Chickens from lines artificially selected for juvenile low and high body weight differ in glucose homeostasis and pancreas physiology

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ABSTRACT

Early pancreatectomy experiments performed in ducks and pigeons at the end of the 19th century revealed that avians, unlike mammals, do not display signs of diabetes. Relative to mammals, birds are considered hyperglycemic, displaying fasting blood glucose concentrations twice that of a normal human. While circulating levels of insulin are similar in avians and mammals, and structure and function of the insulin receptor are also conserved among vertebrate species, birds do not experience deleterious effects of chronic hyperglycemia as observed in mammals. Understanding avian glucose homeostasis, particularly in chickens, has both agricultural and biomedical implications. Improvement of feed efficiency and accelerated growth in poultry may come from a greater understanding of the physiological processes associated with glucose utilization in muscle and fat. The chicken has also recently been recognized as an attractive model for human diabetes, where there is a great need for preventative and therapeutic strategies. The link between type 2 diabetes and obesity, coupled with the inherent hyperglycemic nature of chickens, make chickens artificially selected for juvenile low (LWS) and high (HWS) body weight a favorable model for investigating glucose regulation and pancreas physiology. Oral glucose tolerance and insulin sensitivity tests revealed differences in threshold sensitivity to insulin and glucose clearance rate between the lines. Results from real-time PCR showed greater pancreatic mRNA expression of four glucose regulatory genes (preproinsulin, *PPI*; preproglucagon, *PPG*; glucose transporter 2, *GLUT2*; and

pancreatic duodenal homeobox 1, *Pdx1*) in LWS, than HWS chickens. Histological analysis of pancreas revealed that HWS chickens have larger pancreatic islets, less pancreatic islet mass, and more pancreatic inflammation than LWS chickens, all of which presumably contribute to impaired glucose metabolism. In summary, results suggest that at selection age, there are differences in pancreas physiology that may explain the differences in glucose regulation between LWS and HWS. These data pave the way for future studies aimed at understanding the developmental regulation of endocrine pancreas function in chickens, as well as how aging affects homeostatic control of blood glucose in chickens.

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AUC: Area under the curve

BW: Body weight

d: Day

DOH: Day of hatch

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GLUT: Glucose transporter

h: Hour

HWS: High weight select

HSD: Honest significant differences

IGF-1: Insulin-like growth factor-1

i.p.: Intraperitoneal

IST: Insulin sensitivity test

LWS: Low weight select

OGTT: Oral glucose tolerance test

PB: Pancreas body

Pdx1: Pancreatic duodenal homeobox 1

PH: Pancreas head

PPG: Preproglucagon

PPI: Preproinsulin

RT: Real-time

SEM: Standard error of the mean

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Chapter I

Introduction

The pancreas is unique in that it functions as both an exocrine and endocrine organ. While the exocrine pancreas secretes digestive enzymes into the duodenum, the endocrine pancreas is composed of the islets of Langerhans, which contain specialized hormone-secreting cells. These cells include α-cells, β-cells, δ-cells, and γ-cells, which secrete glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively. The endocrine pancreas plays a critical role in regulating glucose homeostasis by secreting insulin and glucagon in response to high and low blood glucose concentrations, respectively.

Compared to mammals, avians are considered hyperglycemic and do not display signs of diabetes, a phenomenon observed in pancreatectomy experiments conducted over a century ago (Koppanyi et al., 1926). Pancreatectomy experiments led some researchers to suggest the existence of an extrapancreatic source of insulin; a hypothesis suggested as early as 1926 (Koppanyi et al., 1926) and as recent as 1982 (Colca and Hazelwood, 1982). Despite evidence supporting this hypothesis, researchers have been unable to locate an extrapancreatic source of insulin in the chicken (Bagnell et al., 1989). Avian species are also relatively insulin resistant, with the chicken being most refractory to the effects of insulin (Chen et al., 1945). While insulin structure varies slightly among species, the structure of the insulin receptor is highly conserved among vertebrates (Muggeo et al., 1979). In all species, the insulin signaling cascade begins with binding of insulin to cell surface insulin receptors, which are located in a variety of tissues (Muggeo et al., 1979). Insulin regulation in chicken hepatocytes is similar to mechanisms

observed in mammals (Dupont et al., 2004), while there are marked differences in muscle and adipose tissue (Sweazea and Braun, 2005; Duchene et al., 2008; Dupont et al., 2012).

Glucose uptake in peripheral tissues is mediated largely by glucose transporters (GLUTs). In mammals, GLUT4 functions as the primary insulin-dependent glucose transporter in mammals (Seki et al., 2003; Thorens and Mueckler, 2009; Wood and Trayhurn, 2003), while an avian orthologue for *GLUT4* has not been reported. Although several avian GLUTs have been identified, with expression varying among tissues, there appears to be very low expression of all isoforms in skeletal muscle (Kono et al., 2005), raising the question of how chicken skeletal muscle clears glucose from the bloodstream. The physiological role of insulin in regulating glucose uptake in avian skeletal muscle and adipose tissue remains unclear. The implications for understanding these mechanisms are two-fold. From an agricultural perspective, improvement of feed efficiency and accelerated growth may come from a greater understanding of the physiological processes associated with glucose utilization in muscle and fat. This type of research also has implications from a biomedical perspective, particularly obesity and type 2 diabetes, where there is a need for preventative and therapeutic strategies.

White Plymouth Rock chickens that have been artificially selected for juvenile (d 56) low (LWS) and high (HWS) body weight (BW) provide a model for studying the agricultural and biomedical implications of nutrient utilization and carbohydrate metabolism. In the 55th generation of selection, HWS chickens are more than 10-fold heavier than LWS chickens at selection age, with substantial fat accumulation. The HWS are hyperphagic and develop characteristics of metabolic syndrome as juveniles, while the LWS are hypophagic with some

anorexic individuals. Because these lines of chickens display such a wide divergence in food intake and body composition, specifically abdominal fat, they represent an attractive animal model for studying the physiological factors underlying obesity and glucose metabolism.

Almost 30 years ago, Sinsigalli et al. (1987) reported that S_{26} generation HWS chickens were glucose intolerant with mild or moderate hyperinsulinemia, hyperglucagonemia, and perhaps insulin resistant. Zhao et al. (2014) recently reported greater blood glucose concentrations in HWS chickens relative to LWS (S_{54} generation) as early as day of hatch (DOH), as well as at older ages ($\leq$ day 15), even in the fasted state. Because Sinsigalli et al. (1987) observed differences in glucose regulation between LWS and HWS in earlier generations of chickens, and more recent data showed differences in developmental regulation of glucose, there was a strong impetus to evaluate glucose homeostasis in selection age chickens. The following experiments were thus designed to investigate glucose regulation and pancreas physiology in selection age LWS and HWS lines of chickens.

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Chapter II

A review of insulin biology and glucose regulation in chickens

ABSTRACT: Early pancreatectomy experiments in ducks and pigeons performed at the end of the 19th century revealed that avians do not experience diabetes. Additionally, avians are considered hyperglycemic relative to mammals, with chickens displaying fasting blood glucose concentrations twice that of a normal human. Despite having normal circulating plasma insulin concentrations, chickens are also considered insulin resistant. Insulin regulation in chicken hepatocytes is similar to mechanisms observed in mammals, while there are marked differences in muscle and adipose tissue. In addition, an orthologue for GLUT4, the primary glucose transporter associated with insulin sensitive carrier-mediated glucose transport in mammals, has not been identified in the chicken genome. The following is a review of carrier-mediated glucose uptake and insulin signaling in chickens.

Introduction

The pancreas is unique in that it functions as both an exocrine and endocrine organ. Histologically, the exocrine pancreas contains cluster of cells called acini. Each acinus contains acinar cells, surrounding an intercalated duct, which secrete digestive enzymes that drain into the duodenum. The main hormones responsible for regulating pancreatic exocrine secretions are gastrin, cholecystokinin, and secretin. The endocrine pancreas is made up of the islets of Langerhans, distinct portions containing specialized endocrine cells. Pancreatic islet areas

contain α-cells, β-cells, δ-cells, and γ-cells, which secrete glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively. In rodent islets, insulin-secreting β-cells are the predominant cell type, while human islets appear to contain more α-cells (Cabrera et al., 2006; Elayat et al., 1995). While the structure and function of the mammalian endocrine pancreas has been well described, less is known about the avian pancreas; however, islet organization appears to differ significantly among vertebrate species (Bonner-Weir and Weir, 1979). The pancreas plays a critical role in regulating glucose homeostasis by secreting insulin and glucagon in response to high and low blood glucose concentrations, respectively.

Birds display the highest fasting blood glucose concentration among all vertebrates, with concentrations that are more than twice those in mammals (Braun and Sweazea, 2008; Scanes and Braun, 2012). Additionally, birds are relatively insulin resistant; a phenomenon that remains unclear. Several hypotheses have been suggested for why avians are refractory to the deleterious effects of chronic hyperglycemia, a condition that leads to severe oxidative stress in mammals (Scanes and Braun, 2012). Not only do birds produce less reactive oxygen species (superoxide and hydrogen peroxide); endogenous levels of antioxidants, including uric acid, are higher in avian species (Braun and Sweazea, 2008; Ku and Sohal, 1993). Compared to mammals, birds have lower levels of glycated hemoglobin, perhaps due to decreased permeability of avian nucleated erythrocytes, as well as a higher rate of red blood cell turnover (Beuchat and Chong, 1998). These observations have sparked interest in using chickens as a model for human diabetes research (Datar and Bhone, 2011).

How an individual balances glucose metabolism among storage, oxidation, and biosynthesis has been summarized by Stipanuk (2006). While the liver plays a major role in glucose homeostasis through glycogen storage, it is also capable of oxidizing glucose for energy needs, as well as fatty acid and amino acid synthesis. Additionally, hepatocytes can release glucose from glycogen stores, as well as synthesize glucose de novo, during low blood glucose conditions. In skeletal and heart muscle, glucose can also be stored as glycogen, or it can be completely oxidized, while adipose tissue oxidizes glucose for energy or utilizes acetyl CoA for de novo fatty acid synthesis, the specifics of which differ among species. In humans and avian species, the liver serves as the primary site of de novo fatty acid synthesis, whereas in porcine and bovine species it is in adipose tissue. In the brain, glucose is an obligate fuel source and is completely oxidized. The two hormones primarily responsible for glucose regulation are insulin and glucagon, both of which are secreted by the pancreas during periods of high and low blood glucose, respectively. Specifically, glucagon secretion and signaling promotes glucose release, while insulin promotes cellular glucose uptake and utilization in various tissues. Glucose transport is mediated by transporter proteins that vary in tissue expression and mechanism. This review will focus on regulation of carrier-mediated glucose uptake and insulin signaling in chickens.

Historical Perspective

Early pancreatectomy diabetes experiments have been elegantly summarized by Koppanyi et al. (1929). The following is a short synopsis of these historical experiments as described by Koppanyi et al. (1929). Diabetes was first described in dogs at the end of the 19th

century by von Mehring and Minkowski following pancreatectomy experiments. However, pancreas removal in avians was attempted by researchers prior to von Mehring and Minkowski's famous 1889 discovery. Bernard was the first to report pancreatectomy in pigeons and ducks in 1877, and pancreatic duct ligation experiments by Lagendorff (in 1879) in pigeons soon followed. In 1893, Minkowski was the first to describe a different response to pancreatectomy in pigeons and ducks, than the diabetes observed in dogs. Minkowski noted a lack of glycosuria, even following administration of 15 g of sugar. Subsequent experiments on pancreatectomzied ducks also showed that the birds displayed transient hyperglycemia and a lack of glycosuria following pancreas removal (Mirsky et al., 1941; Seitz and Ivy, 1929; Sprague and Ivy, 1936). Giaja was the first to report pancreatectomy experiments in chickens in 1912. He described varying blood glucose concentrations that he considered evidence of hyperglycemia, although no glycosuria was observed. Koppanyi et al. (1929) observed that pancreas removal in chickens resulted in hyperglycemia, glycosuria, and polydipsia within the first week, followed by a return to normal metabolism. Researchers offered two possible explanations that were extensively investigated in following years: (1) another organ may develop the ability to regulate carbohydrate metabolism, or (2) an extrapancreatic source of insulin exists (Koppanyi et al., 1929).

