THE ROLE OF THE SOMATOTROPIC, ADRENOCORTICOTROPIC, AND THYROTROPIC AXES IN BROILER GROWTH AND DEVELOPMENT

LAUREN ALICIA VACCARO

(Under the Direction of LAURA E. ELLESTAD)

ABSTRACT

Commercial genetic selection has produced broilers with high body weights, fast growth rates, and low feed efficiency. Endocrine regulation of growth and metabolism is likely associated with improved broiler performance, but specific effects of the three main hormonal axes regulating these processes are not well understood in birds. The objectives of this research were to (1) identify effects of commercial genetic selection on adrenocorticotropic, thyrotropic, and somatotropic hormonal axes, (2) determine if developmental changes in the somatotropic axis contribute to improved broiler performance, and (3) investigate if thyroid hormones (THs) regulate somatotropic axis activity in muscle cells. Somatotropic, corticotropic, and thyrotropic gene expression was measured in breast muscle and liver of modern Ross 308 and legacy Athens-Canadian Random Bred broilers during embryogenesis and from post-hatch day (d) 10-40. A detailed investigation of developmental somatotropic gene expression was also conducted in Ross 308 broilers from mid-embryogenesis through d21. Circulating hormones were measured post-hatch. In a third study, Quail Muscle Clone 7 (QM7) cells were cultured as undifferentiated myoblasts or differentiated myotubes and treated with triiodothyronine (T_3) or thyroxine (T_4). A decrease in hormones that control basal metabolism and the stress response, as well as a reduction in expression of adrenocorticotropic and thyrotropic signaling genes, was observed in modern

broilers. Though circulating insulin-like growth factors (IGFs) were not different, IGF binding proteins (IGFBPs) were typically upregulated in the liver of modern broilers and downregulated in breast muscle, suggesting that circulating IGFBPs are growth promotive but inhibit muscle development locally. In modern broiler muscle, IGFBPs were largely highest during embryogenesis and lowest post-hatch, suggesting they promote embryonic growth but restrict growth after hatch in muscle tissue. THs regulated expression of select IGFBPs in QM7 cells, and cells were more responsive to T_3 than T_4. Further, undifferentiated cells are likely more responsive to somatotropic and thyrotropic hormonal signaling based on their gene expression profiles. These data suggest that somatotropic and thyrotropic hormonal signaling are important regulators of broiler growth and development, and alterations in their activities as well as crosstalk between these axes contribute to rapid and efficient muscle growth in modern broilers.

INDEX WORDS: Broiler chickens, Athens Canadian Random Bred, growth and development, hormonal signaling, muscle development

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LAUREN ALICIA VACCARO

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LAUREN ALICIA VACCARO

Major Professor: Laura E. Ellestad

Committee: Sammy Aggrey
Jeanna Wilson
Rachel Roberts-Galbraith

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
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CHAPTER 1

INTRODUCTION

Modern meat-type chickens (broilers) are a valuable global food source, and their physiology is the product of decades of genetic selection that has prioritized high body weight, fast growth, and low feed conversion ratio (FCR) [1-6]. In mammalian models, growth and development are well understood to be regulated by the endocrine hormonal axes that are systems of interactions between the hypothalamus, anterior pituitary, and downstream target tissues. Known endocrine axes involved in growth and metabolism include the somatotropic axis, the adrenocorticotropic axis, and the thyrotropic axis [7, 8].

The somatotropic axis facilitates growth and has metabolic effects in bone, muscle, and adipose tissue [9, 10]. Growth hormone releasing hormone (GHRH) is synthesized in the hypothalamus and binds GHRH receptor (GHRHR) [11-14], which induces GH synthesis in the somatotropic cells of the anterior pituitary [15, 16]. The effects of GHRH on GH can be counteracted by somatostatin (SST) and somatostatin receptor 2 (SSTR2) [17]. A major target of somatotropic GH is the liver, which produces insulin-like growth factor 1 (IGF1) and 2 (IGF2) [18, 19]. These hormones signal by binding IGF receptor type 1 (IGFR1) [20]. The IGFs are regulated in turn by the IGF-binding proteins (IGFBPs), a highly conserved protein family [21]. The IGFBPs control growth modulation by physically binding IGFBPs, enhancing or inhibiting IGF affinity for their receptor, extending IGF circulating half-life in plasma, or by acting independently. The IGFBPs can also bind insulin-like growth factor acid-liable subunit (IGFALS), another regulator of the IGFs [22]. The tertiary complex formed between an IGF, IGFBP, and

IGFALS extends the half-life of the IGF in plasma [23]. Activity of the somatotropic axis has several major effects, including increasing muscle accretion and bone growth while decreasing fat deposition [24-26].

The adrenocorticotropic axis is typically most active in environmentally stressful conditions and can induce rapid energy release for the stress response and restrict tissue growth. Upon receiving the correct environmental stimuli [27], the hypothalamic PVN releases CRH [28], which binds the CRH receptor (CRHR) on corticotropes of the anterior pituitary and stimulates production of adrenocorticotropic hormone (ACTH) [28-31]. Activation of CRHR induces ACTH secretion from the anterior pituitary [32, 33], which subsequently causes corticosterone (CORT) release from the adrenal cortex into blood plasma [34]. The actions of CORT are mediated through transcriptional activity of the glucocorticoid receptor [nuclear receptor subfamily 3, group C, member 1 (NR3C1)] [35]. In vertebrates, this signaling pathway increases circulating glucose through metabolic changes while promoting feed consumption. As a result, muscle and bone growth are depressed because of reduced metabolic efficiency [36-38]. The lipophilic nature of CORT prevents it from freely circulating in plasma. Hence, glucocorticoids are often bound to corticosteroid-binding globulin (CBG), also known as transcortin, in circulation [39]. This allows CORT to travel to target tissues throughout the body [40, 41].

The thyrotropic axis controls a variety of biological processes, including skeletal muscle and long bone growth and development, as well as basal metabolism. Thyrotropin-releasing hormone (TRH) is secreted from the hypothalamus in response to changes in environmental conditions and binds TRH receptor (TRHR) [42-44]. This activates thyroid-stimulating hormone (TSH) production in thyrotrophs of the anterior pituitary, which in turn ultimately results in thyroid stimulating hormone production (TSH). [45-47]. After release from the anterior pituitary, TSH

binds thyroid-stimulating hormone receptor (TSHR) on thyroid cells and activates the synthesis and release of the thyroid hormones (THs) [48-50]. Thyroxine (T_4), which is secreted from the thyroid gland in a greater quantity than triiodothyronine (T_3), is less metabolically active than T_3. Therefore, conversion of T_4 to T_3 must occur for THs to have effects. This process is facilitated by the deiodinases, a TH-metabolizing protein family [51, 52]. Therefore, circulating TH levels in plasma are not always indicative of TH activity. The action of T_3 is facilitated by thyroid hormone receptors (THRs) in the nucleus, which act as transcription factors [53]. The THs are transported from circulation into the cytoplasm via TH transporter proteins [54, 55]. These include high-affinity TH transporters such as organic anion transporter1 C1 (OATP1C1), monocarboxylate transporter 8 (MCT8), monocarboxylate transporter 10 (MCT10), and L-type amino acid transporter 1 (LAT1) [56-58].

These endocrine axes also engage in crosstalk, the effects of which can be promotive or inhibitory on their physiological processes. For example, TRH can trigger GH release into plasma, which consequently induces IGF synthesis in the liver [59-61]. Glucocorticoids activate embryonic GH synthesis prior to the maturation of the somatotropic axis in the developing chick [62]. This relationship becomes antagonistic after hatch with CORT inhibiting GH production through reduced transcription of GH [63] in the anterior pituitary or decreased GHR synthesis [64]. Production of TSH can be promoted by CRH [65]. Thus, biological functions can be regulated by hormonal signaling outside of the traditional endocrine axes model, in which hormones facilitate growth, development, and metabolism only by engaging with components of their respective axis.

One experimental model to investigate the effects of these endocrine axes on broiler growth and metabolism is the Athens-Canadian Random Bred (ACRB), a legacy line of broilers. The ACRB line is representative of slower-growing broilers prior to the implementation of intensive

commercial genetic selection [5, 66]. On average, Cobb 500 broilers were 3.5 times heavier than ACRB broilers at 10 weeks. The average FCR of ACRB broilers was reported to be 4.37 (g:g) whereas the FCR of Cobb 500 broilers of the same age was 1.67 [5]. The FCR of the ACRB can be reduced some with the implementation of a modern broiler diet, but diet alone could not induce growth performance resembling that of modern broilers [2]. Modern broilers also have improved yields in comparison to ACRB broilers. On average, Cobb 500 broilers had a hot carcass yield average of 78.84% at 10 weeks of age, whereas the ACRB had a yield of 66.54% [5]. Thus, the ACRB makes an ideal genetic control line to investigate the effects of the somatotropic, adrenocorticotropic, and thyrotropic axes in regulating broiler growth and development.

An additional experimental model that can be utilized are Quail Muscle Clone 7 (QM7) cells, an myogenic line isolated from Japanese quail used to study myogenesis *in vitro* [67]. As breast muscle is an economically valuable tissue in the poultry industry, it is worthwhile to investigate the effects of hormonal signaling on the growth and development of this tissue. The genetic proximity of chickens and Japanese quail, combined with the easy maintenance of QM7 cells in a laboratory setting, make the line an ideal model to study hormonal effects because immortalized muscle cell lines are unavailable in chickens and it is difficult to maintain consistency when isolating chicken primary muscle cells [68]. Experiments can be performed *in vitro* to allow for the precise study of hormonal effects on gene activity without physiological variation observed from bird to bird in *in vivo* models.

Although endocrine systems that control growth and metabolism are poorly understood in birds, they are highly conserved across vertebrates. It is therefore reasonable to postulate that somatotropic, corticotropic, and thyrotropic signaling influences broiler physiology, including muscle development. Thus, understanding the molecular mechanisms of these axes is valuable to

identify their effects on growth and muscle accretion, especially as novel strategies must be developed to maintain efficient meat production in the absence of antibiotics. The goal of this research was to identify which hormonal axis components, such as hormones and their receptors, have been altered by genetic selection and to determine the functional role of hormones and their associated binding proteins in broiler growth and development. This was accomplished by investigating differences in somatotropic, corticotropic, and thyrotropic gene expression between modern and legacy broiler lines in breast muscle and liver tissue, as well as using cultured QM7 cells to determine the effects of the THs on expression of somatotropic and thyrotropic genes and how hormonal crosstalk could impact muscle development.

CHAPTER 2

LITERATURE REVIEW

Overview of endocrine axes regulating growth

The hormonal axes are webs of interactions between the hypothalamus, anterior pituitary, and downstream somatic target tissues that mediate growth and development [69]. Three major axes that affect vertebrate growth are the somatotropic, corticotropic, and thyrotropic axes [7, 8]. The somatotropic axis includes growth hormone releasing hormone (GHRH), growth hormone (GH), GH receptor (GHR), the insulin-like growth factors (IGFs) and IGF receptor (IGFR1), and the IGF binding proteins (IGFBPs). Effects of this axis include inducing growth and skeletal muscle tissue and bone via reducing apoptosis and decreasing fat deposition in adipose [9-16, 18-21]. The major endocrine hormone of the corticotropic axis is corticosterone (CORT) which is released from the adrenal cortex with adrenocorticotropic hormone (ACTH) stimulation following secretion of corticotropin releasing hormone from the hypothalamus. The effects of CORT are mediated by glucocorticoid receptor (NR3C1) and transcortin (CBG). Upon CORT binding to NR3C1, energy is released as part of the stress response, and there is an increase in food consumption, a reduction in feed conversion ratio (FCR), and greater lipogenesis [27-34]. Mechanisms of the thyrotropic axis include thyrotropin-releasing hormone (TRH) synthesis in the hypothalamus which subsequently activates thyroid-stimulating hormone (TSH) [42-47]. The thyroid hormones (THs), thyroxine (T_4) and triiodothyronine (T_3), are released from the thyroid gland after TSH stimulation [48-50]. The THs are regulated at the target tissue level by the TH-metabolizing deiodinases (DIOs) [51, 52].

These signaling systems can regulate growth and metabolism. In endocrine signaling, in which a hormone transmits a biological signal by traveling through the blood to its target tissue. Paracrine signaling occurs when a hormone's target is a local cell type that differs from the cell type the hormone was produced in, whereas autocrine signaling acts upon the cell that originally produced the signal or a nearly identical type of cell [70]. In rarer cases, cell signaling may be direct between adjacent cells through membrane-bound hormones and their receptors. This is known as juxtracrine signaling [71]. The variety of unique hormone signaling systems allow for precise spatial control of growth and metabolism. The function, synthesis, and activity of a particular hormone may change between unique stages of animal development and are sometimes tissue-specific [72].

Somatotropic axis

Hormones and target tissues

Somatotropic hormones are responsible for modulating muscle, bone, and adipose tissue growth. The hypothalamus releases growth hormone releasing hormone (GHRH) [11]. This peptide belongs to the pituitary adenylate cyclase-activating polypeptide superfamily and is highly conserved between vertebrates [73]. The function of hypothalamic GHRH is to induce GH synthesis in the somatotroph cells of the anterior pituitary. Hypothalamic somatostatin (SST) counteracts GHRH and downregulates GH production by binding to SST receptor 2 (SSTR2) in the anterior pituitary [17, 74]. Although extrapituitary functions for GHRH are being investigated [75], in this review GHRH will be discussed only in the context of the somatotropic axis.

Pituitary GH is a key mediator of somatotropic axis activity. Like GHRH, it is highly conserved amongst vertebrates, although the introns of chicken GH DNA sequence are longer than their mammalian homologs [76]. A major target of GH is the liver, which produces primary

effector hormones IGF1 and IGF2 [18, 19]. Outside of the GH-IGF signaling pathway, GH induces metabolic changes independently. For example, GH was found to increase activity in rat liver, potentially by restoring lipase mRNA levels [77]. It also improves lipid utilization in rat skeletal muscle by increasing lipoprotein lipase activity [78]. Both GH and IGF-mediated signaling are required for long bone growth and muscle accretion. When GH signaling is deficient, chickens exhibit a dwarf phenotype characterized by delayed growth, short legs, and reduced body weight [79, 80]. This is caused by a mutated GH receptor (GHR) with reduced GH binding affinity [81].

Plasma GH levels are not always a reliable indicator of growth rate or circulating IGF concentrations in chickens, and administration of GH to broilers frequently yields conflicting results. Historically, circulating GH levels in plasma are higher in slow-growing birds than fast-growing birds [3]. A similar phenomenon exists in domestic turkeys [82]. This appears contradictory to facilitating growth but may be explained by GHR expression. As dwarf broilers have reduced GH-GHR signaling caused by GHR with reduced functionality, slow-growing broilers may have lower GHR expression that the body accommodates for with higher circulating GH. This has been recorded in the literature. A lean broiler line selected for feed conversion was shown to have lower specific GH-GHR binding than a heavy line of fast-growing birds [83]. The authors posited this was the result of GH-induced negative feedback that restricted GHR synthesis. Similarly, modern commercial broiler lines selected for fast growth rates typically have higher levels of GHR mRNA in the liver than slow-growing lines [84]. This indicates an increased sensitivity of hepatic tissue to circulating GH despite lower circulating concentrations.

One action of GH is to increase IGF production in the liver, and they are also synthesized in skeletal muscle and other tissues [85]. Two IGFs have been identified in vertebrates: IGF1 and IGF2. They are similarly sized and have peptide sequences resembling their mammalian

counterparts [20, 86]. Their nomenclature is derived from their structural similarity to insulin, as they share its highly conserved A and B domains [87]. The A and B domains are separated by C and D domains, with structural differences between the IGFs occurring in the C domain specifically [88, 89]. Bone growth and muscle accretion induced by the IGFs occurs via cell proliferation and downregulation of apoptosis following IGF binding to type 1 IGF receptor (IGFR1) [90]. While mammals have a two IGF receptors, only one IGFR has been characterized in avian models [91]. Both IGFs bind IGFR1, although IGF2 binds with a lower affinity than IGF1 [92].

Investigations into the effect of circulating IGFs on broiler growth have led to inconsistent results. In a 2001 study, plasma concentrations of both IGFs were found to be greater in fast-growing birds than slow-growing birds, with increased hepatic *IGF1* expression in fast growing birds [93]. Another study in 2004, however, reported that circulating IGF1 and hepatic *IGF1* did not differ between fast growing, slow growing, and intermediate growing strains, but did increase similarly across all three lines as the birds aged [94]. This could be interpreted as the IGFs having little or no impact on bird growth. When injected *in ovo*, IGF1 was shown to improve bird performance by the second week post-hatch [95]. One interpretation of these inconsistencies is that there are other factors which modulate the effect of IGFs on growth, such as the activity of their receptor or developmental stage.

Importantly, the interaction between IGFs and IGFR1 are mediated by a family of regulator proteins known as the IGF binding proteins (IGFBPs). There are seven IGFBPs in mammals, IGFBP1 through IGFBP7, and six have been identified in birds. While this family of proteins is highly conserved across vertebrates [21, 96-99], IGFBP6 has not been identified in any avian species [100]. Although IGFBP7 is structurally dissimilar from the other IGFBPs and has a low

affinity for the IGFs, it readily binds insulin and prevents insulin receptor activation [101]. Circulating IGFBPs are produced in the liver and can enhance or reduce IGF receptor affinity, lengthen the half-life of an IGF, or alter its tissue specific IGF effects [102, 103]. IGFBPs can also act independently. For example, in the absence of IGFs, IGFBP2 can upregulate apoptosis [104, 105], while IGFBP5 can enhance bone cell proliferation [106]. Each IGFBP has an L, N, and C-domain. In mammalian IGFBPs, variability between family members is introduced in their protein linker region, or L-domain [107]. All three domains are required for proper IGF binding. The N- and C-domains physically sequester the IGF while the L domain is required to maintain binding affinity [101, 108]. The precise mechanism of IGF-IGFBP binding varies across individual IGFBPs. For example, the C-domain of IGFBP4 cannot directly interact with its own N-domain or free IGF1. When IGF1 binds to the N-domain, however, the C-domain shields IGFR1-binding residues on IGF1, preventing IGF1-IGFR1 binding [109]. A similar binding mechanism has been observed in the cooperative activity of IGFBP2's structural domains [110].

The IGFBPs can fine-tune IGF signaling by interacting with other components of the somatotropic axis. They can bind insulin-like growth factor acid-liable subunit (IGFALS), another regulator of IGF action [22]. The tertiary complex formed between an IGF-IGFBP3-and IGFALS extends the half-life of both IGFs in plasma [23, 111]. Although the functionality of chicken IGFALS has not been determined, human and rat IGFALS demonstrate a large degree of structural and functional conservation [112, 113] that likely extends to chickens. Like the IGFs and IGFBPs, IGFALS is primarily synthesized in the liver and its transcription is activated by GH signaling [114]. The IGFBPs are also moderated by proteases [115, 116], which serve as a form of IGF signaling regulation. The enzymatic cleavage of IGFBPs lowers their affinity for IGFs and frees them for to interact with their receptor. All IGFBP proteases are IGFBP-specific, cleaving each

binding proteins' variable L-domains [117]. The IGFBPs modulate growth by binding to IGFs to enhance or reduce receptor affinity, extend the hormone's half-life, or alter tissue specificity [103, 118]. For example, IGFBP1 inhibits protein synthesis in skeletal muscle [119], while IGFBP2 and IGFBP4 inhibit long bone growth [106, 120]. In myoblasts, IGFBP5 has a proliferative effect when bound to IGF1 but an inhibitory effect upon binding IGF2 [121], and IGFBP4 inhibits cellular proliferation of myoblasts only when bound to IGF1 [121]. Additionally, some IGFBPs signal directly without binding an IGF. For example, IGFBP2 independently can upregulate apoptosis [104, 105], while IGFBP5 can independently enhance bone cell proliferation [106]. Therefore, the IGFBPs provide an additional mechanism by which IGF signaling can be regulated and also have direct effects independent of mediating IGF signaling.

Hormonal signaling

Activation of the somatotropic axis begins when GHRH is produced and released from hypothalamic the paraventricular nucleus (PVN) and binds GHRH receptor (GHRHR) on somatotrophs in the anterior pituitary [12-14]. The GHRHR peptide is a transmembrane G-protein coupled receptor (GPCR) [86, 122, 123]. In both chickens and rats, GHRHR is expressed almost exclusively in the pituitary gland and specific to GHRH [124, 125]. When GHRHR is stimulated by GHRH, the coupled Gα stimulatory subunit of the trimeric G-protein complex activates neighboring adenyl cyclase, which facilitates the conversion of ATP to cyclic adenosine monophosphate (cAMP) [126]. The role of cAMP in this pathway is to activate cAMP phosphate kinase A (PKA), which phosphorylates the transcription factor cAMP response element binding protein (CREB) [127, 128]. This binds DNA and initiates GH transcription and GH release from pituitary somatotrophs. The production of GH can be reduced in the anterior pituitary by hypothalamic somatostatin (SST) binding to SST receptor 2 (SSTR2) and subsequent inhibition

of PKA-dependent CREB activation [17, 74]. Like GHRHR, SSTR2 is a transmembrane GPCR [129]. There are two variants, SSTR2A and SSTR2B, that are generated by alternative splicing [130]. Both interact with a G-protein composed of α, β and γ subunits that inhibit adenyl cyclase upon activation [131]. The flux between hypothalamic GHRH and SST production generates distinctive periods of GH up or downregulation and leads to a pulsatile pattern of the hormone in circulation.

GH triggers the production of IGF1 in liver cells by binding GH receptor (GHR), a class I cytokine receptor that transmits its signal via the Janus kinase 2/signal transducer and activator of transcription 5 (JAK2/STAT5) and proto-oncogene tyrosine-protein kinase Src/extracellular signal-regulated kinase (Src/ERK) [132-135]. Expression of *GHR* occurs in chicken liver, muscle, and lymphoid tissues [136, 137]. Transmission of the GH signal requires asymmetrical dimerization of two GHR monomers and subunit rotation within the newly formed dimer [138]. Receptor-associated tyrosine kinase JAK2 phosphorylates the intracellular domain of GHR, then activates STATs 1, 3, and 5b via phosphorylation of its Src homology 2 (SH2) domain [105][139]. Afterwards, STAT5 is translocated to the nucleus as a homodimer [140-143]. After translocation, STAT5 binds nuclear short palindromic γ-interferon-activated sequence (GAS)-like DNA elements [144, 145] and regulates transcription.

The growth inducing and metabolic effects of the IGFs are induced through signaling interactions with IGFR1 or, with much lower affinity, insulin receptor [146], but IGFR1 will remain the primary focus of this review. Of importance to cellular and tissue growth is the phosphatidylinositol-3 kinase (PI3K) signaling cascade that activates the downstream effector Akt/protein kinase B (Akt). When IGF1 binds IGFR1, IGFR1 dimerizes and phosphorylates its intracellular tyrosine residues [147]. This recruits the regulatory subunit of PI3, p85, which bind

IGFR1 via p85's SH2 domains [148, 149]. From here, p85 can recruit catalytic p110 and form a dimer that phosphorylates local phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and recruits Akt to the intracellular side of the plasma membrane [150, 151]. The presence of PIP3 recruits Akt, which then and binds at PIP3K's pleckstrin homology (PH) domain [152]. Once bound, it is phosphorylated at two sites by 3-phosphoinositide-dependent protein kinase 1 and rapamycin complex 2 [153, 154] to become activated.

A number of substrates involved in signaling pathways inducing cell growth can be activated by Akt [155]. One of Akt's substrates is the protein kinase mechanistic target of rapamycin (mTOR) [156]. The activation of mTOR by Akt is not direct, however. Instead, the phosphorylation of tuberous sclerosis 2 (TSC2) is required [157, 158] so mTOR can form a complex with regulatory-associated protein of mTOR (RPTOR) [159]. This complex, known as mTOR complex 1 (mTORC1), is important in facilitating protein synthesis, lipid biosynthesis, and mitochondria biogenesis [160, 161]. These processes are facilitated by mTORC1 activation of transcription factors and translation machinery, such as the sterol-response binding proteins (SREBPs) and S6 kinases (S6Ks), respectively [162, 163].

Biological effects

A considerable amount of descriptive research on the physiological effects of GH in chickens has been performed, but the mechanisms of GH signaling in avian models have yet to be entirely elucidated. Chickens with deficient GH signaling exhibit the dwarf phenotype, characterized by reduced body weight and long bone growth despite no difference in circulating GH between dwarf and full-size birds [164]. This phenotype is induced by a mutated GHR with reduced signaling capacity [81, 165]. This is potentially caused by an inability to activate ERK

due to a confirmational change in the BFG loops, which has been demonstrated in mice [166]. Body weight and bone length are also reduced in hypophysectomized chicks that have their anterior pituitary removed [167]. The effects of hypophysectomy and phenotype of dwarf birds can partially be restored with GH administration [168, 169]. However, the effect of GH is dependent on the delivery method. When administered via intravenous injection at 4 weeks of age, chicken and mammalian GH increase chick body weight and increase circulating IGF1 levels [170]. Pullets given a pulsatile GH injection at eight weeks of age also showed improved feed efficiency and larger carcass weights with increased longitudinal bone growth, but this was not observed in birds that received a continuous treatment via auto syringe pumps [171]. Additional work utilizing pulsatile administration of GH has reported marked increases in body and breast muscle weight [172-174]. This suggests that the pituitary GH release pattern important for affecting growth, not purely levels of circulating GH [175]. This is accomplished by alternating GHRH and SST control of GH production. In the wake of increased GH and IGF1, SST production is upregulated to downregulate GHRH and GH [176].

Cellular proliferation and metabolism of protein and lipids induced by GH has described in humans and other mammalian models [24-26], but these effects are understudied in birds. In chicken skeletal muscle, GH was found to increase expression of *GHR* and genes associated with muscle cell proliferation [177]. Interestingly, the same study found the GH-GHR binding was high as cells proliferated and fused but decreased once differentiation was complete [178]. The effect of GH on lipid metabolism also seems to vary with administration system, bird age, and environment. *In vitro* lipolysis rates increased in male chick hepatocytes aged 1 to 28 days with continuous GH administration in a dose-dependent manner [179]. Pulsatile, 21-day GH administration also reduced fat pad size in three- to eight-week-old male birds [171, 180].

However, body fat content has been observed to increase with GH injections *in vivo* in both three-week and twelve-week-old broilers under constant or pulsatile injection systems [181-183]. This is believed to occur via acetate incorporation into hepatic lipids [184]. One potential mechanism of indirect GHR action is maintenance of mitochondrial function. Knockdown of *GHR* in chicken skeletal muscle both *in vivo* and *in vitro* resulted in reduced expression of mitochondrial biogenesis genes and impaired ATP production [185]. Therefore, birds with reduced GHR activity could have reduced growth because of lower ATP production and fewer mitochondria causing less muscle anabolism.

As GH signaling increases IGF production, it indirectly induces bone and muscle growth via IGF1 [186]. This implies that IGF administration may positively impact bird growth performance. However, direct IGF1 administration did not stimulate growth in male broilers from two to three weeks of age or four-week old female broilers across multiple studies [187-189], despite plasma IGF1 being shown to be greater in fast-growing broilers strains than slow-growing ones [190]. Thus, one can postulate that the effects mediated by IGF1 are regulated by tissue-specific expression of its receptor, intracellular signaling components, or are age dependent. When administered to two-day old embryos, IGF1 increased protein levels [191] by reducing protein catabolism in skeletal muscle [192]. Fast-growing broiler lines have a greater density of IGFR1 in the membrane of skeletal breast muscle cells than slow-growing lines [193], suggesting that cellular sensitivity through the receptor enhances growth sometimes in tandem with circulating hormone levels. This sensitivity may also reflect nutritional status, as increased *IGFR1* expression has been shown in the liver and muscle of fasted one-week and four-week old broilers [194]. Another proposed hypothesis is the ratio of IGF1 production to myostatin (MSTN) production. Rapid muscle growth observed between embryonic day (e) 17 and post-hatch day (d) 0 are

accompanied by a large increase in the IGF:MSTN ratio, favoring IGF1, although the ratio decreased with age [195]. These findings align with MSTN's role as an inhibitor of muscle growth and recorded observations of hepatic *IGF1* expression increasing from embryonic day 13 until four weeks post-hatch [196, 197]. Compared to liver tissue, relatively high levels of both embryonic *IGF1* and *IGF2* have been demonstrated to decrease post-hatch in skeletal muscle, dictating that their effects are likely tissue- and time-dependent, with the primary source of circulating IGF1 produced in the liver [198].

The impact of IGF2 on growth, development, and metabolism are less clear. Fast-growing chickens have higher plasma concentrations of IGF2 than slow-growing ones [199], but IGF2 does not induce weight gain when administered continually into plasma at 4 week for 14 days in female broilers [200]. It also does not reduce protein catabolism as IGF1 does [192], although it does have an anabolic effect on bone and development of the human fetus [201, 202]. Like IGF1, however, an injection of IGF2 in four-week old male chickens has been shown to increase blood glucose levels for a short time, although not as severely [174]. Interestingly, abdominal fat and breast muscle have opposite responses to IGF2 treatment at four weeks of age, with increased fat deposition as muscle growth slows [203], another indicator of the IGFs exhibiting tissue-specific effects. Reduction of muscle growth may occur during chicken embryonic development by microRNA (miRNA)-mediated *IGF2* repression that prevents myoblast differentiation [204]. A potential candidate miRNA is miR-206, which is expressed in chicken skeletal muscle, regulates differentiation, and can alter chicken weight at hatch [205, 206].

The biological effects of the IGFBPs have been most thoroughly investigated in mammals. Mutant constructs of IGFBP3 expressed in Chinese hamster ovary cells and human prostate cancer cells stimulated DNA fragmentation and restricted cellular proliferation in both IGF-dependent

and independent fashions [207]. In porcine embryonic myoblast cells, IGFBP3 and IGFBP5 also restrict proliferation through influencing phosphorylation of the Smad protein and subsequent myostatin activation [208]. What work has been done in chickens on functions of IGFBPs is in the context of embryonic development or prolonged fasting post-hatch. Hepatic *IGFBP1* and *IGFBP2* expression increased during fasting from 16 hours to 5 days in birds at 6 weeks of age and can be lowered by refeeding or an insulin injection [93, 209, 210]. In the pelvic bone cartilage, IGFBP4 has been shown to inhibit both basal and IGF1-mediated bone growth [211]. In chick embryonic fibroblasts (CEFs), rat IGFBP3 inhibits DNA synthesis induced by treatment with IGF1 [212]. Similar effects have been shown when CEFs are treated with recombinant IGFBP1, although it is does not lower DNA synthesis to the degree exhibited by IGFBP3 [213]. As a result, proliferation is prevented. Therefore, the IGFBPs play a role in vertebrate growth and development via IGF signaling regulation and independent actions, and this effect could extend to chickens.

