching second-order neurones is shifted toward anabolism and the default vertebrate state is therefore hunger. On application of satiety signals, AGRP/NPY neurones are quickly inhibited ('switched off'), and POMC/CART neurones are again free to secrete their accumulated α-MSH to compete with now-lowered AGRP second-order neurones and effect an opposite signal to now-lowered NPY. The effect is a quick curb of appetite, and altered metabolism, satiety being the achieved state. This idea of appetite being switched on and off makes inherent sense, because energy intake is an active process in vertebrates. Theoretically, if a vertebrate-analogous melanocortin system existed in an organism whose energy intake was passive, it might be expected that catabolic neurones would fulfil the switching role. Of course, since POMC expression depends on input from medium- and long-term energy signals (e.g. insulin, leptin) in mammals, the speed of the switch depends on bodyweight – in other words, positive energy balance brings about a 'stickiness' in the switch, negative energy balance lubricates the switch, and normal function is resumed at closeness to the bodyweight setpoint. In birds however, which apparently lack functional leptin involvement in central melanocortin regulation, switch speed would be dependent only on the medium-term major endogenous anorexigen insulin, whose expression is tied to blood glucose

concentration. The suggestion that birds lack long-term control of bodyweight contradicts observed data; birds, like other tetrapods, appear to defend a bodyweight setpoint. Insulin sensitivity of catabolic first-order neurones is likely heightened relative to mammals, and certainly birds are known to defend glucose homeostasis better than mammals can, in general. But whether the role of POMC/CART neurones in long-term achievement of bodyweight therefore depends exclusively on fluctuating glucose concentration, or if there are additional longer-term signals which affect catabolic (and anabolic) first-order neurones in birds, remains to be understood.

6.2 The significance of CCKAR

6.2.1 Genomic basis of influence

The existing evidence that CCKAR is intimately linked to bodyweight setpoint is further strengthened by the work described in Chapter 3. There is a convincing association of one particular SNP with bodyweight in the diverse Multistrain line (Figure 3.2), which might be due to close proximity to a perturbed C/EBP-α transcription factor binding site. The DelinvA deletion variant seemed a good candidate for regulation of CCKAR, particularly considering its identification as a CR1 retrotransposon regulatory element, however since any putative effect was linked to all HG haplotype variants in the AIL birds examined in this thesis, it was not possible to properly assess its effect in the AIL. CCKAR haplotype continued to predict a difference bodyweight after 20 AIL generations (Figure 3.4). The novel recombination between markers CCKAR_MnlI and DelinvA (section 3.5.1.3) will provide a resource for narrowing the resolution of causative variants around the CCKAR locus, once enough birds can be generated for experimentation, and this will help in assessing the importance of the candidate SNP. Of course, it is important that the CCKAR recombinant alleles are sequenced to ascertain exactly the crossover position, so that SNPs on either side are linked to the correct genotyping assay. Comparing the recombinant alleles will also enable confirmation that only one recombination event took place (that being a

rare mitotic germline recombination) as otherwise there might be more than two novel alleles, which could complicate future association analyses. If possible, it would be interesting to procure some DNA samples from the Hinai-Dori breed chickens under study in the laboratory of Hideaki Takahashi (see section 3.1.1.2), since there is a clear segregating effect of the locus and it would be interesting to see which variants were common between AIL HG and LG haplotypes and Hinai-Dori HG and LG haplotypes. This would provide additional confidence before investment in more costly genetic engineering of live animals or cells *in vitro*. In addition, genotyping the Multistrain for the YY1 binding site-altering SNP identified in these Hinai-Dori chickens might be illuminating, since the commonness of this SNP in other breeds is not known; for example, it might be rare in non-broiler chickens, but exhibit skewed equilibrium in broilers.

6.2.2 Physiological effects

The strength of association of CCKAR genotype with bodyweight increased in the weeks after hatch but did not become statistically significant until 5 weeks of age in the AIL F_{20}, suggesting that the effect relies on some interaction with the post-hatch environment. Possible explanatory differences are feed intake and locomotive energy expenditure. No effect on relative feed intake was observed in this thesis, however the periods measured were not exhaustive, and it remains possible that a very early difference in relative feed consumption causes divergence of growth trajectories between CCKAR genoptypes. Causative physiological attributes conferred by genotype might still appear during embryonic development, and it would be of value to assess the role of CCKAR, if any, in the prenatal chick. The effect of sex in qualifying some of the physiological differences predicted by CCKAR genotype, and in predicting physiological differences regardless of genotype, ties in with the idea that the physiological effect of CCKAR begins before hatch but is not fulfilled until after hatch, since this is reminiscent of sexual dimorphism. Perhaps therefore differing levels or patterns of CCKAR expression are responsible for some of the phenomenon

of sexually dimorphic growth. In mice, sexually dimorphic expression of CCKAR has recently been demonstrated to predict stereotypic male and female behavioural phenotypes (Xu *et al.*, 2012; Yang *et al.*, 2013).