An interest in glucose regulation and insulin biology research in chickens has continued for over 100 years since Minkowski (1893) first described a lack of diabetes in avians. Because the classic diabetic condition first described by von Mehring and Minkowski (1889) in dogs was later discovered to involve lack of insulin production, researchers were interested in studying an insulin-deficient chicken. In mammals, diabetes experiments often involve cytotoxic destruction

of β-cells by alloxan or streptozotozin administration. In the alloxanized rodent, a redox cycle between alloxan and dialuric acid produces superoxide radicals. Dismutation of the radicals produces hydrogen peroxide, leading to formation of highly reactive hydroxyl radicals. The action of reactive oxygen species, coupled with an increase in cytosolic calcium concentration, results in β-cell destruction. Streptozotozin is transported into the β-cell by GLUT2 and causes DNA alkylation, resulting in activation of poly ADP-ribosylation, thereby leading to depletion of cellular NAD+ and ATP. Phosphorylation of ATP results in the formation of superoxide radicals. Streptozotozin also releases toxic amounts of nitric oxide, thereby inhibiting aconitase activity and leading to DNA damage. As a result, β-cells are destroyed by necrosis (action of alloxan and streptozotozin reviewed by Szkudelski, 2001). However, administration of neither drug has an effect on the β-cells of chickens, and concentrations of blood glucose and insulin remain unchanged (Langslow et al., 1970; Lukens, 1948; Mirsky and Gitelson, 1957; Scott et al., 1945). Therefore, pancreatectomy continued to be a necessary procedure in investigating glucose regulation *in vivo* in chickens. Alternatively, *in vitro* experiments offered an additional option for gaining insight into the effect of pancreatic insulin on blood glucose. *In vitro* stimulation of pancreatic pieces with glucose concentrations ranging from one-half to two times normal chicken glycemic levels (50, 150, 300 mg/dL) had no effect on insulin release (Naber and Hazelwood, 1977). Glucose concentrations of 500 and 700 mg/dL were required to induce only a transient increase in insulin secretion (Naber and Hazelwood, 1977). Failure to stimulate pancreatic insulin release using physiological concentrations of glucose led researchers to question the involvement of insulin in glucose regulation in the chicken. However, experiments in partially pancreatectomized (80%) chickens strongly confirmed the essential role of insulin in glucose utilization (Langslow and Freeman, 1972). Although removal of 80% of the pancreas

did not result in persistent hyperglycemia, chickens exhibited extreme glucose intolerance (Langslow and Freeman, 1972). Researchers also made the novel finding that partial pancreatectomy resulted in decreased plasma insulin concentrations, which was independent of nutritional status (Langslow and Freeman, 1972).

While pancreatectomy remained the best way to study glucose utilization in the chicken, the procedure was associated with a specific, significant disadvantage. Due to the anatomy of the chicken, full pancreatectomy was impossible without causing significant enteric damage. Following removal of 99% of the pancreas, a small splenic remnant remained. While the splenic lobe contains mostly glucagon secreting α-cells, and few insulin producing β-cells, the possibility of the remnant contributing significantly to glucose metabolism remained. Cieslak and Hazelwood (1986) investigated the functional competency of the splenic remnant by evaluating plasma and tissue levels of several pancreatic hormones, including insulin and glucagon, following 99% pancreatectomy. Pancreas removal resulted in immediate enlargement of the splenic lobe, and while insulin and glucagon total content increased over the 16 day experimental period, they were not directly relative to increase in splenic remnant size (i.e. decrease in concentration; Cieslak and Hazelwood, 1986). In addition, release of insulin and glucagon to plasma was subnormal; however, while insulin/glucagon molar ratio was half that of normal chickens, researchers concluded the hormone ratio was adequate to prevent permanent diabetes (Cieslak and Hazelwood, 1986). In a follow-up *in vitro* experiment, researchers assessed the hormone release capability of the splenic remnant following 99% pancreatectomy (Hazelwood and Cieslak, 1989). Sixteen days following 99% pancreatectomy, the enlarged splenic lobe was removed following a 24 hour fast, and incubated *in vitro* with varying

concentrations of glucose (Hazelwood and Cieslak, 1989). In accordance with previous findings, an increase in total content, yet a decrease in hormone (insulin, glucagon, pancreatic polypeptide) concentration was observed (Hazelwood and Cieslak, 1989). Additionally, glucose stimulation resulted in decreased sensitivity of glucagon secretion, while insulin secretory mechanisms were not affected (Hazelwood and Cieslak, 1989). Since previous *in vivo* experiments showed an increase in the sensitivity of insulin release following 99% pancreatectomy, while *in vitro* experiments did not reveal altered insulin sensitivity, researchers concluded that the former results may have been a reflection of increased sensitivity of extrapancreatic insulin sites rather than direct action of the splenic lobe remnant (Hazelwood and Cieslak, 1989).

As mentioned previously, the presence of a non-pancreatic source of insulin and/or accessory β-islet tissue was suggested as early as 1926 (Koppanyi et al., 1926). This hypothesis was investigated by Colca and Hazelwood (1976) who observed a decrease in immunoreactive insulin within 24 hours of pancreatectomy (99%), followed by a return to normal levels on day 2, and supranormal levels on days 3 through 8. Removal of the splenic remnant in a second operation decreased immunoreactive insulin by 50% within 4 hours, which returned to normal levels 20 hours later, and tolbutamide injection resulted in a characteristic hypoglycemic response (Colca and Hazelwood, 1976). Tolbutamide is an aryl sulfonylurea known to lower blood glucose concentrations by inducing electrical activity in pancreatic islets (Henquin and Meissner, 1982), thus increasing cytosolic calcium concentrations and stimulating insulin release (Abrahamsson et al., 1985). Researchers later noted that immunoreactive insulin and glucagon persisted for up to 5 days following pancreatectomy and continued to respond to stimulation by

arginine (administered i.v.; Colca and Hazelwood, 1982), an amino acid known to stimulate glucagon and insulin release even in the absence of glucose (Gerich et al., 1974). Colca and Hazelwood (1982) concluded that results indicated a non-pancreatic reserve of insulin. Despite evidence supporting this hypothesis, researchers have been unable to locate an extrapancreatic source of insulin in the chicken (Bagnell et al., 1989).

Insulin and the Insulin Receptor

While diabetes was first described in dogs at the end of the 19th century, insulin, the main hormone involved in the disease, was not discovered until 1922 (Steiner et al., 1985). As mentioned previously, the endocrine pancreas contains specialized areas referred to as islets of Langerhans, which consist of several different hormone-producing cells, including insulin-secreting β-cells. The insulin hormone is derived from the inactive protein, preproinsulin, which requires post-translational modification (Perler et al., 1980). The preproinsulin gene is transcribed in β-cells, and the resulting mRNA is spliced to produce proinsulin (Perler et al., 1980). Proinsulin consists of A and B peptide chains, linked together by a C peptide (Perler et al., 1980). Enzymatic cleavage of the C peptide produces the mature insulin protein, which crystallizes as granules (Perler et al., 1980). Insulin is stored in pancreatic β-cells and is secreted in response to specific factors, the preeminent stimulus being glucose (reviewed by Steiner et al., 1985). An increase in blood glucose concentration results in secretion of stored pancreatic insulin (Hedeskov, 1980), as well as an increase in preproinsulin transcription (Nielsen et al., 1985; Welsh et al., 1985) and translation (Okamoto, 1981; Permutt, 1974).

While chicken insulin is generally considered to be similar to its mammalian counterpart, specific differences do exist. Smith (1966) was the first to describe the amino acid differences of chicken insulin, as compared to porcine insulin. On the A chain, histidine, asparagine, and threonine replace threonine, serine, and isoleucine at positions 8, 9, and 10, respectively (Smith, 1966). On the B chain, two alanines replace phenylalanine and valine at positions 1 and 2, and serine replaces threonine at position 27 (Smith, 1966). Chicken insulin has enhanced biological potency in both mammals and chickens, which is due to an increased affinity for the insulin receptor (Muggeo et al., 1979; Simon et al., 1974; Simon and Leroith, 1986) and a slower dissociation rate of the chicken insulin receptor complex (Simon et al., 1977). In all species, the insulin signaling cascade begins with binding of insulin to cell surface insulin receptors, which are located in a variety of tissues (Muggeo et al., 1979). While insulin structure varies slightly among species, the structure of the insulin receptor is highly conserved among vertebrates (Muggeo et al., 1979). The insulin receptor is a transmembrane glycoprotein containing two α-subunits and two β-subunits linked by disulphide bonds (Lee and Pilch, 1994). Binding of insulin to ligand binding sites located on the α-subunit stimulates tyrosine-kinase activity of the β-subunit, leading to autophosphorylation of the β-subunit and phosphorylation of tyrosine-specific substrates (Lee and Pilch, 1994; Patti and Kahn, 1998; Zick et al., 1983a, 1983b, 1985).

Mechanisms of Carrier-Mediated Glucose Uptake

Glucose transporters and sodium-dependent glucose transporters (SGLTs) are multi trans-membrane domain proteins that function in carrier-mediated monosaccharide and ion uptake. The GLUT proteins mediate transport through facilitated diffusion, while SGLTs rely on

sodium gradients (Wood and Trayhurn, 2003). At least 14 GLUT isoforms have been identified in mammals (Braun and Sweazea, 2008; Thorens and Mueckler, 2009; Wood and Trayhurn, 2003). GLUT2 and 4 are particularly essential in maintaining glucose homeostasis through insulin signaling. GLUT2 is expressed in pancreatic β-cells, intestinal and kidney epithelial cells, and hepatocytes. In β-cells, GLUT2 plays a critical role in glucose sensing, where oxidation of glucose leads to insulin secretion (Thorens and Mueckler, 2009; Wood and Trayhurn, 2003). GLUT4 is predominantly expressed in skeletal muscle and adipose tissue, where it functions as the primary insulin-dependent glucose transporter in mammals (Seki et al., 2003; Thorens and Mueckler, 2009; Wood and Trayhurn, 2003). Thus, disruption of GLUT4 function is associated with obesity and diabetes (Thorens and Mueckler, 2009). GLUT4 vesicles are intracellularly translocated to the plasma membrane through an insulin signaling-mediated pathway (Braun and Sweazea, 2008; Dupont et al., 2009; Seki et al., 2003; Wood and Trayhurn, 2003). In short, the binding of insulin to the insulin receptor activates insulin receptor substrate (IRS)-1 and phosphoinositide-3 kinase (PI3K), leading to translocation of GLUT4 (Braun and Sweazea, 2008; Dupont et al., 2009). While post-prandial regulation of glucose uptake has been well characterized in mammals, mechanisms of glucose clearance remain unclear in avian species.

Regulation of Glucose Uptake in Chickens

Although avian *GLUT* 1, 2, 3, 5 and 8 have been identified, an avian orthologue for *GLUT4* has not been reported (Carver et al., 2001; Duclos et al., 1993; Kono et al., 2005; Seki et al., 2003). Chicken GLUT2 is highly homologous to the mammalian isoform; however, tissue

specific expression may differ between the two (Kono et al., 2005; Wang et al., 1994). Whereas GLUT2 is predominantly expressed in mammalian pancreas and intestine, expression has been detected in chicken liver, kidney, skeletal muscle (Pectoralis major), hypothalamus, small intestine, and pancreas (Gilbert et al., 2007; Kono et al., 2005; Sumners et al., 2014; Zhang et al., 2013). Zhang et al. (2013) reported that expression of GLUT2 was greater in liver than skeletal muscle or hypothalamus. Further research is needed to determine whether GLUT2 functions in pancreatic glucose sensing in birds, as in mammals. Tissue distribution of other GLUT isoforms has also been investigated in the chicken, where expression was detected in several different tissues (liver, brain, heart, kidney, adipose, testis). Our lab recently investigated mRNA expression of chicken GLUT 1, 2, 3, 8, and 9 in hypothalamus, fat, liver, and skeletal muscle in lines of chickens artificially selected for juvenile low or high body weight. All GLUT isoforms were detected in each tissue, with the exception of GLUT3 expression in the muscle, with differential expression of GLUT 1, 2, and 9 between genetic lines (Zhang et al., 2013).