Several cell growth pathways can be inhibited by IGFBP sequestration of IGFs. The development of human nervous tissue via myelination is restricted by IGFBP1 binding IGF1, and a similar inhibitory effect has been observed when IGFBP1 prevents IGF1 and IGF2 signaling in breast muscle tissue [214, 215]. Long bone growth can be reduced by IGFBP2 via IGF-IGFR signaling inhibition, impairing chondrocyte proliferation [120]. Long bone growth may also be regulated by IGFBP4, which prevents IGF binding to IGFR1 in mouse osteoblasts [106]. The same study also showed that IGFBP5 prevents IGF1 binding to IGFR1, but IGFBP5 could still bind to the surface of mouse osteoblasts independent of both IGF1 and its receptor. In myoblasts, IGFBP5 had a proliferative effect when bound to IGF1 but an inhibitory effect upon binding IGF2, while IGFBP4 remained inhibitory regardless of which IGF was bound [121]. Antiproliferative effects have been induced in cancer cells treated with IGFBP3 [216]. The IGFBPs can also extend the

half-life of circulating IGFs and alter their delivery to tissues [103, 118]. Therefore, the IGFBPs can downregulate skeletal muscle and lone bone growth by IGF-dependent or independent effects, and these are also potential regulatory systems in the chicken.

The IGFBPs also affect cell growth directly in the absence of IGFs. In myoblasts, IGFBP5 prevented IGF1 binding to IGFR1 but could still bind to the cell surface directly [106]. Both IGFBP3 and IGFBP5 have been shown to alter independently the ability of cells to bind to the extracellular matrix in the presence of fibronectin fragments without IGFs [217]. Direct anti-proliferative effects mediated at the cell surface have been identified for IGFBP2, typically through the upregulation of apoptosis [104, 105]. If IGFBP2 enters the cell and travels to the nucleus, however, it has proliferative effects [218]. Independent signaling of IGFBP4 prevents colony formation in colorectal cancer cells [219]. Ultimately, the IGFBPs modulate cellular growth and proliferation via both dependent and IGF independent manners.

Adrenocorticotropic axis

Hormones and target tissues

The hormones of the adrenocorticotropic axis are hypothalamic corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and CORT. The PVN produces CRH in response to environmental stressors [220] such as broiler house temperature, house stacking density, and restriction of water or feed. The secretion of hypothalamic CRH induces ACTH secretion from the anterior pituitary [32, 33], which subsequently induces CORT secretion from the adrenal cortex [34]. This system is highly conserved between mammals and birds [221, 222].

The glucocorticoid CORT is essential in initiating cellular responses involved in mediating the stress response and alters metabolism in response to the stressor. Glucocorticoids are a broad class of steroid hormones synthesized from cholesterol in the adrenal cortex [223]. Cortisol is the

primary circulating glucocorticoid in all mammals except rodents, whereas CORT is the primary circulating glucocorticoid in birds [224] and has an extra hydroxyl group at the seventeenth carbon [225]. The lipophilic nature of CORT prevents it from freely circulating in plasma, and CORT is bound to corticosteroid-binding globulin (CBG), also known as transcortin, in plasma [39]. Most CBG is produced in the liver [226]. In humans, CBG binds cortisol within a hydrophobic pocket [227, 228], allowing it to travel to target tissues throughout the body such as muscle, bone, liver, intestines, and the kidneys, among others [40, 41]. Avian CBG functions similarly, despite the steroid binding site sharing only fifty-percent of its amino acid residues with mammalian CBG [229, 230].

Hormonal signaling

In response to an external stressor [27], the hypothalamic PVN releases CRH [28], which binds CRH receptor (CRHR). Two types have been identified in chickens (CRHR1 and CRHR2) [30, 31]. Both are GPCRs that transmit the CRH signal, although CRHR2's affinity is much higher for CRH than that of CRHR1 [31]. Like GHRH, CRHRs are GPCRs [231] that activate several intracellular pathways, most frequently the cAMP/PKA signaling pathway that is responsible for inducing ACTH production by the sequential cleavage of precursor molecule pro-opiomelanocortin (POMC) by pro-hormone convertase 1/3 (PC1/3) [232-234]. The CRHRs potentially enhance ACTH production through dimerization with the arginine vasotocin VT2 receptor (VT2R) [235], as well.

Once ACTH is secreted from the anterior pituitary, it has two functions: initiating CORT production from the adrenal cortex and repressing CRH secretion from the hypothalamus [236, 237]. Like CRH, the ACTH signal is transmitted into the cell via a GPCR, melanocortin-2 receptor (MC2R) [238]. Little work on the MC2R signaling mechanism has been completed in chickens,

but its sequence is relatively conserved with mammalian MC2R, suggesting similar function [239]. In humans, MC2R facilitates steroidogenesis and secretion by the cAMP/PKA pathway [240]. Interestingly, MC2R requires several accessory proteins, known as the melanocortin receptor accessory proteins (MRAPs), to function [241]. Three have been identified as essential for MC2R function in zebrafish and one in chicken [242, 243]. The role of these accessory proteins is to transport MC2R from its site of production in the endoplasmic reticulum to the cell membrane [244, 245] before ACTH signaling occurs.

Glucocorticoid signaling is mediated via the glucocorticoid receptor (NR3C1), a ligand activated transcription factor. First, CORT diffuses into cells upon reaching its target tissue and acts intracellularly. In the cytoplasm, NR3C1 is bound in a complex composed of essential chaperones heat shock protein (HSP) 90 and HSP70, and nonessential chaperones Hop, HSP40, and p23 [246]. Receptor inactivation is induced by HSP70, which causes the receptor to partially unfold. Reactivation is reliant on ATP hydrolysis on HSP90 bound to the receptor and is mediated by histone deacetylase 6 (HDAC6) [247, 248]. When CORT binds activated NR3C1, NR3C1 is released from the complex and enters the nucleus to bind a glucocorticoid response element (GRE) within the regulatory region of its target genes, upregulating or downregulating gene expression [35, 249]. Changes in gene expression are tissue dependent. The secretion of CORT from the adrenal cortex also acts as a negative feedback system in the hypothalamus and on pituitary corticotrophs, reducing CRH and ACTH secretion [250].

Biological effects

The adrenocorticotropic axis induces changes in metabolism, bone formation, and nervous system function [36]. During embryogenesis, CORT induces expression of GH as pituitary somatotrophs mature [251-253]. The mother hen also transfers CORT from her plasma to the yolk

before an egg is laid [254]. Fast-growing White Recessive Rock chickens had not only higher yolk CORT levels than the slow-growing Yellow Feathered chickens, but also greater levels of CORT-metabolizing enzymes 11β-hydroxysteroid dehydrogenases (11β-HSDs) and 20-hydroxysteroid dehydrogenase (20-HSD) [255]. This supports the notion that CORT has a growth-promotive effect in the chicken embryo.

Post-hatch, CORT has a growth inhibitory effect [38]. One well-understood response to glucocorticoids in both mammals and birds is the release of glucose into the bloodstream from liver and breast muscle glycogen stores, and this has been demonstrated in birds receiving dietary CORT supplements for both short and long terms [256]. A similar response can also be achieved using a subcutaneous CORT injection, where blood glucose concentration increased for three hours [257]. The release of glucose provides the body with an energy source under stressful conditions, but the increased energy expenditure that follows results in lower body mass and poorer feed efficiency with higher feed consumption [38]. Breast and thigh mass were lower percentages of total body weight, whereas abdominal fat and liver percentages increased alongside greater fat accumulation following CORT administration [36, 37, 258]. In bone, reduced cellular proliferation at the growth plate [259]. In muscle, increased levels of proteolysis have been observed after CORT treatment, alongside increased cholesterol uptake in muscle [260, 261]. Therefore, the post-hatch actions of CORT can result in reduced broiler performance by inhibiting growth of economically valuable tissues and reducing feed utilization.

Thyrotropic axis

Hormones and target tissues

The thyrotropic axis regulates basal metabolism and tissue growth. In the hypothalamic PVN, thyrotropin-releasing hormone (TRH) is secreted in response to environmental changes such as

temperature or nutritional status [42, 43, 262]. This tripeptide highly conserved across vertebrates and, like many other neuroendocrine signaling peptides, it activates production of the glycoprotein thyroid-stimulating hormone (TSH) from thyrotrophs of the anterior pituitary upon TRH binding [46, 263]. This hormone is composed of two subunits: the alpha glycoprotein subunit (aGSU) and TSH-beta subunit (TSHB). These subunits are individually transcribed and translated but covalently linked via carbohydrate bonds post-translation. The alpha subunit is conserved across TSH, follicle-stimulating hormone, and luteinizing hormone [264], but the beta subunit is unique to each and conveys that particular hormone's biological activity [44]. TSH binds the thyroid-stimulating hormone receptor on thyroid cells and activates the synthesis and release of thyroid hormones [48].

Two thyroid hormones are produced from thyroglobulin and secreted by the thyroid gland, triiodothyronine (T_3) and thyroxine (T_4) [265]. Of the two, T_4 is the gland's major product and the primary thyroid hormone found in circulation. Comparatively, T_4 is less active because of a lower affinity for TH receptors [49]. Both THs are bound in circulation by transthyretin (TTR), albumin, and thyroxine-binding globulin (TBG) [266] due to their hydrophilic amine structure. The TH binding proteins make the THs soluble in plasma and therefore ensure that the THs reach their target tissues by preventing their partitioning into lipid membranes. Once it reaches its target tissue, T_4 is locally converted to T_3 by the removal of an iodine from the molecule's outer ring by the enzymes deiodinase 1 (DIO1) and deiodinase 2 (DIO2) [51, 52]. However, T_3 production can also occur in the liver and this is where more circulating T_3 is produced [267]. Of the two molecules, T_3 has the greater affinity for receptors. It preferentially binds the nuclear receptors, thyroid hormone receptors alpha and beta (THRA and THRB), after being transported into the cell or converted from T_4 intracellularly. To ensure proper hormonal signaling, T_3 can also be locally

inactivated by DIO1 and deiodinase 3 (DIO3) in target tissues if converted into 3,5-diiodo-L-thyronine (T_2). Additionally, DIO3 can also prevent T_4 from being converted to T_3 by metabolizing T_4 directly to reverse T_3 (rT_3) [268, 269].

The deiodinases are one level of tissue-specific regulation of the THs. This results in plasma TH not always indicating TH activity, as DIO expression varies across tissue types. This has been demonstrated in the chicken embryo. Hepatic *DIO3* is highest prior to embryonic day 19 but decreases afterwards. Comparatively, *DIO3* expression in the kidney is lower and constant [268]. Embryonic measurements of *DIO3* mRNA denoted it was the most widespread throughout developing chick tissues including the brain, lung, liver, skin, skeletal muscle, and intestine, but no *DIO1* was detected in skeletal muscle and *DIO2* was only found in the brain [270]. Reduced levels of liver *DIO3* were observed in developing chicks injected with GH at embryonic day 18 and day of hatch, which consequently could T_3 breakdown [271]. Fasting eight-day-old broilers had increased *DIO3* in the liver but lowered it in the kidneys, although no change was observed for *DIO1* between tissues [272]. Levels of *DIO2* mRNA measured by Northern blot showed greater levels of *DIO2* in the brain and liver of seventeen-day-old chickens compared to the skeletal muscle, intestines, or kidneys [273]. *DIO3* was comparable to liver in isolated intestinal tissue from sixty-week-old chickens administered rT_3 [274]. Like other components of the thyrotropic axis, the deiodinases are conserved between mammalian and avian models [275].

THRs are located within in the cell nucleus, and act as ligand-activated transcription factors when bound by T_3 [53]. The THs are transported from circulation into the cytoplasm via TH transporter proteins [54, 55]. These include high-affinity TH transporters such as organic anion transporter1 C1 (OATP1C1), monocarboxylate transporter 8 (MCT8), monocarboxylate transporter 10 (MCT10), and L-type amino acid transporter 1 (LAT1) [56-58]. These primarily

transport T_4, which is converted to T_3 intracellularly. Homologs for all four of these transporters have been identified in chickens [276]. Only MCT8 is a dedicated TH transporter, with a high preference for T_4 and T_3. The other transporters, while having varying affinities for the THs, are also capable of transporting other biological molecules such as amino acids, lactate, and pyruvate [277]. All THRs have a nuclear localization signal (NLS) [278] that is essential for proper transport to the nucleus, but this may work in tandem with specific peptides on the N-terminus [279]. Once THs bind to THRs at TH response elements (TREs), confirmational changes occur with co-factors and transcription factors recruited to the DNA for transcriptional regulation [280].

Hormonal signaling

The central regulatory cells of the thyrotropic axis are TRH-releasing neurons in the PVN. These neurons are essential for normal axis function. Humans with a nonfunctional TRH receptor (TRHR) demonstrate hypothyroidism, [281] similarly to mice lacking *TRH* altogether [282]. The production of TRH can be controlled by a multitude of hormones. Frequently, its production is regulated by the THs as part of a negative feedback loop. When TH levels are high, *TRH* transcription is low, and vice-versa [283]. Other *TRH* transcription regulators include leptin, [284], α-melanocyte-stimulating hormone (α-MSH), and noradrenaline (NA) [285]. After synthesis in the hypothalamus, TRH released into the portal vessels activates TSH production in the thyrotrophs of the anterior pituitary via TRHR-1, a GPCR [286]. This receptor, as well as TRHR-3, has been identified in chickens [287, 288]. The binding of TRH to TRHR-1 activates several different signaling cascades through the second messengers cAMP or inositol trisphosphate (IP_3) [45], including activation of CREB, AP-1, and Elk-1 [47].

The role of TSH is primarily to simulate TH production from the thyroid gland. Receptors for TSH are located on the basolateral plasma membrane of the follicular epithelium [289]. The

TSH receptor (TSHR) is a GPCR. Once activated, thyroglobulin is transported from the outer colloid layer to thyroid follicular cells [265]. Thyroglobulin is a glycoprotein that functions as both a scaffold and precursor molecule for TH synthesis [290] and has been found in chickens during embryogenesis [291] and post-hatch [292]. Thyroglobulin transcription is induced by TSH [293]. To produce THs, tyrosyl residues on thyroglobulin are iodinated to create monoiodotyrosine (MIT) and (diiodotyrosine) DIT. Afterwards, iodothyronine is formed by linking different iodotyrosyl residues together, and it is cleaved from the thyroglobulin scaffolding [294]. Whether T_4 or T_3 is produced depends on which residues are linked together: two DIT create T_4, while one DIT and one MIT form T_3 [295]. The release of TH is historically understood to occur by passive diffusion from the gland [296]. However, plasma TH concentrations have been shown to be depressed when TH transporters, such as MTC8, function poorly [297]. This suggests that passive diffusion and active transport are both required to maintain normal plasma TH levels.

The mechanism behind TH signaling is multifold. Previously, the role of the THRs in TH signaling was briefly discussed. These receptors are activated by binding THs, primarily T_3 [298], intracellularly, enhancing or suppressing transcription [299, 300] of specific genes via binding to their TREs. Activation induces a confirmational change in the receptor that enhances its affinity for TREs [301]. Each TRE is composed of one or more palindromic half-sites [302, 303] and THRs may bind to these sites as monomers, homodimers, or THRA/THRB heterodimers to differentially regulate transcription from a single TRE [304]. The effects of activated THRs are potentiated using nuclear hormone receptor-associated proteins (TRAPs) [305, 306]. The second major mechanism supporting TH signaling is the DIO system. The DIOs are not directly involved in cell signaling as they do not bind a receptor, but are essential because of the ability to regulate TH availability and activity due to their ability to metabolize THs by removing an iodine [307]. This is typically

tissue-specific, but DIO1 is produced in great amounts in the mammalian and avian liver [308]. Plasma DIOs maintain circulating TH levels as part of a negative feedback loop to TH signaling [309]. This is accomplished by DIOs in the plasma membrane of hepatocytes which maintain required T_3 levels in the blood [310]. The spatial distribution of DIO expression also allows for tissue-specific metabolism of thyroid hormones. For example, the developing chick embryo expresses *DIO3* most widely and it can be found in the thyroid, lung, brain, pituitary, heart, liver, spleen, gonads, skin, muscle, intestine, bursa, and kidneys. Comparatively, *DIO1* was in all of these tissues except for the brain, thyroid, skin, and muscle. Transcripts of *DIO2* were only expressed in the brain [270].

Biological effects

The thyrotropic axis regulates both metabolism and growth in higher vertebrates [49, 50], and this has been demonstrated in the chicken. Baseline TH activity must be maintained for proper growth, as weight gain and long bone growth are diminished in hypothyroid and thyroidectomized birds [311]. However, TH activity also appears to reach a threshold in which it becomes growth inhibitive, and this has been demonstrated in several contexts. For example, T_3 administration was shown to inhibit chondrocyte proliferation and expansion of the bone growth plates [312]. Such restriction of growth plate development prevents bones from elongating. Muscular dystrophy induced by T_3 has also been shown to reduce muscle growth [313]. One possible explanation of this phenomenon of reduced growth when TH signaling surpasses basal levels is energy usage inefficiency. This is because T_3 induced greater O_2 consumption via enhanced oxidative respiration prevents energy from being converted into tissue [314]. In broilers, T_3-supplemented diets significantly reduced male and female bird body weights and increased FCR by 28 days of age [315]. Birds fed T_3-supplemented diets from 12 to 21 days of age also had smaller body weights,

although there was no change in FCR. Other birds in this experiment that were fed selenium-supplemented diets for the same period, which is required for DIO activity [316], had smaller body weights due to increased protein breakdown in the presence of excess T_3 [317]. Plasma T_3 and FCR were also greater in male broilers fed low-energy (2800 kilo-calorie) diets [318]. In the context of broiler performance, lower T_3 reduces BMR, resulting in less energy used for heat production and more energy available for deposition as muscle and fat.

Regulation of the THs is orchestrated by TH transporters and deiodinases in target tissues, which in turn impacts metabolism that is dependent on T_3. This is well understood in mammals, but less so in chickens. Investigations into TH metabolic control have often yielded conflicting results in birds. For example, fasting experiments in male chickens resulted in decreased plasma T_3, while refeeding increased T_3 levels afterwards [319, 320], possibly by increased hepatic DIO3 activity [321, 322]. Levels of THs are also tied to heat stress resistance. Three-week old heat-stressed broilers without hypothyroidism injected with T_3 and T_4 had lower survival rates than those with hypothyroidism [323]. This is likely caused by an increase in basal metabolism, which generates heat, after TH injection. Administration of TRH, which should stimulate TH production, caused no effect on bird performance when administered via drinking water [324], whereas another study showed a 14% increase in bird growth rate when administered in feed [325]. Therefore, growth and metabolism in broilers is likely not merely the result of TH signaling or circulating TH levels, but also their availability and regulation by THRs and DIOs.

Developmental and functional crosstalk between axes

Endocrine axes do not exist in isolation. While each facilitates its own "top-down" regulation beginning at the hypothalamus, hormones associated with each axis have been observed to trigger responses in other pathways, as well. The effects of those responses may be promotional

or inhibitory. Together, these interactions create a “crosstalk” between hormonal systems that regulate growth, metabolism, and development.

Hormonal control of GH outside of the somatotropic axis has been demonstrated previously. Administration of TRH to broilers and dwarf chickens increased GH levels in the plasma [59-61]. Embryonic somatotrophs also release GH in the presence of TRH during late embryonic development, indicating that TRH triggers GH secretion both during embryonic and post-hatch development [326]. The THs also regulate GH secretion in birds, inhibiting GH release stimulated by TRH as part of the negative feedback loop [327]. Similarly, IGF1 can inhibit GH release triggered by TRH [59]. The transcription of hepatic GHR, however, is upregulated by THs and affects IGF1 production as a consequence [328]. Therefore, the thyrotropic axis tends to potentiate somatotropic activity.

The glucocorticoids are capable of inducing GH secretion during embryogenesis. Injection of chick embryos with CORT at day 11 of embryogenesis increased the number of GH secreting cells [253]. In primary embryonic chick pituitary cells, CORT activates GH transcription and GH protein synthesis while inducing chick somatotropic differentiation in tandem with GHRH [72, 329, 330]. In chickens, CORT stimulates somatotroph development via GH secretion in tandem with THs. Primary pituitary cells collected at day 11 of embryogenesis yielded GH-producing somatotrophs when treated with CORT and THs [331]. This requires the action of both THs and CORT, as TH treatments alone in vitro were unable to stimulate somatotroph development from embryonic chick pituitary cells [332]. The injection of T_3 and T_4 into fertilized chicken eggs also increased somatotroph size [332]. After hatching, however, CORT becomes antagonistic to GH production. This occurs through reduced transcription of *GH* [63] in the anterior pituitary or decreased synthesis of *GHR* in target tissues such as chondrocytes [64] in mammals.

Glucocorticoids and GH are frequently involved in modulating TH metabolism. Both GH and ACTH inhibit DIO3, increasing T_3 levels [333, 334]. Glucocorticoid administration to chick embryos has been shown to increase plasma T_3 but reduce circulating T_3 levels in juvenile birds. Injections of CRH into chicken embryos have been shown to increase T_4 and T_3 concentrations [335]. This was demonstrated in chick embryos, juveniles, and adult chickens during perfusion studies where CRH stimulated TSH production [336]. Broadly, this work illustrates that endocrine axes regulate biological activity by utilizing different hormonal systems as part of organismal growth and development. The relationships are contextual in the developing chicken. For example, CORT has a synergetic effect on GH during embryo development, promoting somatotroph development and GH production. This relationship becomes antagonistic after hatching when CORT begins to downregulate GH production.

Overview of muscle development

It is important to understand the cellular mechanisms of muscle growth and renewal in broiler chickens, as muscle is an economically valuable tissue. Muscle development occurs through the proliferation of satellite cells [337]. Typically, satellite cells reside in an inactive state where they do not divide [338]. They can, however, begin to proliferate to maintain the satellite stem cell pool or to initiate muscle repair [339]. Satellite cells in active proliferation and their descendants are referred to as myoblasts [338, 340]. Myoblasts are characterized by their rapid expression of myogenic transcription factors such as of myoblast determination protein 1 (MyoD) and myogenic factor 5 (MYF5) [341, 342]. For muscle fibers to grow, myoblasts must mature into contractile tissue, which requires the formation of myotubes. Myotubes are long, multinuclear cells that do not divide and are formed from many fused myoblasts [337, 343]. Myoblast fusion requires the myoblast to exit the cell cycle and myogenic regulatory factors (MRFs) are upregulated [344-346].

Preliminary myotubes will fuse with additional myoblasts to form mature myotubes [347]. Adult animals typically have a set number of muscle fibers that are formed from mature myotubes. Muscle growth occurs when these fibers increase in size [348].

In chickens, muscle fiber development is largely completed by hatching. The proliferation and differentiation of myoblasts into myotubes occurs during embryogenesis and the number of muscle fibers a chick has is set at hatch [349, 350]. Muscle hypertrophy post-hatch requires satellite cells to fuse with an existing myotube and increase its number of nuclei [351]. It also requires an increase in protein synthesis such that the rates of protein degradation do not surpass it [349]. Newly-hatched chicks exhibit rapid muscle growth as satellite cells quickly divide, synthesize DNA, and fuse with myotubes [178]. This cannot be accomplished without a suitable energy source from feed, and chicks restricted from feed for twenty-four- or forty-eight-hours post-hatch show reduced muscle growth compared to those who received feed immediately [178, 352]. The rapid growth period ends by three to four weeks post-hatch, but growth continues at a reduced rate [353]. Therefore, the most critical period for muscle development in the chick is shortly after hatch.

A commonly used experimental model to study myogenesis *in vitro* is the Quail Muscle Clone 7 (QM7) cell line. It is an immortalized myogenic cell line isolated from *Coturnix japonica* (Japanese quail) fibrosarcoma. These cells can be maintained in a monoculture, as undifferentiated myoblasts or induced to form multinuclear myotubes when cultured with reduced serum media [67]. Japanese quail have a close genetic proximity to chickens and thus QM7 cells are suitable replacements for immortalized myoblast lines, which are unavailable in chickens [68]. The culture of primary myoblasts from chickens is also unreliable.

The effect of hormonal signaling on muscle development

Myogenesis is regulated in part by hormonal signaling, and muscle growth is affected by the activity of the somatotropic and thyrotropic axes. Although most work regarding these hormonal effects has been completed in mammalian models, several studies have investigated their actions in chickens. Treatment of chicken satellite cells with human IGF1 and IGF2 stimulates DNA synthesis [354], which implies that the IGFs stimulate muscle hypertrophy by activating these cells and subsequently inducing fusion with existing myotubes. The IGFBPs, however, prevent these effects by sequestering IGF applied to chicken satellite cells and reduce DNA synthesis *in vitro* [355]. The IGFs also appear to function in embryonic chicken myogenesis. The delivery of a retrovirus designed to induce IGF1 overexpression to the embryonic chick hindlimb caused an increase in muscle fibers [356].

The THs can have growth-promotive effects, but they are context specific and vary with developmental stage. Embryonic chicken thigh myoblasts *in vitro* did not divide with the addition of T_4, but they transitioned to myotubes and remained in a differentiated state longer than cells without treatment [357]. This suggests that during embryonic development, T_4 functions to facilitate myoblast differentiation. Juvenile chickens fed a T_3-supplemented diet at 0.1 mg/kg showed increased thigh muscle growth from zero to six weeks, but growth was depressed from six to eight weeks [358]. Male chickens with hypothyroidism induced from two days post-hatch to eight weeks of age had lower fresh muscle weight and less DNA content than controls or those who received T_4 supplementation [359]. Therefore, TH activity is likely required for proper muscle growth in younger chickens, but these effects diminish as the birds age. These studies collectively demonstrate that both the somatotropic and thyrotropic axes are involved in muscle development, although their specific actions are likely linked to developmental stage.

The effect of genetic selection on the modern broiler

Research significance of heritage breeds

The three axes previously discussed are widely accepted to be major players in growth, development, and metabolism in mammals. As these systems are highly conserved across vertebrates, it stands to reason they play a similar role in birds. This creates interesting questions regarding their influence on the physiology of modern broiler chickens. The modern broiler is the product of a seventy years of artificial genetic selection, with an emphasis on production efficiency [360]. The endocrine systems responsible for growth and metabolism are likely influenced by such selection, but little-to-no work has been done investigating this in the context of modern commercial broilers.

One tool with immense value in investigating the effects of genetic selection on broilers is the use of legacy broiler lines. A legacy line, in the context of broiler production, is a genetic variety that reflects the physiology and behavior of birds from previous decades before the implementation of commercial genetic selection. While metabolism and organ response to high growth rates have been compared between broilers and layers [361], this does not elucidate how genetic selection has transformed broiler physiology since broilers and layers are selected for different traits.

The performance of legacy strains has previously been compared to that of modern broilers. The breast muscle mass of the New Hampshire X Barred Columbian (UIUC), a line maintained at University of Illinois, Urbana, and representative of inbred broilers from the 1940's [362], was 9% of total body weight, whereas in modern commercial Ross 708 birds hatched in 2009 maintained under the same conditions, it represented 18% of total body weight [363]. The same study found that FCR in Ross 308 was significantly greater than that of the UIUC, although FCR values were

not presented. Positive and negative growth factors have also been found to be differentially enriched in the breast muscle tissue of the UIUC in comparison to Ross. Two legacy broiler lines are maintained at the University of Alberta representing broilers from 1957 and 1978, respectively [364]. These birds were significantly smaller than Ross 308 chickens hatched in 2005: the 1957 broilers reached over 20% of the Ross 308's body weight by 42 days of age, whereas the 1978 broilers were over 40% of the Ross 308's body weight at the same day [364]. The 1957 broilers had an FCR of 2.882 (g:g) at 42 days of age, while the FCR 1978 broilers was 1.899 (g:g) and the Ross 308's was 1.674 (g:g) [364]. The performance traits of another legacy line, the Athens Canadian Random Bred (ACRB) were also compared to 2012 Cobb 500 broilers. By ten weeks of age, Cobb 500 broilers were nearly four times bigger than ACRB broilers [5]. A conclusion to make from these comparisons is that legacy broiler lines have reduced growth capacity than modern broiler lines, and that modern broiler performance is caused by a greater number of decades dedicated to genetic selection of performance traits.

The Athens-Canadian Random Bred

The ACRB is a valuable tool in studying the impacts of genetic selection on the modern broiler. It is a legacy population of meat-type chickens maintained at the University of Georgia in Athens since 1958, derived from the Ottawa Meat Control Strain of the Canada Department of Agriculture. It was developed from three commercial and one experimental bird strain and is maintained via artificial insemination instead of natural mating. The most prominent physical characteristics of the ACRB are their white feathering and four comb patterns: rose, pea, comb, and walnut-type [66]. Legacy lines such as the ACRB are valuable because they provide a control that can be utilized to determine the effect of genetic selection on the physiology and performance of modern broilers.

The physiology and behavior of ACRBs differs greatly from that of modern broilers. In 1994, two studies were conducted comparing performance between ACRB and Arbor Acres (AA) lines, using diets which were traditionally formulated in 1957 and 1991. The AA were a commercial line in 1991. In both studies, the 1991 diet did not enhance performance of the ACRB birds to be comparable to that of the AA birds [6, 365]. This study was performed again in 2003, with the Ross 308 commercial strain in use at the time instead of AA, and similar results were obtained [2]. A further comparative study, utilizing the 2012 Cobb 500, noted that modern birds were ten grams heavier than legacy birds at hatch. The average FCR of ACRB birds was 4.37 whereas the FCR of Cobb 500 broilers of the same age was 1.67 but differences were greatest between 6 and 8 weeks. [5] ACRB behavior also differed greatly from Cobb 500 birds [5]. Legacy chicks show heightened levels of activity and perching, with increased activity potentially reducing weight gain efficiency.

The ACRB has also been employed as a control in physiological studies to further understand how modern broilers respond to stress or disease. When subjected to heat stress, modern broiler growth was severely depressed compared to the ACRBs [1]. However, in another study that investigated the response of broilers to osteochondrosis, no differences were observed in the prevalence of lesions between ACRB and modern broiler lines [366]. Incidences of woody breast syndrome and muscle lesions are also uncommon in ACRB compared to modern broilers.

Observational studies using the ACRB have also indicated developmental differences between modern and legacy broilers, alongside their divergence in growth performance and disease resistance. Modern-type AA broiler eggs were 50% heavier than ACRB eggs, and developing modern broiler embryos had smaller hearts, heavier livers, and greater T_3 concentration in the blood plasma [367]. This research demonstrates that the ACRB legacy line may be utilized

as an effective control in studying the development, performance, and disease resistance of modern broilers.

Rationale and Objectives

The improved production efficiency of today's broilers is the result of decades of genetic selection practices by the industry, though it is unclear how these have impacted endocrine systems regulating growth and metabolism. Three of these endocrine systems are the somatotropic, adrenocorticotropic, and thyrotropic axes. There are several open questions about how these systems function in birds, as is evidenced by contradictory results found in the literature. A deeper understanding of physiological processes regulating growth and metabolism may allow for the development of strategies to continue to improve broiler production efficiency, such as providing novel targets for future selection programs and finding ways to harness these systems to optimize growth and metabolic efficiency. Utilizing a legacy line of broilers representing birds prior to the advent of intensive selection as a genetic baseline is a way to determine how select endocrine systems have been impacted, thus experiments comparing legacy and modern commercial broilers provide an avenue to accomplish this. Additionally, ontogenic investigations into the somatotropic axis are valuable because they provide greater understanding of somatotropic processes that have been historically understudied in avian models.