CCKAR seems to predict a difference in the investment of stored energy in chickens, in that the relative size of metatarsal bone and visceral organs depends on genotype at the CCKAR locus (section 3.4.2). Perhaps the most obvious effect is that the gallbladder of HG birds is relatively enlarged. This observation tempts the thought that CCKAR might play a role in development or tissue remodelling in the gallbladder. It might of course be that these respond to the bile load produced by the liver, and still the role of disparate CCKAR expression in bile production, if any, remains to be elucidated. Should this be investigated further, CYP7A1 might be a prime candidate measurement of a gene involved in bile production, since it is common to most bile salt synthesis pathways (Russell, 2003).

6.3 PP-fold hormone dynamics

6.3.1 Novel findings and interpretation

The published article presented in Chapter 4 describes the distribution of PYY and PPY mRNA expression to a higher level of resolution than seen before. The respective responses of PYY and PPY transcription to disparate short- and long-term nutritive states are also described. Relative upregulation of PPY transcription is dependent on sustained positive energy balance, whereas PYY is implicated as a short-term satiety factor. Together with the concordant identification of the pancreas as the major site of PYY production, nutrient-responsive changes in PYY expression might indicate an important role for PYY in regulating insulin production. In chickens, unlike mammals, PYY is not cleaved by DPP-IV, so its receptor specificity does not change to favour Y_2. Upon phylogenetic analysis of translated preproPYY sequences (section 4.5), this seems to be the norm for non-mammalian vertebrates, although some exceptions do exist. At this stage, any role for PYY in regulating insulin

production in chickens is conjectural and based on recent mammalian observations (Guo *et al.*, 1988; Bertrand *et al.*, 1992; Shi *et al.*, 2015; Ramracheya *et al.*, 2016). Another consequence of the ability of DPP-IV to cleave mammalian PYY_{1-36} is that specificity of PYY_{3-36} for the Y_1 receptors expressed by ARC anabolic and catabolic neurones is reduced, and so enteroendocrine and pancreatic PYY might have lost function as direct regulators of the central melanocortin system in mammals. The same however might not be true for most non-mammalian vertebrates, including poultry, so PYY could constitute a major regulator of satiety response. Indeed Aoki and colleagues (2017) observed reduced feeding in chicks administered intravenous PYY_{1-37}. A caveat to this theory is that the likelihood of native periphery-derived PYY regulating neurones directly via the bloodstream is speculative since the distribution of sequestering Y_1 receptors in avian vasculature is yet to be assessed.

6.3.2 Future work

The novelty of the chicken PYY gene sequence – the first directly evidenced avian PYY gene sequence – and the other avian PYY mRNA sequences described in section 4.5, means that many opportunities exist for pioneering investigation of the roles and regulation of PYY in avian species. It would however be appropriate, because of the above inferences, to begin by examining the likelihood of glycaemic regulation by pancreatic PYY-mediated regulation of insulin production. The most probable receptor mediating such an effect would be Y_1, extrapolating from observations made in mammals (Shi *et al.*, 2015). Immunohistochemical delineation of this receptor's distribution would therefore be appropriate, to determine whether a specific pancreatic role is likely. Additionally, the effect of exogenous Y_1 receptor agonist/antagonist molecules on blood glucose would be interesting to observe in chickens, perhaps in parallel with exogenous application of PYY or NPY. Since the aforementioned mammalian studies do not agree on the direction of the PYY effect, it will be interesting to see whether information from the chicken might weigh in on determining the likely endogenous role of pancreatic PYY.

It is also fascinating to learn that pancreatic PYY secretion depends on stimulation by CCK in humans (Degen *et al.*, 2007), and it would be appropriate to ascertain whether the same is true for non-mammals. It might be possible to quantify PYY gene expression in tissue slices from the assay of pancreatic exocrine secretion described in section 3.3.2.5, although the nature of this experiment might mean considerable variability in the results.

6.4 CCK-gastrin hormone dynamics

CCK was originally considered a peripherally-produced hormone which acts locally to stimulate digestive function. However, CCK is now also known to act indirectly via vagal afferent signal to the NTS, and possibly directly in the bloodstream, to inform the central melanocortin system of nutritional status. The discovery therefore that the basal hypothalamus produced large amounts of CCK (Figure 5.2) could suggest that involvement in a reciprocal vagal pathway might be a major endogenous function of CCK in chickens. The interplay between mammalian CCK and PYY might offer an implied role for PYY as a downstream effector in this reciprocal vagal efferent CCK signal, perhaps acting to regulate insulin in response to central signalling. Of course if PYY is found to act directly at ARC neurones, this would imply an interplay loop whereby peripheral PYY and central CCK were regulating each other's function.