While chicken GLUT expression varies among tissues, there appears to be very low expression of all isoforms in skeletal muscle, which raises the question of how skeletal muscle clears glucose from the bloodstream (Kono et al., 2005). Sweazea and Braun (2005) observed that glucose uptake was diminished in skeletal muscles isolated from English sparrows that were incubated with phloretin, a non-specific glucose transporter inhibitor, indicating the presence of one or more glucose transporters. Further evidence for an insulin-dependent glucose transporter in avian skeletal muscle was revealed when glucose transport across the cell membrane of skeletal muscle extracts increased in tissues from chickens that were injected with insulin (Tokushima et al., 2005). Chickens also showed decreased mRNA expression of GLUT2 and 3

following intraperitoneal (i.p.) injection of insulin, which is suggestive of an insulin-dependent mechanism (Zhang et al., 2013). The physiological role of insulin in regulating glucose uptake in avian skeletal muscle and white adipose tissue is unclear, as pharmacological doses of insulin were required to mediate these effects under experimental conditions.

Insulin Function in Chickens

Insulin is secreted by pancreatic β-cells in response to elevated glucose and regulates plasma glucose concentration by stimulating cellular glucose uptake. While the insulin signaling pathway and regulation of GLUT4 has been well characterized in mammals, little is known about insulin function in avian species. Although concentrations of circulating chicken insulin are similar to those found in mammals (Simon et al., 2011), as mentioned previously, chicken insulin has a higher binding affinity for the mammalian insulin receptor than human or porcine insulin (Simon et al., 1977). In mammals, at least eleven intracellular substrates of the insulin receptor have been identified (Dupont et al., 2009), while only two have been identified in the chicken; IRS-1 and Shc (Dupont et al., 1998a, 1998b). An IRS-2 homolog has also been predicted from the chicken genome, but tissue expression has not yet been reported (Dupont et al., 2009). Several components of typical insulin pathways have also been characterized in chicken liver and muscle (phosphatidylinositol-3' kinase (PI3K), protein kinase B (Akt), extracellular signal-regulated protein kinase 2 (MAPK ERK2), GSK3, 70 kDa ribosomal protein S6 kinase (P70S6K) and S6 ribosomal protein; Dupont et al., 2012).

Resistance to insulin in avians, as evidenced by the requirement of large amounts of exogenous insulin to produce hypoglycemic convulsions, was noted as early as 1923 (Chen et al., 1945). Among avian species, chickens appear to be most insulin resistant, as compared to pigeons, ducks, and canaries (Chen et al., 1945). Despite the similar potencies of chicken and mammalian insulin in vitro (Goodridge, 1968; Hazelwood et al., 1968; Langslow and Hales, 1969), as well as similar half-lives in chicken plasma (Langslow and Freeman, 1972), chickens require a dose of 50 i.u./kg body weight to produce mild hypoglycemia (Heald et al., 1965; Langslow et al., 1970; Lepkovsky et al., 1967). Additionally, injected insulin results in an increase in the concentration of plasma free fatty acids in chickens, which is the opposite of what has been observed in mammals (Heald et al., 1965; Langslow et al., 1971; Lepkovsky et al., 1967). In addition to being relatively insensitive to glucose concentration, the chicken β-cell does not produce a sustained increase in insulin release in response to prolonged or repetitive stimuli, suggesting poor insulinogenic reserve (Naber and Hazelwood, 1977). In addition, the chicken pancreas contains one-fifth the amount of insulin of mammalian pancreas (Kimmel et al., 1968), and in great contrast to mammals, fasting (up to 72 hours) has little effect on plasma insulin concentrations (Langslow et al., 1970). Some insulin signaling pathway components are refractory to stimulation (Akiba et al., 1999; Simon, 1988; Tokushima et al., 2005), and it appears that in chickens the regulatory mechanisms associated with insulin signaling are tissue specific (Dupont et al., 2012). Insulin regulation in chicken hepatocytes is similar to mechanisms observed in mammals (Dupont et al., 2004), while there are marked differences in muscle and adipose tissue (Duchene et al., 2008; Dupont et al., 2012; Sweazea and Braun, 2005). Sweazea and Braun (2005) found that skeletal muscle isolated from English sparrow was resistant to glucose uptake when incubated with insulin and insulin-like growth factor (IGF)-1,

both of which are activators of the mammalian insulin-mediated glucose pathway. Moreover, when tissues harvested from insulin-injected mourning doves were incubated with glucose, there was no difference in glucose uptake between control and insulin-injected tissues (Sweazea et al., 2006). In avian muscle, early insulin signaling steps (IRS-1 and PI3K) are insensitive to insulin, while downstream elements (Akt, P70S6K, S6 ribosomal protein) respond typically (Duchene et al., 2008; Dupont et al., 2012). In chicken adipose tissue, despite possessing typical insulin cascade elements (Dupont et al., 2012), *in vivo* insulin immuno-neutralization does not alter insulin signaling steps (Dupont et al., 2009). Although all steps appear insensitive to insulin in chicken adipose tissue (Dupont et al., 2012), it is possible that insulin exerts an effect through a pathway other than PI3K. However, it is also important to note that compared to mammals, PI3K activity is 15 to 20 times higher in avian muscle, and 2 to 3 times higher in avian liver and adipose tissue (Dupont et al., 2012). Lastly, most recent research showed that insulin response in broiler chickens is age-dependent during both embryonic and post-hatch development (Franssens et al., 2014).

Implications

Although decades of research have revealed that birds are hyperglycemic and relatively insulin resistant, the role of insulin in regulating glucose homeostasis and mechanisms underlying the adaptations to chronic hyperglycemia remains unclear. The implications for understanding these mechanisms, as well as their relation to obesity are two-fold. From an agricultural perspective, improvement of feed efficiency and accelerated growth may come from a greater understanding of the physiological processes associated with glucose utilization in

muscle and fat. This type of research also has implications from a biomedical perspective, particularly obesity and type 2 diabetes, where there is a need for preventative and therapeutic strategies.

Agricultural

In the agricultural industry, broiler chickens represent one of the most advanced and effective examples of selective breeding. In the late 1960s, the poultry industry began employing artificial selection to improve growth rate and feed efficiency. Within approximately 30 years following the initial impetus, both time to slaughter and feed consumption decreased by 50% (Olsson et al., 2006). Not only has slaughter weight increased, but chickens have larger pectoral muscle mass and increased meat yield (Havenstein et al., 2003a, 2003b). Unfortunately, such a rapid increase in body weight has resulted in some musculoskeletal abnormalities and poor walking ability (i.e. leg weakness), as well as ascites (Julian, 1998; Paxton et al., 2013). While selective breeding alone has been tremendously successful in producing quality meat chickens in a short period of time, while utilizing minimal resources, there is still room for improvement. Increasing meat yield and feed efficiency without negatively affecting musculoskeletature would signify another great improvement for the poultry industry. To achieve this, a greater understanding of nutrient utilization is required. The crucial role of pancreatic hormones in regulating carbohydrate metabolism makes discerning the underlying mechanisms of insulin signaling and glucose uptake, particularly in muscle and adipose tissue, of great importance.

Biomedical

As of 2011, diabetes currently affects 20.9 million people, is the 7th leading cause of death in the US, and incurs a total annual healthcare cost of $174 billion dollars (CDC, 2011). Additionally, in 2009-2010 over one third of the US adult population were classified as obese, a trend that has had a significant increase over the last decade, and is a major predisposing factor for developing type 2 diabetes (Ogden et al., 2012). In mammals, chronic hyperglycemia is almost always associated with serious deleterious effects on the body, but in contrast, even older birds do not appear to exhibit ill effects of prolonged hyperglycemia (Braun and Sweazea, 2008). With the growing concern of hyperglycemia and insulin resistance in humans, chickens have recently been recognized as an attractive model for studying diabetes (Datar and Bhonde, 2011). NIH has recently launched a research initiative stating that, "The domesticated chicken is the premier non-mammalian research model organism" (NIH). In fact, genetic mapping has revealed there is greater homology between humans and chickens than humans and mice (Burt et al., 1999). The chicken was the first avian genome to be sequenced and analyzed (International Chicken Genome Sequencing Consortium, 2004), and as such the chicken genome is the most well-annotated and serves as a reference for all other birds. Generally speaking, the use of chickens for biomedical research has many advantages. Currently, chickens serve as a model for investigating atherosclerosis, hypertension, cholesterol metabolism, bone development, pathology, and cancer (Datar and Bhonde, 2011). The short generation interval (21 days of incubation), combined with ease of artificial insemination, allows for efficient and straightforward research protocols. Historically, chicken eggs have proved to be an invaluable resource in studying embryogenesis and development, therefore presenting an attractive possibility for conducting gestational diabetes experiments. In fact, the chicken chorioallantoic membrane model is currently utilized in studying hyperglycemia-induced angiogenesis (Larger

et al., 2004). Specifically regarding regulation of glucose metabolism through insulin signaling, chickens and humans both produce a single form of insulin, while rats and mice secrete two (Perler et al., 1980). Despite differences between the effect of insulin in chickens and mammals, the mechanism of pancreatic insulin release by calcium-mediated exocytosis is similar (Flotzer et al., 1982; King and Hazelwood, 1976; Naber and Hazelwood, 1977). Transplanting embryonic chick pancreas in mice has also been successful in reversing experimentally induced diabetes (Eloy et al., 1979). Additionally, researchers have shown that isolated chick islets are suitable for screening of hypoglycemic agents and islet banking (Datar et al., 2006; Datar and Bhonde, 2010) and that the shell-less chick embryo culture system may be useful in studying glucose-induced malformations similar to those observed in mammalian embryos (Datar and Bhonde, 2005).

White Plymouth Rock chickens that have been artificially selected for juvenile (day 56) low and high BW provide a model for studying the agricultural and biomedical implications of nutrient utilization and carbohydrate metabolism. In the 55th generation of selection, HWS chickens are more than 10-fold heavier than LWS chickens at selection age, with substantial fat accumulation. The HWS are hyperphagic and develop characteristics of metabolic syndrome as juveniles, while the LWS are hypophagic with some anorexic individuals. Because these lines of chickens display such a wide divergence in food intake and body composition, specifically abdominal fat, they represent an attractive animal model for studying the physiological factors underlying obesity and glucose metabolism.

Almost 30 years ago, Sinsigalli et al. (1987) reported that S_{26} generation HWS chickens were glucose intolerant with mild or moderate hyperinsulinemia, hyperglucagonemia, and perhaps insulin resistant. Zhao et al. (2014) recently reported greater blood glucose concentrations in HWS chickens relative to LWS (S_{54} generation) as early as DOH, as well as at older ages ($\leq$ day 15), even in the fasted state. Because Sinsigalli et al. (1987) observed differences in glucose regulation between LWS and HWS in earlier generations of chickens, and more recent data showed differences in developmental regulation of glucose, there was a strong impetus to evaluate glucose homeostasis in selection age chickens. The following experiments were thus designed to investigate glucose regulation and pancreas physiology in selection age LWS and HWS lines of chickens.

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Chapter III

Chickens from lines artificially selected for juvenile low and high body weight differ in glucose homeostasis and pancreas physiology

ABSTRACT: Artificial selection of White Plymouth Rock chickens for juvenile (day 56) body weight resulted in two divergent genetic lines; hypophagic low weight (LWS) chickens and hyperphagic obese high weight (HWS) chickens, with the latter more than 10-fold heavier than the former at selection age. A study was designed to investigate glucose regulation and pancreas physiology in selection age LWS and HWS chickens. Oral glucose tolerance and insulin sensitivity tests revealed differences in threshold sensitivity to insulin and glucose clearance rate between the lines. Results from real-time PCR showed greater pancreatic mRNA expression of four glucose regulatory genes (preproinsulin, *PPI*; preproglucagon, *PPG*; glucose transporter 2, *GLUT2*; and pancreatic duodenal homeobox 1, *Pdx1*) in LWS, than HWS chickens. Histological analysis of pancreas revealed that HWS chickens have larger pancreatic islets, less pancreatic islet mass, and more pancreatic inflammation than LWS chickens, all of which presumably contribute to impaired glucose metabolism.

Introduction

Compared to mammals, avian species are considered hyperglycemic, with fasting blood glucose (BG) concentrations typically twice that of non-diabetic humans (Braun and Sweazea, 2008; Scanes and Braun, 2012). Studies investigating insulin secretion in response to glucose perfusion found that chicken pancreatic β-cells are relatively glucose insensitive (King and

Hazelwood, 1976). In addition to high concentrations of circulating glucose, relative insulin resistance is common among birds, including chickens (Chen et al., 1945), despite having "normal" plasma insulin concentrations (Simon et al., 2011). Insulin induced hypoglycemia is achieved in avians with relatively high doses of insulin (Simon, 1988). Although circulating glucagon concentrations are higher in birds than mammals, and glucose homeostasis in chickens appears to rely more heavily on glucagon secretion than insulin (reviewed by Scanes and Braun, 2012), insulin is still thought to play a major role in glucose metabolism in birds (Dupont et al., 2008; 2009; 2012).