Another goal of this research was to expand understanding of TH signaling, particularly with regards to crosstalk with the somatotropic axis in the context of broiler muscle cell growth and differentiation. This work will provide insight into the biological processes regulating meat production and can be used to develop novel strategies to improve the efficiency of this process.

Therefore, we had two primary hypotheses. Firstly, long-term commercial genetic selection has affected endocrine systems involved in mediating growth and metabolism, causing

alterations to those systems leading to desirable growth traits. Secondly, that the somatotropic and thyrotropic axes exhibit hormonal crosstalk that potentially influences muscle growth and development.

Therefore, the specific objectives of this research were:

1. To identify effects of commercial genetic selection on adrenocorticotropic, thyrotropic, and somatotropic hormonal axes.
2. To determine if developmental changes in the somatotropic axis contribute to improved broiler performance.
3. Investigate if THs regulate somatotropic axis activity in avian muscle cells.

CHAPTER 3

EFFECTS OF GENETIC SELECTION ON ACTIVITY OF CORTICOTROPIC AND THYROTROPIC AXES IN MODERN BROILER CHICKENS[1]

[1]Vaccaro, L.A., T.E. Porter, and L.E. Ellestad. 2022. *Domestic Animal Endocrinology* 78:106649
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Abstract

Commercial selection for meat-type (broiler) chickens has produced economically valuable birds with fast growth rates, enhanced muscle mass, and highly efficient feed utilization. The physiological changes that account for this improvement and unintended consequences associated with them remain largely unexplored, despite their potential to guide further advancements in broiler production efficiency. To identify effects of genetic selection on hormonal signaling in the adrenocorticotropic and thyrotropic axes, gene expression in muscle and liver and post-hatch circulating hormone concentrations were measured in legacy [Athens Canadian Random Bred (ACRB)] and modern (Ross 308) male broilers between embryonic days (e) 10 and e18 and post-hatch days (d) 10 and d40. No interactive effects or main effects of line were observed for adrenocorticotropic gene expression during either developmental period, although age effects appeared for corticosteroid-binding globulin in liver during embryogenesis and post-hatch and glucocorticoid receptor in both tissues post-hatch. There was a main line effect for circulating corticosterone (CORT), with levels in ACRB greater than those in Ross. Several thyrotropic genes exhibited line-by-age interactions during embryonic or post-hatch development. In liver, embryonic expression of thyroid hormone receptor beta (THRB) was greater in ACRB on e12, and deiodinase 3 (DIO3) levels were greater in Ross on e14 and e16. In juvenile liver, deiodinase 2 (DIO2) expression was greater in ACRB on d10 but greater in Ross on d20, while DIO3 was higher in ACRB on d30 and d40. Levels of thyroid hormone receptor alpha (THRA) mRNA exhibited a main line effect, with levels greater in ACRB juvenile breast muscle. Several thyrotropic genes exhibited main age effects, including DIO2 and DIO3 in embryonic breast muscle, THRA and THRB in post-hatch liver, and DIO2 in post-hatch breast muscle. Circulating triiodothyronine (T3) displayed a main line effect, with levels in Ross significantly reduced as compared to ACRB.

These findings suggest that in modern broilers, a decrease in levels of hormones that control basal metabolism (T3) and the stress response (CORT), as well as altered expression of genes regulating thyroid hormone activity, could contribute to lower heat production, reduced stress response, and altered nutrient partitioning, leading to more efficient feed utilization and faster, more productive growth.

Introduction

Economically valuable traits such as body weight, growth rate, and feed conversion ratio (FCR) in modern broiler (meat-type) chickens are the product of decades of commercial genetic selection [1-6] and are regulated, in part, through hormonal interactions between the hypothalamus, anterior pituitary, and downstream target tissues. The adrenocorticotropic and thyrotropic axes are two such hormonal systems that likely play a role in selection-driven changes [7, 8], though their specific contributions to the production efficiency of modern broilers are not well known, in part because effects of corticosterone (CORT) and thyroid hormone (TH) administration on bird physiology are inconsistent across previous work [3, 4, 258, 324, 325, 368-370]. Systems governing endocrine axis activity, such as hormone receptor expression, hormone availability, and hormone bioactivity play a critical role mediating effects of these axes in key target tissues. As such, it is important to consider tissue-specific expression of hormone receptors, chaperones, and enzymes mediating hormone-receptor affinity to contextualize endocrine signaling on a broader scale.

The adrenocorticotropic axis regulates vertebrate metabolism through secretion of CORT from adrenal cortical cells and can induce rapid release of energy and restrict tissue growth. These effects are mediated mainly through transcriptional activity of the glucocorticoid receptor [nuclear receptor subfamily 3, group C, member 1 (NR3C1)][35]. Approximately 80% of CORT is bound to corticosteroid-binding globulin (CBG) in plasma [371]. As only free CORT can enter cells to interact with NR3C1, CBG determines the activity and intensity of CORT signaling in target tissues [372]. Glucocorticoid signaling increases available energy and feed consumption in vertebrates while reducing muscle and bone growth [36-38], all of which depress metabolic efficiency and feed conversion into economically valuable tissues. The treatment of chicken

skeletal muscle with CORT increases cholesterol uptake, proteolysis, gluconeogenesis, and lipogenesis [260, 261], while decreasing protein synthesis and glucose uptake [257-259, 373].

The thyrotropic axis controls basal metabolic rate (BMR), thermoregulation, and development of muscle and bone [49, 50] through the action of THs, thyroxine (T_4) and triiodothyronine (T_3), secreted from the thyroid glands. This axis is thought to have been altered by domestication, since the thyroid-stimulating hormone receptor (THSR) locus was detected as one of three selective sweeps identified in domesticated chickens, with virtually all domesticated strains carrying the same allele [374]. While a positive relationship between T_3 and BMR has been demonstrated in chickens using fasting and refeeding experiments [319, 320], other work investigating thyrotropic control of metabolism has yielded conflicting results. One study demonstrated that thyrotropin-releasing hormone (TRH) caused no effect on bird performance when administered intermittently via drinking water between 2 and 21 days of age, despite increasing plasma T_4 [324]. Another showed a 14% increase in bird growth rate when TRH was administered in feed between 3 and 6 weeks of age [325], alongside higher plasma T_3 and diminished T_4. Changes in circulating THs in each study suggest that the TRH was bioavailable when administered orally, but the results suggest that plasma T_4 or T_3 concentrations may not always be indicative of TH effects or that their effects are dependent on developmental stage. While a relatively low amount of biologically active T_3 is produced by the thyroid gland, higher levels are derived by local conversion of T_4 to T_3 by the activity of deiodinase 2 (DIO2) [51, 52]. T_3 signals are subsequently potentiated by thyroid hormone receptors alpha (THRA) and beta (THRB), which function as nuclear transcription factors. In addition to the above factors that promote T_3 signaling, other deiodinases such as DIO3 deactivate T_3 and convert T_4 to biologically inactive reverse T_3 (rT_3) [270, 320]. Thus, regulation of thyroid hormone signaling is tightly

controlled within tissues by deiodinase activity, and holistic examination of thyrotropic axis activity requires investigation into their expression.

The economic value of chicken as an affordable protein source makes it an important species to investigate endocrine control of growth and metabolism. A model useful for identifying the role of genetic selection in producing the physiology of the modern broiler is the Athens Canadian Random Bred (ACRB) population, a legacy line reflective of broilers from the mid-1950's [66], prior to the beginning of intensive commercial selection. The ACRB birds are a slow-growing, smaller strain with a higher FCR than modern broilers [5]. Their small size is reflected in their total body weight as well as proportional weight of breast and leg muscle. A modern broiler diet reduces ACRB FCR, but it is still greater than that of modern broilers [6], indicating that modern broiler FCR is partially influenced by genetic differences in physiology. Therefore, the objective of this study was to investigate differences in adrenocorticotropic and thyrotropic activity between modern and legacy broilers, including circulating concentrations of hormones and expression of hormone receptors and their regulatory proteins in key metabolic tissues.

Materials & Methods

Animals and tissue collection

Two separate experiments were conducted in which tissues were collected and analyzed from male ACRB and modern Ross broilers. The first experiment was conducted during embryonic development, and the second experiment was conducted after hatch. During each experiment, birds of both lines were incubated, hatched, and raised concurrently. Only male birds were used in the present study to simplify data interpretation, as it is known that differences in growth metabolism exist between the sexes and preliminary work from our lab has shown that sex effects exist in thyrotropic and corticotropic neuroendocrine gene expression as early as mid-

embryonic development (Ellestad and Porter, unpublished data). All animal procedures were approved by the University of Georgia and University of Maryland Institutional Animal Care and Use Committees.

Embryonic development

Fertile eggs from ACRB and Ross 308 broiler lines were incubated under standard conditions (37.5°C, 60% relative humidity, rotation every 2 – 3 h) at the same time and in the same incubator, with the day eggs were set defined as embryonic day (e) 0. Embryos were weighed, euthanized, and skin, liver, and breast muscle (*Pectoralis major*) collected on e10, e12, e14, e16, and e18 from 12 embryos at each time point. Skin tissue was kept on ice and stored at -20°C prior to genomic DNA extraction for molecular sexing. Liver and breast muscle tissues were flash frozen in liquid nitrogen and stored at -80°C prior to total RNA extraction for gene expression analysis.

Post-hatch juvenile development

Embryonated ACRB and Ross 308 eggs were incubated as previously described. At hatch, birds from both lines were sexed, and males of each line were in raised in separate floor pens located in the same room (n = 8 floor pens per line) with free access to water and a standard modern commercial three-phase diet. Birds were fed starter diet (21.3% crude protein, 1.2% digestible lysine, 3050 kcal/kg metabolizable energy, 0.95% calcium and 0.48% available phosphorus) from post-hatch day (d) 0 – d14, grower diet (19.6% crude protein, 1.09% digestible lysine, 3120 kcal/kg metabolizable energy, 0.85% calcium and 0.43% available phosphorus) from d14 – d28, and finisher diet (17.9% crude protein, 0.98% digestible lysine, 3170 kcal/kg metabolizable energy, 0.75% calcium and 0.38% available phosphorus) from d28 – d42. Body and feeder weights were determined for each pen on d7, d14, and d42 and used to determine body weight gain (BWG), feed intake (FI), and FCR between d7 and d42.

Blood and tissues were sampled from one bird in each pen on d10, d20, d30, and d40 (n=8 birds per line at each time point). Blood was collected from the brachial vein into heparinized tubes and stored on ice until centrifugation at 1,500 x *g* for 10 minutes at 4°C before long-term storage at -20°C prior to analysis of circulating hormone levels. Following blood collection, birds were weighed, euthanized, and liver and breast muscle tissues were collected, flash frozen in liquid nitrogen, and stored at -80°C prior to total RNA extraction for gene expression analysis.

Molecular sexing of embryos

To determine embryo sex, genomic DNA (gDNA) was isolated from skin tissue using the QIAamp Fast DNA Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Concentration of gDNA in each sample was determined using a NanoDrop 2000 spectrophotometer (ThermoFisher, Waltham, MA) and integrity was ensured using gel electrophoresis. Sex of each embryo was determined by PCR amplification of chromo-helicase-DNA binding protein using 2550F (5'-GTTACTGATTCGTCTACGAGA-3') and 2718R (5'-ATTGAAATGATCCAGTGCTTG-3') primers, which generate a single band for males and two bands for females [375]. Reactions (25 µl) were conducted with 2X GoTaq Green DNA master mix (Promega, Madison, WI) and contained 0.4 µM each forward and reverse primer and 100 ng gDNA template. The PCR cycling conditions were as follows: 95°C for 10 minutes, followed by 30 cycles of 95°C for 30 seconds, 48°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. Only tissue samples from male embryos were used to assess gene expression.

Reverse transcription-quantitative PCR

RNA isolation and reverse transcription

Total RNA was isolated with the RNeasy Mini kit (Qiagen) utilizing modified versions of the manufacturer's protocol for lipid-rich (liver) or fibrous (breast muscle) tissues as described below. Liver tissue samples were mechanically homogenized in 1 mL QIAzol reagent (Qiagen) for 30 sec and incubated for 5 min at room temperature before addition of 200 µL chloroform followed by 15 sec of vigorous shaking. All samples were incubated for an additional 3 min at room temperature and centrifuged at 4°C for 15 min at 12,000 x *g*. Afterwards, 600 µL of 70% ethanol was added to the supernatant of each sample before the remainder of the isolation was carried out according to the manufacturer's instructions.

Breast muscle tissue samples were mechanically homogenized for 30 sec as directed by the manufacturer, after which they were allowed to sit for 2 min at room temperature before addition of 1,080 µL deionized water and 20 µL Proteinase K (Qiagen). Samples were incubated for 10 min at 55°C in a shaking water bath and centrifuged for 3 min at 10,000 x *g* prior to addition of 900 µL 100% ethanol to the supernatant. The remainder of the isolation procedure was carried out according to manufacturer's instructions.

Isolated RNA was quantified using a Take3 Epoch microplate spectrophotometer (BioTek, Winooski, VT) and run on a denaturing gel to verify integrity. Reverse transcription reactions (20 µl) were performed using 1 µg total RNA, 5 µM Random Hexamers (Thermoisher), 200 units M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA), 0.5 mM dNTPs, and 8 units RNaseOUT (Invitrogen, Carlsbad, CA). Identical reactions excluding the reverse transcriptase enzyme were performed using RNA pools made from all samples to control for gDNA contamination. Reactions were diluted 10-fold prior to qPCR analysis, with final 500-fold dilutions generated for 18s ribosomal rRNA (18s) detection.

Primer design

Intron-spanning primers from Integrated DNA Technologies (IDT, Coralville, IA) were designed using Primer Express Software (Applied Biosystems, Foster City, CA) with the following parameters: melting temperature between 58-60°C, 40-60% GC content, 18-30 nucleotides in length, and amplicon length of 100-150 base pairs. The amplification efficiency of each primer pair was determined by analyzing six serial dilutions of pooled liver and muscle cDNA by qPCR. Amplification efficiency was calculated from the slope of the linear regression line that resulted from graphing cycle threshold (Ct) versus $\log_2$-transformed dilution using the following equation: efficiency = $(10^{(-1/\text{slope})}-1)$ [376, 377]. Primer sequences and calculated amplification efficiencies are listed in **Table 3.1**.

Quantitative PCR

Transcripts were analyzed in duplicate using qPCR reactions (10 µl) that consisted of 2 µl diluted cDNA, 5 µl 2X PowerUp SYBR Green Master Mix (ThermoFisher), and 400 nM each forward and reverse primer. Cycling was performed using a StepOne Plus Real-Time PCR System (Applied Biosystems) with the following conditions: 50°C for one min, 10 min at 95°C, followed by 40 cycles of 95°C at 15 sec, 30 sec at 58°C, and 30 sec at 72°C, and a post-amplification disassociation curve analysis to ensure amplification of a single product. Transcripts were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in liver and 18s in breast muscle. Data were transformed and normalized using the equation $(2^{\Delta Ct})_{target}/(2^{\Delta Ct})_{GAPDH\ or\ 18s}$, where $\Delta Ct = Ct_{no\ RT} - CT_{sample,}$ and are expressed relative to the line with the highest expression level at a single age (equal to 100%) using equations described previously [378-381].

Hormone assays

CORT enzyme-linked immunosorbent assay (ELISA)

Plasma (50 µl) was extracted twice with 250 µl diethyl ether. Ether was allowed to evaporate overnight, and each sample was reconstituted in 250 µl ELISA buffer (Cayman Chemical, Ann Arbor, MI) before storage at -20°C. All samples were analyzed in duplicate on a VICTOR3 Multilabel Plate Reader (Perkin Elmer, Waltham, MA) using a Corticosterone ELISA Kit (Cayman Chemical), which has a sensitivity limit of 8.192 pg/mL. The fractional maximum binding was logit-transformed, and the amount of hormone in each sample was calculated using a linear standard curve. Intra- and inter-assay coefficient of variations (CVs; %) were determined to be 6.46 and 10.57, respectively.

TH radioimmunoassays (RIAs)

Thyroid hormones were measured by T_3 and T_4 coated-tube RIA Kits (MP Biomedicals, Irvine, CA) per the manufacturer's instructions with modifications as previously described [382, 383]. Briefly, samples were incubated at 4°C for 16 h instead of at 37°C for 1 h following addition of radioactive tracer, and standard curves were extended to 0.03 ng/mL (T_3) and 1.5 ng/mL (T_4) by performing a series of 2-fold dilutions of the highest standards with steroid-free serum. Samples were diluted 1:4 (T_3) or run undiluted (T_4) and analyzed using volumes recommended by the kit manufacturer. After tracer was decanted, all tubes were allowed to dry for 48 h prior to determining the amount of radioactivity bound to each tube by counting for 1 min in a Wallac Wizard Model 1470 Gamma Counter (Perkin Elmer). Calculations for sample hormone levels performed for both assays were identical to those used for the CORT ELISA. Assay sensitivities were 3.125 ng/mL (T_3) and 1.5 ng/mL (T_4), with intra-assay CVs of 10.44 and 10.89, respectively.

Statistical analysis

Data were analyzed via a two-way analysis of variance (ANOVA) using the Fit Model Procedure of JMP Pro 14 (SAS Institute, Cary, NC). When ANOVA indicated a significant line,

age, or line-by-age effect (P≤0.05), *post hoc* multiple means comparisons were performed at the appropriate level using the test of least significant difference. When line-by-age interactions were not significant (P>0.05), main effect means for line and age were determined. Main effect P-values determined by ANOVA for embryonic RT-qPCR are listed in **Table 3.3** and **3.4**, while post-hatch P-values for RT-qPCR, ELISAs, and RIAs are presented in **Table 3.5** and **3.6**.

Results

Growth performance

Embryonic development

Embryonic body weight was measured at each age tissues were collected. A significant line-by-age effect was observed, and Ross embryos were significantly heavier than ACRB embryos from e14 onwards, and by e18, Ross embryos were approximately 20% heavier than ACRB (**Figure 3.1A**; **Table 3.2**; P≤0.05). These data suggest that differences in endocrine systems regulating growth and metabolism might manifest during the latter third of the 21-day embryonic developmental period.

Post-hatch juvenile development

A significant line-by-age interaction was observed after hatch, as Ross weights were three-fold greater than ACRB on d10 and this pattern was maintained until d40 (**Figure 3.1B**; **Table 3.2**; P≤0.05). Final body weight and total FI, body weight gain BWG, and FCR (g FI/g BWG) of the lines between d7 and d42 of juvenile development were also compared (**Table 3.2**). Ross birds had higher FI, BWG, and final body weight than ACRB throughout this period (P≤0.05), and FCR of ACRB was significantly higher than that of Ross birds (P≤0.05). Taken together, these results demonstrate that physiological differences between modern and legacy lines persist across

developmental stages and discrepancies in performance become more pronounced post-hatch as the modern birds grow substantially faster than the legacy birds.

Adrenocorticotropic axis

Glucocorticoid hormones can change energy consumption via altered nutrient uptake and utilization. As such, expression of genes responsible for mediating glucocorticoid signaling were compared between modern and legacy broilers during embryonic and juvenile development. Additionally, circulating CORT levels in juveniles were compared. Expression patterns in the corticotropic axis of both lines during embryonic development are shown in **Figure 3.2**, and main effect means are shown in **Table 3.3** (line) and **Table 3.4** (age). Juvenile expression patterns are shown in **Figure 3.3**, post-hatch CORT levels are depicted in **Figure 3.4**, and main effect means for parameters without significant interactive effects are shown in **Table 3.5** (line) and **Table 3.6** (age).

Embryonic gene expression

No significant line-by-age effects or main effects of line were observed for hepatic gene expression in the corticotropic axis during embryonic development. A main effect of age on *CBG* was detected, where expression in both lines was consistent from e10 to e16 but decreased on e18 (**Figure 3.2A**; **Table 3.4**; $P \leq 0.05$). Hepatic *NR3C1* exhibited no difference in expression across lines or ages (**Figure 3.2B**; **Tables 3.3** and **3.4**). Expression of *CBG* mRNA was not detected in embryonic breast muscle, which is consistent with the literature regarding vertebrate CBG production [384], and no significant line-by-age interactions or main effects of line or age were observed in muscle during embryogenesis for any of the remaining genes measured (**Figure 3.2C**; **Tables 3.3** and **3.4**).

Post-hatch juvenile development

Gene expression

There were no significant line-by-age or line effects for hepatic *CBG* and *NR3C1* mRNA expression in juvenile male broilers (**Figure 3.3**), though both genes exhibited a main effect of age (**Table 3.6**; P≤0.05). In both lines, expression of *CBG* in liver decreased between d10 and d20 and increased to intermediate levels at d30 and d40 (**Figure 3.3A**; **Table 3.6**; P≤0.05). Levels of *NR3C1* mRNA in the liver of both lines increased from d10 to d20, decreased at d30, and increased again on d40 (**Figure 3.3B**; **Table 3.6**; P≤0.05). Consistent with embryonic measurements and previous observations [385, 386], *CBG* mRNA was not detected in breast muscle at any age. Significant line-by-age or main effects of line were not observed for *NR3C1* in the breast muscle (**Tables 3.5** and **3.6**), though there was a significant main effect of age (**Tables 3.6**; P≤0.05). Expression of *NR3C1* in both ACRB and Ross breast muscle increased between d10 and d20 and remained consistently higher on d30 and d40 (**Figure 3.3C**; **Table 3.6**; P≤0.05).

Circulating CORT

A significant line-by-age effect was not observed for circulating CORT levels during juvenile development (**Figure 3.4**), but significant main effects of line and age were detected (**Tables 3.5** and **3.6**; P≤0.05). Overall, plasma CORT levels were higher in ACRB (**Figure 3.4** and **Table 3.5**; P≤0.05), and circulating levels gradually decreased from d10 to d40 in both lines (**Figure 3.4**; **Table 3.6**; P≤0.05). These data suggest that CORT-induced changes to energy utilization in modern broilers has been altered from their legacy counterparts. This might lead to increased weight gain and efficiency of feed nutrient use in modern broilers, as less energy from the diet is being diverted from productive growth.

Thyrotropic axis

The expression of THRs and circulating TH were compared between legacy and modern broilers to determine the effect of genetic selection on thyrotropic gene expression and hormone concentration, as the THs control BMR, thermoregulation, and the development of bone and muscle tissue. Deiodinase expression was also compared between broiler lines, given their ability to control tissue-specific TH signaling. Embryonic expression patterns of thyrotropic genes in both lines are shown in Figures 3.5 and 3.6, and main effect means are shown in **Table 3.3** (line) and **Table 3.4** (age). Expression data from juveniles are presented in **Figures 3.7** and **3.8**, while circulating TH concentrations are displayed in **Figure 3.9**. Main effect means are shown in **Table 3.5** (line) and **Table 3.6** (age).

Embryonic gene expression

Hepatic *THRB* exhibited a significant line-by-age effect in which ACRB expression was 2.5-times greater than Ross on e12 (**Figure 3.5B**; $P \leq 0.05$), while *THRA* approached a significant interactive effect with difference in expression on e12 resembling that of *THRB* (**Figure 3.5A**; P=0.078). No additional main effects of age or line were detected in the liver, but expression patterns of *THRA* also approached significance for an age effect, with substantially lower levels on e16 and e18 (**Figure 3.5A**; **Tables 3.3** and **3.4** P=0.0528). In breast muscle, significant interactive or main effects were not detected for *THRA* or *THRB* between e10 and e18 (**Figure 3.5C and 3.D**; **Tables 3.3** and **3.4**).

While there were no significant interactive or main effects for hepatic expression of *DIO1* during embryogenesis (**Figure 3.6A**; **Tables 3.3** and **3.4**), there was a significant line-by-age interaction for hepatic *DIO3*, where expression in Ross liver was 2-fold higher than in ACRB on e14 and e16 (**Figure 3.6B**; $P \leq 0.05$). Hepatic expression of *DIO2* was undetected between e10 and e18. No significant line-by-age interactions or main effects of line were observed for *DIO2* and

DIO3 in the breast muscle during embryonic development (**Figures 3.6C** and **D**; **Tables 3.3** and **3.4**). However, an age effect was noted for both genes; in both legacy and modern birds, their expression levels were consistent from e10 to e12 but decreased afterwards at each age until e18 (**Figure 3.6C** and **D**; **Tables 3.3** and **3.4**; P≤0.05). The expression of *DIO1* was not detected in the breast muscle during this period, which is consistent with previous observations [269].

Post-hatch juvenile development

Gene expression

Expression levels of *THRA* and *THRB* mRNA in the liver did not exhibit line-by-age effects or main effects of line between d10 and d40 (**Figure 3.7A** and **B**). However, both demonstrated main effects of age (**Table 3.6**; P≤0.05). Hepatic *THRA* increased in both lines between d10 and d20, decreased on d30, and increased again on d40 (**Figure 3.7A**; **Table 3.6**; P≤0.05). The expression of *THRB* in ACRB and Ross liver decreased between d10 and d20 but recovered to d10 levels on d30 and before further increasing to the highest levels on d40 (**Figure 3.7B**; **Table 3.6**; P≤0.05). A significant interactive effect or main effect of age was not observed for either *THRA* or *THRB* in the breast muscle (**Figure 3.7C** and **D**; **Tables 3.5** and **3.6**). However, *THRA* exhibited a main effect of line, whereas overall expression was greater in ACRB (**Figure 3.7C**; **Table 3.5**; P≤0.05).

No significant line-by-age interactions or main effects of line or age were observed for hepatic *DIO1* expression (**Figure 3.8A**; **Tables 3.5** and **3.6**). However, significant line-by-age effects were exhibited for *DIO2* and *DIO3* (**Figure 3.8B** and **C3**; P≤0.05). Expression of *DIO2* in Ross liver was approximately 5-fold higher than in ACRB liver on d10 but decreased to one-fourth of ACRB expression levels on d20 (**Figure 3.8B**; P≤0.05). Hepatic *DIO3* expression was greater in ACRB than Ross on d30 and d40 (**Figure 3.8C**; P≤0.05). In breast muscle, *DIO1* expression was not detected at any age post-hatch. Only a main effect of age was observed for *DIO2*

expression in breast muscle, in which expression decreased between d10 and d20, rose to d10 levels on d30, and remained elevated on d40 (**Figure 3.8D**; **Table 3.6**; P≤0.05). However, *DIO3* mRNA did exhibit a significant line-by-age effect, in which expression was 2-fold greater in Ross than ACRB on d20 (**Figure 3.8E**; P≤0.05), in part due to an apparent developmental delay in the increase in expression in legacy birds.

Circulating thyroid hormones

For both THs, no significant line-by-age effects were observed (**Figure 3.9**). Circulating T_3 exhibited main effects of both line and age **(Figure 3.9A**; **Tables 3.5** and **3.6**; P≤0.05). Overall, Ross T_3 levels were lower than those in ACRB (**Table 3.5**; P≤0.05), and plasma T_3 was 2.5-fold lower on d20 than on other ages (**Figure 3.9A**; **Table 3.6**; P≤0.05). Levels of T_4 did not display a main effect of line but did exhibit a main effect of age, in which they increased in both lines approximately 2-fold between d10 and d20 and remained elevated on d30 and d40 (**Figure 3.9B**; **Table 3.6**; P≤0.05).

Discussion

Genetic selection for economically valuable traits has been an essential tool used to improve the efficiency of poultry production on a global scale [387, 388] and has likely affected hormonal systems controlling growth and metabolism, as has been observed in the dairy, beef, and pork industries [388-392]. Thus, it is important to investigate the impact of commercial genetic selection on broiler endocrine systems, as this could provide additional information regarding markers to use in genetic selection programs as well as targets for alternative strategies to enhance meat production efficiency. The present study examined the activity of adrenocorticotropic and thyrotropic axes in modern and legacy male broilers during embryonic and juvenile development to identify how these endocrine systems may have been affected by commercial selection. The

results suggest that decades of selection have altered aspects of both axes in economically important tissues, contributing to the improvement in production characteristics.

Since only males were examined, some of the observed effects on gene expression and circulating hormone levels could be sex-specific and might differ in females, particularly as they approach sexual maturity. Differences in circulating CORT between sexes has been previously documented [393], and CORT [394] and THs [395] are known to affect pullet reproductive function. Understanding how selection has affected these axes in females should allow further advancements to be made that balance reproductive efficiency with improvements in growth efficiency. Further, as both lines were fed a modern commercial-type diet, it is possible that nutrient requirements of the ACRB birds were exceeded, and this may have contributed to some of the observed differences in gene expression and circulating hormones. However, given the substantial improvement in growth performance of modern birds over legacy birds when both lines were fed the same diet, as observed here and elsewhere [6], it is likely that many of the differences uncovered in this study are true physiological changes driven by selection.

The adrenocorticotropic axis regulates metabolism and energy use. Specifically, CORT signaling increases energy expenditure and redirects nutrient distribution between tissues such as muscle and adipose [38, 396]. The signaling action of CORT is mediated through NR3C1 and CBG. As such, we investigated expression of these genes to elucidate the potential sensitivity of liver and breast muscle cells to CORT, as well as circulating CORT levels in juvenile broilers. No line-by-age interactive effects were observed for *CBG* or *NR3C1* expression in either tissue across both experiments, suggesting that overall patterns of these genes during different phases of broiler development have not been impacted by genetic selection. However, Ross 308 manifested with lower CORT levels than ACRB, suggesting that modern broilers have more efficient energy

utilization and storage than their legacy counterparts. Physiological differences between lines induced by the adrenocorticotropic axis are likely tied to circulating CORT levels in tandem with signaling regulation. Reductions in weight gain, body weight, and FCR in chickens treated with CORT are well documented, and these effects cannot be compensated for by a high-energy diet [385, 386, 397]. Skeletal muscle growth in 28-day old broilers was depressed by CORT treatment due to reduced protein synthesis and increased protein turnover [258, 373]. Elevated CORT levels have also been linked to reduced chondrocyte proliferation and long bone growth [259, 398]. Thus, the higher body weights and larger skeletons of modern broilers may be caused, in part, by reduced adrenocorticotropic axis activity that allows for increased protein synthesis and bone growth.

Though significant differences in expression of *CBG* between the lines were not observed, higher levels of plasma CORT in ACRB birds might suggest proportional differences in CBG bound- versus free CORT between the lines. More specifically, fractionally greater unbound CORT in ACRB plasma might raise CORT signaling levels as *CBG* expression does not increase to compensate [399]. Expression of *NR3C1* post-hatch was highest on d40 in both the liver and breast muscle, when circulating CORT was lowest in both lines. Others have shown that hepatic *NR3C1* negatively correlates with circulating CORT [400]. Greater *NR3C1* mRNA levels may indicate heightened tissue sensitivity to CORT in the face of decreased plasma hormone levels, as increased mRNA could be indicative of increased NR3C1 protein levels. It is necessary to maintain a certain degree of CORT sensitivity when hormone concentrations are reduced, as the animal must maintain homeostatic balance of glucocorticoid signaling so the body can respond to short-term stressors and reallocate nutrients appropriately [401].