The results obtained for nutrient-responsive gastrin expression from the chicken antrum are somewhat unaccountable in the context of dynamic gastrin expression in mammals. It is proposed that, over this short time-scale, gastric acid dilution in fed birds might have relatively lowered gastrin expression, however further testing, perhaps with a fasted group treated with antacid, could further clarify this point. In any case, the regulation of gastrin expression over a longer-term disparate feeding study would be of value in determining the endogenous role(s) of chicken gastrin. The antrum was however confirmed as the major site of gastrin production – almost to the exclusion of all other tissues tested, thereby corroborating previous immunohistochemical results. Indeed, the receptor mediating CCK-stimulated

pancreatic PYY release should be identified as a priority, since the anatomical proximity of the antrum to the pancreas might implicate gastrin as a regulator of insulin production, if this pathway depends on CCKBR. Perhaps elevated gastrin expression under short-term nutrient restriction acts to inhibit insulin production in this way. If however the pathway depends of CCKAR, the observed increase in bodyweight in CCKAR-deficient chickens might be the result of sustained misregulation of glucose homeostasis. Indeed, the relative importance of insulin and PYY between mammals and birds might be skewed by the lack of leptin as a long-term adiposity signal in birds.

6.5 Application of knowledge to the poultry industry

The link between establishing mechanisms controlling growth, and improvement of poultry management practice can seem tenuous. However, this is likely a symptom of the relatively poor characterisation of avian energy homeostasis thus far. In recent years however, research into energy homeostasis and hormonal response to nutrient intake have produced useful insight for both the study and management of poultry. For example, measurement of AGRP has become an accepted index for hunger in the field of poultry energy balance (Dunn *et al.*, 2012; Boswell & Dunn, 2017). Such an index can be used to quantify the effectiveness of emerging welfare-ameliorative management strategies. Hormonal response to inclusion of soluble fibre in diets has been shown not to mimic increased feed provision, in terms of hormonal response (Reid *et al.*, 2017). On the other hand, inclusion of insoluble fibre does seem to have an inhibitory effect on hunger (Nielsen *et al.*, 2011), and inclusion of such insoluble fibres also improves reproductive health in broiler breeders (Moradi *et al.*, 2013). Furthermore, specific regulatory trends for hormones involved in energy homeostasis have been demonstrated to affect reproductive physiology in broiler breeders (Briere *et al.*, 2011; Mcderment *et al.*, 2012), some of which might be used in genetic selection programmes. Developing a fuller understanding of avian energy homeostasis is therefore of demonstrable value to any poultry production facility, particularly broiler breeding farms.

6.6 General conclusion

Although the precise nature of the influence of disparate CCKAR expression on growth phenotype remains elusive, it is clear that the physiological mechanisms for increased growth are complex. Pulling together information from Chapters 3, 4 and 5, it seems likely that identifying the site of hypothalamus-derived CCK action (and precise region of expression) might be key to understanding the role of CCK signalling in determining bodyweight setpoint, particularly since the brain appears to constitute the major source of CCK. If CCKAR is the receptor which mediates CCK-dependent pancreatic PYY regulation, the effect on growth might come about by means of long-term glucose imbalance.

Interplay between CCK and PP-fold peptide signalling should be assessed to determine whether CCK might act at pancreatic CCKARs – or indirectly via efferent vagal signalling – to stimulate downstream PYY and/or PPY transcription. This response, or alternatively that of gastrin at CCKBRs, might be critical in the defence of glucose homeostasis in birds. Furthermore, the insensitivity of non-mammalian PYY to DPP-IV cleavage might facilitate its role in pancreatic defence of glucose homeostasis, since PYY stimulates insulin production via the Y_1 receptor, for which PYY_{3-36} exhibits vastly lowered affinity. Likewise, circulating PYY_{1-36} (or galliforme PYY_{1-37}) might be of greater importance in direct regulation of the central melanocortin system in non-mammalian vertebrates, since inhibition and stimulation, respectively, of AGRP/NPY and POMC/CART neurones is dependent on Y_1-mediated signalling.

In conclusion, there is clearly much to learn about hormonal control of energy balance in birds, however pursuing a fuller explanation could help improve the welfare of avian livestock, as well as providing a valuable non-mammalian example from which to infer vertebrate trends. Explication of the potential interdependence of PP-fold and CCK-gastrin hormone family members in defending glucose homeostasis and overall bodyweight setpoint should take priority.

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Appendix 1

Non-standard reagents and solutions

Non-standard supplied reagents and solutions

Table A.I.1 overleaf shows details of all non-standard supplied reagents and solutions.

Non-standard prepared reagents and solutions

Recipes for all non-standard prepared solutions are shown below.

0.1M PBS with 4% (w/v) paraformaldehyde (PFA): Heat ≈2g NaOH in DEPC-H_2O (250ml total volume). Add 20g PFA and stir until dissolved. Add 200ml DEPC-H_2O and 50ml 1M PBS. Chill to 4°C, adjust pH to 7.4 with HCl.