In mammals, chronic hyperglycemia is almost always associated with serious deleterious effects on the body, but in contrast, even older birds do not appear to exhibit ill effects of prolonged hyperglycemia (Braun and Sweazea, 2008). With the growing concern of hyperglycemia and insulin resistance in humans, chickens have recently been recognized as an attractive model for studying diabetes (Datar and Bhonde, 2011). Furthermore, it has been well documented that obesity is often associated with impaired glucose metabolism.

Long –term artificial selection of White Plymouth Rock chickens for juvenile (day 56) BW resulted in two highly divergent genetic lines; low weight and obese high weight chickens. In the 55th generation of selection, HWS chickens were more than 10-fold heavier than LWS chickens at selection age, with substantial fat accumulation (Dunnington et al., 2013). The HWS are hyperphagic and develop characteristics of metabolic syndrome as juveniles, while the LWS are hypophagic with some anorexic individuals. Because these lines of chickens display such a

wide divergence in food intake and body composition, specifically abdominal fat, they represent an attractive animal model for studying the physiological factors underlying obesity.

Almost 30 years ago, Sinsigalli et al. (1987) reported that S_{26} generation HWS chickens were glucose intolerant with mild or moderate hyperinsulinemia, hyperglucagonemia, and perhaps insulin resistant. The experiments reported here were designed to investigate glucose regulation and pancreas physiology at selection age in the LWS and HWS lines of chickens after ~30 generations of continued selection since the Sinsigalli et al. (1987) publication.

Materials and Methods

Animals

The LWS and HWS lines were established from a common founder population generated by crosses among seven inbred lines of White Plymouth Rock chickens, and have been maintained as closed populations by continuous selection. Detailed descriptions of the breeding and maintenance of the lines has been previously published (Dunnington and Siegel, 1996; Marquez et al., 2010; Dunnington et al., 2013). Chickens used in the present experiments were obtained from the Paul S. Siegel Poultry Research Center at Virginia Tech. They were from the S_{55} generation, and were provided feed and water *ad libitum* with continuous light. The diet has remained the same throughout selection, and contains 3% crude fat, 6% crude fiber, 20% crude protein and 2,685 kcal metabolizable energy/kg in mash form. All procedures were carried out in accordance with the guidelines established by the National Research Council and were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Statistics

All data were analyzed in JMP 10 Pro using the Fit Model platform. Effects included in each analysis are outlined below. Interactions were analyzed by ANOVA and differences among treatments were detected by Tukey's honest significant difference (HSD). Significance was accepted at $P \leq 0.05$ and results are reported as LS means $\pm$ standard error of the mean (SEM).

Oral Glucose Tolerance Test (OGTT)

At 58 day (d) of age, LWS and HWS chickens (equal number males and females) were fasted for 16 hours (h) and randomly assigned to one of two treatment groups: glucose (n = 12/line) or vehicle (n = 8/line). Glucose treated chickens received a glucose bolus (2 g/kg BW; 20% w/v H_2O) by oral gavage, while vehicle treated chickens received an equivalent volume of water. Blood glucose concentration was measured at 0, 5, 15, 30, 60, 120, 240, and 300 min relative to treatment (0, 5, 15, and 30 min for vehicle treated chickens) via small brachial blood vessels and using a handheld glucometer as previously described (Smith et al., 2011; Zhao et al., 2014; Agamatrix, Inc., Salem, NH). Area under the curve (AUC; calculation described previously by Gilbert et al., (2011) and glucose clearance rates (15-120 min) were calculated. Data for glucose and vehicle treated chickens were analyzed separately; effects in the statistical model included line, time, and sex and all interactions (analyzed by ANOVA).

Insulin Sensitivity Test (IST) #1

Following a six day glucose wash-out period, the same chickens used in the OGTT were fasted for 16 h and subjected to an IST at 64 d of age. Chickens previously treated with glucose

were assigned to the insulin treatment group (n = 12/line), and the rest were again assigned to the vehicle treatment group (n = 6 HWS, n = 5 LWS). Insulin treated chickens received 80 µg/kg BW human insulin (Sigma-Aldrich, St. Louis, MO; diluted in 1X PBS) via i.p. injection, while vehicle treated chickens received an equivalent volume of 1X PBS. Concentrations of BG were measured at 0, 5, 15, 30, 60, and 120 min in both insulin and vehicle treated chickens, as described above. Re-feeding was initiated immediately following the 120 min sampling time in insulin treated chickens, and BG concentration was measured at 180 min. Data for insulin and vehicle injected chickens were analyzed separately and effects included line, time, and sex and all interactions among them (analyzed by ANOVA). The BG measurements for the 180 min sampling time were not included in the model.

Insulin Sensitivity Test (IST) #2

To further investigate the difference in BG concentrations between LWS and HWS following re-feeding, a second IST was performed with d 49 chickens using similar procedures. Once again, an equal number of males and females were injected with either 80 µg/kg BW insulin or an equivalent volume of PBS (n = 12), and BG concentration was measured at 0, 30, 60, and 120 min. Birds were offered feed for 1 h and BG concentration was measured again at 180 min. During the re-feeding period, we recorded which birds were eating and which were not. Data for insulin and vehicle injected chickens were analyzed separately. Two analyses were performed; the first included BG concentrations at 0, 30, 60, and 120 min, and effects included line and time and the interaction between them. The second analysis involved the difference between BG concentrations at 180 and 120 min, while the third analysis involved the

insulin treated HWS chickens that included 180-120 values of eaters (those that consumed food after 120 min) and non-eaters. Both the second and third analyses were by one-way ANOVA.

Pancreatic mRNA Expression of Glucose Regulatory Genes

At 65 d of age, the chickens used in the OGTT and IST #1 were euthanized, their pancreas immediately removed, weighed, and sectioned into pancreas head (PH) and body (PB). The proximal 1/3 (containing the pancreatic duodenal duct) was considered the PH, while the remaining 2/3 were considered the PB. Tissue samples were snap frozen in liquid nitrogen, and stored at -80 °C until analysis. Extraction of RNA was performed using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol for animal tissue, with some modifications (5% β-mercaptoethanol in buffer RLT). Frozen pancreas tissue was pulverized using a metal mortar continuously exposed to liquid nitrogen, and a pestle that had been pre-cooled by liquid nitrogen. Approximately 30 mg of pulverized tissue was transferred to a tube containing the RLT buffer. Samples were vortexed, and remaining steps were performed as recommended in the RNeasy Mini Handbook (beginning at step 4), including the optional on-column DNase digestion. Eluted RNA samples were stored at -80 °C until further analysis. RNA quality was evaluated by 1% agarose-formaldehyde gel electrophoresis, and RNA concentration was determined using a Nanophotometer Pearl (Implen Inc., Westlake Village, CA) at 260/280/230 nm. Complementary DNA was reverse transcribed using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), diluted 1:30 and stored at -20 °C until use. Primers (see Table 1) were designed using Primer Express 3.0 (Applied Biosystems) software, synthesized by Eurofins MWG Operon (Huntsville, AL), and tested for amplification efficiency prior to use. Quantitative real-time (RT)

PCR was performed using an Applied Biosystems 7500 Fast PCR machine and Fast SYBR green (Applied Biosystems). Samples were run in triplicate in 96-well plates and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Relative mRNA expression was quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and average ΔCt values of PB from LWS chickens served as the calibrator. Data were analyzed separately for each gene, and effects included line, sex, and tissue and all interactions.

Pancreatic Islet Mass Quantification

At 56 d of age, pancreata were collected from 20 chickens (n = 10/line; equal number of males and females). Samples were rinsed with PBS, fixed in 10% neutral buffered formalin (4 °C overnight incubation), and subjected to graded ethanol dehydration. Samples were paraffin embedded, cut in 5 µm sections, mounted on slides (3-4 sections/slide), and stained with hematoxylin and eosin. Microscopic histological evaluation was performed using Nikon NIS-Elements Advanced Research software (Nikon, Melville, NY) and the Large Image Stitching method, which as cited by Ying and Monticello (2006), "involves 'tile-by-tile' acquisition over the entire tissue section followed by a stitching of images to form an entire digital slide". Total cross sectional area was quantified using the automated measurement feature by employing the intensity threshold method. Islet areas were traced using the manual draw feature, and the area of each was quantified. Data were expressed as percent islet area ([islet area/pancreas area] × 100), total islet mass ([% islet area][pancreas weight]), total islet mass as a percentage of BW ([% islet area][pancreas weight] / BW), and islet size. During histological evaluation of islet mass, areas of aggregated lymphocytes were noted, and later quantified. Since lymphocytic aggregates are typically associated with areas of inflammation, total pancreatic inflammation, as

a percentage of BW, was calculated as: [(% lymphocytic aggregates)(pancreas weight)]/BW. Two separate data analyses were performed. In the first, effects included line, tissue (PH vs. PB), sex and the interactions among them. The second analysis involved values for the total pancreas sample (PH and PB combined), and effects included line, sex, and the interactions between them.

Results

Oral Glucose Tolerance Test (OGTT)

There was no sex effect for any of the traits measured. The LWS chickens responded more quickly to the glucose bolus, having a peak in BG concentration at 15 min post-gavage, whereas HWS chickens did not peak until 30 min (Figure 1A), with a difference between lines at 15 min ($P < 0.0001$), but not at 30 min or other time points. The LWS chickens were more efficient in clearing circulating glucose from the bloodstream, with an average glucose clearance rate of 1.72 %/min, vs. 0.66 %/min for HWS chickens ($P = 0.0006$; Figure 1B). The HWS chickens tended to have a greater AUC compared to LWS ($P = 0.0565$; Figure 1C). For vehicle treated chickens, HWS had higher BG concentrations than LWS at 0 min ($P = 0.0113$; Figure 1D).

Insulin Sensitivity Test (IST) #1

Results for BG from vehicle treated chickens were not significant (Figure 2A), nor was there an effect of sex in any of the analyses. Insulin treatment was associated with a significant time × line interaction ($P = 0.0476$) on BG (Figure 2B). The LWS chickens responded to insulin

more quickly than HWS chickens, with a more pronounced reduction in BG after injection during the first 60 min. From 60 to 120 min, BG decreased more in HWS than in LWS. Following 1 h of re-feeding, LWS chickens appeared to show a compensatory response, with BG concentrations at 180 min exceeding baseline levels. In contrast, BG concentrations in HWS chickens failed to return to fasting levels (~200 mg/dL).

Insulin Sensitivity Test (IST) #2

Results from the second IST were similar to the first, in that in LWS there was a prompt decline in BG during the first 60 min, after which concentrations plateaued. Again, HWS chickens showed a more dramatic decrease in BG between 60 and 120 min, where concentrations differed between the lines at 120 min ($P = 0.0005$; Figure 2C). Responses of both lines following 1 h of re-feeding were similar to those observed in IST #1. At 180 min, LWS chickens had BG concentrations nearly double that of initial baseline levels, whereas the change in BG concentration of HWS chickens from the 120 min time point was essentially nil, resulting in a difference between LWS and HWS ($P < 0.0001$) between 180 and 120 min. The difference in BG concentration between 180 and 120 min was also different between vehicle treated LWS and HWS chickens ($P = 0.0120$; Figure 2D). However, it should be noted that while vehicle treated LWS chickens did not experience a compensatory increase in glucose, as observed in insulin treated chickens, their HWS counterparts showed a much greater increase in BG between 120 and 180 min than insulin treated HWS chickens, with changes (120-180) of 70.3 and 4.7 mg/dL, respectively. To ensure that the difference in BG at 180 min was a true effect of genetic line, a one-way ANOVA was performed on data from insulin treated HWS chickens to evaluate the effect of eating. There was no difference between "eaters" and "non-eaters" ($P = 0.4922$).

Pancreatic mRNA Expression of Glucose Regulatory Genes

Pancreatic mRNA abundance of the four genes investigated was greater in LWS than HWS (*PPI*, $P < 0.0001$; *PPG*, $P = 0.0021$; *GLUT2*, $P = 0.0011$; *Pdx1*, $P < 0.0001$; Figures 3 A, B, C, D), respectively. There was a three-way interaction of line × sex × tissue on *PPI* mRNA, where expression was greatest in the PB of LWS males ($P = 0.0094$; Figure 3E). There was a two-way interaction of sex × tissue on *PPG* mRNA where abundance was lowest in the PB of all males ($P = 0.0104$; Figure 3F). A main effect of sex was observed for *GLUT2* mRNA, with expression higher in females than males (1.19 ± 0.09 vs. 0.79 ± 0.09, respectively; $P = 0.0017$). There was a tissue × sex interaction for expression of *Pdx1* mRNA, where in females expression was greater in the PB than in the PH ($P = 0.0028$; Figure 3G). There was also a line × tissue interaction on *Pdx1* mRNA, with greater expression in LWS than HWS in both regions of the pancreas, with a greater accentuated difference between lines in the PH ($P = 0.0436$; Figure 3H).