The thyrotropic axis is important in the context of genetic selection of broilers due to its roles in thermoregulation, basal metabolism, and bone and muscle growth [49, 50], and a genomic

region important for chicken domestication that contains THSR, an important regulator of TH secretion, has been identified [374]. Increased metabolic rate and restriction of long bone development limit muscle accumulation and therefore could impact meat yield and quality. The availability of TH's is unique across tissues and dependent on local deiodinase activity [268]. Therefore, a multifaceted investigative approach, achieved here by determining circulating THs alongside expression of THRs and DIOs, is valuable when studying the effect of genetic selection on thyrotropic axis activity and how it might contribute to improved production efficiency in modern broilers. Maintenance of basal metabolism by T_3 is facilitated by THR-mediated transcriptional regulation, which becomes possible due to ligand-induced conformational changes of the bound receptor [402, 403]. Multiple isoforms have been identified for each receptor [404], although THRA isoform 1 has the greatest affinity for T_3 [280]. The isoforms of THRB also modulate gene expression, alongside maintaining the TH negative feedback loop when bound to T_3 [405, 406]. Expression of post-hatch *THRA* mRNA was elevated in ACRB muscle at all ages throughout this study. This could lead to increased energy expenditure as heat loss in this metabolically active tissue, thus greater FCR. Alternatively, THRs have been demonstrated to exhibit thyroid-hormone independent down-regulation of gene expression [407-410], so increased expression in the breast muscle of ACRB birds may serve to downregulate genes associated with muscle cell proliferation and differentiation. Hepatic *THRB* expression was lower in Ross 308 on e12. A THRB homolog with a predicted T_3 binding site has been previously identified in chicken and may function similarly to THRA in this developmental context [411]. Thus, reduced *THRB* in Ross could result in dampened expression of THR-regulated genes in the liver or induce a weaker negative feedback response, maintaining TH synthesis and production.

The effects of THs are typically regulated by means of the deiodinase enzymatic activity, which can control the bioactive levels of TH in tissue. The relevance of tissue-specific TH signaling can be further understood in the context of deiodinase activity. In the breast muscle of embryos from both lines, expression of *DIO2* and *DIO3* declined towards hatch. This occurs alongside known downregulation of pituitary thyroid-stimulating hormone (TSH) expression and secretion [62, 412, 413]. As TSH induces production of THs into the blood, reducing *DIO2* and *DIO3* expression, and consequently, enzyme activity could serve to maintain baseline TH signaling as T_4 conversion to T_3 and T_3 conversion to T_2 are decreased, respectively.

Tissue-specific deiodinase activity can contribute to both endocrine and paracrine TH activity, in which the liver is thought to primarily control circulating TH availability while the muscle is thought to modulate local TH action in that tissue. While DIO1 and 2 typically convert T_4 to T_3, activating TH signaling, DIO3 inactivates T_3 by converting it to chemically inert thyronine (T_2) and T_4 by converting it to rT_3 [274, 414-416]. Significant line-by-age interactive effects were observed for hepatic *DIO3* expression in both experiments and for *DIO2* in the liver after hatch, suggesting that genetic selection may have contributed to broad developmental differences in regulating bioactivity of TH via endocrine action. Expression of *DIO3* was greater in Ross 308 liver during embryogenesis but was reduced on d30 and d40 as compared to ACRB. This suggests that endocrine T_3 deactivation mediated by *DIO3* is delayed during ACRB development, whereas this could occur much earlier the Ross 308. This could lead to a reduction in T_3 just prior to hatch in modern broilers as compared to their legacy counterparts, contributing to the difference in body weight that occurs beginning on e14 and allowing for rapid growth after hatch.

Hepatic expression of *DIO2* was greater in Ross on d10 but this pattern was reversed on d20 when expression became significantly greater in ACRB. Hepatic DIO2 activity should contribute to the balance of circulating THs. Circulating T_4 increased and T_3 decreased on d20 in both lines. While *DIO2* expression in Ross liver was reduced at this age, potentially leading to reduced conversion of T_4 to T_3, hepatic *DIO2* in ACRB did not exhibit a similar decrease on d20, and this appears contradictory to observed circulating T_3 levels. However, expression of *DIO2* decreased in breast muscle of both lines on d20, suggesting that decreased *DIO2* in ACRB breast muscle tissue might contribute to the drop in plasma T_3 on that age in legacy broilers. The deiodinases have unique expression profiles throughout the body, allowing for tight control of local TH signaling[417]. The results above could suggest that in Ross birds, T_3 produced in the breast muscle is free to signal in a paracrine fashion and promote local muscle growth, while in ACRB more of this T_3 is released as an endocrine signal, resulting in less paracrine activity and reduced breast muscle development. This suggests that the tissue-specific expression patterns of the DIOs, and therefore their local function, have been altered by genetic selection in Ross broilers. Thus, metabolic activity mediated by the THs can occur via endocrine maintenance of plasma THs or tissue-specific paracrine control of their action. These modes of TH signaling would appear to have changed due to genetic selection to allow for enhanced muscle accretion in modern broilers.

Circulating plasma T_3 levels can be a biological indicator of metabolic rate and therefore energy consumption [320]. Legacy juveniles had greater plasma T_3 levels than Ross 308. This indicates that lower TH signaling may exist in modern broilers, possibly contributing to more productive growth. For example, T_3 inhibits chondrocyte proliferation and expansion of the bone growth plates [312]. Restriction of growth plate development prevents bones from elongating while muscular dystrophy induced by T_3 reduces muscle growth [313], both of which likely

contribute to the small size of and lower body weights of legacy broilers. With higher T_3, more energy is also lost as heat [314], which prevents it from being accumulated as muscle or adipose. Increased *DIO3* expression observed in juvenile hepatic ACRB tissue may be a result of lower GH sensitivity in this slower-growing line [3], as it is understood that GH decreases hepatic DIO3 [271]. Increased DIO3 might also be required to manage higher circulating T_3 in ACRB birds. Taken together, these findings indicate that genetic selection may have altered the concentration and activity of circulating THs by enhancing or reducing expression of their nuclear receptors and regulatory proteins, which coalesce into decreased T_3 levels in the Ross broilers. In turn, the BMR of modern broilers may have been lowered, ultimately resulting in improved efficiency of feed nutrient use in terms of energy stored as muscle or bone growth, as reflected in a reduced FCR.

In summary, we found that the concentration of circulating hormones and expression levels of genes belonging to the adrenocorticotropic and thyrotropic axes differed between male legacy and modern broilers. Glucocorticoid signaling is likely reduced in Ross 308 due to lower CORT levels in the line. Additionally, differences in post-hatch expression of *THRA*, *DIO2*, and *DIO3* between the lines implicate these genes in affecting broiler metabolism by controlling tissue-specific T_3 availability, potentially making these genes targets for marker-assisted selection by industry breeders or other novel strategies to improve broiler production. This research illustrates the importance of understanding functional roles of endocrine systems on bird growth and metabolism and provides targets within these systems that may be utilized to further enhance broiler production efficiency.

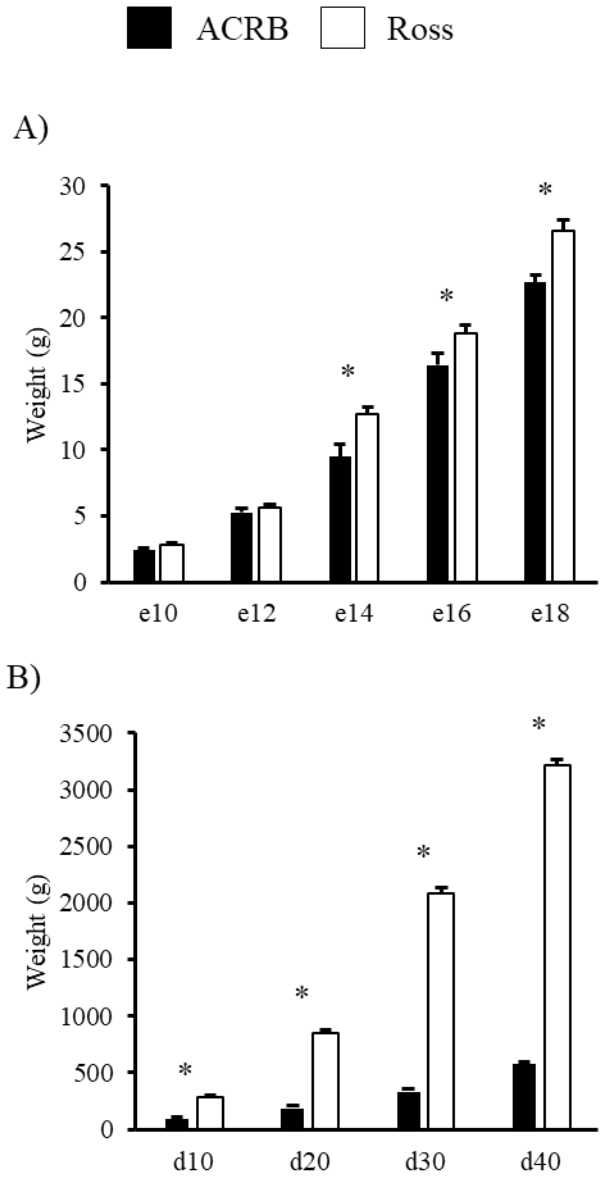

Figure 3.1. Body weights (g) of legacy ACRB and modern Ross 308 male broilers on (A) embryonic days (e) 10, 12, 14, 16, and 18 and (B) post-hatch days (d) 10, 20, 30, and 40. Significant line-by-age interactions were detected, and the presence of an asterisk (*) indicates a significant difference in expression between the lines at indicated ages during embryogenesis ($P \leq 0.05$; n=4 replicate birds per line per age) or juvenile development ($P \leq 0.05$; n=8 replicate birds per line per age).

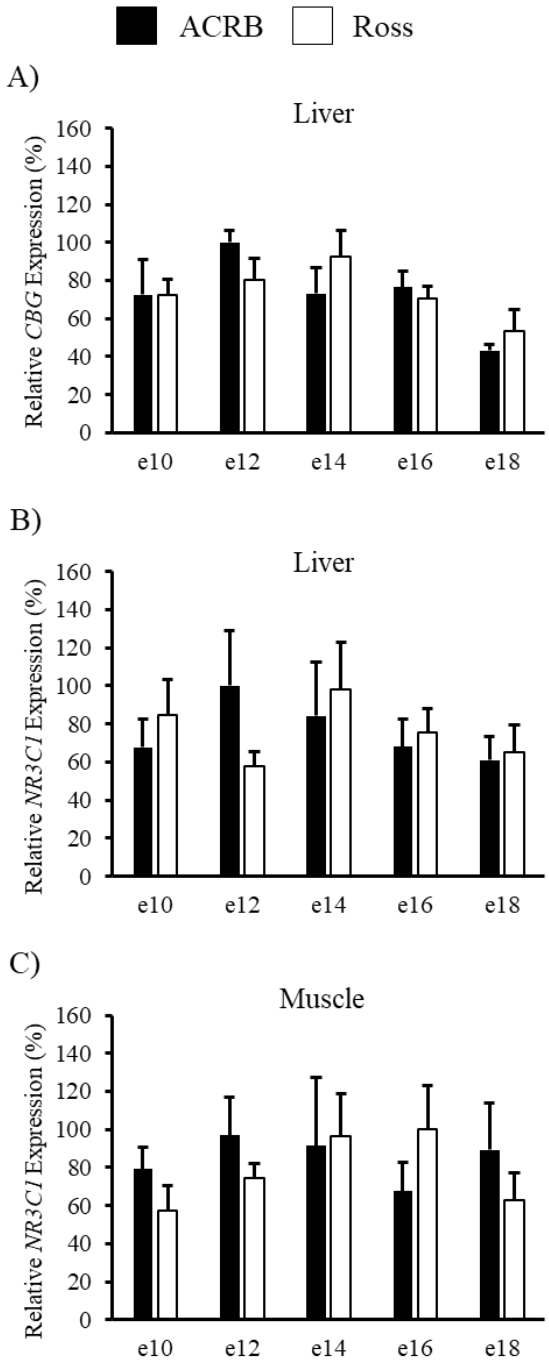

Figure 3.2. Relative mRNA expression of (A) *CBG* in liver, (B) *NR3C1* in liver, and (C) *NR3C1* in breast muscle on embryonic days (e) 10, 12, 14, 16, and 18 in legacy ACRB and modern Ross 308 male broilers. Expression of *CBG* in breast muscle was undetected. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* in liver and *18S* RNA in breast muscle (n=4 replicate birds per line per age). The data (mean + SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). No significant line-by-age interactive effects were observed for (A) *CBG* in liver (P=0.5899), (B) *NR3C1* in liver (P=0.1031), and (C) *NR3C1* in breast muscle (P=0.4645), and main effect means for line and age for all genes are presented in Tables 3.5 and 3.6, respectively.

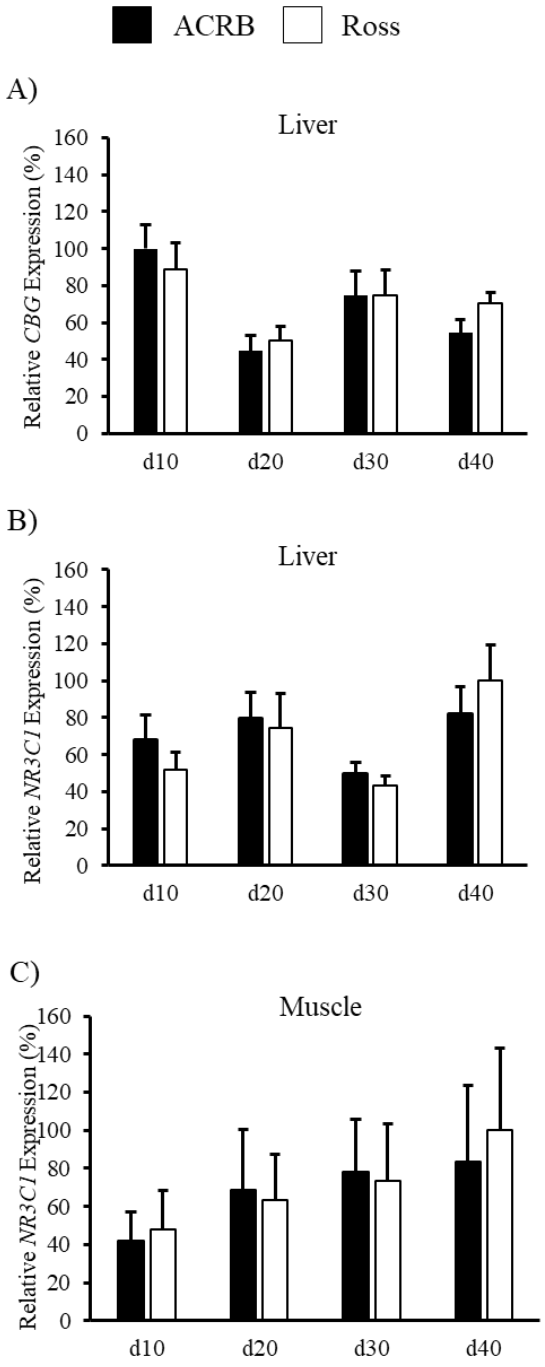

Figure 3.3. Relative mRNA expression of (A) *CBG* in liver, (B) *NR3C1* in liver, and (C) *NR3C1* in breast muscle on post-hatch days (d) 10, 20, 30, and 40 in legacy ACRB and modern Ross 308 male broilers. Expression of *CBG* in breast muscle was undetected. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* in liver and *18S* RNA in breast muscle (n=8 replicate birds per line per age). The data (mean + SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). No significant line-by-age interactive effects were observed for (A) *CBG* in liver (P=0.2563), (B) *NR3C1* in liver (P=0.4312), and (C) *NR3C1* in breast muscle (P=0.8862), and main effect means for line and age for all genes are presented in Tables 3.7 and 3.8, respectively.

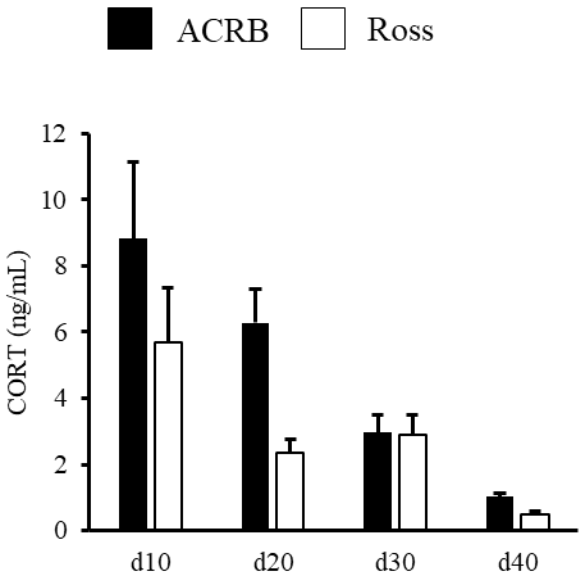

Figure 3.4. Circulating CORT concentrations in legacy ACRB and modern Ross 308 male broilers on post-hatch days (d) 10, 20, 30, and 40. No significant line-by-age interactive effects were observed (P=0.1889), and main effect means of line and age for all genes are presented in Tables 3.7 and 3.8, respectively.

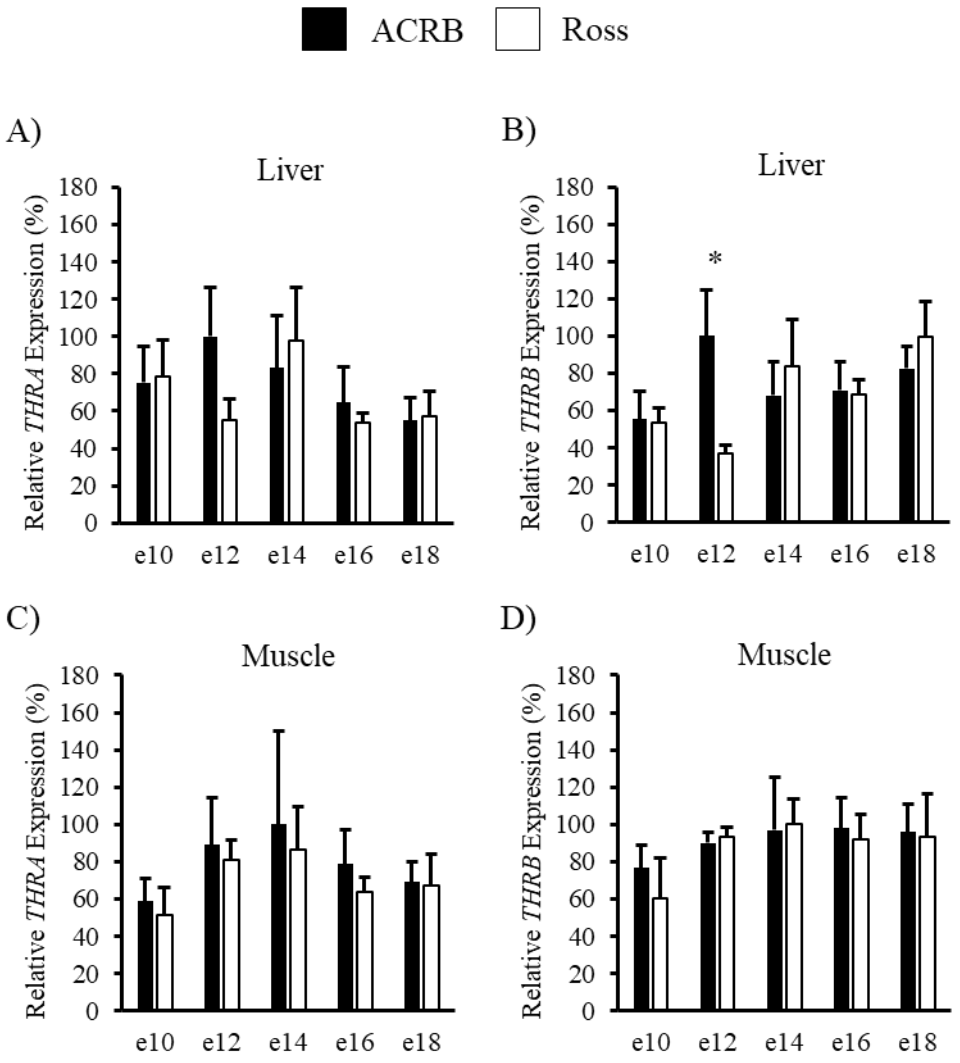

Figure 3.5. Relative mRNA expression of (A) *THRA* in liver, (B) *THRB* in liver, (C) *THRA* in breast muscle, and (D) *THRB* in breast muscle on embryonic (e) days 10, 12, 14, 16, and 18 in legacy ACRB and modern Ross 308 male broilers. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* in liver and *18S* RNA in breast muscle (n=4 replicate birds per line per age). The data (mean + SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). A significant line-by-age interaction was detected for (B) *THRB* in liver (P=0.0317), and the presence of an asterisk (*) indicates a significant difference in expression between the lines at the indicated age (P≤0.05; n=4). No significant interactive effects were detected for (A) *THRA* in liver (P=0.0788), (C) *THRA* in breast muscle (P=0.09919), and (D) *THRB* in breast muscle (P=0.7739). For genes with no significant interactive effects, main effects means of line and age are presented in Tables 3.5 and 3.6, respectively.

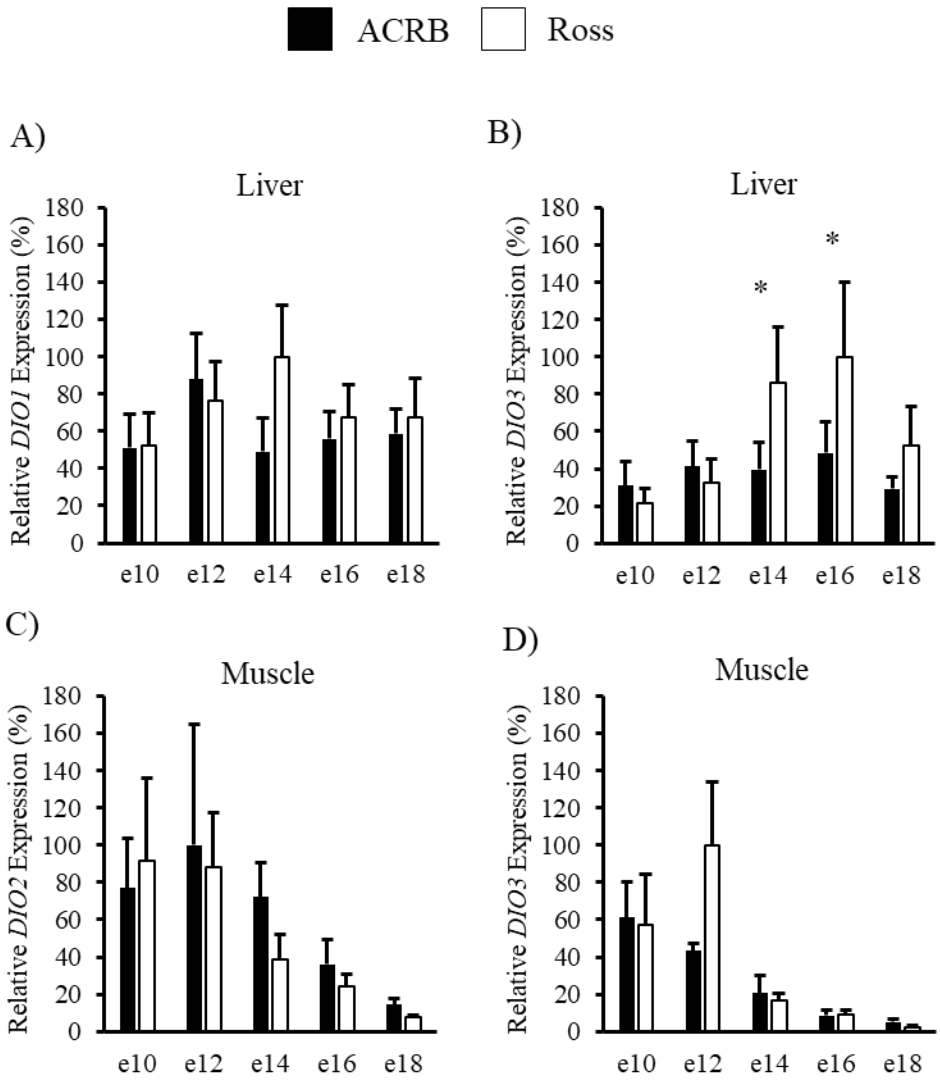

Figure 3.6. Relative mRNA expression of (A) *DIO1* in liver, (B) *DIO3* in liver, (C) *DIO2* in breast muscle, and (D) *DIO3* in breast muscle on embryonic days (e) 10, 12, 14, 16, and 18 in legacy ACRB and modern Ross 308 male broilers. Expression of *DIO2* in liver and *DIO1* in breast muscle was undetected. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* in liver and *18S* RNA in breast muscle (n=4 replicate birds per line per age). The data (mean + SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). A significant line-by-age interaction was detected for (B) *DIO3* in liver (P=0.008), and the presence of an asterisk (*) indicates a significant difference in expression between the lines at the indicated ages (P≤0.05). No significant interactive effects were detected for (A) *DIO1* in liver (P=0.3060), (C) *DIO2* in breast muscle (P=0.6474), and (D) *DIO3* in breast muscle (P=0.3426). Main effect means of line and age are presented in Tables 3.5 and 3.6, respectively.

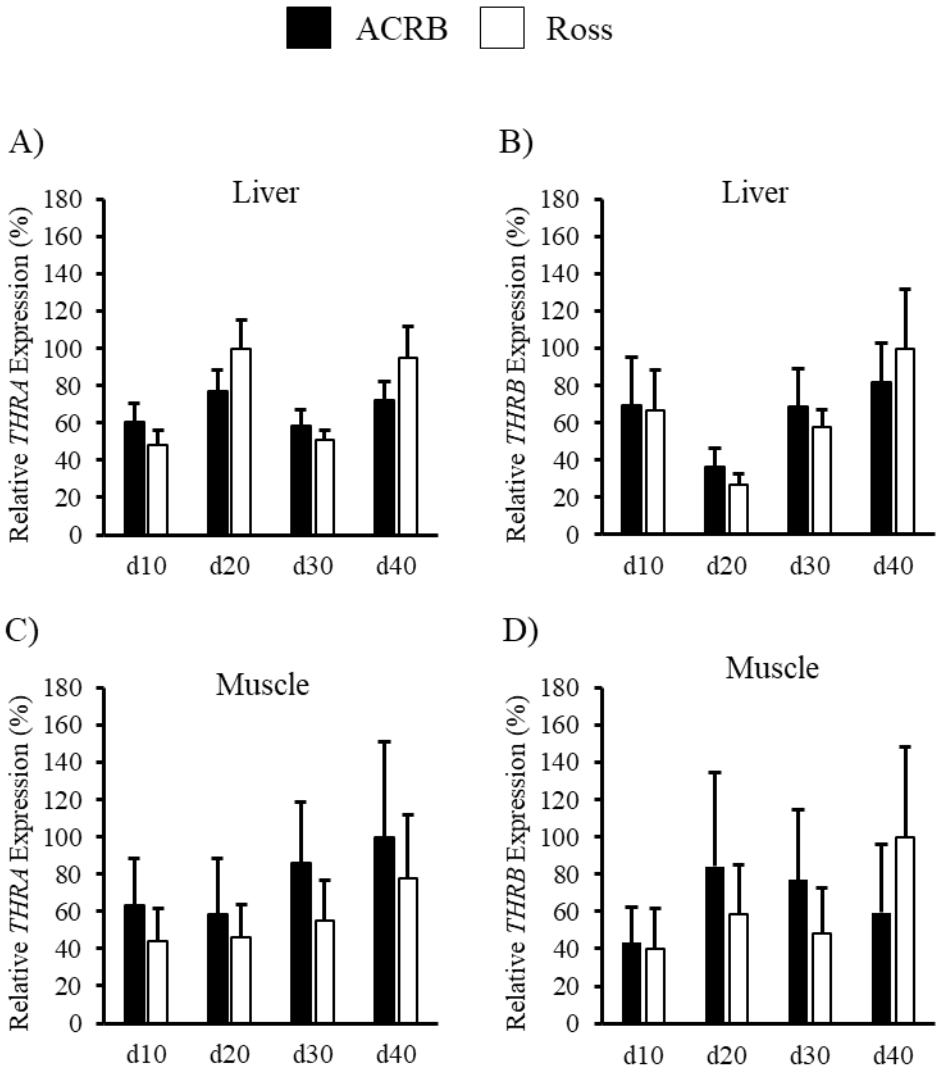

Figure 3.7. Relative mRNA expression of (A) *THRA* in liver, (B) *THRB* in liver, (C) *THRA* in breast muscle, and (D) *THRB* in breast muscle on post-hatch days (d) 10, 20, 30, and 40 in legacy ACRB and modern Ross 308 male broilers. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* in liver and *18S* RNA in breast muscle (n=8 replicate birds per line per age). The data (mean + SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). No significant line-by-age interactive effects were observed for (A) *THRA* in liver (P=0.1022), (B) *THRB* in liver (P=0.6003), (C) *THRA* in breast muscle (P=0.8057), and (D) *THRB* (P=0.7034) in breast muscle, and main effect means of line and age for all genes are presented in Tables 3.7 and 3.8, respectively.

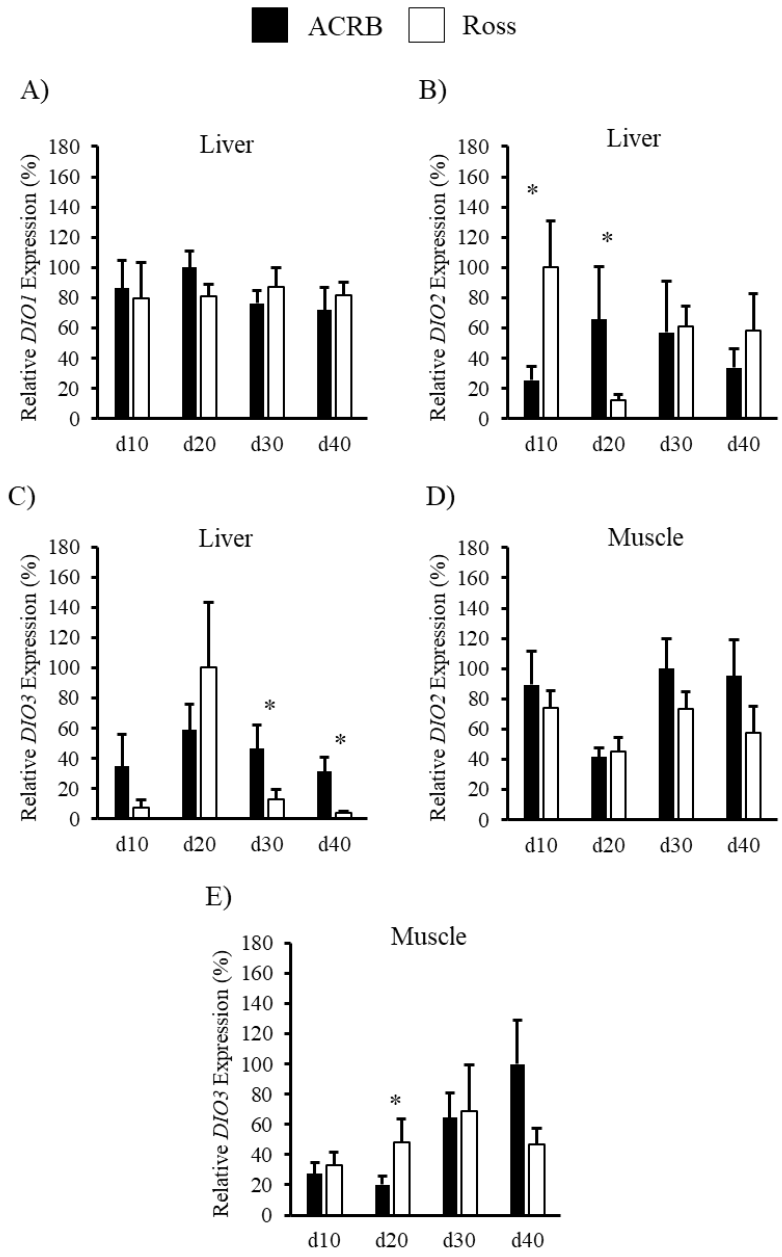

Figure 3.8. Relative mRNA expression of (A) *DIO1* in liver, (B) *DIO2* in liver, (C) *DIO3* in liver, (D) *DIO2* in breast muscle, and (E) *DIO3* in breast muscle on post-hatch days (d) 10, 20, 30, and 40 in legacy ACRB and modern Ross 308 male broilers. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* in liver and *18S* RNA in breast muscle (n=8 replicate birds per line at each age). The data (mean + SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). No significant line-by-age interactions were detected for (A) *DIO1* (P=0.4071) in liver, and main effects for line and age for these genes were presented in Tables 3.7 and 3.8, respectively. Significant line-by-age interactions were detected for (B) *DIO2* (P=0.022) and (C) *DIO3* (P=0.021) in liver, and (E) *DIO3* (P=0.058) in breast muscle, and the presence of an asterisk (*) indicates a significant difference in expression between the lines at indicated ages (P≤0.05).