1M phosphate-buffered saline (PBS): Dissolve 115g Na_2HPO_4, 29.64g $NaH_2PO_4.2H_2O$ and 8.5g NaCl in 800ml H_2O. Adjust volume to 1L and autoclave.

10X dNTP mix: Add 20µl each dNTP from dNTP set (Thermo Fisher Scientific, MA, USA) to 920µl H_2O.

Diethyl pyrocarbonate water (DEPC-H_2O): Add 1ml diethyl pyrocarbonate to 999ml H_2O and agitate vigorously for 2min. Vent for 2h in a fume hood then autoclave.

Hybridisation buffer: Mix the following reagents: 3.5g NaCl, 149mg Tris, 200mg BSA, 100mg Ficoll, 100mg PVP, 2µl 250mM EDTA, 5ml 25% dextran sulphate, 250mg NaPPI, 200µl 25µg.µl^{-1} yeast tRNA (cat# R9001, Sigma-Aldrich, Dorset, England), 250µl 20µg.µl^{-1} yeast total RNA (cat# R7125, Sigma-Aldrich, Dorest, England)10mg salmon testes DNA, 5mg Poly(A), 10ml formamide in a total aqueous volume of 50ml.

Iodine-mix: Mix 20ml iodine-reagent with 1880ml H_2O and 20ml N-HCl.

Iodine-reagent: Dissolve 1.5g potassium iodide and 0.15g iodine in H_2O (total volume 50ml).

Scott's Tap Water Substitute (STWS): Dissolve 20g Mg_2SO_4 and 3.5g $NaHCO_3$ in H_2O (final volume 1L).

Standard Sodium Citrate (SSC) (20X): Dissolve 175.4g NaCl and 88.2g $Na_3C_6H_5O_7$ in H_2O (final volume 1L). Dilute this 20X stock to appropriate concentration before use.

TEA-AA solution: Add 7.45ml triethanolamine to 500ml H_2O and mix. Adjust pH to 8.0 with NaOH/HCl. Add 1.5 ml acetic anhydride immediately before use.

Reagent name	Kit name	Concentration	Manufactu
^{35}S-labelled dATP	-	12.5mCi·ml^{-1}	PerkinElme
Brilliant III Ultra-fast SYBR green qPCR MM	Brilliant III Ultra-fast SYBR green qPCR MM	2X	Agilent Tec
Cobalt Chloride ($CoCl_2$)	Terminal Deoxynucleotidyl Transferase (TdT)	2.5mM	Sigma Aldri
DNAzol	-	as supplied	Thermo Fis
Exonuclease I (ExoI)	-	20U·µl^{-1}	New Englar
FastStart buffer w/0mM $MgCl_2$	FastStart Taq	10X	Roche, Bas
FastStart buffer w/20mM $MgCl_2$	FastStart Taq	10X	Roche, Bas
FastStart Taq	FastStart Taq	5U.µl^{-1}	Roche, Bas
Green Buffer	Terminal Deoxynucleotidyl Transferase (TdT)	10X	Sigma Aldri
$MgCl_2$	FastStart Taq	25mM	Roche, Bas
MnlI	-	5U·µl^{-1}	New Englar
NEBuffer 4	-	10X	New Englar
Quick-Load 100bp DNA ladder	-	50µg·ml^{-1}	New Englar
ROX reference dye	Brilliant III Ultra-fast SYBR green qPCR MM	500X	Agilent Tec
Shrimp Alkaline Phosphatase (SAP)	-	1U·µl^{-1}	New Englar
SYBR Safe DNA gel stain	-	10,000X	Thermo Fis
Terminal Deoxynucleotidyl Transferase (TdT)	Terminal Deoxynucleotidyl Transferase (TdT)	20U·µl^{-1}	Sigma Aldri
Trizol	-	as supplied	Thermo Fis
-	2nd Generation 5'/3' RACE	-	Roche, Bas
-	Brilliant III Ultra-fast SYBR green qPCR MM	-	Agilent Tec
-	Direct-zol RNA Miniprep	-	Zymo Rese
-	dNTP set	2mM each dNTP	Thermo Fis
-	FastStart Taq	-	Roche, Bas
-	High Capacity cDNA Reverse Transcription	-	Applied Bio
-	QIAquick Nucleotide Removal	-	Qiagen NV,
-	QIAquick PCR Purification	-	Qiagen NV,

Table A.I.1 – Details of non-standard supplied reagents and solutions

Appendix 2

Oligonucleotide primers and probes

Table A.II.1 overleaf contains details of all primers and probes used for work described in this thesis.