Pancreas Weight and Histology

Pancreata were heavier in HWS than LWS on both d 65 and 56 (3.20 ± 0.09 vs. 1.12 ± 0.09 and 3.29 ± 0.07 vs. 0.89 ± 0.07, respectively; $P < 0.0001$). However, pancreas, as a percentage of BW, was heavier in LWS than in HWS on both d 65 and 56 ($P < 0.0001$; Figures 4 A and B, respectively). The HWS chickens had greater total islet mass than LWS (1.98 ± 0.1 vs. 0.54 ± 0.1, respectively; $P < 0.0001$), when calculated as absolute weight; however, this calculation does not account for the larger absolute organ weight of HWS than LWS pancreata. Thus, when expressed as a percentage of BW (% BW), LWS chickens had greater total islet mass than HWS ($P = 0.0002$; Figure 4C). Pancreata of HWS chickens contained larger islet

areas than LWS pancreata ($P < 0.0001$; Figure 4D), but relative islet mass (% islet area) was similar (0.58 ± 0.05 vs. 0.60 ± 0.05 for LWS and HWS, respectively; $P = 0.7174$). The PB also contained larger islets than the PH ($P = 0.0405$; Figure 4E). Lastly, inflammation was greater in HWS than LWS ($P < 0.0001$; Figure 4F). Figures 5 A and B contain representative images of pancreatic islets and lymphocyte aggregates in LWS and HWS chickens, respectively.

Discussion

Glucose Tolerance and Insulin Sensitivity

Differences in glucose regulation and insulin sensitivity between LWS and HWS chickens were first noted by Sinsigalli et al. (1987) during generation S_{26}. Results from the OGTT were similar to those observed by Sinsigalli et al. (1987), in that LWS chickens were more efficient in clearing BG. However, Sinsigalli et al. (1987) noted a greater peak in glucose in HWS, while we observed the opposite, with a greater peak in LWS. Sinsigalli et al. (1987) also found that LWS tended to have higher blood glucose concentrations than HWS, while our experiments revealed greater glucose concentrations in HWS, particularly at baseline (time 0) of the OGTT. Although glucose concentrations of vehicle chickens did not differ significantly between LWS and HWS during either IST, HWS consistently tended to have greater values. Thus, while HWS chickens continue to exhibit glucose intolerance, the magnitude in the HWS line has increased over the past 30 generations of divergent selection for juvenile body weight.

Following re-feeding during the first IST, BG concentrations for LWS chickens were greater than those at 0 min, while there was no increase in BG to fasting levels in HWS. To

further investigate the differences in BG concentrations following re-feeding, a second IST was performed. The second IST was performed similarly to IST #1, with some differences; vehicle treated chickens were also subjected to 1 h re-feeding, and their eating status recorded. Results of IST #2 were consistent with those observed during IST #1. In response to re-feeding, LWS chickens displayed a compensatory increase in BG, with average concentrations almost twice those of fasting values, whereas average BG concentrations for HWS barely increased from the 120 min time point. Without further investigation, one may make two assumptions regarding the compensatory increase in BG in LWS: the high average BG concentration of LWS at 180 min is a normal response to re-feeding following a fast, or the differences in BG between LWS and HWS at 180 min is a result of eating vs. not eating. However, vehicle treated LWS chickens did not display such a drastic increase in BG following re-feeding, and although significantly different, BG concentrations of vehicle treated LWS and HWS chickens at 180 min did not display the three-fold difference observed in their insulin treated counterparts. Additionally, there was no significant difference in BG concentration of "eaters" vs. "non-eaters" in insulin treated chickens. These results suggest that both the compensatory increase in BG observed in LWS insulin treated chickens following 1 h re-feeding, as well as the extreme difference in BG between LWS and HWS following re-feeding, was a true interactive effect of insulin treatment and genetic selection for juvenile body weight.

Our results clearly demonstrate a difference in threshold response to insulin between the lines. Smith et al. (2011) also reported a different threshold response in food intake and BG to central insulin in LWS and HWS. Smith et al. (2011) observed greater decreased food intake in LWS than HWS in response to central insulin administration, suggesting that hyperphagia in

HWS may be a result of diminished central insulin sensitivity. While these findings support central regulation of endocrine pancreas function, our results also implicate peripheral regulation, but perhaps with different modes of action on food intake. During central insulin administration, HWS continued to eat for a longer period than LWS (Smith et al., 2011). While food was withheld for the majority of the ISTs reported here, some HWS refused to eat during the 1 h re-feeding period, while all LWS ate. Moreover, Smith et al. (2011) reported decreased BG in LWS compared to HWS following central insulin administration, and suggested the effect of insulin on glucose was more likely a result of decreased food intake. Whereas we observed much greater BG concentrations in LWS than HWS following peripheral insulin administration and 1 h of re-feeding, irrespective of food intake. Taken together, these results suggest both central and peripheral regulation of endocrine pancreas function in LWS and HWS chickens.

Pancreatic mRNA Expression of Glucose Regulatory Genes

Messenger RNA expression of the four genes investigated (preproinsulin, *PPI*; preproglucagon, *PPG*; glucose transporter 2, *GLUT2*; and pancreas and duodenal homeobox 1, *Pdx1*) was significantly greater in LWS than HWS. Preproinsulin and PPG are inactive precursors to insulin and glucagon, respectively (Perler et al., 1980; Hasegawa et al., 1990; Simon et al., 2004). Glucose transporter 2 is an insulin independent glucose transporter expressed in the small intestine, liver, kidney, fat, skeletal muscle, hypothalamus, and pancreas of chickens (Kono et al., 2005; Zhang et al., 2013). Pancreas and duodenal homeobox 1 is a transcription factor involved in the development and maturation of pancreatic β-cells (Kitamura et al., 2002). These results are especially compelling when correlated with pancreatic islet mass. That HWS had lower mRNA expression is accentuated by the observation that they also had

greater absolute total islet mass, and yet mRNA expression was still greater in LWS. Lower pancreatic expression of insulin-related genes in HWS may play a role in their differential response to insulin. It is relevant to note the results for *PPG*, which displayed a three-fold difference in mRNA expression between LWS and HWS. Since insulin and glucagon work in concert to maintain glucose homeostasis through negative feedback of one another, that both *PPI* and *PPG* are significantly greater in LWS than HWS, is compelling. While plasma glucagon concentrations are naturally higher in avian species compared to mammals (reviewed by Scanes and Braun, 2012), Sinsigalli et al. (1987) observed higher endogenous glucagon and insulin levels in HWS, compared to LWS. Greater plasma insulin concentrations, coupled with higher BG in HWS chickens, suggested diminished insulin sensitivity of peripheral tissues (Sinsigalli et al., 1987). Unfortunately, endogenous levels of plasma insulin and glucagon were not measured in the present study. Without this information, it is difficult to conclude if continued genetic selection has resulted in greater plasma glucagon levels in LWS chickens, as mRNA data suggests. However, low glucagon concentrations in HWS would corroborate IST results, in that it was more difficult for HWS chickens to control glucose homeostasis following insulin injection. It is also possible that like insulin, glucagon sensitivity of peripheral tissues is diminished in HWS, and that Sinsigalli et al. (1987) observed that the enhanced ability of LWS to metabolize glucose was not directly associated with greater plasma insulin levels. Additionally, higher mRNA expression of both *GLUT2* and *Pdx1* in LWS chickens substantiates our OGTT and IST results. Glucose transporter 2 is a high capacity, low affinity transporter, and plays an important role in glucose sensing in pancreatic β-cells. Perhaps a superior ability of LWS β-cells to function in glucose sensing may help explain the higher glucose clearance rate observed in LWS chickens during the OGTT. While Pdx1 is especially important during

embryonic development for proper pancreatic development, it is also essential for β-cell survival later in life. Experiments with mouse and human pancreatic islets have shown that insufficient *Pdx1* expression results in increased apoptosis (Johnson et al., 2003; Johnson et al., 2006). Perhaps the low expression of *Pdx1* in HWS chickens may lead to decreased β-cell survival, risking reduced insulin production. Future studies should focus on insulin immunostaining in the pancreas to quantify β-cells.

While it is known that in rodents islets are differentially distributed along the length of the pancreas (Elayat et al., 1995), examining the mRNA expression of glucose regulatory genes in different regions of the pancreas provided further insights on transcriptional regulation of chicken glucose homeostasis. There was significantly greater mRNA expression of *PPI* in the PB, and although not significant greater mRNA expression of *PPG* in the PH. To our knowledge, distribution of pancreatic α- and β-cells has only been previously investigated once in broiler chickens (Ruffier et al., 1998). Both isolation of functional chicken pancreas islets (Ruffier et al., 1998) and immunocytochemical studies in rat pancreata (Elayat et al., 1995) have revealed a higher distribution of α-cells in the PH, and a greater concentration of β-cells in the PB. Our results provide evidence for a similar distribution of pancreatic endocrine cells in the pancreas of LWS and HWS chickens, in that α-cells are responsible for glucagon secretion, while β-cells produce insulin.

Pancreas Weight and Histology

While pancreas weights for LWS and HWS chickens have been reported for younger ages (DOH, d 8 and 21), there are no reports of pancreas weight at selection age (d 56). Nitsan

et al. (1991) observed no differences in absolute or relative pancreas weights between the lines on DOH (S_{30} generation). Similarly, relative pancreas weights between LWS and HWS did not differ on d 8 or 21 (O'Sullivan et al., 1992). In contrast, we found significant differences in both absolute and relative pancreas weight between the lines. At both ages, absolute pancreas weight was greater in HWS chickens, but LWS had significantly heavier pancreata relative to BW. Furthermore, relative pancreas weight was negatively correlated with both BW and fasting BG on d 65 (data not shown). Studies on human pancreas have reported decreased organ weights in individuals suffering from or predisposed to type 1 diabetes (Fonseca et al., 1985; Campbell-Thompson et al., 2013). Since type 1 diabetes is characterized by hyperglycemia and insulin resistance, among other factors, it may be possible to correlate the relatively lighter pancreas of HWS chickens with differences in glucose homeostasis.

There are few reports of pancreatic islet histology in chickens. To account for the greater absolute pancreas weight of HWS chickens, total islet mass was calculated as a percentage of BW. As expected, LWS had greater total pancreatic islet mass (% BW). Further experiments are required for complete understanding of these results (i.e. immunostaining for β-cells), but several inferences can be made. Obesity studies in humans have observed a positive correlation between obesity and β-cell mass, particularly in insulin-resistant individuals (Weir et al., 1990). However, it is widely understood that individuals suffering from insulin-dependent diabetes have a decreased number of pancreatic β-cells (Weir et al., 1990). Our data show that the HWS have larger islets than LWS, also supporting the idea that HWS are obese and respond differently to insulin. Furthermore, throughout the course of disease progression, diabetics typically display an initial increase in β-cell mass, followed by a significant decline (reviewed by Swenne, 1992). In

regard to pancreas physiology, the greater islet size but reduced total islet mass (% BW) in HWS than LWS chickens at selection age may be indicative of a state similar to that observed in obesity. The similarities in pancreas weights between young LWS and HWS chickens previously reported (Nitsan et al., 1991; O'Sullivan et al., 1992), could suggest that the decreased islet mass observed in selection age HWS chickens develops later in life as a result of chronic obesity. It is also possible that the observed results are associated with the 25 generations of continued genetic selection since the S_{30} generation.