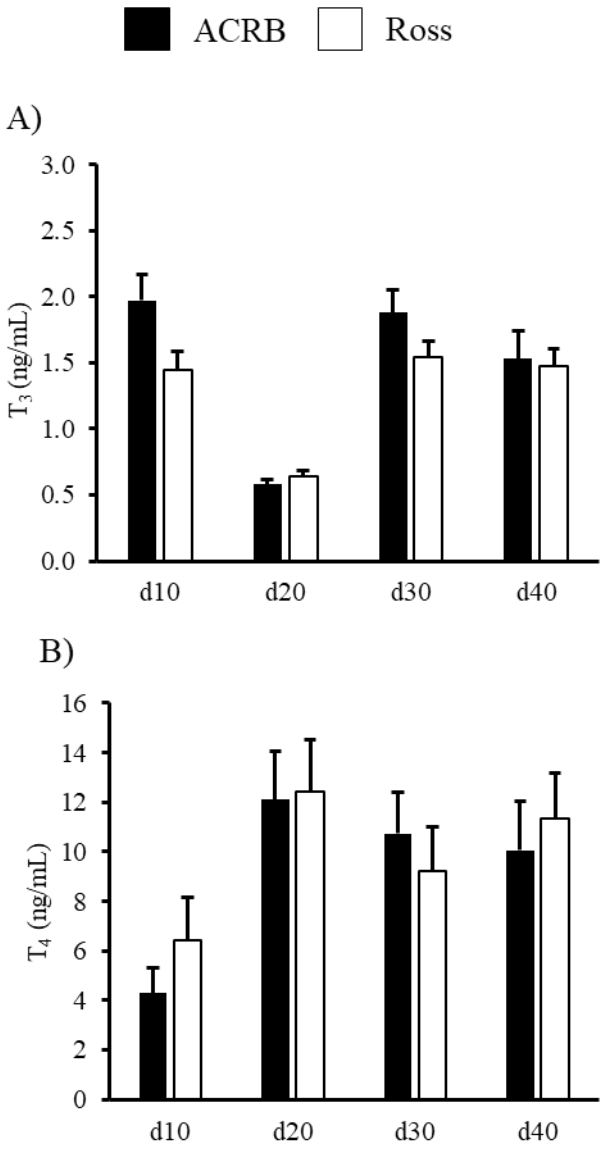

Figure 3.9. Circulating (A) T_3 and (B) T_4 in legacy ACRB and modern Ross 308 male broilers on post-hatch days (d) 10, 20, 30, and 40 as determined by T_3 and T_4 RIA (n=8 replicate birds per line at each age). No significant line-by-age interactive effects were observed for (A) T_3 (P=0.1895) or (B) T_4 (P=0.7638), and main effect means of line and age for all hormones are presented in Tables 3.7 and 3.8, respectively.

Table 3.1. Primers used for reverse transcription-quantitative PCR.

Gene Symbol	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Transcript ID[a]**	**Primer Efficiency**
Hormone receptors				
NR3C1	TCGTGAAAAGAGAAGACTCA	AAAAACGTCTGGAAGCAAAAGC	80964	1.06
THRA	CTTCAACCTGGACGACACC	ACGTCTCCTGGCACTTCTCT	52263	1.12
THRB	ACCTGGGCATGTCTCTTTCT	CAGGAGGAAACCCTCTTGAC	31162	1.12
Hormone binding proteins				
CBG[b]	GGATTGGCACATTTGACTTG	TGCTGGCAGAATTAGAAACG		0.93
Deiodinases				
DIO1	CGAAGAAGCTCACGCAGTAG	TCCTCAAGGCTTCTGTGATTT	17472	1.09
DIO2	TGGAACAGCTTCCTCCTGG	TATTGCTGCCATCATTGCCC	94775	1.06
DIO3[b]	CCTCATCCTCAACTTCGG	GATGTACACCAGCAGGAA		1.03
Reference genes				
GAPDH	AGCCATTCCTCCACCTTTGAT	AGTCCACAACACGGTTGCTGTAT	23323	1.02
18s[b]	AGCCTGCGGCTTAATTTGAC	CAACTAAGAACGGCCATGCA		0.96

[a]Transcript identification from Ensembl chicken genome assembly GRCg6a (http://www.ensembl.org/Gallus_gallus/Info/Index) preceded by ENSALGT000000.
[b]These sequences are not on the assembled chicken genome and primers were designed based on sequences in GenBank with the following accession numbers: *CBG* – KU180444; *DIO3* – NM_001122648; *18S* – AF173612.

Table 3.2. Feed intake (FI), body weight gain (BWG), feed conversion ratio (FCR; g FI/f BWG), and final body weight (BW) of ACRB and Ross 308 broilers.

	d7 – d42 FI (g)	**d7 – d42 BWG (g)**	**d7 – d42 FCR**	**d42 BW (g)**
ACRB	2197.2±103.3[b]	586.1±11.6[b]	3.8±0.2[a]	652.5±12.7[b]
Ross	4949.1±92.3[a]	3154.9±47.9[a]	1.6±0.02[b]	3327.6±48.5[a]

[abc]Values that do not share a common letter are significantly different ($P \leq 0.05$; n=8).

Table 3.3. Main effect means[1] (±SEM) of line for gene expression in embryonic male broilers when a line-by-age interaction was not present.

	ACRB	Ross 308	P-value
Corticotropic Axis (Figure 2)			
Liver			
CBG	99.1±8.8	100±9.9	0.6666
NR3C1	99.9±11.1	100±13.1	0.6410
Muscle			
NR3C1	100±10.7	93.4±11.8	0.6072
Thyrotropic Axis (Figures 5 and 6)			
Liver			
THRA	100±12.4	90.6±10.6	0.5668
DIO1	83.5±10.8	100±12.7	0.2404
Muscle			
THRA	100±13.1	88.7±8.6	0.5681
THRB	100±7.1	94.9±7.8	0.5360
DIO2	100±27.3	89.9±22.3	0.3277
DIO3	67.5±15.3	100±30.6	0.7934

[1]Main effects are expressed relative to the highest line (100%).
[abc]Values that do not share a common letter are significantly different (P≤0.05).

Table 3.4. Main effect means[1] (±SEM) of age for gene expression in embryonic male broilers when a line-by-age interaction was not present.

	e10	e12	e14	e16	e18	P-value
Corticotropic Axis (Figure 2)						
Liver						
CBG	80.1±13.5[a]	100±13.8[a]	81.5±14.8[a]	72.3±10.9[a]	47.6±8.7[b]	<0.0077
NR3C1	94.1±17.2	97.2±21.7	100±24.2	78.6±14.6	69.2±13.7	0.3673
Muscle						
NR3C1	77.9±13.6	100±16.8	96.4±22.9	87.0±19.2	79.7±19.2	0.8077
Thyrotropic Axis (Figures 5 and 6)						
Liver						
THRA	84.9±14.1	85.6±17.5	100±20.8	65.2±10.3	61.9±9.2	0.0528
DIO1	62.9±14.3	100±18.6	90.9±21.9	75.3±13.0	76.9±14.3	0.2810
Muscle						
THRA	59.3±9.1	92.2±13.9	100±23.1	77.3±10.6	74.1±8.8	0.3589
THRB	68.1±12.3	92.9±3.7	100±12.2	96.3±10.1	95.9±11.4	0.1734
DIO2	90.9±25.6[a]	100±35.1[a]	56.1±12.1[ab]	32.1±7.6[b]	12.3±2.1[c]	0.0003
DIO3	82.2±21.3[a]	100±26.7[a]	25.8±5.3[b]	12.4±2.1[c]	5.7±1.1[d]	<0.0001

[1]Main effects of age are expressed relative to the age with the highest expression level (100%).
[abc]Values that do not share a common letter are significantly different (P≤0.05).

Table 3.5. Main effect means[1] (±SEM) of line for gene expression and circulating hormones in juvenile male broilers when a line-by-age interaction was not present.

		ACRB	Ross 308	P-value
Corticotropic Axis (Figure 3)				
Liver				
	CBG	100±8.2	97.9±9.3	0.3657
	NR3C1	100±9.2	96.4±11.4	0.4568
Muscle				
	NR3C1	93.8±19.7	100±21.2	0.7071
CORT		4877.6±860.8[a]	2830.3±576.3[b]	0.0135
Thyrotropic Axis (Figures 7 and 8)				
Liver				
	THRA	91.2±6.0	100±8.8	0.6385
	THRB	100±14.0	98.1±14.8	0.7983
	DIO1	98.5±7.6	100±7.3	0.9995
Muscle				
	THRA	100±19.2[a]	72.9±13.5[b]	0.0034
	THRB	100±23.4	95.2±21.6	0.6765
	DIO2	77.9±6.7	100±10.9	0.3570
T_3		1.5±0.1[a]	1.3±0.1[b]	0.0428
T_4		9.3±1.0	9.9±0.9	0.6579

[1]Main effects are expressed relative to the highest line (100%).
[abc]Values that do not share a common letter are significantly different (P≤0.05).

Table 3.6. Main effect means[1] (±SEM) of age for gene expression and circulating hormones in juvenile male broilers when a line-by-age interaction was not present.

	d10	d20	d30	d40	P-value
Corticotropic Axis (Figures 3 and 4)					
Liver					
CBG	100±9.9[a]	47.4±6.4[c]	78.9±9.8[b]	62.5±6.5[b]	<0.0001
NR3C1	65.7±8.9[bc]	84.4±12.7[ab]	51.1±4.4[c]	100±13.0[a]	0.0017
Muscle					
NR3C1	48.3±13.1[b]	71.2±20.5[a]	82.0±21.4[a]	100±31.1[ab]	0.0193
CORT	7870.2±1455.9[a]	4101.2±744.2[b]	2670.8±392.9[bc]	763.7±110.7[c]	<0.0001
Thyrotropic Axis (Figures 7 - 9)					
Liver					
THRA	61.5±7.1[b]	100±10.9[a]	61.9±5.69[b]	94.7±11.0[a]	<0.0001
THRB	74.8±17.9[b]	34.9±5.9[c]	69.4±12.0[b]	100±20.4[a]	<0.0001
DIO1	91.7±16.3	100±7.9	90.5±8.3	85.0±9.2	0.3659
Muscle					
THRA	61.9±16.8	58.8±18.7	80.1±22.8	100±33.0	0.1485
THRB	51.5±16.4	86.7±33.2	77.2±28.1	100±37.3	0.3343
DIO2	93.9±12.9[a]	49.9±5.9[b]	100±13.8[a]	89.8±17.9[ab]	0.0249
T_3	1.7±0.1[a]	0.6±0.0[b]	1.7±0.1[a]	1.5±0.1[a]	<0.0001
T_4	5.4±1.0[b]	12.2±1.4[a]	9.9±1.2[a]	10.7±1.3[a]	0.0029

[1]Main effects are expressed relative to the highest age (100%).
[abc]Values that do not share a common letter are significantly different (P≤0.05).

CHAPTER 4

THE EFFECT OF GENETIC SELECTION ON SOMATOTROPIC GENE EXPRESSION IN COMMERCIAL MODERN BROILERS: A POTENTIAL ROLE FOR INSULIN-LIKE BINDING PROTEINS IN REGULATING BROILER GROWTH & BODY COMPOSITION[1]

[1]Vaccaro, L.A., T.E. Porter, and L.E. Ellestad. 2022. *Frontiers in Physiology* 13:935311
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Abstract

The somatotropic axis influences growth and metabolism, and many of its effects are a result of insulin-like growth factor (IGF) signaling modulated by IGF-binding proteins (IGFBPs). Modern commercial meat-type (broiler) chickens exhibit rapid and efficient growth and muscle accretion resulting from decades of commercial genetic selection, and it is not known how alterations in the IGF system has contributed to these improvements. To determine the effect of genetic selection on somatotropic axis activity, two experiments were conducted comparing legacy Athens Canadian Random Bred and modern Ross 308 male broiler lines, one between embryonic days 10 and 18 and the second between post-hatch days 10 and 40. Gene expression was evaluated in liver and breast muscle (Pectoralis major) and circulating hormone concentrations were measured post-hatch. During embryogenesis, no differences in IGF expression were found that corresponded with difference in body weight between the lines beginning on embryonic day 14. While hepatic IGF expression and circulating IGF did not differ between the lines post-hatch, expression of both IGF1 and IGF2 mRNA was greater in breast muscle of modern broilers. Differential expression of select IGFBPs suggests their action is dependent on developmental stage and site of production. Hepatic IGFBP1 appears to promote embryonic growth but inhibit post-hatch growth at select ages. Results suggest that local IGFBP4 may prevent breast muscle growth during embryogenesis but promote it after hatch. Post-hatch, IGFBP2 produced in liver appears to inhibit body growth, but IGFBP2 produced locally in breast muscle facilitates development of this tissue. The opposite appears true for IGFBP3, which seems to promote overall body growth when produced in liver and restrict breast muscle growth when produced locally. Results presented here suggest that paracrine IGF signaling in breast muscle may contribute to overall growth and muscle accretion in chickens, and

that this activity is regulated in developmentally distinct and tissue-specific contexts through combinatorial action of IGFBPs.

Introduction

Growth and body composition in vertebrates are controlled by several highly conserved endocrine axes [69, 418]. In particular, the somatotropic axis is known to regulate growth and development of mammals via cellular proliferation and metabolic effects in muscle, bone, and adipose tissue [9, 10]. However, its physiological impact on these processes is not as well understood in birds. Particularly lacking is information regarding how local production of insulin-like growth factor (IGF) 1 and IGF2 in tissues such as muscle impacts growth and body composition and how IGF-binding proteins (IGFBPs) regulate both endocrine and paracrine IGF signaling.

The key effector hormones in the somatotropic axis include IGF1 and IGF2 [20], which are synthesized in the liver upon growth hormone receptor (GHR) activation [18, 19, 85, 133, 134]. A dwarf phenotype is observed in chickens deficient in GHR signaling [79-81], and this is partially caused by decreased hepatic IGF production [197]. On the cellular level, IGFs downregulate apoptosis while increasing cellular proliferation by binding the type 1 IGF receptor (IGFR1) [90, 91, 146]. This would imply a direct relationship between IGF signaling and growth in chickens, but studies have been inconclusive. Direct IGF1 administration did not stimulate growth in two to three week-old male chickens [187, 189] or four week-old females [188]. Increased hepatic *IGF1* mRNA expression has been observed in chickens selected for high body weight [93], but not consistently [94]. Similarly, fast-growing chickens had greater plasma IGF2 [199], but IGF2 did not induce weight gain when directly administered [200]. Studies investigating levels of growth hormone (GH), which is classically thought to induce IGF secretion from the liver, also yield results inconsistent with the idea that increased somatotropic activity always leads to increased growth. Pituitary GH expression was greater between three and seven weeks of age in male broilers with lower body weight as compared to those with a higher body weight [419], and the percentage

of GH-secreting cells in slow-growing chickens was greater at five weeks of age, though fast-growing embryos secreted more GH per hour [420]. Circulating GH was also found to be higher in chickens selected for egg production (layers) than those selected for meat production (broilers), despite layers growing slower and having lower body weights [421].

Cellular effects induced by IGF signaling are regulated by IGFBPs. These proteins are highly conserved across vertebrates [21, 96-99], although IGFBP6 has not been retained in birds. Growth modulation occurs when an IGFBP physically binds an IGF to enhance or reduce receptor affinity, extend the hormone's half-life, or alter its tissue specificity [103, 118]. For example, IGFBP1 inhibits protein synthesis in skeletal muscle [119], while IGFBP2 and IGFBP4 inhibit long bone growth [106, 120]. In myoblasts, IGFBP5 has a proliferative effect when bound to IGF1 but an inhibitory effect upon binding IGF2 [121]. Additionally, some IGFBPs can act independently. For example, IGFBP2 can upregulate apoptosis [104, 105], while IGFBP5 can enhance bone cell proliferation [106]. As both ligand-dependent and ligand-independent effects of IGFBPs are important in growth regulation, their actions may contribute to the enhanced growth and muscle accretion of commercial modern broiler chickens.

The commercial modern broiler is raised specifically for meat production has an increased growth rate, greater body weight, reduced feed conversion ratio (FCR; g feed intake/g body weight gain), and higher meat yields [1-6], all of which are the result of decades of artificial genetic selection by the poultry industry. A useful experimental model to investigate the impact of the somatotropic axis on broiler growth and body composition is the comparison of modern, commercially selected broilers with non-selected ones. Athens Canadian Random Bred (ACRB) legacy broilers are representative of slower-growing, lower body weight birds prior to the beginning of intensive commercial broiler selection [5, 66, 422]. Administration of a modern diet

to ACRBs reduced their FCR some but not to the point of a commercial broiler and did not increase growth or body weight [6], which makes them an ideal genetic control strain. In a recent study where ACRB were compared with Ross 308 commercial modern broilers to identify effects of genetic selection on the corticotropic and thyrotropic axes, it was reported that Ross 308 body weights were significantly greater than those for ACRB beginning during the last week of embryogenesis, and this difference continued throughout juvenile development [418]. FCR of ACRB was also significantly higher than of Ross 308, reflecting the improved efficiency of feed nutrient use in commercial modern broilers. Together, these results suggest that physiological changes induced by genetic selection begin to appear mid-embryogenesis. Given the conservation of the somatotropic axis across species and its importance in mediating tissue growth and development in mammals, it is likely that IGFs, their receptors, and IGFBPs are linked to improvements in commercial modern broiler growth efficiency. Therefore, the objective of this study was to determine the effect of genetic selection on mRNA expression and circulating hormone concentrations within the somatotropic axis by comparing these parameters between commercial modern Ross 308 and legacy ACRB broiler lines.

Materials & Methods

Animals and tissue collection

Samples used for this study were collected from male ACRB and Ross 308 broilers during the same two experiments described in a previously published study [418]. The first experiment was conducted during embryogenesis, and the second was conducted during post-hatch juvenile development. All experimental procedures using animals were conducted in accordance with University of Georgia and University of Maryland Institutional Animal Care and Use guidelines.

In the first experiment, skin, liver, and breast muscle (*P. major*) were collected from 12 embryos of each line on embryonic days (e) 10, 12, 14, 16, and 18, with e0 being the day eggs were placed in the incubator. All eggs were incubated under identical conditions. The sex of each embryo was determined by PCR analysis of the sexually dimorphic chromo-helicase-DNA binding protein [375] using genomic DNA extracted from skin tissue, as previously described [418]. Liver and breast muscle from four male embryos of each line at each age (n=4) were used for gene expression analysis as described below.

In the second experiment, males of each line were raised separately in floor pens (n=8 floor pens per line) under identical environmental conditions with free access to water and an identical three-phase diet typical for commercial modern broiler production. Liver, breast muscle (*P. major*), and plasma were collected from one bird per pen (n=8 per line) on post-hatch days (d) 10, 20, 30 and 40 as previously described [418]. Liver and breast muscle were used for gene expression analysis, while plasma was used to evaluate circulating hormone levels, as described below.

*Reverse transcription-quantitative P*CR (RT-qPCR)

Total RNA was isolated from liver and breast muscle using RNeasy Mini kits (Qiagen) with modifications for lipid-rich or fibrous tissues, respectively, and analyzed by RT-qPCR as previously described [418]. Briefly, total RNA (1 µg) was reverse transcribed with random hexamer primers (ThermoFisher, Waltham, MA) and M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA). Resulting cDNA was amplified by qPCR using intron-spanning primers (**Table 4.1**; Integrated DNA Technologies, Coralville, IA) designed with Primer Express software (Applied Biosystems, Foster City, CA). Serial dilutions of pooled liver and muscle cDNA were analyzed by qPCR to determine amplification efficiency for each primer pair, which was calculated using the following equation: efficiency = $(10^{(-1/\text{slope})}-1)$ [376, 377].

Transcripts in liver were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and those in muscle were normalized to 18s ribosomal rRNA (18s rRNA). The equation $(2^{\Delta Ct})_{target}/(2^{\Delta Ct})_{GAPDH\ or\ 18s}$, where $\Delta Ct = Ct_{no\ RT} - CT_{sample}$, was used to transform and normalize data as previously described [378-381, 418]. Each transcript's line-by-age interactive data are expressed relative to the line and age with the highest mRNA level, and main effect data are expressed relative to the line or age with the highest mRNA level. As a result, the line-by-age, line, or age value with the highest expression level was 100% in all cases.

IGF enzyme-linked immunosorbent assays (ELISAs)

Samples were analyzed in duplicate on a VICTOR3 Multilabel Plate Reader (Perkin Elmer, Waltham, MA) using commercially available competitive-binding ELISAs (Cusabio, Houston, TX) for IGF1 and IGF2, which have sensitivity limits of 125 and 62.5 pg/ml, respectively. ELISAs were performed according to manufacturer's instructions with the modification that plates were incubated for 18 h at 4^{o}C instead of 60 min at 37^{o}C after adding the standards or samples and biotinylated IGF. Intra and inter-assay coefficient of variations (CVs) for IGF1 ELISAs were determined to be 4.023 and 6.479, respectively. Intra and inter-assay coefficient of variations (CVs) for IGF2 ELISAs were determined to be 10.0 and 34.6, respectively.

Statistical analysis

Data were analyzed with a two-way analysis of variance (ANOVA) using the Fit Model Procedure of JMP Pro 14 (SAS Institute, Cary, NC). When ANOVA indicated a significant line-by-age effect, line effect, or age effect ($P \leq 0.05$), *post hoc* multiple means comparisons were performed using the test of least significant difference. Main effect means were only calculated and analyzed when there was not a significant interaction ($P > 0.05$).

Results

IGF and hormone receptor expression during embryonic development

Levels of mRNA for IGFs and somatotropic hormone receptors in embryonic ACRB and Ross liver are shown in **Figure 4.1**. Expression of *GHR* did not exhibit a significant line-by-age effect in embryonic liver (**Figure 4.1A**; P>0.05), but a near significant main effect of line was observed in which Ross 308 had elevated expression as compared to ACRB (**Table 4.2**; P=0.0640). A significant main effect of age for *GHR* was also detected in liver, with levels significantly and steadily increasing between e10 and e18 (**Table 4.3**; P≤0.05). No significant differences in expression between lines or at different ages were detected for liver *IGF1* during embryogenesis (**Figure 4.1B**; **Tables 4.2** and **4.3**; P>0.05). Significant line-by-age interactive effects were detected for *IGF2* and *IGFR1* in liver, however. *IGF2* was approximately 2-fold greater in Ross on e10 and e14, but a transient decrease in expression in Ross on e12 with a concomitant increase in ACRB expression resulted in reduced levels of Ross *IGF2* at this age (**Figure 4.1C**; P≤0.05). A similar though less prominent expression pattern was observed for liver *IGFR1*, with levels in ACRB being approximately two-fold greater than Ross on e12 (**Figure 4.1D**; P≤0.05).

As shown in **Figure 4.2**, no significant line-by-age interactions were detected for any of these genes in embryonic breast muscle (**Figure 4.2A – 4.2D**; P>0.05). However, *GHR*, *IGF1*, and *IGFR1* exhibited age main effects in this tissue (**Table 4.3**; P≤0.05). Expression of *GHR* increased in both lines between e10 and e14 and remained elevated thereafter (**Table 4.3**; P≤0.05). Expression of *IGF1* began to significantly decrease at e18 (**Table 4.3**; P≤0.05). Expression of *IGFR1* dropped between e14 and 16 and remained low on e18 (**Table 4.3**; P≤0.05). No main effect of age for *IGF2* was observed in breast muscle (**Table 4.3**; P>0.05).

IGF and hormone receptor expression during post-hatch development

Expression levels of somatotropic hormones and receptors in ACRB and Ross post-hatch liver are presented in **Figure 4.3**. Only *GHR* exhibited a significant line-by-age interaction, in which expression was two-fold greater in Ross liver at both d30 and d40 (**Figure 4.3A**; $P \leq 0.05$). No line-by-age interactions or main effects of line were observed *IGF1*, *IGF2*, or *IGFR1* (**Figure 4.3B – 4.3D**; $P > 0.05$), but they exhibited main age effects (**Tables 4.2** and **4.3**; $P \leq 0.05$). Expression of *IGF1* in both Ross and ACRB liver increased steadily between d10 and d30 and remained elevated through d40 (**Table 4.3**; $P \leq 0.05$), whereas *IGF2* increased between d10 and d20 before decreasing on d30 and returning to intermediate levels at d40 (**Table 4.3**; $P \leq 0.05$). Hepatic expression of *IGFR1* exhibited a similar pattern to *IGF2* and went up between d10 and d20, was reduced on d30, and increased again on d40 (**Table 4.3**; $P \leq 0.05$).

Levels of these genes in post-hatch breast muscle are shown in **Figure 4.4**. No significant interactive effects were detected for *GHR* and *IGF1* (**Figure 4.4A** and **4.4B**; $P > 0.05$), but each exhibited main line effects. Expression was higher overall in ACRB breast muscle for *GHR*, whereas *IGF1* mRNA levels were greater in Ross breast muscle (**Table 4.4**, $P \leq 0.05$). *GHR* also displayed a main effect of age, increasing from d10 to d20 and remaining stable through d40 in this tissue (**Table 4.4**; $P \leq 0.05$). Additionally, *IGF1* approached significance for a main effect of age, where breast muscle expression increased between d10 and d40 (**Table 4.5**; $P = 0.0531$). *IGF2* did demonstrate a significant line-by-age interactive effect, in which expression was two-fold greater in Ross breast muscle on d20 and increased to five-fold greater on d40 (**Figure 4.4C**; $P \leq 0.05$). A significant interactive effect was not observed for *IGFR1* mRNA in breast muscle (**Figure 4.4D**; $P > 0.05$), but it approached significance for a main effect of age. Expression increased from d10 to d20, decreased at d30, and returned to d20 levels on d40 (**Table 4.**5; $P = 0.0683$).

Circulating IGFs in post-hatch plasma

Figure 4.5 shows circulating concentrations of IGF1 and IGF2 in post-hatch broilers, which were determined because of their ability to regulate overall body growth and induce cellular growth and proliferation in breast muscle. There was no significant line-by-age effect for IGF1 (**Figure 4.5A**; P>0.05), although there was a main effect of age. Levels of IGF1 increased between d10 and d20 and remained elevated through d40 (**Table 4.5**; P≤0.05). Circulating IGF2 approached significance for a line-by-age effect, in which IGF2 was greater in Ross at d10 and d20 but higher in ACRB on d40 (**Figure 4.5B**; P=0.0647). IGF2 also exhibited a main effect of age, with circulating levels peaking on d20 in both lines (**Table 4.5**; P≤0.05).

IGFBP expression during embryonic development

The liver is a major producer of IGFBPs [103], and this protein family is essential for controlling IGF signaling, thus regulates IGF effects on myogenic growth [121, 208]. Relative IGFBP expression levels measured in embryonic ACRB and Ross liver are presented in **Figure 4.6**. *IGFBP1* exhibited a significant line-by-age interaction, where ACRB expression at e12 was 4-fold greater than Ross but the opposite was observed at e16 when Ross expression was 2.5-fold greater than ACRB (**Figure 4.6A**; P≤0.05). *IGFBP2* did not exhibit an interactive effect (**Figure 4.6B**; P>0.05), but expression in liver was low from e10 to e12 and increased steadily thereafter through e18, indicating a main age effect (**Table 4.3**; P≤0.05). *IGFBP3* exhibited a significant interactive effect and expression was approximately 2-fold greater in Ross liver than in ACRB liver on both e14 and e16 (**Figure 4.6C**; P≤0.05). No interactive effects or main effects of line or age were observed for *IGFBP4* in this tissue (**Figure 4.6D**; **Tables 4.2** and **4.3**; P≤0.05). *IGFBP5* also did not have a significant interactive effect (**Figure 4.6E**; P>0.05), but it approached significance for a main effect of line where hepatic ACRB expression was greater than that in Ross (**Table 4.2**;

P=0.094). Age was also significant for liver *IGFBP5* expression, increasing between e10 and e14 and decreasing on e16 and e18 (**Table 4.5**; P≤0.05). *IGFBP7* displayed a nearly significant line-by-age interaction in embryonic liver (**Figure 4.6F**; P=0.0697) and was greater in Ross than ACRB on e14. Additionally, its expression increased from e10 to e14, denoting a main effect of age (**Table 4.3**; P≤0.05).

The *IGFBPs* did not display any significant interactive effects in embryonic breast muscle (**Figure 4.7**; P>0.05). *IGFBP1* and *IGFBP 7* exhibited a main effect of age, with expression decreasing or increasing between e10 to e18, respectively (**Table 4.3**; P≤0.05). No significant main effects of line or age were observed for *IGFBP2*, *IGFBP3*, or *IGFBP5* (**Tables 4.2** and **4.3**; P>0.05). A line main effect was detected for breast muscle *IGFBP4*, in which levels in Ross were significantly lower (**Table 4.2**; P≤0.05).

IGFBP expression during post-hatch development

IGFBP expression in post-hatch liver is shown in **Figure 4.8**. Only *IGFBP1* exhibited a significant line-by-age interaction (**Figure 4.8A**; P≤0.05), whereas the remaining IGFBPs did not (**Figure 4.8B – 8F**; P>0.05). Levels of ACRB *IGFBP1* mRNA were 4-fold higher than Ross at d20 (**Figure 4.7A**; P≤0.05) and numerically lower than Ross on d10 and d30. Main effects of line and age were observed for *IGFBP2* and *IGFBP3*, whereas *IGFBP4* only had a main effect of age. Liver expression of *IGFBP2* was greater in ACRB, while expression of *IGFBP3* was greater in Ross (**Table 4.4**; P≤0.05). *IGFBP2* was 10- to 30-fold higher on d20 than other age, and *IGFBP3* expression on d20 and d40 was almost twice that of d10 and d30 (**Table 4.5**; P≤0.05). After a 5-fold increase in expression between d10 and d20, *IGFBP4* remained high through d40 (**Table 4.5**; P≤0.05). *IGFBP5* and *IGFBP7* also exhibited main effects of line and age. Expression of both genes were significantly greater in Ross liver (**Table 4.4**; P≤0.05), and their expression increased

approximately 2-fold between d10 and d20 and then decreased to intermediate levels of d30 and d40 (**Table 4.5**; P≤0.05).