#	Name	Sequence	Type	Application	Target
1	AR_CCK_ISH1	TTCCCTAGGACAGAGAACCTCCC-AGTGGAACCTTTCCGGGCTTG	Probe	CCK in situ hybridisation	XM_418814
2	CCK_F1	CAGCAGAGCCTGACAGAACC	Primer	CCK qPCR	XM_418814
3	CCK_R4	CCTGTGTGTGGGATCAATGC	Primer	CCK qPCR	XM_418814
4	CCKAR_5RACE_GSP2	AGCAAAGCAGTGATGTTGGT	Primer	CCKAR 5' RACE	XM_420751
5	CCKAR_intron4R	GTGTAGGACAGCAGGTGGAT	Primer	CCKAR loc. seq./5' RACE	XM_420751
6	CCKAR_delinv_A(r)	TTCACGACCTCACTGATCCG	Primer	CCKAR locus sequencing	
7	CCKAR_down5k_A-F	CACCCATGCATGTAAAGGGC	Primer	CCKAR locus sequencing	
8	CCKAR_down5k_A-R	GTCTCATCCTGCAGCCTGA	Primer	CCKAR locus sequencing	
9	CCKAR_down5k_B-F	ACGGGATTTAGTTCGTAACAGTG	Primer	CCKAR locus sequencing	
10	CCKAR_down5k_B-R	GTTACCAACCTGTTCTGCTCA	Primer	CCKAR locus sequencing	
11	CCKAR_down5k_C-F	TCAGATACTGCTCTCGATGGA	Primer	CCKAR locus sequencing	
12	CCKAR_down5k_C-R	AGGCTGCCTTGGATATCTACC	Primer	CCKAR locus sequencing	
13	CCKAR_down5k_D-F	ATCAGCAGCCTCCACATCAT	Primer	CCKAR locus sequencing	
14	CCKAR_down5k_D-R	TTCTGGGTAGTCTCGTGTGG	Primer	CCKAR locus sequencing	
15	CCKAR_down5k_E-F	CAGGCAGAACGTAGCATTGT	Primer	CCKAR locus sequencing	
16	CCKAR_down5k_E-R	TGCATTCAAAAGGGAAGGGA	Primer	CCKAR locus sequencing	
17	CCKAR_down5k_F-F	AAAACAATGCAGTCCAGGGG	Primer	CCKAR locus sequencing	
18	CCKAR_down5k_F-R	TTAAAACCATGCTGCTCCGG	Primer	CCKAR locus sequencing	
19	CCKAR_down5k_H-F	TTCACCAACAGCCCACTAC	Primer	CCKAR locus sequencing	
20	CCKAR_F3	CATTTGAAAACAGCAGAAGCA	Primer	CCKAR_MnlI genotyping	
21	CCKAR_altR3	CTGCTGAATGACATCACTTGG	Primer	CCKAR_MnlI genotyping	
22	CCKAR_intron1F	GCTTTGCTGTGTGATATCCTCT	Primer	CCKAR locus sequencing	
23	CCKAR_intron1R	ACAATACAGCAGGATCCGGA	Primer	CCKAR locus sequencing	
24	CCKAR_intron2BF	GAACCAGTACAAATAAAGGCTGT	Primer	CCKAR locus sequencing	
25	CCKAR_intron2BR	TGAAAGCAGAAGGAAGGCAC	Primer	CCKAR locus sequencing	
26	CCKAR_intron2CF	CACCAATCAAGCAGGACAAGT	Primer	CCKAR locus sequencing	
27	CCKAR_intron2CR	AGCGTACATGTTTCCAGATGG	Primer	CCKAR locus sequencing	
28	CCKAR_intron2DF	TAATGCTCTGCCTCTTCTGC	Primer	CCKAR locus sequencing	
29	CCKAR_intron2DR	GCTGAAATGTGCAGAATCGG	Primer	CCKAR locus sequencing	
30	CCKAR_intron3BF	TGCTTCAACTGGTGCTGAGA	Primer	CCKAR locus sequencing	
31	CCKAR_intron3BR	GCCTCAAGATGACTGCTCAC	Primer	CCKAR locus sequencing	
32	CCKAR_intron3CF	GCAAGCTGGTCCCTTTCAC	Primer	CCKAR locus sequencing	
33	CCKAR_intron3CR	ACCTGTATCCTTCCTCCTCAC	Primer	CCKAR locus sequencing	
34	CCKAR_intron4F	TTGATACCAGCCAGAGAAGATC	Primer	CCKAR locus sequencing	
35	CCKAR_up5k_B-F	TCTGCTCTGCCCTTGTCAG	Primer	CCKAR locus sequencing	
36	CCKAR_up5k_B-R	GGGTTTGTTGCCAGATCTTTC	Primer	CCKAR locus sequencing	
37	CCKAR_up5k_C-F	TCCACAGAATCAATGGCCTT	Primer	CCKAR locus sequencing	
38	CCKAR_up5k_C-R	GGGATGTTGGGAATTTTAGGCA	Primer	CCKAR locus sequencing	
39	CCKAR_up5k_D-F	ACATTTCTCTAGACTACCTGCAG	Primer	CCKAR locus sequencing	