We also observed a striking difference between LWS and HWS chickens in the number of lymphocyte aggregations present in the pancreas. In humans, pancreatitis is characterized histologically, in part, by lymphoplasmacytic infiltration (Kawaguchi et al., 1991). Aggregation of B-lymphocytes has also been reported in the pancreas of type 2 diabetes mouse models (New Zealand Obese; Junger et al., 2002). While type 1 diabetes has long been considered an autoimmune disease marked by β-cell loss, more recent experiments investigating immune-related β-cell destruction have continued to correlate both insulin and non-insulin dependent diabetes with pancreatic inflammation (Mueller et al,. 1996; Cameron et al., 2000; Donath et al., 2003; Kolb and Mandrup-Poulsen, 2005; Eizirik et al., 2009). While the HWS chickens may not exhibit all signs or symptoms of a human diabetic, they undoubtedly share some aspects of this multifaceted disease. Quantification of lymphocyte aggregation during the current experiment revealed significantly greater pancreatic inflammation (% BW) in HWS than LWS. Considering the well documented correlation of pancreatic inflammation with human diabetes, it is reasonable to associate these data with the impaired glucose metabolism and insulin sensitivity observed in the HWS line.

Conclusion

In summary, experimental results showed that LWS and HWS display differences in threshold sensitivity to insulin and glucose clearance rate. The HWS chickens also have lower pancreatic mRNA expression of glucose regulatory genes (*PPI*, *PPG*, *GLUT2*, *Pdx1*), which suggests glucose homeostasis differs at the transcriptional level between the genetic lines. Gene expression results also provide evidence that LWS and HWS chicken PH contains more α-cells, and PB more β-cells, as observed in rodents and broiler chickens. Histological analysis of LWS and HWS pancreas revealed that HWS chickens have larger pancreatic islets, less pancreatic islet mass, and greater pancreatic inflammation; all of which presumably contribute to impaired glucose metabolism. Further studies should focus on similar parameters in both younger and older chickens, as well as an investigation of glucose homeostasis in regards to liver physiology.

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Table 1. Chicken primer sequences

Gene	Primer[1]	Accession No.
PPI[2]	For: 5' – CCTCTTCTGGCTCTCCTTGTCTT – 3' Rev: 5' – TGGTTGGCAGCTGCATAGC – 3'	X58993
PPG[3]	For: 5' – GCACTAAAAGAAATGGCCAACAAG – 3' Rev: 5' – GCTGATCCGGGAATTTGTCA – 3'	Y07539
GLUT1[4]	For: 5' – GAAGGTGGAGGAGGCCAAA – 3' Rev: 5' – TTTCATCGGGTCACAGTTTCC – 3'	NM_207178
Pdx1[5]	For: 5' – CCGACGGATGAAATGGAAGA – 3' Rev: 5' – CTCGGGATCAGCGCTGTT – 3'	XM_001234635
GAPDH[6*]	For: 5' – CCTAGGATACACAGAGGACCAGGTT – 3' Rev: 5' – GGTGGAGGAATGGCTGTCA – 3'	NM_204305

[1]For = forward primer; Rev = reverse primer. Sequences were generated using Primer Express 3.0 (Applied Biosystems, Foster City, CA) software and synthesized by Eurofins MWG Operon (Huntsville, AL).
[2]Preproinsulin
[3]Preproglucagon
[4]Glucose transporter 2
[5]Pancreatic and duodenal homeobox 1
[6]Glyceraldehyde 3-phosphate dehydrogenase
*Endogenous control

Figure 1

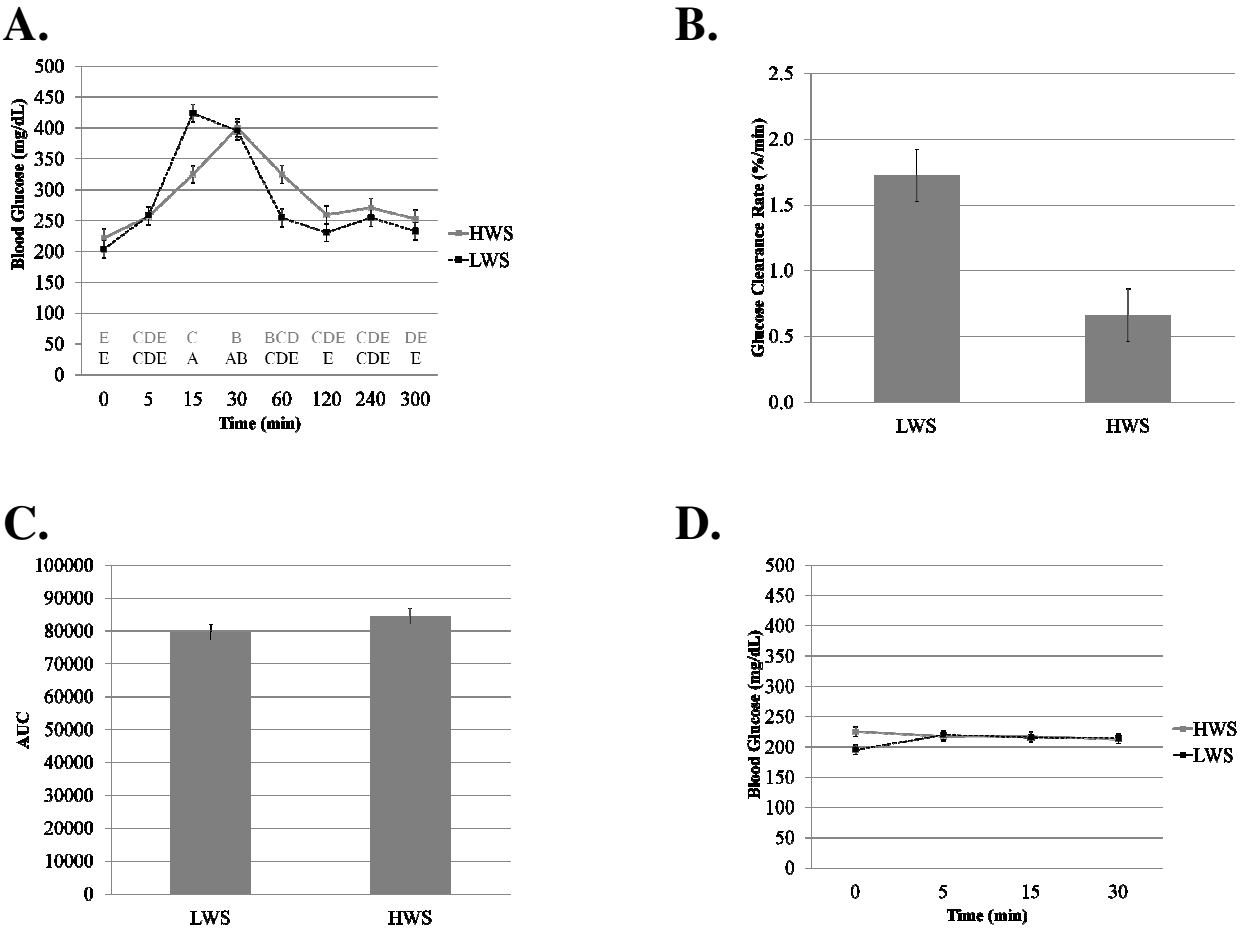


Figure 1. Effect of genetic line on glucose tolerance in chickens (d 58) selected for low (LWS) or high (HWS) body weight.

A. LWS and HWS chickens (n = 12/line) were fasted for 16 h and orally gavaged with glucose (2 g/kg BW; 20% w/v H_2O). Blood glucose (BG) concentrations (mg/dL) were measured using a handheld glucometer at the indicated time points. Different letters between all combinations of genetic line and time represent significant differences, $P \leq 0.05$. Line × time interaction, $P < 0.0001$.

B. Glucose clearance rate (15-120 min; %/min) during OGTT. $P = 0.0006$.

C. Area under the curve (AUC) calculated from glucose curve. $P = 0.0565$.

D. Vehicle treated LWS and HWS chickens (n = 8) were orally gavaged with H_2O (equivalent volume), and BG concentrations were measured at the indicated time points. Main effect of line, HWS > LWS, $P = 0.0113$. Line × time, $P = 0.0561$.

Figure 2

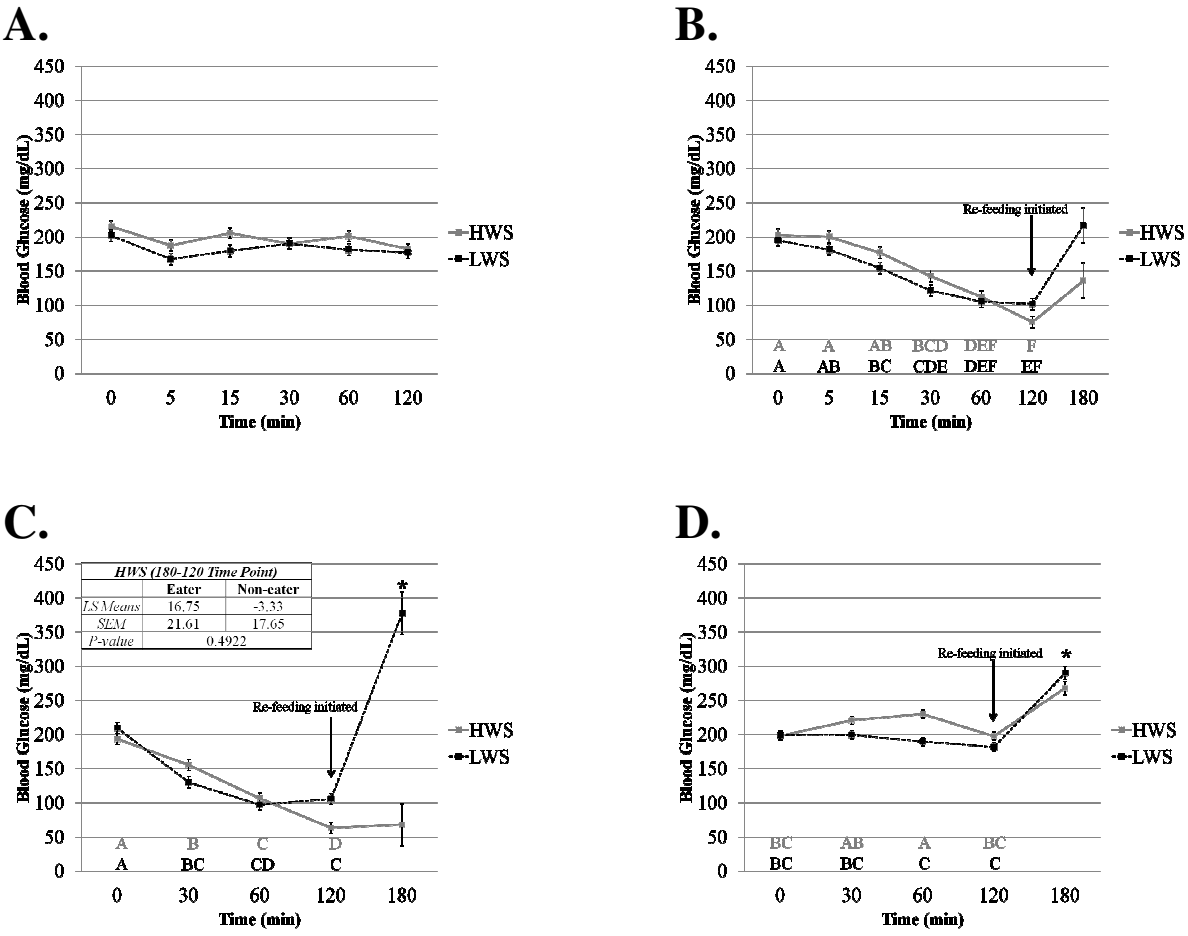


Figure 2. Effect of genetic line on insulin sensitivity in chickens selected for low (LWS) or high (HWS) body weight.

A. Vehicle treated LWS (n = 5) and HWS (n = 6) chickens (d 64) were fasted for 16 h and injected i.p. with 1X PBS (equivalent volume). BG concentrations were measured at the indicated time points. Line × time, $P = 0.3350$.

B. LWS and HWS chickens (d 64; n = 12) were fasted for 16 h and injected i.p. with insulin (80 μg/kg BW). Blood glucose (BG) concentrations (mg/dL) were measured using a handheld glucometer at the indicated time points. Re-feeding was initiated following the 120 min measurement. Statistical analysis did not include 180 min time point. Different letters between

all combinations of genetic line and time represent significant differences, $P \leq 0.05$. Line × time, $P = 0.0476$.

C. LWS and HWS chickens (d 49; n = 12) were fasted for 16 h and injected i.p. with insulin (80 μg/kg BW). BG concentrations (mg/dL) were measured at the indicated time points. Re-feeding was initiated following the 120 min measurement, and 180 min measurements were analyzed separately (see materials and methods for details). Different letters between all combinations of genetic line and time represent significant differences, $P \leq 0.05$. Line × time, $P = 0.0005$. $^{*}P < 0.0001$.