Figure 9 illustrates IGFBP mRNA levels in post-hatch breast muscle. *IGFBP1* did not have a significant interactive effect (**Figure 4.9A**; P>0.05) or line main effect (**Table 4.4**; P>0.05) but did exhibit a main effect of age. Expression increased approximately 5-fold between d10 and d20 and was reduced about 2-fold at later ages (**Table 4.5**; P≤0.05). *IGFBP2* displayed a significant line-by-age interaction in post-hatch breast muscle and was higher in Ross than ACRB at d40 (**Figure 4.9B**; P≤0.05). No significant interactive effects were determined for *IGFBP3*, *IGFBP4*, *IGFBP5*, or *IGFBP7* (**Figure 4.9C – 4.9F**; P>0.05), but each demonstrated a main effect of line (**Table 4.4**; P≤0.05). Apart from *IGFBP3*, which was higher in ACRB breast muscle, expression was greater in Ross (**Table 4.4**; P≤0.05). Additionally, *IGFBP4*, *IGFBP5*, and *IGFBP7* expression differed significantly across ages. *IGFBP4* expression increased between d10 and d30 and remained high on d40 (**Table 4.5**; P≤0.05). Levels of *IGFBP5* mRNA were lower at d10 and d20 than d30 and d40 (**Table 4.5**; P≤0.05). Expression of *IGFBP7* increased significantly after d10 and remained high thereafter (**Table 4.**5; P≤0.05).

Discussion

The highly conserved nature of the somatotropic axis in vertebrates implies that it plays an important functional role in the growth and development of birds, though how it contributes to the improvements in growth rate and meat production efficiency made through genetic selection of commercial broilers is still not known. Thus, this study examined if components of the somatotropic axis, including hormones, hormone receptors, and hormone binding proteins, differed between a genetic control line (ACRB) and a commercial modern broiler line (Ross 308) during embryonic and post-hatch development. The results suggest that selection has impacted

local IGF signaling in breast muscle more than endocrine IGF signaling, and that IGFBPs play an important role in modulating somatotropic axis activity in a tissue-specific manner to affect growth. Multiple lines of evidence from this study suggest that classical somatotopic axis activity might not play a major role in driving chicken embryonic growth, in large part because embryonic IGF levels are likely not influenced by circulating GH. Pituitary GH in chickens increases during the last half of embryonic development [62, 423-426], around the time that the birds used in this study began diverging in body weight. It was previously shown that Ross embryos were significantly heavier by e14, and body weight differences between the lines continued to increase through d40 [418]. In liver and breast muscle, neither *GHR* nor *IGF1* expression differed between the lines during embryonic development, suggesting that GH stimulation of IGF1 is not driving the observed differences in growth. While liver *IGF2* mRNA was higher in Ross 308 on e14, this was not maintained on e16 and 18 despite Ross embryos growing at a faster rate. *GHR* was observed to increase in liver and breast muscle during this period in both lines. However, this increase was accompanied by either no change or inconsistent changes in liver *IGF1*, *IGF2*, and *IGFR1* or a decrease in *IGF1* and *IGFR1* in breast muscle, suggesting that *IGF1*, *IGF2*, and *IGFR1* production are not dependent on GH during late embryonic development. It has been suggested that the somatotropic axis is not fully established until after hatch [62, 419], and this study provides further evidence that IGF production is likely not GH-dependent in the embryonic somatotropic axis.

Heightened expression of *GHR* mRNA in liver and muscle throughout late embryonic development may be used for GH binding protein (GHBP) synthesis, which is made by cleaving off GHR's extracellular domain [427, 428]. Human GHBPs form a complex with GH [429], and this may similarly occur in chickens. As pituitary GH production increases late in chicken

embryonic development, GHBP might sequester it until target tissues like liver and muscle are responsive to GH after the somatotropic axis is fully established.

It has been reported that pituitary and plasma GH levels are lower in fast-growing birds after hatch [3, 419, 430]. Hepatic *GHR* expression was greater in Ross than ACRB on d30 and d40, and this may reflect a need for increased GH sensitivity to compensate for reduced circulating GH relative to the slower-growing ACRB birds. This could be accomplished by providing additional plasma membrane binding sites for GH and/or by increasing its half-life in plasma via GHBP action. Ultimately, however, higher *GHR* in Ross liver does not appear to contribute to increased hepatic *IGF1* or *IGF2* expression or circulating IGF levels in relation to those parameters in to ACRB.

Levels of *IGF1* and *IGF2* mRNA were greater in post-hatch Ross breast muscle as compared to ACRB, suggesting these hormones support the rapid muscle growth observed in commercial modern broilers. Together with the observation that hepatic and circulating IGFs did not differ between the lines, these results indicate that differential paracrine IGF signaling may impact growth on a tissue-specific basis and contribute to the faster growth and increased muscle accretion in modern birds. Our findings align with the previously proposed theory that IGF signaling in chicken muscle acts in a paracrine fashion, contributing to hypertrophy in a manner similar to mice, rats, and rabbits [431-433].

The IGFBP family mediates IGF effects by enhancing or dampening IGF signaling. This occurs by either increasing IGF-receptor affinity, physically sequestering it to prevent receptor binding, or extending IGF's half-life in circulation. Additionally, many IGFBPs can act independently to induce cellular activity [18, 19, 85, 133, 134]. Our results suggest that effects of some IGFBPs on broiler growth may differ between embryonic and post-hatch development.

Expression of *IGFBP1* was greater in ACRB liver at e12 but increased in Ross liver at e16. This correlates with the difference in embryonic body weight between the lines previously observed beginning on e14 [418]. Here, elevated IGFBP1 may serve to transport IGF in circulation, as liver *IGF2* in the embryo was greater in Ross at e10 and e14 and could facilitate growth during the last week of embryogenesis. In the liver of post-hatch ACRBs, however, *IGFBP1* was greater at d20, when broilers are growing most rapidly. Work performed in mice indicates IGFBP1, when produced in the liver, limits growth [434-436], and it could act similarly in post-hatch chickens. Combined, these results indicate that IGFBP1 function may change across developmental stages in broilers, in turn altering bird physiology by promoting IGF signaling during embryogenesis and inhibiting it during certain stages of juvenile post-hatch development.

IGFBPs function in an endocrine fashion when secreted into plasma from the liver but a paracrine one when produced locally in peripheral tissues [437]. While levels of *IGFBP4* in liver did not differ between the lines at any stage, differential expression of *IGFBP4* in breast muscle suggests it may act locally to regulate growth of this tissue and, like IGFBP1, may have opposing effects during embryonic and post-hatch developmental stages. In embryonic development, elevated *IGFBP4* mRNA in ACRB breast muscle suggests in acts in an inhibitory manner. This would be consistent with previous reports that IGFBP4 inhibited growth of mouse skeletal muscle [438, 439]. The effect in breast muscle is likely to be IGF-dependent, because IGFBP4 inhibits cellular proliferation of myoblasts only in the presence of IGF1 [121]. Since expression of *IGF1* and *IGF2* mRNA in breast muscle did not differ between the lines, it is possible that elevated IGFBP4 in ACRB reduces IGF signaling in this tissue through its sequestration. On the other hand, during post-hatch development, IGFBP4 appears to act in a paracrine manner to stimulate breast muscle growth. Levels of *IGFBP4* mRNA in Ross breast muscle post-hatch were almost twice that

of ACRB, as were *IGF1* and *IGF2* mRNA. This indicates that, in post-hatch breast muscle, IGFBP4 could work to perpetuate IGF signaling through increasing the hormones' half-life and/or facilitating their access to IGFR1.

IGFBP7 may also regulate skeletal muscle generation in chickens based on results presented here. IGFBP7 has been shown to limit cell cycle activation in mice, protecting against satellite cell exhaustion to ensure long-term muscle growth [440]. Increased *IGFBP7* mRNA was observed in Ross broiler breast muscle post-hatch, suggesting it could work in a similar manner to promote muscle growth after hatch by maintaining a healthy satellite cell population. This could contribute to greater breast muscle yield in commercial modern broilers [5, 66, 363] by supporting the satellite cell population and facilitating their differentiation during muscle accretion.

Within the same developmental stage, the effects of a singular IGFBP can also change depending on whether it acts in an endocrine or paracrine manner. Hepatic post-hatch *IGFBP2* was greater in ACRB, aligning with inhibitory IGFBP2 action observed in zebrafish where it reduced cell proliferation during fasting [441]. However, *IGFBP2* was greater in post-hatch Ross breast muscle later in development. Since IGFBP2 has been shown to induce chicken primary myoblast proliferation [442], this might mean that endocrine IGFBP2 released from post-hatch liver inhibits overall body growth but paracrine IGFBP2 activity in breast muscle facilitates its growth. Data presented here suggest that the inverse may be true for IGFBP3, which has a promotive effect on IGF signaling in mammals when acting in an endocrine manner by extending their half-life in the blood [443] but may inhibit breast muscle growth by acting in paracrine manner. *IGFBP3* mRNA was greater in Ross embryonic liver at e14 and e16, ages at which they start increasing in size relative to ACRBs. Thus, when synthesized in the liver, IGFBP3 could extend IGF signaling by maintaining IGFs in the blood of Ross embryos and contribute to their

larger size that begins around late embryogenesis. Importantly, elevated hepatic *IGFBP3* in Ross birds continued post-hatch, playing into its established role as a metabolic regulator [444] and suggesting it may also impact body composition and feed efficiency in chickens. Post-hatch *IGFBP3* was reduced in Ross muscle compared to ACRB, suggesting that it may negatively regulate muscle accretion through direct sequestration of IGFs or in another manner. Together, these results are indicative that IGFBPs act in a tissue-specific manner to control IGF signaling through both endocrine and paracrine mechanisms and can have both inhibitory and stimulatory effects depending on their mode of action, as has been observed in mammals.

Like *IGFBP3*, hepatic *IGFBP5* and *IGFBP7* mRNA levels were higher in post-hatch Ross broilers, indicative of an endocrine effect by these proteins that promotes bird growth and muscle accretion. In mice, it was shown that single knockouts for *IGFBP3*, *IGFBP4*, or *IGFBP5* showed little growth impairment, while triple knockout mice were significantly smaller with reduced fat pad accumulation and less skeletal muscle [445]. This indicates that some IGFBPs exhibit functional redundancy in regulating growth and metabolism in mammals, and a similar phenomenon might exist in birds.

To summarize, we found that expression levels of select somatotropic genes differed between male legacy and commercial modern broilers. Although there were no differences in circulating IGFs, elevated *IGF1* and *IGF2* in post-hatch Ross muscle suggests that paracrine IGF signaling contributes to the increased breast muscle size of commercial modern broilers. Control of IGF signaling by IGFBPs likely also differs between commercial modern and legacy broilers and plays a role in regulating chicken growth. It was observed that select IGFBPs appear to play distinct, and sometimes opposing, growth-promoting or growth-inhibiting roles in a developmental and tissue-specific manner and that functional redundancy among the IGFBPs may exist. In

conclusion, these results suggests that rapid growth and increased muscle accretion in commercial modern broilers is achieved not through increased levels of circulating IGFs but by changing local IGF expression to affect paracrine IGF activity, specifically in muscle. This activity is further regulated through combinatorial action of IGFBPs, which appear to make up a robust control system acting to support growth within different developmental and physiological contexts.

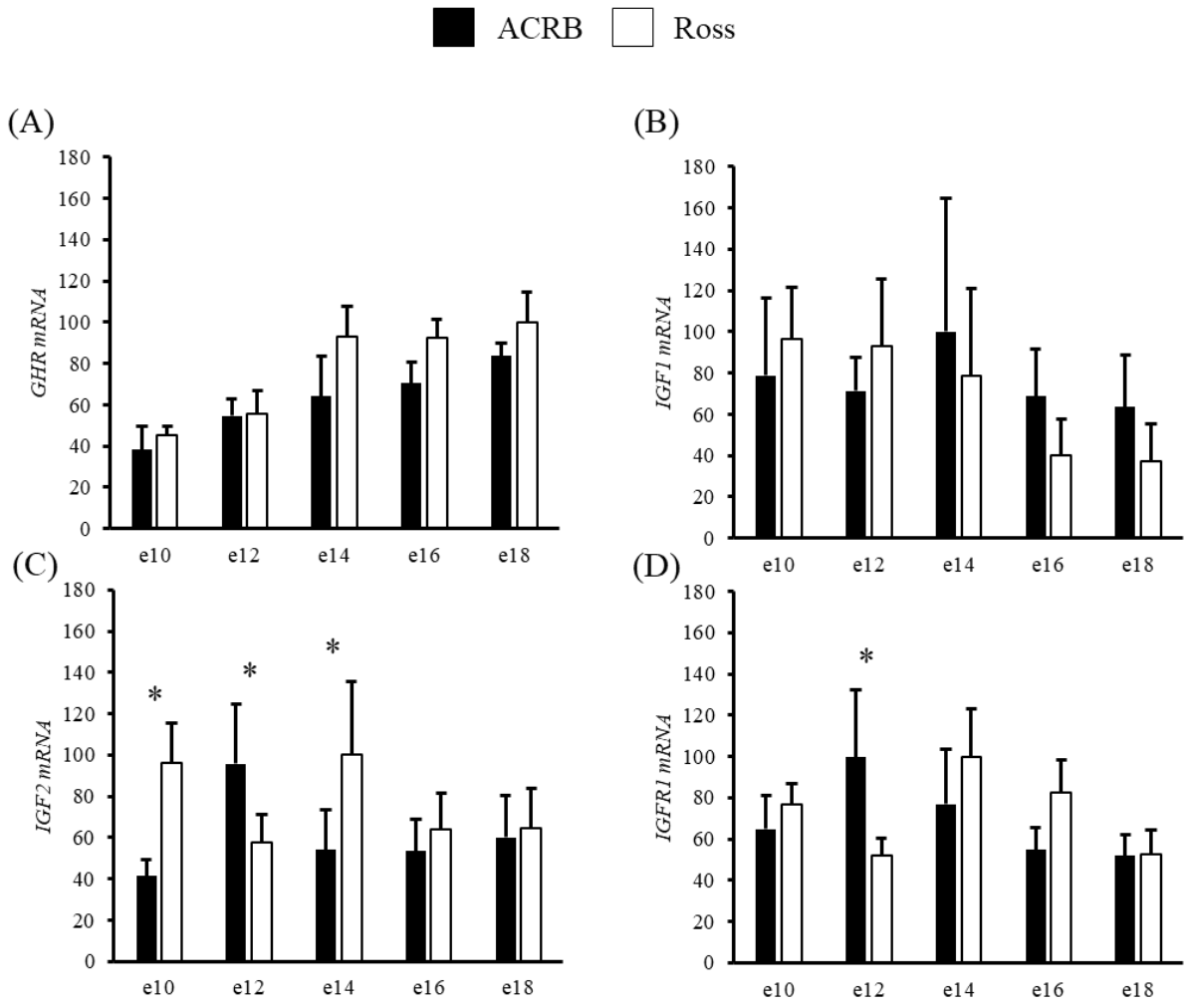

Figure 4.1. Relative mRNA expression of (A) *GHR*, (B) *IGF1*, (C) *IGF2*, and (D) *IGFR1* in liver on embryonic days (e) 10, 12, 14, 16, and 18 in legacy ACRB and modern Ross 308 male broilers. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* mRNA (n=4 replicate birds per line at each age). The data (mean + SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). No significant line-by-age interactions were detected for (A) *GHR* (P=0.7777) or (B) *IGF1* (P=0.7562), and main effect means for line and age for these genes are shown in Tables 4.2 and 4.3, respectively. Significant line-by-age interactions were identified for (C) *IGF2* (P=0.0003) and (D) *IGFR1* (P=0.0235), and the presence of an asterisk (*) indicates a significant difference in expression between the lines at those ages (P≤0.05).

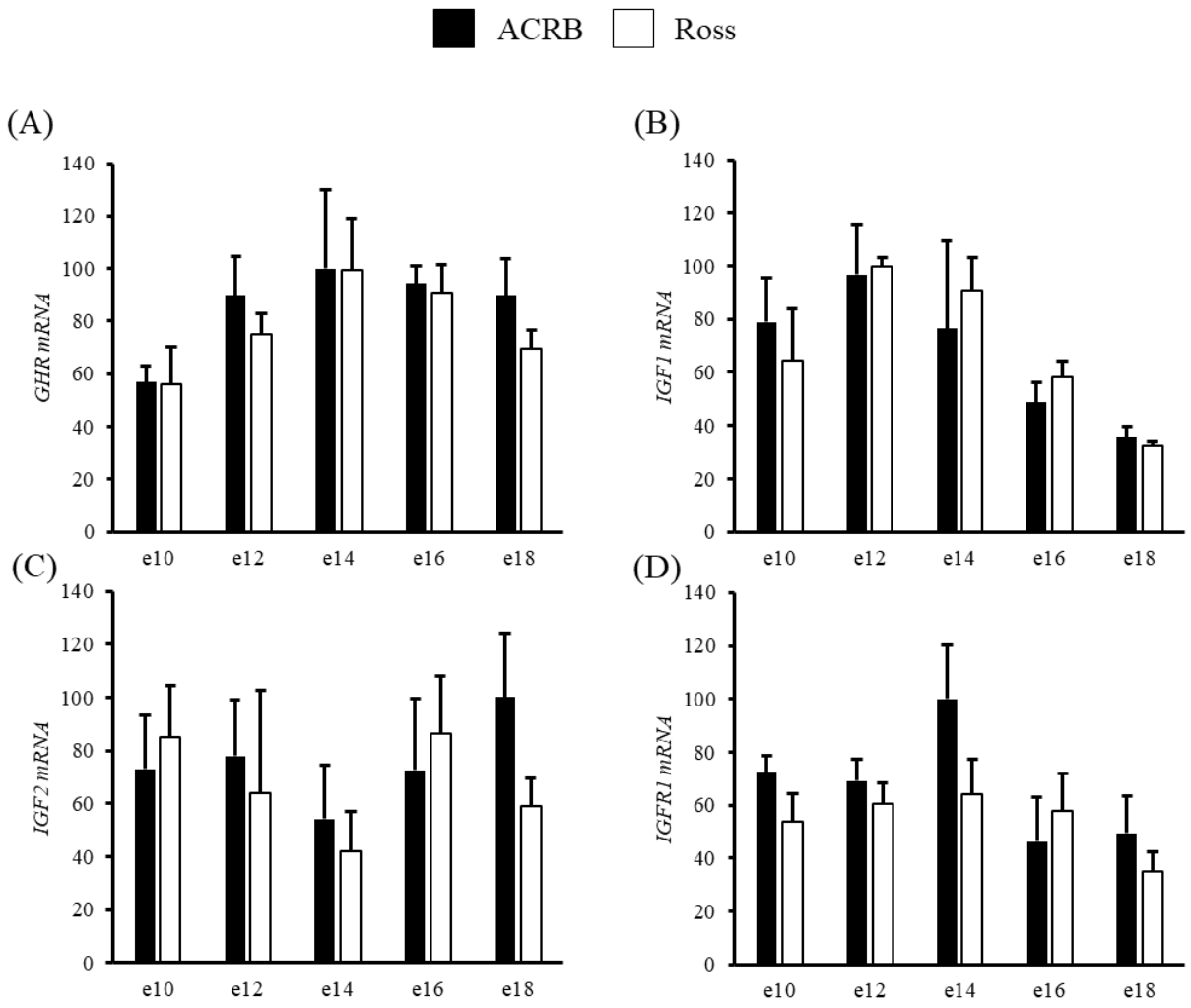

Figure 4.2. Relative mRNA expression of (A) *GHR*, (B) *IGF1*, (C) *IGF2*, and (D) *IGFR1* in breast muscle on embryonic days (e) 10, 12, 14, 16, and 18 in legacy ACRB and modern Ross 308 male broilers. Relative expression levels were measured using RT-qPCR and normalized to *18S* RNA (n=4 replicate birds per line at each age). The data (mean + SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). No significant line-by-age interactions were observed for (A) *GHR* (P=0.9321), (B) *IGF1* (P=0.5901), (C) *IGF2* (P=0.6246), or (D) *IGF1R* (P=0.4752), and main effect means of line and age all genes are presented in Tables 4.2 and 4.3, respectively.

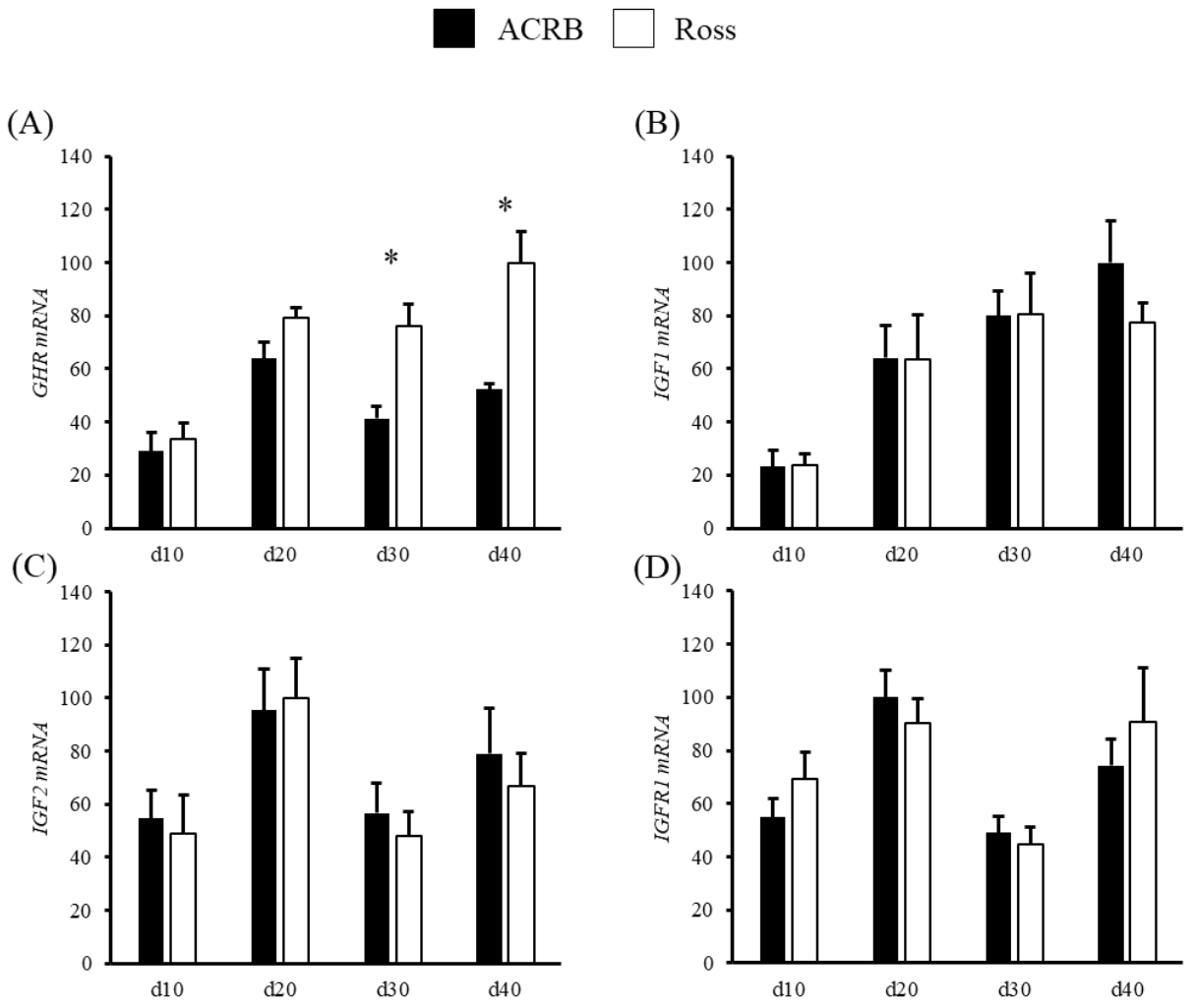

Figure 4.3. Relative mRNA expression of (A) *GHR*, (B) *IGF1*, (C) *IGF2*, and (D) *IGFR1* in liver on post-hatch days (d) 10, 20, 30, and 40 in legacy ACRB and modern Ross 308 male broilers. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* mRNA (n=8 replicate birds per line at each age). The data (mean + SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). A significant line-by-age interaction was detected for (A) *GHR* (P=0.0446), and the presence of an asterisk (*) indicates a significant difference in expression between the lines at the indicated age. No significant line-by-age interactions were detected for (B) *IGF1* (P=0.6890), (C) *IGF2* (P=0.8688), or (D) *IGFR1* (P=0.7405), and main effect means of line and age for these genes are presented in Tables 4.4 and 4.5, respectively.

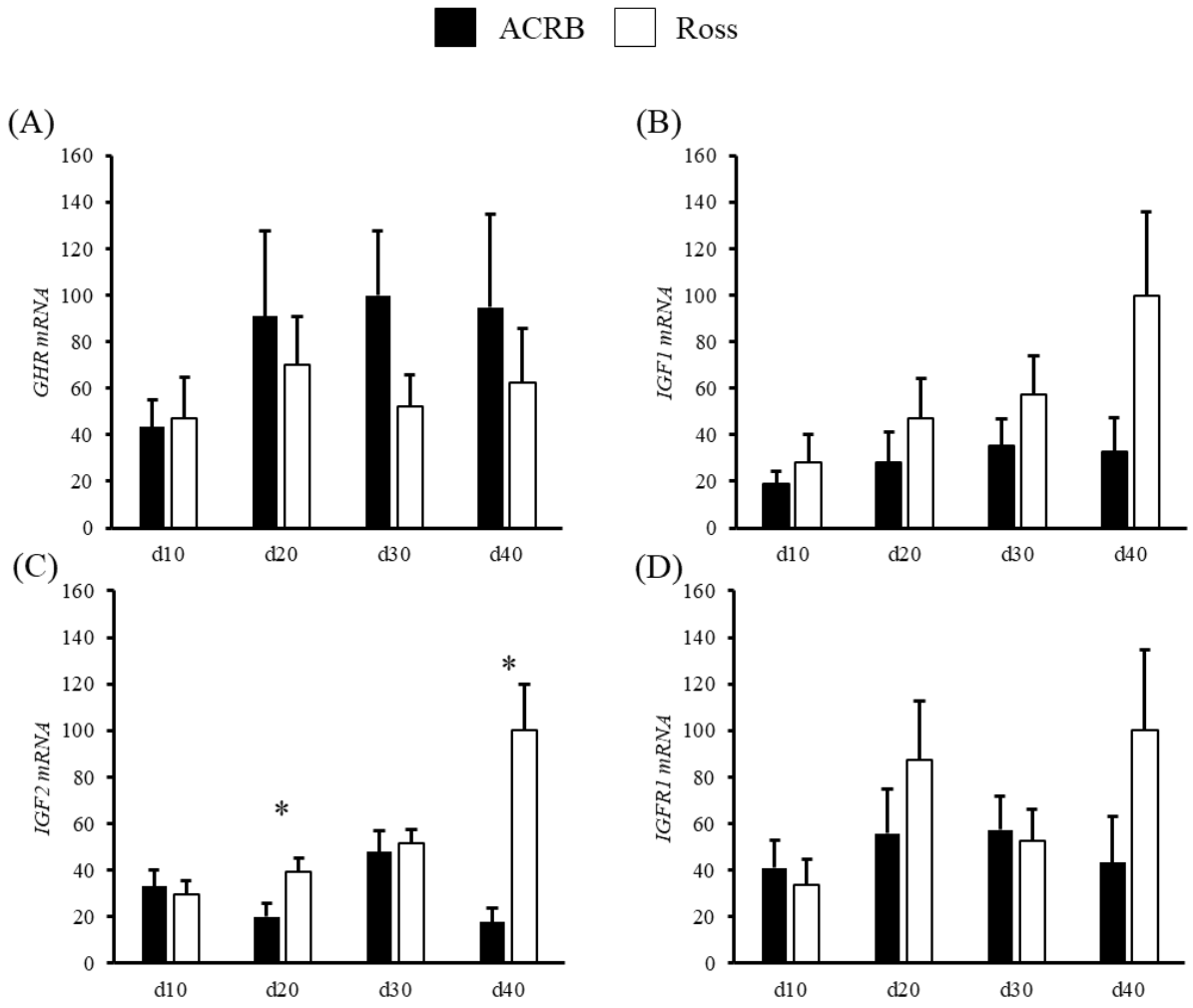

Figure 4.4. Relative mRNA expression of (A) *GHR*, (B) *IGF1*, (C) *IGF2*, and (D) *IGFR1* in breast muscle on post-hatch days (d) 10, 20, 30, and 40 in legacy ACRB and modern Ross 308 male broilers. Relative expression levels were measured using RT-qPCR and normalized to *18S* RNA (n=8 replicate birds per line at each age). The data (mean + SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). No significant line-by-age interactions were detected for (A) *GHR* (P=0.5112), (B) *IGF1* (P=0.1424), or (D) *IGF1R* (P=0.1258), and main effect means of line and age for these genes are presented in Tables 4.4 and 4.5, respectively. A significant line-by-age interaction was detected for (C) *IGF2* (P=0.0111), and the presence of an asterisk (*) indicates a significant difference in expression between the lines at the indicated age.

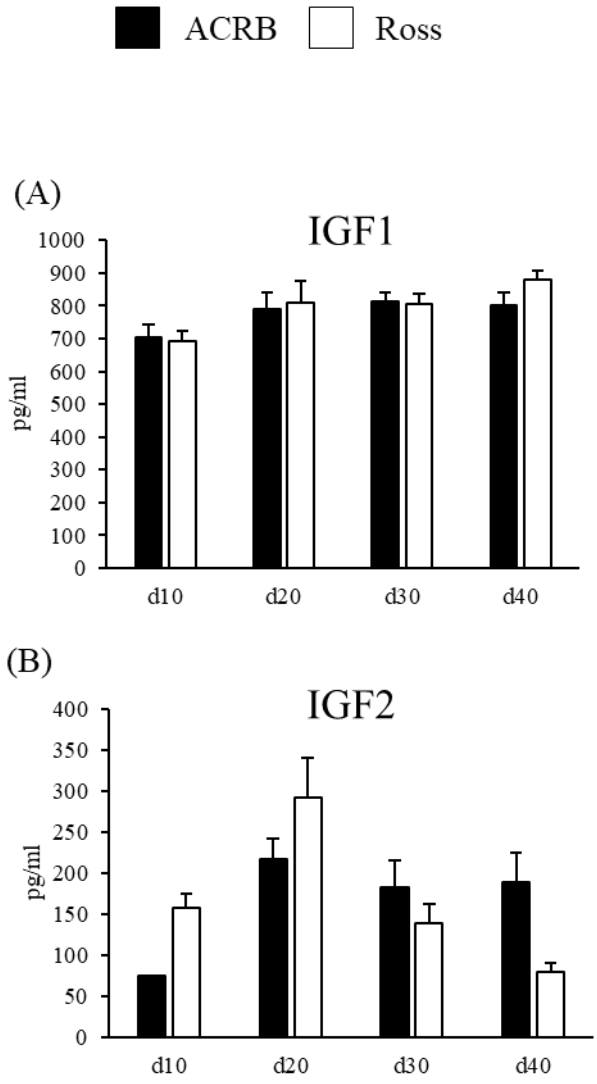

Figure 4.5. Circulating (A) IGF1 and (B) IGF2 in legacy ACRB and modern Ross 308 male broilers on post-hatch days (d) 10, 20, 30, and 40 as determined by ELISA (n=8 replicate birds per line at each age). No significant line-by-age interactions were observed for (A) IGF1 (P=0.7065) or (B) IGF2 (P=0.0647), and main effect means of line and age are presented in Tables 4.4 and 4.5, respectively.