40	CCKAR_up5k_D-R	CTGGCTCTTCATCTCAAAGGT	Primer	CCKAR locus sequencing	
41	CCKAR_up5k_E-F	AATCCAGCTCAGTCCAGGAC	Primer	CCKAR locus sequencing	
42	CCKAR_up5k_E-R	ATAGCTGAGACAAGGCTTCC	Primer	CCKAR locus sequencing	
43	CCKAR_up5k_F-F	TGCCAGAAAGAACCAGGAGA	Primer	CCKAR locus sequencing	
44	CCKAR_up5k_F-R	CTTCCATGAGCACTGTGGC	Primer	CCKAR locus sequencing	
45	CCKAR_up5k_H-F	ACGAAGCTGAAAACACATCCA	Primer	CCKAR locus sequencing	
46	CCKAR_up5k_H-R	ATCCCAAACGTCTGAGTGGC	Primer	CCKAR locus sequencing	
47	CCKAR_up5k_I-F	AGTTTTGGCATCTTAGACTGGA	Primer	CCKAR locus sequencing	
48	CCKAR_up5k_I-R	ACTTGCAAACAGGATGTGCA	Primer	CCKAR locus sequencing	
49	CCKAR_up5k_K-F	AGTACAGAAAGGACATTGAGGTG	Primer	CCKAR locus sequencing	
50	CCKAR_up5k_K-R	GGTCTTCCTCTGGATCTGCT	Primer	CCKAR locus sequencing	
51	CCKAR_up5k_L-F	AAGAGATTGCTGCAGTTACGA	Primer	CCKAR locus sequencing	
52	CCKAR_up5k_L-R	TACTCTGACCTGCTGCAAAC	Primer	CCKAR locus sequencing	
53	CCKAR_up5k_M-F	CCATTCCAACTCTCCAAGCA	Primer	CCKAR locus sequencing	
54	CCKAR_up5k_M-R	GTGAGAAGAGACCAACCCCA	Primer	CCKAR locus sequencing	
55	CCKAR_up5k_N-F	AAAAGGGCCTGGAGATTATCA	Primer	CCKAR locus sequencing	
56	CCKAR_up5k_N-R	CATTGTGTTGAGGGACATGG	Primer	CCKAR locus sequencing	
57	CCKAR_up5k_O-F	CCCAAACATCCAACTTCCAA	Primer	CCKAR locus sequencing	
58	CCKAR_up5k_O-R	TTTTGTTGTTTGACTCTTGCTCTT	Primer	CCKAR locus sequencing	
59	CCKARupstreamF	TACCCTTGAGGCTGGAAATG	Primer	CCKAR locus sequencing	
60	CCKARupstreamR2	ACCCTCTTCTGTTACTGGCC	Primer	CCKAR locus sequencing	
61	CCKAR_delinvA_genoF	GCTTGTTCTGTAGGTTCTGTTGT	Primer	CCKAR_DelinvA	
62	CCKAR_delinvA_genoR	TGTATGAGGGAAGCTGCGC	Primer	CCKAR_DelinvA	
63	AR_GAST_ISH1	ATGAGGCCGAGGAACACCTTCG-TCTTCATGGCTCACGCTGCTGCT	Probe	GAST in situ hybridisation	NM_205400
64	ARgastrinF2	GCTTCATCTCCCGCTTCCT	Primer	GAST qPCR	NM_205400
65	ARgastrinR2	GCTTTATTGCGGGACCAGAG	Primer	GAST qPCR	NM_205400
66	LBR-F	GGTGTGGGTTCCATTTGTCTACA	Primer	LBR qPCR	NM_205342
67	LBR-R	CTGCAACCGGCCAAGAAA	Primer	LBR qPCR	NM_205342
68	NDUFA1-F1	ATGTGGTACGAGATCCTGCC	Primer	NDUFA qPCR	NM_001302115
69	NDUFA1-R1	TTCTCCAGACCCTTGGACAC	Primer	NDUFA qPCR	NM_001302115
70	AR_aPP_ISH1	GTGACCACGTTGAGGTACTGCT-GGAGGTCGTTGTAGAAGCGGATG	Probe	PPY in situ hybridisation	NM_204786
71	PPY 02 Primer F	TCTACAACGACCTCCAGCAG	Primer	PPY qPCR	NM_204786
72	PPY 03 Primer R	CTCTTCGCACAGCACCCG	Primer	PPY qPCR	NM_204786
73	PYY-GSP2	GATGGGCTGCACTGACACT	Primer	PYY 5' RACE	MF455303
74	PYY-GSP3	TGACCAGGTTGATGTAATGGC	Primer	PYY 5' RACE	MF455303
75	AR_PYY-ISH1	TGCTGCGCTTCCCATACCGCTG-CCGCGTGACCAGGTTGATGTAAT	Probe	PYY in situ hybridisation	MF455303
76	PYY-ARF1	TTACATCAACCTGGTCACGC	Primer	PYY qPCR	MF455303
77	PYY-ARR3	TCAGACCACAGCGCATCACT	Primer	PYY qPCR/5' RACE	MF455303
78	YWHAZ_F	GTGGAGCAATCACAACAGGC	Primer	YWHAZ qPCR	NM_001031343
79	YWHAZ_R	GCGTGCGTCTTTGTATGACTC	Primer	YWHAZ qPCR	NM_001031343