D. Vehicle treated LWS and HWS chickens (d 49; n = 12) were fasted for 16 h and injected i.p. with 1X PBS (equivalent volume). BG concentrations were measured at the indicated time points. Re-feeding was initiated following the 120 min measurement, and 180 min measurements were analyzed separately (see materials and methods for details). Different letters between all combinations of genetic line and time represent significant differences, $P \leq 0.05$. Line × time, $P = 0.0085$. $^{*}P = 0.0120$.

Figure 3

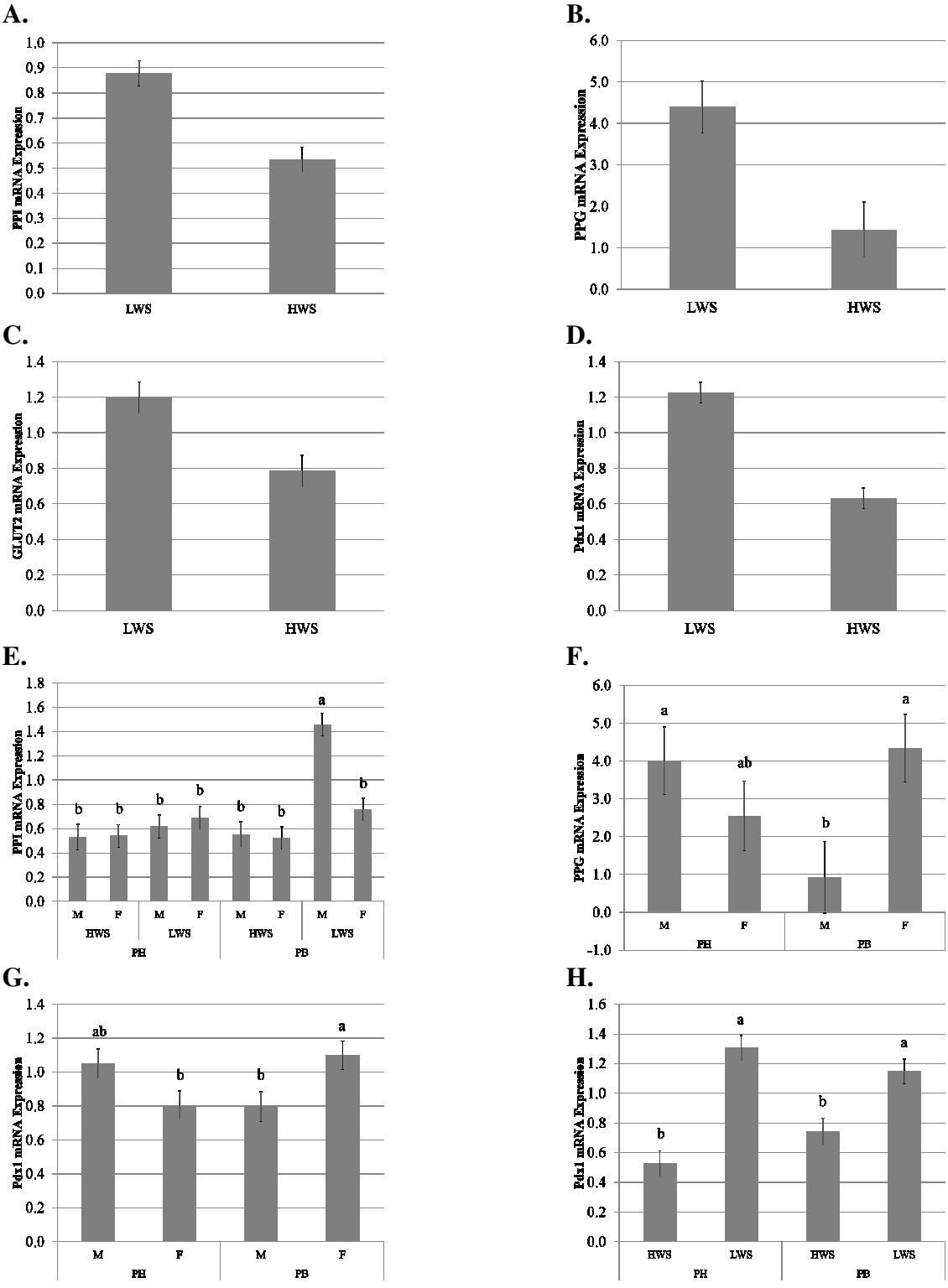

A.
PPI mRNA Expression
LWS
HWS
B.
PPG mRNA Expression
LWS
HWS
C.
GLUT2 mRNA Expression
LWS
HWS
D.
Pdx1 mRNA Expression
LWS
HWS
E.
PPI mRNA Expression
M F M F M F M F
HWS LWS HWS LWS
PH PB
F.
PPG mRNA Expression
M F M F
PH PB
G.
Pdx1 mRNA Expression
M F M F
PH PB
H.
Pdx1 mRNA Expression
HWS LWS HWS LWS
PH PB

Figure 3. Effect of genetic line on mRNA expression of pancreatic glucose regulatory genes in chickens selected for low (LWS) or high (HWS) body weight.

Pancreata were sampled from LWS and HWS chickens (d 65) and pancreatic mRNA expression was investigated using real-time PCR ($2^{-\Delta\Delta Ct}$ method).

A. $P < 0.0001$

B. $P = 0.0021$

C. $P = 0.0011$

D. $P < 0.0001$

E. Bars represented by different letters differ significantly, $P \leq 0.05$. Line × tissue × sex, $P = 0.0094$.

F. Bars represented by different letters differ significantly, $P \leq 0.05$. Tissue × sex, $P = 0.0104$.

G. Bars represented by different letters differ significantly, $P \leq 0.05$. Tissue × sex, $P = 0.0028$.

H. Bars represented by different letters differ significantly, $P \leq 0.05$. Line × tissue, $P = 0.0436$.

Figure 4

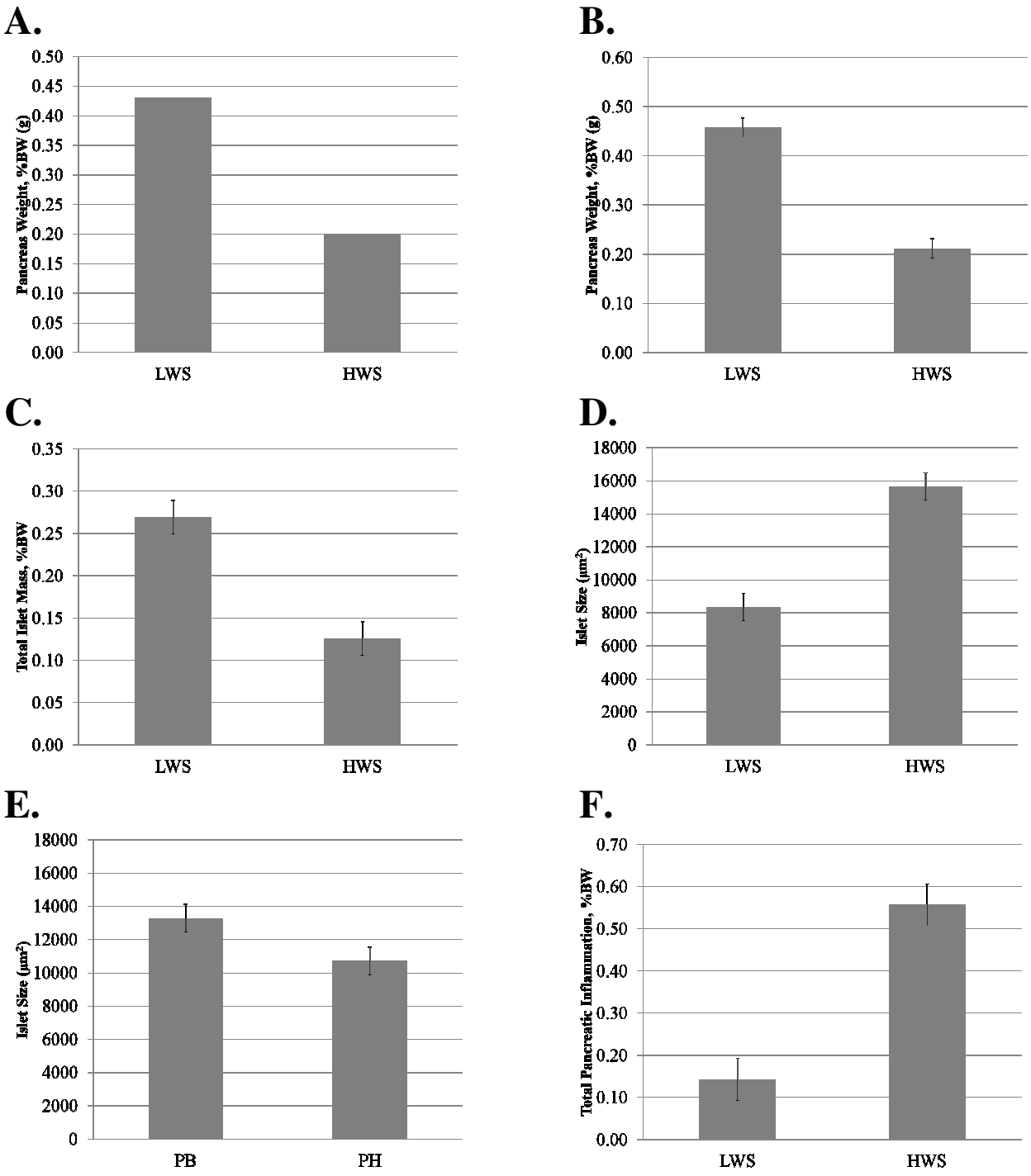


Figure 4. Effect of genetic line on pancreas physiology in chickens selected for low (LWS) or high (HWS) body weight.

A. Relative pancreas weight (% BW) of LWS and HWS chickens on d 65, $P < 0.0001$.

B. Relative pancreas weight (% BW) of LWS and HWS chickens on d 56, $P < 0.0001$.

C. Total pancreatic islet mass (% BW) of LWS and HWS chickens on d 56, $P = 0.0002$. Calculation details are outlined in materials and methods.

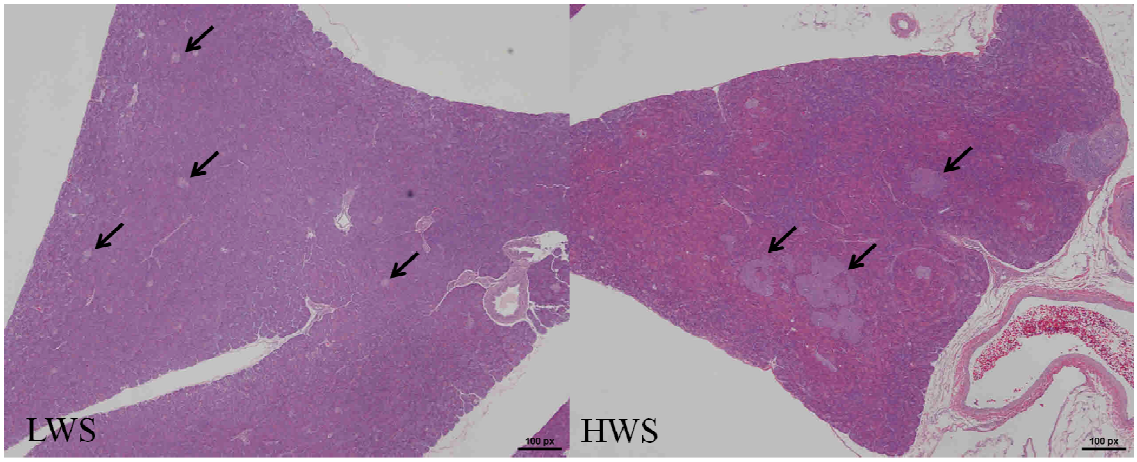
D. Pancreatic islet size (μm^2) in LWS and HWS chickens on d 56, $P < 0.0001$.

E. Pancreatic islet size (μm^2) in the pancreas body (PB) and pancreas head (PH) on d 56, $P = 0.0405$.

F. Total pancreatic inflammation (% BW) of LWS and HWS chickens on d 56, $P < 0.0001$. Calculation details are outlined in materials and methods.

Figure 5

A.



B.