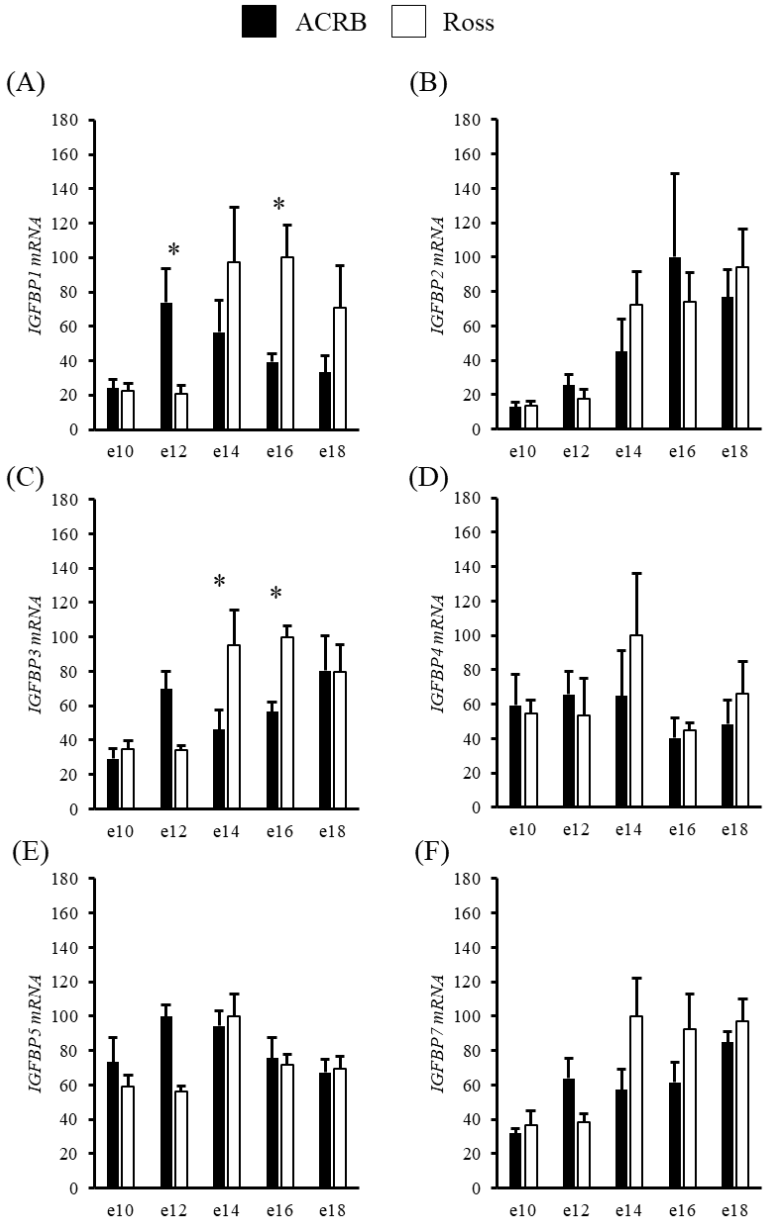

Figure 4.6. Relative mRNA expression of (A) *IGFBP1*, (B) *IGFBP2*, (C) *IGFBP3*, (D) *IGFBP4*, (E) *IGFBP5*, and (F) *IGFBP7* in liver on embryonic (e) days 10, 12, 14, 16, and 18 in legacy ACRB and modern Ross 308 male broilers. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* mRNA (n=4 replicate birds per line at each age). The data (mean + SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). Significant line-by-age interactions were detected for (A) *IGFBP1* (P=0.0038) and (C) *IGFBP3* (P=0.0080), and the presence of an asterisk (*) indicates a significant difference in expression between the lines at the indicated age (P≤0.05). No significant line-by-age interactions were detected for (B) *IGFBP2* (P=0.3060), (D) *IGFBP4* (P=0.2942), (E) *IGFBP5* (P=0.1055), or (F) *IGFBP7* (P=0.0697), and main effect means of line and age for these genes are presented in Tables 4.2 and 4.3, respectively.

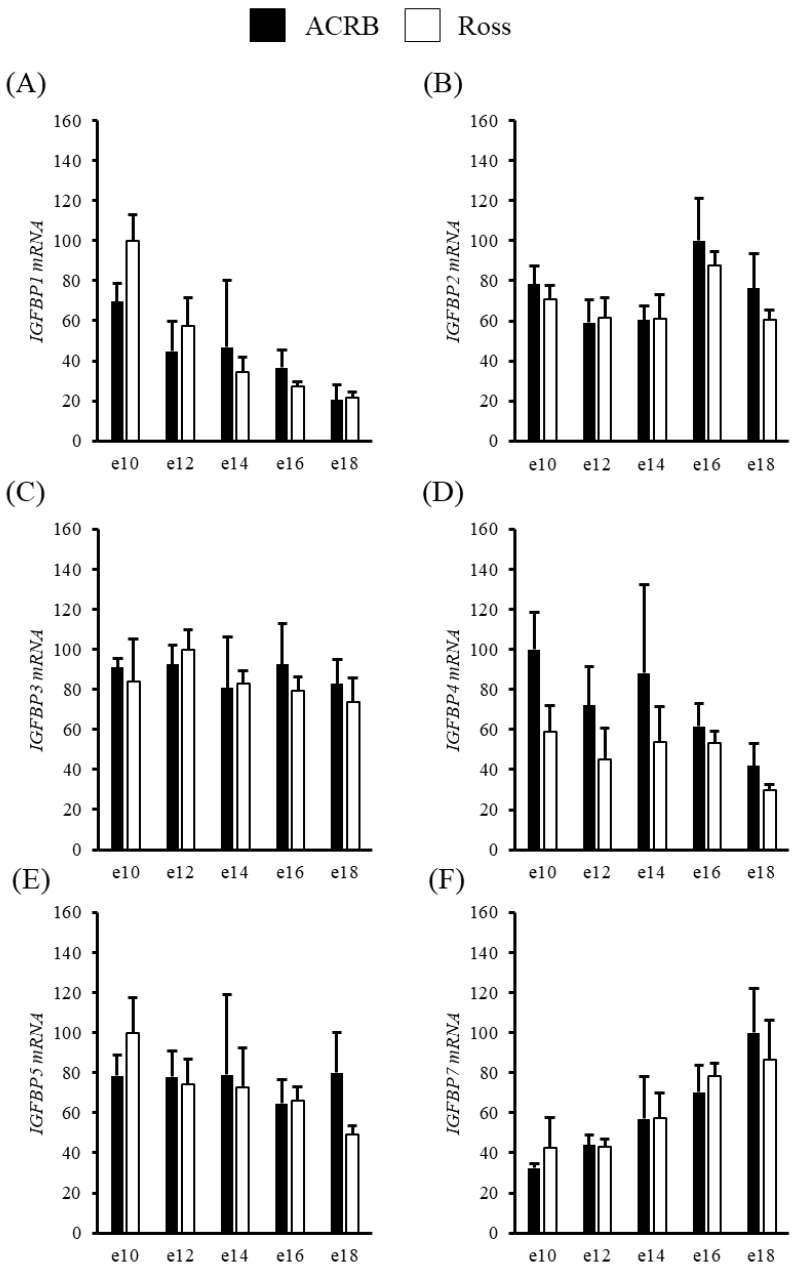

Figure 4.7. Relative mRNA expression of (A) *IGFBP1*, (B) *IGFBP2*, (C) *IGFBP3*, (D) *IGFBP4*, (E) *IGFBP5*, and (F) *IGFBP7* in breast muscle on embryonic days (e) 10, 12, 14, 16, and 18 in legacy ACRB and modern Ross 308 male broilers. Relative expression levels were measured using RT-qPCR and normalized to *18S* RNA (n=4 replicate birds per line at each age). The data (mean + SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). No significant line-by-age interactions were detected for (A) *IGFBP1* (P=0.8032), (B) *IGFBP2* (P=0.9609), (C) *IGFBP3* (P=0.8806), (D) *IGFBP4* (P=0.8715), (E) *IGFBP5* (P=0.6831), or (F) *IGFBP7* (P=0.9480), and main effect means of line and age for all genes are presented in Tables 4.4 and 4.5, respectively.

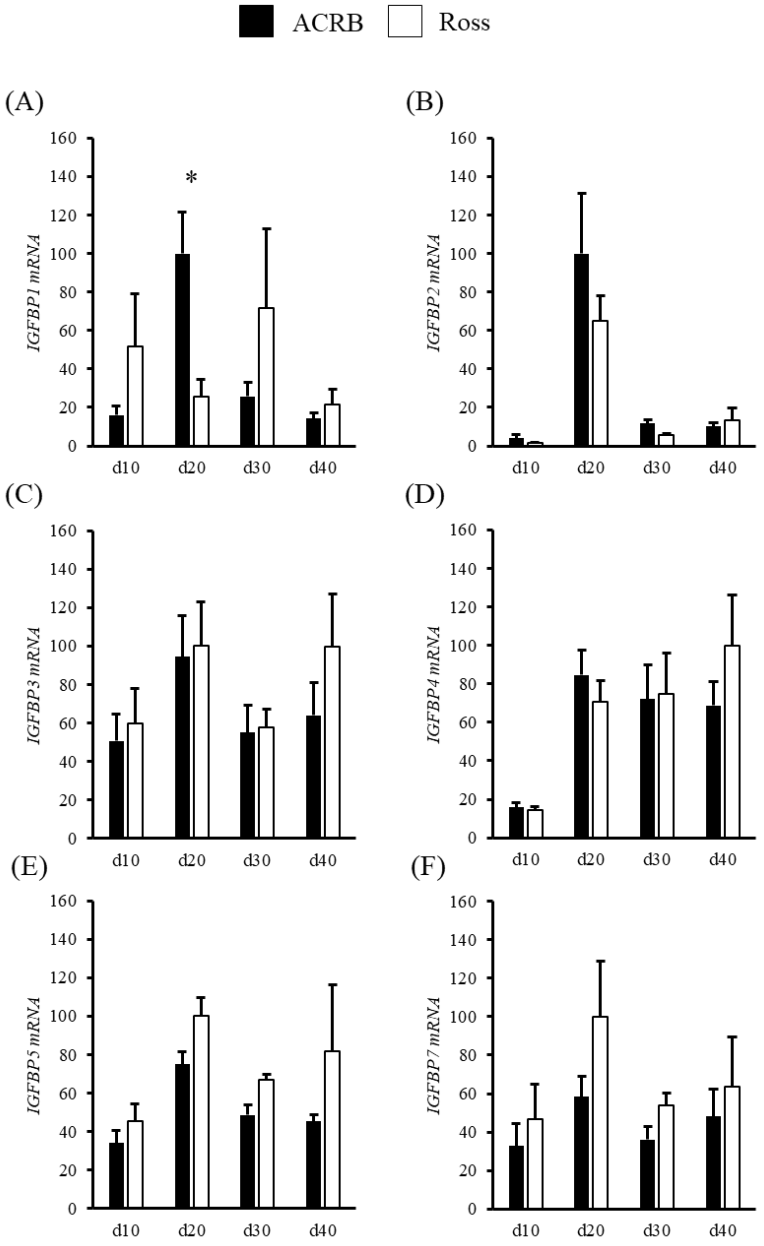

Figure 4.8. Relative mRNA expression of (A) *IGFBP1*, (B) *IGFBP2*, (C) *IGFBP3*, (D) *IGFBP4*, (E) *IGFBP5*, and (F) *IGFBP7* in liver on post-hatch days (d) 10, 20, 30, and 40 in legacy ACRB and modern Ross 308 male broilers. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* mRNA (n=8 replicate birds per line at each age). The data (mean + SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). A significant line-by-age interaction was detected for (A) *IGFBP1* (P=0.0014), and the presence of an asterisk (*) indicates a significant difference in expression between the lines at the indicated age (P≤0.05). No significant line-by-age interactions were detected for (B) *IGFBP2* (P=0.5051), (C) *IGFBP3* (P=0.5261), (D) *IGFBP4* (P=0.5834), (E) *IGFBP5* (P=0.8311), or (F) *IGFBP7* (P=0.8716), and main effect means of line and age for these genes are presented in Tables 4.4 and 4.5, respectively.

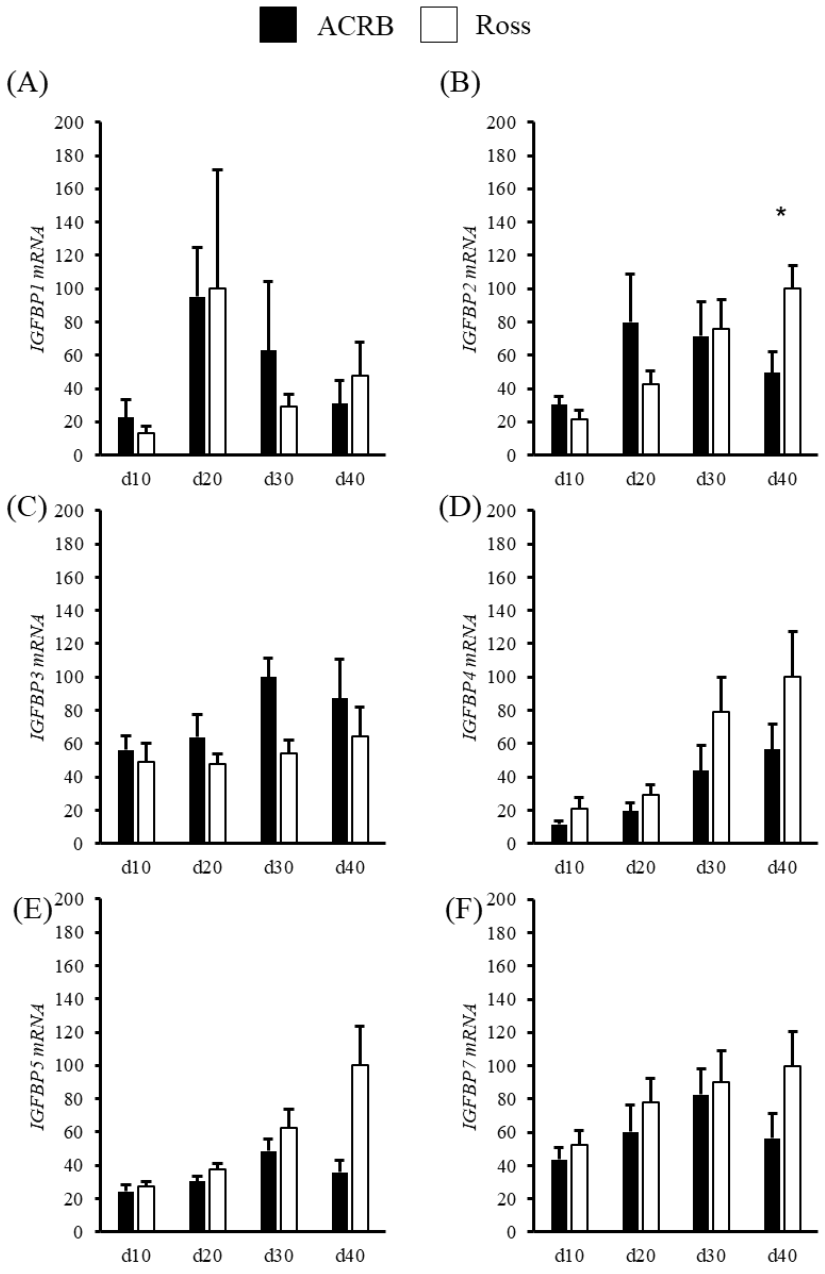

Figure 4.9. Relative mRNA expression of (A) *IGFBP1*, (B) *IGFBP2*, (C) *IGFBP3*, (D) *IGFBP4*, (E) *IGFBP5*, and (F) *IGFBP7* in breast muscle on post-hatch days (d) 10, 20, 30, and 40 in legacy ACRB and modern Ross 308 male broilers. Relative expression levels were measured using RT-qPCR and normalized to *18S* RNA (n=8 replicate birds per line at each age). The data (mean + SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). A significant line-by-age interaction was identified for (B) *IGFBP2* (P=0.0022), and the presence of an asterisk (*) indicates a significant difference in expression between the lines at those ages (P≤0.05). No significant line-by-age interactions were detected for (A) *IGFBP1* (P=0.3093), (C) *IGFBP3* (P=0.7127), (D) *IGFBP4* (P=0.6558), (E) *IGFBP5* (P=0.1711), or (F) *IGFBP7* (P=0.4647), and main effect means of line and age for these genes are presented in Tables 4.4 and 4.5, respectively.

Table 4.1. Primers used for reverse transcription-quantitative PCR.

Gene Symbol	Forward primer (5'-3')	Reverse primer (5'-3')	Transcript ID[1]	Efficiency
IGFs				
IGF1	TGAGCTGGTTGATGCTCTTC	AGCCTCCTCAGGTCACAACT	20816	0.99
IGF2	AGTCAGAGCGTGACCTCTCC	CTGCGAGCTCTTCTTCTGC	53800	1.05
Hormone receptors				
GHR	TGCTGATTTTTCCTCCTGTG	GGCTGGCTAAGATGGAGTTC	23973	1.08
IGF1R	TGGGGACCTCAAAAGTTACC	ATCCCATCAGCAATCTCTCC	74990	1.04
Hormone binding proteins				
IGFBP1	CAGAGAAGTGGAGGGGACAT	CTTCTGGGGATCCAGGAAT	47713	
IGFBP2	ATCACAACCACGAGGACTCA	GAGGGAGTAGAGGTGCTCCA	18698	0.96
IGFBP3	TTGAGTCCTAGGGGTTTCCA	ATATCCAGGAAGCGGTTGTC	82156	1.02
IGFBP4	AACTTCCACCCCAAGCAG	AATCCAAGTCCCCCTTCAG	68153	0.96
IGFBP5	CTGAAGAGCAGCCAGAGGAT	TTGTCCACACACCAACACAG	38163	0.98
IGFBP7	ATGTGACAGGAGCACAGATCTACCT	TCTGGATACCATACTGTCCTCGAAT	61018	0.95
Reference genes				
GAPDH	AGCCATTCCTCCACCTTTGAT	AGTCCACAACACGGTTGCTGTAT	23323	1.00
18s[2]	AGCCTGCGGCTTAATTTGAC	CAACTAAGAACGGCCATGCA	173612	0.96

[1] Transcript identification from Ensembl chicken genome assembly GRCg6a (http://www.ensembl.org/Gallus_gallus/Info/Index) preceded by ENSGALT000000.
[2]Sequence for 18S rRNA is not on the assembled chicken genome, and primers were designed based on the sequence in GenBank (accession number AF173612).

Table 4.2. Means[1] (±SEM) and ANOVA P-values of the line main effect for somatotropic gene expression in embryonic male ACRB and Ross 308 broilers.

		ACRB	Ross 308	P-value
IGFs and Receptors				
Liver (%)[2]				
	GHR	80.6±7.6	100±8.9	0.0640
	IGF1	75.2±14.9	100±34.5	0.7004
Muscle (%)[2]				
	GHR	100±7.9	90.5±7.4	0.3378
	IGF1	93.1±12.1	100±9.9	0.7055
	IGF2	100±12.9	88.2±13.5	0.4571
	IGF1R	100±10.9	84.5±7.7	0.2150
IGFBPs				
Liver (%)[2]				
	IGFBP2	95.5±22.4	100±17.7	0.6238
	IGFBP4	87.3±11.4	100±14.7	0.3633
	IGFBP5	100±6.1	86.8±5.8	0.0940
	IGFBP7	82.1±7.5	100±12.4	0.2619
Muscle (%)[2]				
	IGFBP1	99.8±20.1	100±15.8	0.7343
	IGFBP2	100±9.4	91.2±5.6	0.6339
	IGFBP3	100±7.2	95.9±6.1	0.6978
	IGFBP4	100±13.7[a]	69.7±8.0[b]	0.0354
	IGFBP5	100±10.5	97.2±8.9	0.8773
	IGFBP7	100±13.3	96.3±10.0	0.7269

[1]Means are only presented for data where a significant line-by-age interaction was not present and were calculated between embryonic day 10 and 18 for each line.
[2]Data within each gene are expressed relative to the line with the highest mRNA level (equal to 100%).
[a,b]Values within each gene that do not share a common letter are significantly different ($P \leq 0.05$).

Table 4.3. Means[1] (±SEM) and ANOVA P-values of the age main effect for somatotropic gene expression in embryonic male ACRB and Ross 308 broilers.

	e10	e12	e14	e16	e18	P-value
IGFs and Receptors						
Liver (%)[2]						
GHR	45.5±6.1^{c}	60.0±7.0^{bc}	85.5±13.6^{ab}	88.68±8.3^{ab}	100±8.8^{a}	0.0023
IGF1	51.3±12.4	48.12±10.2	100±50.7	31.84±8.4	29.59±8.9	0.4101
Muscle (%)[2]						
GHR	56.5±8.0^{b}	82.66±8.3^{a}	100±15.3^{a}	92.8±5.8^{a}	81.3±8.9^{a}	0.0243
IGF1	71.8±12.7^{ab}	100±8.9^{a}	85.9±14.6^{ab}	54.1±4.9^{bc}	34.9±2.3^{c}	0.0006
IGF2	96.9±15.9	86.2±24.8	57.2±13.8	96.2±19.8	100±19.3	0.4383
IGF1R	77.9±8.9^{abc}	81.6±6.9^{ab}	100±15.9^{a}	65.6±12.9^{bc}	54.5±10.8^{c}	0.0446
IGFBPs						
Liver (%)[2]						
IGFBP2	15.3±2.1^{c}	24.9±4.7^{c}	67.6±15.5^{b}	100±27.9^{ab}	98.0±15.1^{a}	<0.0001
IGFBP4	69.1±11.0	72.3±14.4	100±26.3	51.9±7.0	69.4±13.8	0.5605
IGFBP5	68.2±8.0^{b}	80.2±9.3^{ab}	100±7.5^{a}	75.8±6.5^{b}	70.3±4.9^{b}	0.0271
IGFBP7	37.5±4.7^{c}	56.3±8.2^{b}	86.5±15.5^{a}	84.5±13.7^{a}	100±7.9^{a}	<0.0001
Muscle (%)[2]						
IGFBP1	100±11.4^{a}	58.9±11.2^{ab}	44.2±26.0^{bc}	36.7±5.4^{bc}	24.2±4.6^{c}	0.0068
IGFBP2	78.8±5.7	64.5±7.4	64.9±7.20	100±11.2	74.3±10.6	0.0808
IGFBP3	90.2±12.0	100±6.7	85.1±10.7	89.2±10.6	81.9±8.5	0.6923
IGFBP4	100±16.9	76.4±16.4	89.4±26.8	75.2±7.8	48.1±8.6	0.0866
IGFBP5	100±12.1	83.8±9.3	82.9±20.4	71.9±6.9	73.6±13.7	0.4908
IGFBP7	40.6±8.8^{c}	46.1±3.3^{c}	60.7±11.2^{bc}	78.8±7.5^{ab}	100±15.1^{a}	0.0009

[1]Means are only presented for data where a significant line-by-age interaction was not present and were calculated across both lines at each embryonic day (e).
[2]Data within each gene are expressed relative to the age with the highest mRNA level (equal to 100%).
[abc]Values that do not share a common letter are significantly different (P≤0.05).

Table 4.4. Means[1] (±SEM) of the line main effect for gene expression and circulating hormones in post-hatch male broilers.

		ACRB	Ross 308	P-value
IGFs and Receptors				
Liver (%)[2]				
	IGF1	100±11.1	92.0±10.5	0.6546
	IGF2	100±10.2	92.3±10.1	0.4426
	IGF1R	94.4±7.3	100±9.4	0.826
Muscle (%)[2]				
	GHR	100±18.2[a]	71.4±11.4[b]	0.0447
	IGF1	48.4±9.4[b]	100±20.2[a]	0.0009
	IGF1R	71.1±11.2	100±17.8	0.242
IGFBPs				
Liver (%)[2]				
	IGFBP2	100±32.9[a]	67.9±18.3[b]	0.0073
	IGFBP3	83.4±10.8[b]	100±13.3[a]	0.0444
	IGFBP4	92.8±11.8	100±15.7	0.9186
	IGFBP5	69.0±5.2[b]	100±12.9[a]	0.0234
	IGFBP7	66.3±8.5[b]	100±16.5[a]	0.0027
Muscle (%)[2]				
	IGFBP1	100±27.5	97.2±38.7	0.3532
	IGFBP3	100±10.1[a]	70.08±7.4[b]	0.0041
	IGFBP4	54.1±10.19[b]	100±18.05[a]	0.0333
	IGFBP5	60.6±5.5[b]	100±14.3[a]	0.0125
	IGFBP7	75.2±8.7[b]	100±10.6[a]	0.0308
Hormones				
IGF1 (pg/mL)[3]		776.7±21.5	796.7±24.4	0.5014
IGF2 (pg/mL)[3]		190.9±15.9	167.7±19.8	0.7571

[1]Means are only presented for data where a significant line-by-age interaction was not present and were calculated between post-hatch day 10 through 40 for each line.
[2]Data within each gene are expressed relative to the line with the highest mRNA level (100%).
[3]Circulating hormone data are expressed as absolute concentration.
[ab]Values that do not share a common letter are significantly different (P≤0.05).

Table 4.5. Means[1] (±SEM) of the age main effect for gene expression and circulating hormones in post-hatch male broilers.

	d10	d20	d30	d40	P-value
IGFs and Receptors					
Liver (%)[2]					
IGF1	26.6±3.8^{c}	72.0±11.3^{b}	90.6±9.8^{ab}	100±9.9^{a}	<0.0001
IGF2	52.9±9.0^{c}	100±10.7^{a}	53.5±7.3^{bc}	74.5±10.5^{ab}	0.007
IGF1R	65.1±6.6^{bc}	100±6.9^{a}	49.3±4.6^{c}	86.7±11.7^{ab}	0.0002
Muscle (%)[2]					
GHR	56.6±12.4^{b}	100±24.8^{a}	95.2±20.3^{a}	97.1±27.7^{ab}	0.0260
IGF1	33.7±9.3	55.6±15.9	67.6±14.9	100±31.6	0.0531
IGF1R	51.0±10.7	98.6±22.2	74.8±13.1	100±29.2	0.0683
IGFBPs					
Liver (%)[2]					
IGFBP2	3.4±1.2^{c}	100±20.6^{a}	10.4±1.5^{b}	14.1±4.1^{b}	<0.0001
IGFBP3	56.7±11.6^{b}	100±15.6^{a}	57.9±8.5^{b}	84.2±16.6^{a}	<0.0001
IGFBP4	18.1±1.7^{b}	92.1±9.9^{a}	86.9±16.0^{a}	100±17.3^{a}	<0.0001
IGFBP5	45.3±6.6^{c}	100±7.4^{a}	66.2±4.24^{b}	72.6±19.9^{b}	0.0006
IGFBP7	50.3±13.3^{c}	100±20.0^{a}	56.9±6.3^{b}	70.5±18.2^{b}	0.0393
Muscle (%)[2]					
IGFBP1	19.4±6.5^{c}	100±41.9^{a}	49.4±22.3^{ab}	42.4±13.4^{b}	0.0011
IGFBP3	68.4±8.9	71.8±9.3	100±11.6	97.4±18.5	0.1052
IGFBP4	19.4±4.4^{c}	30.4±5.1^{b}	75.7±16.1^{a}	100±21.4^{a}	<0.0001
IGFBP5	36.5±3.6^{b}	48.6±3.4^{b}	79.1±9.5^{a}	100±21.5^{a}	0.0003
IGFBP7	55.2±6.5^{b}	80.5±12.6^{a}	100±13.8^{a}	92.2±15.9^{a}	0.0029
Hormones					
IGF1 (pg/mL)[3]	698.3±26.1^{b}	798.3±42.7^{a}	811.3±18.7^{a}	839.8±26.3^{a}	0.0096
IGF2 (pg/mL)[3]	145.5±13.8^{b}	247.9±27.5^{a}	164.8±21.5^{b}	139.2±23.6^{b}	0.0042

[1]Means are only presented for data where a significant line-by-age interaction was not present and were calculated across both lines at each post-hatch day (d).
[2]Data within each gene are expressed relative to the age with the highest mRNA level (100%).
[3]Circulating hormone data are expressed as absolute concentration.
[abc]Values that do not share a common letter are significantly different (P≤0.05).

CHAPTER 5

DYNAMIC CHANGES IN INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN EXPRESSION OCCUR BETWEEN EMBRYONIC AND EARLY POST-HATCH DEVELOPMENT IN BROILER CHICKENS[1]

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Abstract

Changes in somatotropic gene expression, particularly insulin-like growth factor binding proteins (IGFBPs), could contribute to efficient growth and rapid muscle accretion in commercial broilers. Previously, it was demonstrated that somatotropic gene expression has been altered by commercial genetic selection of broiler chickens but function of these genes during distinct developmental stages has yet to be elucidated. Therefore, the objective of this study to was to evaluate somatotropic gene expression in liver and breast muscle (*Pectoralis major*) between embryonic day (e) 12 and post-hatch d21 and circulating hormone concentrations were determined on post-hatch ages. Liver *IGF1* rose rapidly post-hatch. In muscle, *IGF1* exhibited a dynamic expression pattern and decreased from e14 to e20, returned to e14 levels at d5, and decreased again at d14. The mRNA levels of several IGFBPs changed between embryogenesis and post-hatch. Liver *IGFBP2* increased from e12 to e20 but returned to e12 levels on d1 and remained low. Conversely, liver *IGFBP4* expression was greater post-hatch than during embryogenesis. In breast muscle, expression of both *IGFBP2* and *IGFBP4* was reduced after hatch. Expression of select IGFBPs was also depressed in liver during the peri-hatch period between e20 and d1. Liver *IGFBP1*, *IGFBP3*, *IGFBP5*, and *IGFBP7* mRNA levels all decreased around this time and returned to embryonic levels by d3. Circulating insulin-like growth factor (IGF) 1 and 2 levels did not change between hatch and d21. These data suggest that IGF effects are likely modulated by *IGFR1* and IGFBP expression rather than changes in circulating hormone levels, with promotion or restriction of IGF-receptor binding regulating growth. Additionally, most IGFBPs synthesized in the liver may have growth-promotive effects, as their expression largely returns to embryonic levels post-hatch and steadily increases after hatch. Those produced in breast muscle appear to have broadly growth-promotive effects during embryogenesis but restrict growth of this tissue after hatch, as

they are downregulated to facilitate local IGF signaling. The differences observed in IGFBP expression between juvenile and post-hatch also suggest changes in IGFBP function or activity after the metabolic switch from the yolk as a primary energy source.