Primers in rows 5 – 62 were used to target gDNA
See table A.III.1 for details of CCKAR locus se

Table A.II.1 – Details of all primers and probes

Appendix 3

CCKAR locus sequencing fragments

Table A.III.1 contains details of all CCKAR locus fragments sequenced as part of the work described in Chapter 3. Additional sequencing information (exonic regions) for each haplotype was provided by Ian Dunn.

Fragment #	Position (galGal4:chr4)	Forward primer	Reverse primer
1	72810159-72810988	CCKAR_up5k_O-F	CCKAR_up5k_O-R
2	72810938-72811727	CCKAR_up5k_N-F	CCKAR_up5k_N-R
3	72811659-72812443	CCKAR_up5k_M-F	CCKAR_up5k_M-R
4	72812237-72812940	CCKAR_up5k_K-F	CCKAR_up5k_K-R
5	72812837-72813536	CCKAR_up5k_B-F	CCKAR_up5k_B-R
6	72813386-72814051	CCKAR_up5k_C-F	CCKAR_up5k_C-R
7	72813905-72814594	CCKAR_up5k_D-F	CCKAR_up5k_D-R
8	72814454-72815107	CCKAR_up5k_E-F	CCKAR_up5k_E-R
9	72814943-72815579	CCKAR_up5k_F-F	CCKAR_up5k_F-R
10	72815334-72816259	CCKAR_up5k_L-F	CCKAR_up5k_L-R
11	72816044-72816682	CCKAR_up5k_H-F	CCKAR_up5k_H-R
12	72816520-72817142	CCKAR_up5k_I-F	CCKAR_up5k_I-R
13	72817057-72818145	CCKARupstreamF	CCKARupstreamR2
14	72818473-72819322	CCKAR_intron1F	CCKAR_intron1R
15	72819438-72820426	CCKAR_intron2DF	CCKAR_intron2DR
16	72820270-72821112	CCKAR_intron2BF	CCKAR_intron2BR
17	72820923-72821750	CCKAR_intron2CF	CCKAR_intron2CR
18	72821549-72822440	CCKAR_intron3CF	CCKAR_intron3CR
19	72822265-72823112	CCKAR_intron3BF	CCKAR_intron3BR
20	72823019-72823973	CCKAR_intron4F	CCKAR_intron4R
21	72824952-72825928	CCKAR_down5k_A-F	CCKAR_down5k_A-R
22	72825866-72826816	CCKAR_down5k_B-F	CCKAR_down5k_B-R
23	72826753-72827723	CCKAR_down5k_C-F	CCKAR_down5k_C-R
24	72827584-72828583	CCKAR_down5k_D-F	CCKAR_down5k_D-R
25	72828489-72829435	CCKAR_down5k_E-F	CCKAR_down5k_E-R
26	72829341-72830330	CCKAR_down5k_F-F	CCKAR_down5k_F-R
27	72830200-72831909	CCKAR_down5k_H-F	CCKAR_delinv_A(r)

Table A.III.1 – CCKAR locus sequencing fragments

AIL CCKAR haplotypes

The below sequences are the full CCKAR high growth (HG) and low growth (LG) associated haplotypes derived from sequencing across the CCKAR locus in the AIL (section 3.3.1.2) in FASTA format. Selected features of the CCKAR gene are highlighted: 5' UTR (green), exons (blue) and 3' UTR (pink). The first transcribed base according to the novel TSS (Section 3.4.1.4) is highlighted red. Variants used for standard genotyping (CCKAR_MnlI SNP & DelinvA deletion) are highlighted yellow.