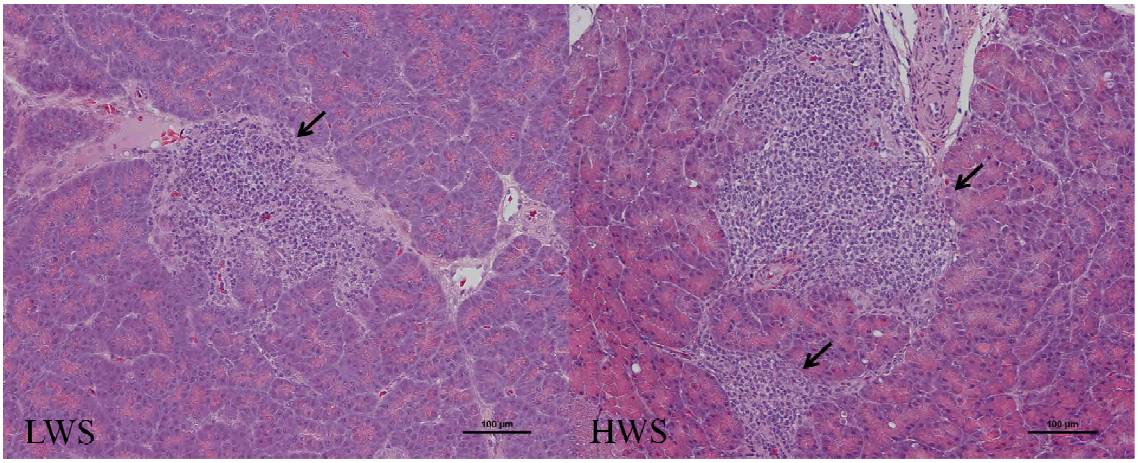


Figure 5.

A. Representative image of pancreatic islets in LWS and HWS chickens on d 56. Arrows are pointing to examples of islet areas.

B. Representative image of pancreatic lymphocyte aggregates (inflammation) in LWS and HWS chickens on d 56 (20X). Arrows are pointing to examples of lymphocyte aggregates.

Supplementary Table 1: Pancreatic Islet Mass Quantification.
Analysis of variance and LS means ± SEM.

Effect	No. Islets	% Islet Area	Total Islet Mass	Islet Density	Islet Size
Line					
LWS[1]	34.4	0.58	0.27	0.000023	8339.69
HWS[2]	45.1	0.60	0.99	0.000017	15634.22
SEM[3]	2.41	0.05	0.05	1.09×10^{-6}	848.22
P-value	0.0035	0.7174	< 0.0001	0.0002	< 0.0001
Tissue					
Pancreas Body	43.9	0.60	0.84	0.000021	13267.26
Pancreas Head	35.6	0.58	0.41	0.000020	10706.64
SEM	2.41	0.05	0.05	1.09×10^{-6}	848.22
P-value	0.0213	0.7819	< 0.0001	0.6135	0.0405
Sex					
Male	39.2	0.62	0.69	0.000019	12444.66
Female	40.3	0.56	0.56	0.000021	11529.25
SEM	2.41	0.05	0.05	1.09×10^{-6}	848.22
P-value	0.7378	0.3481	0.0715	0.4171	0.4510
L[4] x T[5]	0.4595	0.5950	0.0078	0.3817	0.2349
L x S[6]	0.3772	0.5565	0.6832	0.0342	0.1751
S x T	0.8727	0.6550	0.7345	0.9717	0.8390
L x T x S	0.8727	0.1723	0.2467	0.7547	0.6854

[1]Low weight select chickens
[2]High weight select chickens
[3]Standard error of the mean
[4]Line
[5]Tissue
[6]Sex

Chapter IV

Epilogue

Although first described over 120 years ago, the chronic hyperglycemic state of avians remains ambiguous. Currently, little is known regarding insulin signaling in chickens, particularly downstream elements, and a specific insulin sensitive glucose transporter has yet to be identified. Because a better understanding of glucose regulation in chickens has both agricultural and biomedical implications, the LWS and HWS lines serve as an attractive experimental model. Additionally, the divergence in body weight, particularly abdominal fat, between the lines provides an opportunity for investigating the role of adipose tissue in basic carbohydrate metabolism, and the link between obesity and diabetes.

When working with the LWS and HWS chicken lines, an important question that continuously arises is whether a specific difference is a direct result of genetic selection, or if the disparity has been acquired secondarily. For example, we observed larger and fewer islets in selection age HWS chickens. It is possible that this phenomenon is an inherent characteristic of the HWS line; a direct result of selection, but may also be indicative of a progressive disease-like condition. In humans, progression of diabetes is often associated with increased islet size; the body's attempt to compensate for increased blood glucose concentrations. In late-stage diabetes, however, β-cell apoptosis leads to a significant decrease in islet mass. Because we have only investigated pancreas physiology in selection age chickens, if enlarged islets in HWS is indicative of some sort of disease-like progression, we don't know what stage selection age represents. It is possible that very young HWS will have islets similar in size to young LWS. It is also possible that large islets in HWS may be an inherent characteristic of selection, meaning

that even young HWS have large islets. Another possibility is that older HWS will have even larger islets, which may be indicative of continued disease-like progression. Lastly, at some point, older HWS may experience a state similar to late-stage diabetes, in which islets would be smaller, indicating β-cell apoptosis. The larger islets observed in HWS chickens is simply one example of how investigating selection age chickens only tells a part of the story. To better understand how long-term artificial body weight selection affects glucose regulation and pancreas physiology, it is important to also examine pancreatic gene expression and pancreas histology in younger and older birds.

Early on in my dissertation project, some of the first experiments that we performed were the OGTT and IST, the results of which laid the ground work for subsequent studies. During the first IST, one of the most interesting observations was the compensatory increase in blood glucose in LWS chickens following re-feeding. Furthermore, the observation of this phenomenon was serendipitous. We had not planned to re-feed, but did so out of necessity to prevent severe hypoglycemia. We then measured blood glucose at one hour post-re-feeding. Because the finding was serendipitous, we had not planned on recording feed consumption following re-feeding. Thus, one of the main reasons that we decided to perform the second IST was to allow for the measurement of blood glucose concentrations 1 hour post re-feeding in both control and experimental chickens, and also to collect information on the feeding status of individual chickens. We hypothesized that the compensatory increase in blood glucose in LWS following re-feeding was not simply a result of eating vs. not eating, but was a physiological response induced prandially. The second IST allowed us to conclude that both the compensatory increase in blood glucose observed in LWS insulin treated chickens following 1 hour re-feeding,

as well as the extreme difference in blood glucose between LWS chickens and HWS chickens following re-feeding, were true interactive effects of insulin treatment and genetic selection for juvenile body weight. While results of the OGTT and IST led us to hypothesize that LWS chickens would display greater mRNA expression of glucose regulatory genes, which we later observed, the three-fold difference in PPG expression between LWS and HWS was surprising. While we did not measure endogenous levels of circulating glucagon, it is possible that the ~30 years of continued selection since Sinsigalli et al. observed greater concentrations of glucagon in HWS chickens, has resulted in LWS chickens now having greater production and blood concentrations of glucagon. Furthermore, we also observed a compensatory increase in blood glucose in LWS in response to delayed access to feed at hatch. Intriguingly, the compensatory increase was observed at day 15 post-hatch, even though those individuals had been fully fed after receiving access to feed at 72 hours post-hatch. This is indicative of an epigenetic effect, although it is beyond the scope of this dissertation to speculate on the possible mechanisms. From a physiological perspective, it is possible that LWS chickens are relying more on glucagon than insulin for glucose homeostasis, which may explain why the LWS were relatively more glucose tolerant and displayed a difference in threshold sensitivity to insulin as compared to the HWS. This hypothesis is corroborated by evidence that glucagon appears to play a critical role, more so than insulin, in regulating glucose metabolism in chickens. In fact, the few reported cases of diabetes in avians has been due to excessive glucagon concentrations, rather than decreased insulin.

While the endocrine pancreas undoubtedly plays a critical role in regulating glucose homeostasis and carbohydrate metabolism, the involvement of other tissues and organs is

essential. The liver acts as an important site of glycogen storage, as well as de novo glucose, and in chickens and humans, fatty acid synthesis. Skeletal and heart muscle also store and oxidize glucose, while adipose tissue plays a role in glucose oxidation. To develop a more complete picture of the differences in glucose regulation between the lines, it would be interesting to study the expression of genes involved in carbohydrate metabolism in liver, muscle, and adipose tissue. Not only could the effects and interactions of genetic line and tissue be examined, but also of age. As mentioned previously, it would be relevant to investigate whether differences in gene expression are present at hatch, prior to digestion of feed and fat accumulation, and how those differences are affected over time.

As mentioned in Chapter II, chickens have recently been recognized as an attractive model for conducting diabetes research. While the advantages of using chickens as model organisms was discussed previously, this brings up the question of whether HWS chickens may be suitable as a model for human diabetes and obesity. Unfortunately, the complex and multifaceted nature of both conditions makes this question difficult to fully address. While I am sure there are many critics when it comes to using a completely different species as a model for a human disease, I think it is important to note that in many of the common mammalian animal models of diabetes, the condition is chemically induced. While using the HWS line as a model for studying hyperglycemia does bring up the issue of crossing species, an advantage is that the chickens inherently have high blood glucose concentrations. Additionally, while spontaneous diabetes animal models do exist (e.g. the Chinese hamster and BB/Wistar rat), they do not exhibit excessive adiposity, a condition commonly related to diabetes in humans. The HWS chickens would be advantageous in that they display both spontaneous diabetes and obesity.

Currently, some strains of chickens are already being used as a model for human obesity. The obese strain of chickens display a hereditary spontaneous autoimmune thyroiditis, which closely resembles Hashimoto's disease in humans, and the lean and fat chickens have been selected for abdominal fat tissue weight. Again, while obese chicken models may not be perfect, neither are most common mammalian models. I think the story of the ob/ob mouse is a perfect example of this. While the mutated leptin gene was implicated in extreme obesity in mice, the mutation was found to be very rare in humans. Lastly, I think there is a subtle difference in using the term "model" and simply using an animal to perform experiments that may have implications for certain human diseases and conditions. Even if the HWS line was not utilized as a true model of diabetes, understanding why chickens do not suffer from the deleterious effects of chronic hyperglycemia may lay the groundwork for developing effective therapeutic strategies in treating human diabetes. Similarly, continuing to investigate the traits associated with highly divergent body weight between the lines may one day have implications for human obesity.

Appendix A

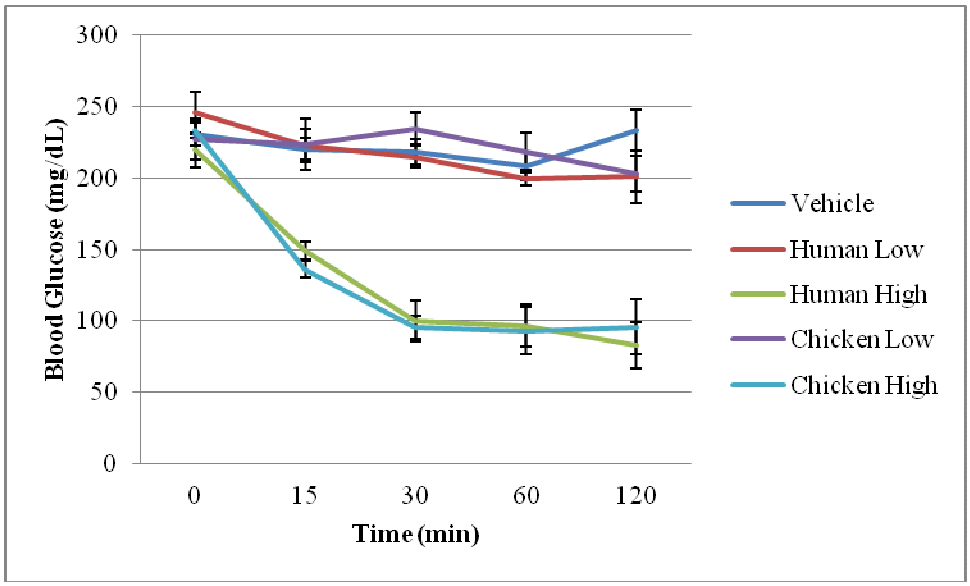


Figure A.1. Comparison of the effect of human and chicken insulin on blood glucose in chickens.

Cobb chickens (d 5) were injected i.p. with a low (0.08 µg/kg BW) or high (80 µg/kg BW) dose of either human or chicken insulin. Insulin sensitivity was assessed by sampling blood glucose concentrations at the indicated time points, to ensure a similar response to either form of insulin.