>CCKAR_HG

GATTATCACTATATAAGAGCAAGAGTCAAACAACAAAATACTTAATTGTGTACAGCAAAATACATAGACACAAGTCTGACTTAAA
ATCTTACTTTCATCCTAAGAGTCCTGGAACTTGAGCAGCACAGCCCAGTACCTCACCATCTCAGTACCCTTCCAACTCTTCCGTG
CAAAATCATGACAACAGAAAAACAGCAAAATTCTAAACATTATATAATCTTTTGTTTTTATTCAATCAAAATTCTACTTGGATTC
TTTCAAGGTGATTGTTTTAATTCTTTGTGGAGATTTCTGGTAGGAGTTCATTGAAGTTGTATCTATGTTGTAACCAGTAGAAATA
GTAAAAAGGCCTTCTAAATGAGACACGCATCCCCTAACACTTCTAGAGGGTCAACAAGGCCAAGTGCAGGTAGTGTACTTGGGTC
TGGACACAACATCACAGAACCAAAATTGCAGGCATGGAAGGGACTTCAAGAGATCATCGAGTCCAATCCTCCTGCTAAAGCAGGT
TCCCTAAATTAGATAGCATGGATGGGTGTCCAGATAGGTCTTGAATGTCTCCATAGAAGGAGACTCTACACCCTCTCTGTGCAAC
CCATTCCAGTGCTCCATCACCCTTACCACAAAGAAGTTCTTTTGCATGATTGTATGGAACTTCCTATGTTCAGGCTTTCATAGAA
TCATAGAATCACCAAGGTTGGAAAAGACCTGCAAGATCATCCATTCCAGCTCTCCAAGCAATTGCCTGTCACCAATAGTTCTGAC
TAAACCATGTCCCTCAACACAATGTCTAAACGTTCCTTGAACACCTCCAGGACTGGTGATTCCTATCACTAAGCACCACCGAGAA
GAGTCTGGCCTCATCCATTTGCCTCCCACCTCCCTTTAGGTATTTATAAGCACTAATCAAATCCCTCCTCATTCTTTTCCCCAGA
CTGAAGAGACCTTGGTTACCCTGGGGCAATCCCAGGTATGAGTACAGACTGGCAGAAGAACTTAGAGGATCCCTGTGGAGAAGGG
CTTTTGGGGTCTGGTGGATGTCTTTAGTGACTGGAAAGCTGCCTAGAGGCTGGAGAGCTGCCTGATGGAAAGTGACCTTGGTGTC
ACTTGGAGTGATGGACAGTTGGCTGAATATGAGTCAGCAGTGTGCCCAGGTGGCCAAGAAGACCAGTGGCATCCTGGCTTGTATC
AGGAACGGTGTGGTAAGCAGGACTAGGGAAGTAATCCTGCCCCCTGCCCCCTCACCATGCCCCTCAGCATTGGTGAGGCCTCACC
TCAGGTACTATGTTCAGTTTTGAGTGTCTGAGTACAGAAAGGACATTGAGGTGCTGGAGCAGGTCCAAAGAATGGCAACAAGGCT
TGTGAAGGGCTTGGAGAATATGCCCTACAAAGAGAGACTGAAGGAACTGGGGCTGTTTAGTCTGGGGAAAAGAAGGCTGAGGGCA
GACCTTATTGCTCTCTTCCAATATCTGAAAGGTGCTTACAGTAAGACTGGGGTTGGTCTCTTCTCACTGGTGACAGGACGAGGAG
AAATGGCCTCAAGTTGCGCCAGGGTAAGGTTAGGTTGGATATCAGGAAACACTTCTTTACAGAAAGGTTGTTAAGCACTGGAATA
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GGTGGCCAGCAAGGTGAAGGAGTTGATTGTTCCCTTCTGCTCTGCCCTTGTCAGGCCCTATCTGGAGTACTACATCTAGGCATGG
TACCCCCAGCACAAGAAAGACACAGAGCTGCTAGAGCAGATCCAGAGGAAGACCACAAAGAGCCTGAAGGGTTTATTTTCTTTTG
TAAACACTTACACTGGCCCATTGAGTGACCTGTGCCAAATCACTTCCCTAGGCTTCATTTTTTCATCTCTAAATTGGAACTCATA
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TTAGCAGCGTCCAGATCATCCGTCAGAATGATCTGTATAGTCTGTAACGGTCTGTACAGTCCTCACTACACAGCTTTTTGTTAGC
AGTGGTACCTGAAAGGACAAAAATAGTCAGAAGCAGAAGTAGGTACAATCTGCAGAGCTGGATGTTAGAGCAAGGGCTTTGTAAG
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GACACACCTCTGTATGCTGTGCTGTACCCCTTTTGATTGTGGAGGCTGAGCCAGGAGTTTCAAAATGCCAGCACTGTGACCCTTT
AACTTACGCCCTTGAGAACTTTGATGTAATGTGAAAGTACACACCTAAACTAGGAATGGGAAATCAACATCTTTTTGTAGATAGA
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ATCACCTATGCTCTTACTCTTGAATTTTAAGATGTAAGAAGACATATTCAATTACATATGATTTGTGTAAAGCAAAAGGTAACAT
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Appendix 4

Article as published (see Chapter 5)

The final published version of the above article became available before final submission of this thesis and is attached overleaf.