Introduction

The modern commercial broiler chicken is capable of rapid growth and muscle accretion during juvenile development [1-6]. The molecular mechanisms behind these traits have yet to be fully elucidated, and they are associated with highly conserved endocrine systems known to regulate vertebrate growth and metabolism. One of these systems is the somatotropic axis that is generally understood to induce growth via cellular proliferation and protein accretion in muscle and bone tissue [9, 10, 69, 446]. Many of these processes are indirectly induced by growth hormone (GH) through increased insulin-like growth factor (IGF) 1 and IGF2 production and signaling. Circulating IGFs are synthesized in the liver [18-20, 85] and influence growth by downregulating apoptosis and increasing cellular proliferation after binding the type I IGF receptor (IGFR1) [90, 91, 146].

In mammals, IGF1 contributes to growth and adult body weight [447], and a lack of IGF1 is typically associated with dwarfism [448]. However, the direct relationship between IGF signaling and growth in birds is unclear. Administration of IGF1 did not stimulate growth in two- to three-week-old male chickens [187, 189] or four-week-old female chickens [188]. Increased hepatic *IGF1* mRNA expression has been observed in birds selected for high body weight [93], but not consistently [94]. Similarly, fast-growing chickens had greater plasma IGF2 [199], but IGF2 did not induce weight gain when directly administered [200]. Circulating IGF1 concentrations did not differ between modern commercial Ross 308 and legacy Athens Canadian Random Bred (ACRB) broilers, despite Ross 308 chickens having significantly greater body weights post-hatch [446, 449].

The IGFs are regulated by IGF-binding proteins (IGFBPs). This family of proteins is highly conserved across vertebrates [21, 96-99], although IGFBP6 does not appear to be present in birds.

Modulation of IGF action occurs when an IGFBP binds an IGF to enhance or reduce receptor binding affinity, extend the hormone's half-life, or alter tissue specificity [103, 118, 450]. Several effects of IGFBPs on IGF action have been observed in mammalian and some avian models and many are tissue-specific and context-dependent. For example, IGFBP1 was shown to inhibit protein synthesis in human skeletal muscle [119], and IGFBP2 and IGFBP4 were shown to suppress long bone growth in mouse cells and embryonic chick wing, respectively [106, 120]. In rat myoblasts, IGFBP5 has a proliferative effect when bound to IGF1 but an inhibitory effect when bound to IGF2 [121]. Additionally, some IGFBPs appear to act independently. For example, IGFBP2 can upregulate apoptosis in sarcoma and breast cancer cells [104, 105], while IGFBP5 can enhance bone cell proliferation in embryonic chick wing without binding to an IGF [106]. As the somatotropic axis is highly conserved across vertebrates and the IGFBP family exhibits a multiplicity of functions, it is important to understand their impact on economically important traits in poultry, such as muscle accretion and feed efficiency.

We previously measured circulating IGF1 and IGF2, as well as mRNA expression of IGFs and IGFBPs in the liver and breast muscle tissue of modern commercial (Ross 308) and legacy [Athens-Canadian Random Bred (ACRB)] broiler lines during embryonic and juvenile development [446]. Although no differences were observed in circulating IGFs between lines, IGFBP levels diverged during embryogenesis and post-hatch. The liver is thought to be the primary source of IGFBPs in plasma, and local production of IGFBPs in muscle could mediate IGF action in this tissue. Unchanged circulating IGF levels across broiler lines with differing body weights (BWs) and growth rates suggest that rapid growth effects of IGFs could be facilitated by the IGFBPs. Liver *IGFBP1* was greater in Ross 308 embryos on e14, and *IGFBP4* increased in Ross 308 on e14 and e16. Post-hatch, *IGFBP3*, *IGFBP5*, and *IGFBP7* levels were greater in Ross 308

liver. Typically, IGFBPs secreted into plasma extend the half-life of circulating IGFs, suggesting that some IGFBPs synthesized in the liver work to potentiate effects of these hormones. Observed *IGFBP* expression levels were reduced for Ross 308 in the breast muscle in some instances, however. Embryonic breast muscle tissue in Ross exhibited reduced *IGFBP4* compared to ACRB, but *IGFBP4* was greater in the same tissue after hatch. Similarly, Ross 308 also exhibited increased *IGFBP5* and *IGFBP7* post-hatch in skeletal muscle. Only *IGFBP3* was reduced in Ross 308 muscle compared to ACRB muscle during post-hatch. This could denote that certain IGFBPs enhance or inhibit muscle accretion when secreted locally. Holistically, these results suggest that IGFBPs likely modulate broiler growth and development and select IGFBPs regulate overall body growth and muscle accretion in modern broilers.

Somatotropic axis activity initiates between e12 and e16 in chickens and continues developing into the early post-hatch period [62, 419, 426]. As prior work indicates, expression of certain IGFBPs changes during embryonic and post-hatch development [446]. Thus, the influence of IGFBPs on regulating broiler growth may have distinct effects across these periods. These effects might be linked to traits observed in modern commercial broilers such as rapid growth rate and muscle accretion and improved feed efficiency. Therefore, the objective of this study was to evaluate somatotropic gene expression in liver and breast muscle of commercial broiler chickens from mid-embryogenesis through three weeks post-hatch to determine whether dynamic changes in somatotropic gene expression contribute to economically valuable traits.

Materials & Methods

Animals and tissue collection

Tissues used in this study were collected from embryonic and post-hatch male Ross 308 broilers hatched from a breeder flock raised at the University of Georgia's Poultry Research Center farm.

All procedures using animals were approved by the University of Georgia's Institutional Animal Care and Use Committee.

Fertile Ross 308 eggs were obtained and incubated under standard conditions (37.5°C and 60% humidity, rotation every 2 – 3 h), with the day eggs were set defined as embryonic day (e) 0. After hatching, birds were raised in floor pens (n = 6 pens) with free access to water and a two phase commercial-type broiler diet. Birds were fed starter (21.3% crude protein, 1.2% digestible lysine, 3050 kcal/kg metabolizable energy, 0.95% calcium and 0.48% available phosphorus) from d0 – d14 and grower (19.6% crude protein, 1.09% digestible lysine, 3120 kcal/kg metabolizable energy, 0.85% calcium and 0.43% available phosphorus) from d14 – d21.

On e12, e14, e16, e18, and e20, embryos were humanely euthanized. Skin, liver, and breast muscle were collected from 12 embryos at each time point. The sex of each embryo was determined by PCR analysis of the sexually dimorphic chromo-helicase-DNA binding protein [375] using genomic DNA extracted from skin tissue, as previously described [449]. Liver and breast muscle from six male embryos at each age (n = 6) were used for gene expression analysis as described below.

One bird was selected from each floor pen at post-hatch d0 (day of hatch), d1, d3, d5, d7, d10, d14, and d21 from which blood and tissues were harvested. Blood was collected from a cardiac puncture into heparinized tubes, stored on ice until centrifugation at 1,500 x *g* for 10 minutes at 4°C, and stored at -20°C prior to analysis of circulating hormone levels. After blood collection, birds were euthanized by cervical dislocation, and sexed by visual identification of the gonads and only males were used for liver and muscle collection (n = 6). Tissues were flash frozen in liquid nitrogen and stored at -80°C prior to total RNA extraction for gene expression analysis.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated from liver and breast muscle using RNeasy Mini kits (Qiagen) with modifications for lipid-rich or fibrous tissues, respectively, and analyzed by RT-qPCR as previously described [446, 449]. Briefly, 1 µg total RNA was reverse transcribed with random hexamer primers (ThermoFisher, Waltham, MA) and M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA). Resulting cDNA was amplified by qPCR using intron-spanning primers (Table 1; Integrated DNA Technologies, Coralville, IA) designed with Primer Express software (Applied Biosystems, Foster City, CA). Serial dilutions of pooled liver and muscle cDNA were analyzed by RT-qPCR to determine amplification efficiency for each primer pair, which was calculated using the following equation: efficiency = $(10^{(-1/\text{slope})}-1)$ [376, 377].

Transcripts were normalized to 18s ribosomal rRNA (18s rRNA). The equation $(2^{\Delta Ct})_{target}/(2^{\Delta Ct})_{GAPDH\ or\ 18s}$, where $\Delta Ct = Ct_{no\ RT} - CT_{sample}$, was used to transform and normalize data as previously described [378-381, 446, 449]. Data are expressed relative to the age with the highest mRNA level. As a result, the age with the highest expression level is 100% in all cases.

IGF enzyme-linked immunosorbent assays (ELISAs)

Samples were analyzed in duplicate on a VICTOR3 Multilabel Plate Reader (Perkin Elmer, Waltham, MA) using commercially available competitive-binding ELISAs (Cusabio, Houston, TX) for IGF1 and IGF2, which have sensitivity limits of 125 and 62.5 pg/ml, respectively. ELISAs were performed according to manufacturer's instructions, with the modification that plates were incubated for 18 h at 4ºC instead of 60 min at 37ºC after adding the standards or samples and biotinylated IGF. All samples were analyzed in a single ELISA plate for IGF1 and two ELISA plates for IGF2. The intra-assay CV for the IGF1 ELISA was determined to be 8.07, and the and intra-assay and inter-assay CVs for IGF2 were determined to be 17.93 and 15.06 and 37.87, respectively.

Statistical analysis

Data were analyzed with a one-way analysis of variance (ANOVA) using the Fit Model Procedure of JMP Pro 14 (SAS Institute, Cary, NC). When ANOVA indicated a significant effect of age, *post hoc* means comparisons were performed using the test of least significant difference. All differences were considered significant at $P \leq 0.05$.

Results

IGF and hormone receptor expression

Distinct developmental expression patterns were detected for *IGF1*, *IGF2*, *IGFR1*, and *GHR* in the liver (**Figure 5.1**; $P \leq 0.05$). Expression of *IGF1* began to increase on d5 and continued steadily rising through d21 (**Figure 5.1A**). Levels of *IGF2* decreased from e12 to e16, increased transiently on e20 before droping again on d0, and then steadily increased after hatch (**Figure 5.1B**). Unlike *IGF1* and *IGF2, IGFR1* expression dropped between e12 and d0 and increased to intermediate levels on d5, after which it remained constant (**Figure 5.1C**). Expression of *GHR* did not change beween e12 and 18, decreased ten-fold between e18 and d1, and then steadily increased again after hatch through d21 (**Figure 5.1D**).

As in liver, expression of these genes was also dynamic in breast muscle between mid-embryonic development and three weeks post-hatch (**Figure 5.2**; $P \leq 0.05$). A cyclical expression pattern was observed for *IGF1* mRNA, with a transient decrease observed in the peri-hatch period and a second decline between d7 and d21 (**Figure 5.2A**). Levels of *IGF2* increased slightly between e12 and e16 and remained at that level with the exception of subtle and inconsistent decreases observed on d5, d7, and d14 (**Figure 5.2B**). Expression of *IGFR1* in breast muscle was highest on e12 and 14, decreased through d3, and remained at that level through d21 (**Figure**

5.2C). Expression of *GHR* exhibited a similar pattern, though there was a transient increase on d1 and d3 and the overall difference in expression across the ages was smaller (**Figure 5.2D**).

IGFBP expression

Dynamic expression patterns were exhibited between the developmental stages for all IGFBPs produced in the (**Figure 5.3**; $P \leq 0.05$). There was a transient decrease in *IGFBP1* at d1 and expression of this gene at other ages was relatively stable (**Figure 5.3A**). Expression of *IGFBP2* increased from e12 to d0 but dropped sharply between d0 and d1 and remained low through d21 (**Figure 5.3B**). A decrease was observed for *IGFBP3* from e18 to d0 and it remained at low-to-intermediate levels after hatch (**Figure 5.3C**). Only *IGFBP4* expression increased consistently in the liver throughout embryogenesis and after hatch. Its levels increased between e16 and e20 and again at d5. Though not statistically significant, levels on d21 were substantially higher on d21 than the other post-hatch ages (**Figure 5.3D**). A decrease in *IGFBP5* was observed at and just after hatch, but expression was restored to embroynic levels by d5 (**Figure 5.3E**). Much like *IGFBP1* and *IGFBP5*, *IGFBP7* decreased transiently from after e20 before increasing from d3 to d5 (**Figure 5.3F**).

Expression of all IGFBPs except IGFBP7 changed between developmental stages in breast muscle (**Figure 5.4**; $P \leq 0.05$). Transcripts of *IGFBP1* could not be detected in this tissue, which is consistent with our previous findings [446]. Levels of *IGFBP2* expression increased between e12 and e16. However, it began to decrease again on e20 through d3 and remained low during the first three weeks of post-hatch development (**Figure 5.4A**). Levels of *IGFBP3* diminished between e14 and e16 and returned to e14 levels at d1. A second decrease occurred at d5 and its expression remained relatively lower through d10 (**Figure 5.4B**). Levels of *IGFBP4* decreased from e12 to e20 and dropped substantially again on d3, so overall they were lower after hatch than during

embryogenesis (**Figure 5.4C**). For *IGFBP5*, expression steadily declined from e20 to d3 and tended to be lower between d3 and d21 than at earlier ages (Figure 4D). No significant effect of age was detected for *IGFBP7* (**Figure 5.4E**; P>0.05).

Circulating IGFs in post-hatch plasma

Circulating IGF concentrations did not change during juvenile development (**Figure 5.5**; P>0.05). However, changes in levels of IGF2 approached significance (**Figure 5.5B**; P=0.0661), with concentrations appearing to rise between d1 and d3 before dropping after d7.

Discussion

The somatotropic axis is an important regulator of mammalian growth and development. As a highly conserved endocrine axis, its functions likely extend to birds as well. Therefore, it is reasonable to speculate that it is critical for the proper development and rapid growth of commercial broiler chickens. Previously, expression of genes associated with the somatotropic axis were observed to differ between a modern commercial broiler line, the Ross 308, and a legacy broiler line, the ACRB [446]. These results indicate that the somatotropic axis is involved in the growth and development of modern broilers to such a degree that its activity has been altered by commercial genetic selection. However, its functions during critical development periods, including mid-to-late embryogenesis and early post-hatch, have not been fully elucidated. The somatotropic axis becomes active beginning of the last week of embryogenesis, although IGF does not appear GH-dependent until post-hatch, when the axis is fully mature [62, 426, 446]. Thus, evaluating somatotropic gene activity from mid-embryonic through early post-hatch development may provide insight into the axis' functions during these distinct developmental periods.

In this study, the expression of somatotropic genes were measured in male Ross 308 commercial broilers from mid-embryonic development through three-weeks post-hatch. Levels of

IGF1 in liver were low during embryogenesis compared to post-hatch when they increased greatly. In breast muscle, *IGF1* levels diminished between embryogenesis and the peri-hatch period but rose again in the first week post-hatch. Comparatively, *IGF2* was present during embryonic and post-hatch development at similar levels in both tissues. Like *IGF2*, *IGFR1* was greatest in liver and breast muscle during the last week of embryogenesis and decreased following hatch. Expression of hepatic *GHR* was lowered only during peri-hatch period. In breast muscle, it was noticeably reduced by the first post-hatch week compared to levels during embryogenesis. The IGFBPs also demonstrated dynamic changes in expression between developmental stages, particularly during the peri-hatch period when mRNA levels of several dropped transiently. This was observed for *IGFBP1*, *IGFBP2*, *IGFBP3*, *IGFBP5*, and *IGFBP7* in liver, as well as *IGFBP2* and *IGFBP4* in breast muscle. Other IGFBPs increased post-hatch, such as hepatic *IGFBP4* and breast muscle *IGFBP3*. Despite these dynamic changes in expression in liver and breast muscle, circulating IGF1 and IGF2 concentrations in plasma did not change throughout the first three weeks post-hatch. Collectively, these results provide further evidence that IGFBPs are the major controllers of IGF signaling, not circulating IGF plasma levels [446]. In addition, the dynamic fluctuations in IGFBP expression observed may indicate that the activity of these proteins varies based on developmental stage.

Of the two IGFs, IGF1 is typically thought to be the more important regulator of post-natal growth in mammals [447]. Our results revealed that in broilers, hepatic *IGF1* expression was low during embryonic development and early post-hatch, until d3, after which it increased through d21. This pattern is consistent with those previously reported, with the highest levels occurring during the 2.5 to 3.5-week period in which weight gain is most rapid in broilers [446, 451]. Low embryonic expression of *IGF1* in the liver coincided with greater *GHR* in the same tissue.

Specifically, *GHR* decreased from e18 to e20, but returned to mid-embryonic levels at d7. This indicates that GH signaling via GHR likely does not control IGF1 production until after hatch, when the somatotropic axis becomes fully active. However, *IGF2* is expressed in the liver a week prior to hatch at levels comparable to those post-hatch [62, 419]. These results suggest that IGF2 is the primary stimulator of IGF-induced growth during embryogenesis, and it potentially has a reduced role in post-hatch growth and development as IGF1 increases [452], similarly to mammals. Further evidence for this comes from the observation that *IGFR1* levels were greater in embryonic liver and breast muscle prior to hatch. As IGFR1 has a lower affinity for IGF2 than IGF1 [453], higher *IGFR1* expression in these tissues during embryogenesis would increase their sensitivity to IGF2 signaling. The decrease in *IGFR1* post-hatch may indicate the development of negative feedback in the somatotropic axis, further supporting that maturation of this endocrine axis does not occur until after hatch. Thus, the transition from embryonic to post-hatch growth and development is accompanied by a switch in the primary signaling IGF; IGF2 is the primary facilitator of IGF-induced growth prior to hatch, and IGF1 inherits this role after hatch.

The production of *IGF1* in breast muscle also suggests that local synthesis of IGF1 is critical for growth of this tissue, as has been previously observed [446]. A drop in *IGF1* levels in muscle was observed between e12 and e20, and between e20 and d3, *IGF1* levels increased greatly again. This was followed by a decrease at d10 with no restoration in expression levels by d21. Comparatively, IGF2 may assist in maintaining muscle growth, as expression of *IGF2* in breast muscle rose between e12 and e16 but expression remained constant thereafter. Thus, rapid muscle accretion induced by satellite muscle cell proliferation observed shortly post-hatch in broilers could be facilitated by locally produced IGF1, whereas IGF2 might perform a role in maintaining muscle tissue throughout both embryogenesis and post-hatch [178]. Additionally, *IGFR1*

expression may change in the wake of increased paracrine IGF1 signaling. By d3, *IGFR1* levels decreased in breast muscle, whereas *IGF1* increased during that time. This suggests that *IGFR1* mRNA production diminishes post-hatch in breast muscle following increased IGF1 production, potentially as part of a negative feedback loop.

The IGFBPs can inhibit growth through interference with IGF signaling by preventing access to IGFR1 [103]. Both IGF1 and the IGFBPs found in circulation are primarily produced in the liver. Therefore, diminished expression of multiple IGFBPs in liver post-hatch could allow for greater access of circulating IGFs to IGFR1, ultimately promoting growth. In the liver, both *IGFBP2* and *IGFBP3* decreased at or shortly after hatch and remained relatively low through d21. This suggests that hepatic IGFBP2 and IGFBP 3 have an inhibitory effect on post-hatch growth. Data from the present study suggests that endocrine IGFBP2 and IGFBP3 have an inhibitory effect on post-hatch growth in chickens. Further, the transition from embryonic development to post-hatch requires a metabolic switch from yolk lipoprotein to carbohydrates sourced from a corn-based diet [454, 455]. This suggests that IGFBP2 and IGFBP3 may have an inhibitory effect of glucose homeostasis or alter carbohydrate metabolism, as well as depressing growth. Decreased gene expression of these IGFBPs, then, allows for greater IGF signaling.

Similar growth-inhibitory effects in broiler breast muscle may also be caused by IGFBP2 and IGFBP4. Like locally produced IGFs, these IGFBPs potentially act in a paracrine fashion in this muscle, implying their ability to inhibit growth could be tissue specific. Overexpression of *IGFBP2* in mice slows the development of myofibers, causing total lower body protein and reduced muscle mass [456, 457]. In this study, *IGFBP2* was observed to increase between e12 and e16 but began to decrease afterward to very low levels throughout the post-hatch period. If IGFBP2 acts similarly in avian muscle, a reduction in expression would be required to facilitate rapid

muscle accretion observed after hatch. Levels of *IGFBP4*, which decreased between e14 and d3, is also involved in myofiber development, particularly in blocking differentiation in mouse myoblasts [458-460]. Taken together, IGFBP2 and IGFBP4 appear to act in a paracrine, inhibitory fashion in breast muscle prior to hatch and are subsequently downregulated to allow for rapid muscle growth after hatch.

Select IGFBPs promote or inhibit IGF action, as well as have independent effects [104, 105]. The results observed in this study suggest that IGFBP4 functions as a promoter or inhibitor of growth in a tissue-specific manner. Throughout embryonic and post-hatch development, *IGFBP4* mRNA levels increased in liver and decreased in breast muscle. When released into circulation from the liver, IGFBPs bind to IGFs in plasma to extend their half-life. Therefore, higher *IGFBP4* expression in broiler liver post-hatch suggests it may promote growth via stabilizing it in circulation and, therefore, promoting IGF signaling, similar to mammals [439]. However, *IGFBP4* mRNA decreased at d3 in breast muscle. A reduction in local IGFBP4 activity in broiler muscle could facilitate growth of this tissue, as less IGFBP4 would allow for increased IGF access to IGFR1. This inhibitory effect of IGFBP4 in mammals has been established to occur via paracrine signaling, including acting as an IGF antagonist in mouse smooth muscle cells [438, 461, 462] and rat skeletal muscle cells [463]. These data and our results suggest that IGFBP4 has multiple roles in the context of growth, including both positive and negative IGF interactions that may be tissue-specific [464], and these effects appear conserved between mammals and birds.

The effects of IGFBPs on IGF signaling can affect biological processes outside of cellular proliferation and differentiation. Hepatic *IGFBP1*, *IGFBP5*, and *IGFBP7* decreased from e20 to d1, only to return to embryonic levels within the first week of hatch. These changes during the peri-hatch period may be the result of the known metabolic switch from pre- to post-hatch. Most

of the energy utilized by the chick embryo is sourced from lipoproteins in the yolk. This occurs until around d3 [454], after which energy is purely obtained from a carbohydrate-based diet [455]. The IGFBP downregulation observed peri-hatch coincides with this metabolic transition, as the IGFs begin to function in glucose homeostasis via GH suppression [465]. Overexpression of human *IGFBP3* in fasted mice induced hyperglycemia and impaired glucose tolerance [466]. In mouse models where *IGFBP3* was deleted, mice had larger livers and higher body weights than controls [444, 467]. Once the somatotropic axis becomes fully mature early post-hatch, IGFBPs can also begin to function as regulators of IGF signaling.

Circulating IGF levels did not change significantly during post-hatch development, although IGF2 tended towards a decrease over time. This suggests that, as IGF1 concentrations stay relatively stable in plasma, its effects are modulated by IGFBP activity and IGFR1 sensitivity. The slight decrease observed in circulating IGF2 suggests that IGF-induced growth is primarily carried out by signaling and modulation of IGF1. The IGFBPs may alter endocrine IGF signaling by directly binding to IGFs and either preventing them from binding IGFR1 or facilitating their transport to IGF-sensitive tissues expressing the receptor [103, 118, 450]. Therefore, the IGFBPs are critical for controlling endocrine IGF action. However, as both IGF1 and IGF2 were detected in breast muscle tissue, locally produced IGFs that signal in a paracrine fashion are likely important for breast muscle growth.

Results presented here indicate that somatotropic gene expression in broilers changes dynamically in a tissue-specific manner between embryogenesis and three-weeks post-hatch, which suggests these genes function in regulating broiler growth, development, and metabolism during these periods. The primary signaling IGF may switch from IGF2 during embryogenesis to IGF1 post-hatch, a consequence of a fully mature somatotropic axis. The effect of IGFBPs on

growth likely differs depending on developmental stage, tissue of origin, and mode of action. Certain IGFBPs synthesized in the liver, such as IGFBP4, appear to be promotive for IGF signaling, potentially through extending IGF half-life in circulation. Although a transient decrease in expression of these IGFBPs decreased at peri-hatch, this may be caused by the switch in nutrient utilization beginning at late embryogenesis. The rebound in hepatic IGFBP levels during juvenile development could still be indicative of a net-positive impact on growth. However, in breast muscle, most IGFBPs more likely function in an inhibitory fashion in a paracrine manner. The reduction in breast muscle IGFBP expression during the peri-hatch period and the first three weeks post-hatch may allow for enhanced sensitivity to circulating and paracrine IGFs, which is necessary to induce the rapid post-hatch growth of this tissue typical for modern commercial broilers. However, the strength of this effect may vary across members of the protein family. In conclusion, differential activity of the IGFBPs between developmental stages reinforces the idea they are the critical regulators of IGF signaling that contributes to broiler growth and development.

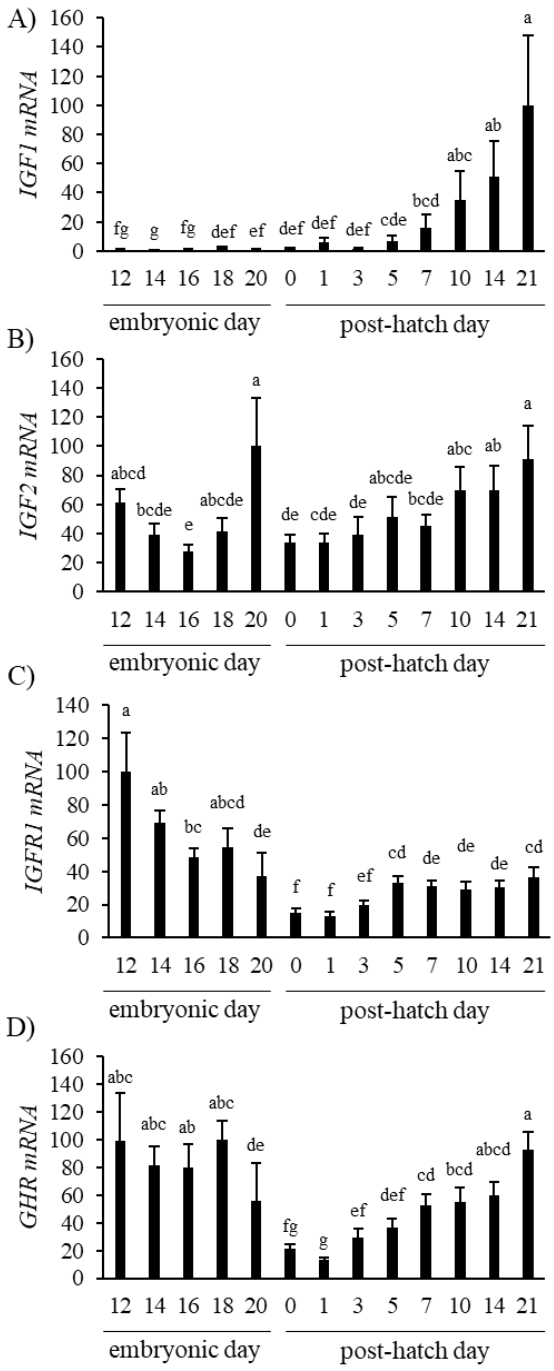

Figure 5.1. Relative mRNA expression of (A) *IGF1*, (B) *IGF2*, (C) *IGFR1*, and (D) *GHR* in liver on embryonic days (e) 12, 14, 16, 18, and 20, day of hatch (d0), and post-hatch days (d) 1, 3, 5, 7, 10, 14, and 21 in Ross 308 male broilers. Relative expression levels were measured using RT-qPCR and normalized to *18S* RNA. The data (mean + SEM) are expressed relative to the age with the highest expression level (equivalent to 100%). Data were analyzed by one-way ANOVA followed by Fisher's least significant difference test. All genes exhibited an effect of age, and values without a common letter are significantly different ($P \leq 0.05$; n = 6 replicate birds at each age).

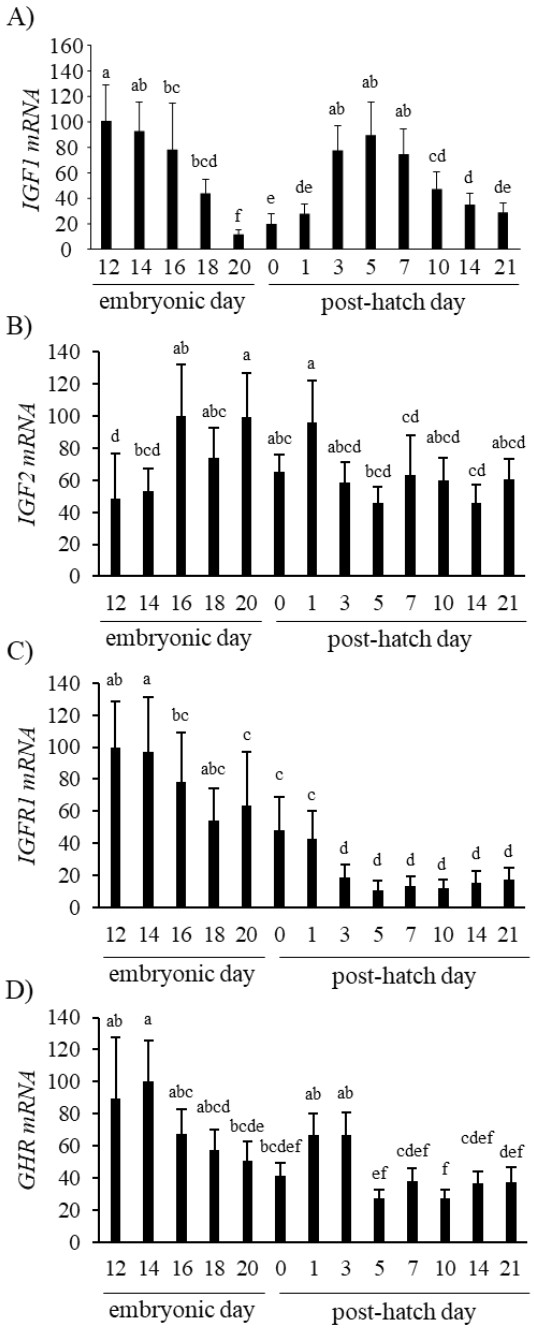

Figure 5.2. Relative mRNA expression of (A) *IGF1*, (B) *IGF2*, (C) *IGFR1*, and (D) *GHR* in breast muscle on embryonic days (e) 12, 14, 16, 18, and 20, day of hatch (d0), and post-hatch days (d) 1, 3, 5, 7, 10, 14, and 21 in Ross 308 male broilers. Relative expression levels were measured using RT-qPCR and normalized to *18S* RNA. The data (mean + SEM) are expressed relative to the age with the highest expression level (equivalent to 100%). Data were analyzed by one-way ANOVA followed by Fisher's least significant difference test. All genes exhibited an effect of age, and values without a common letter are significantly different ($P \leq 0.05$; n = 6 replicate birds at each age).

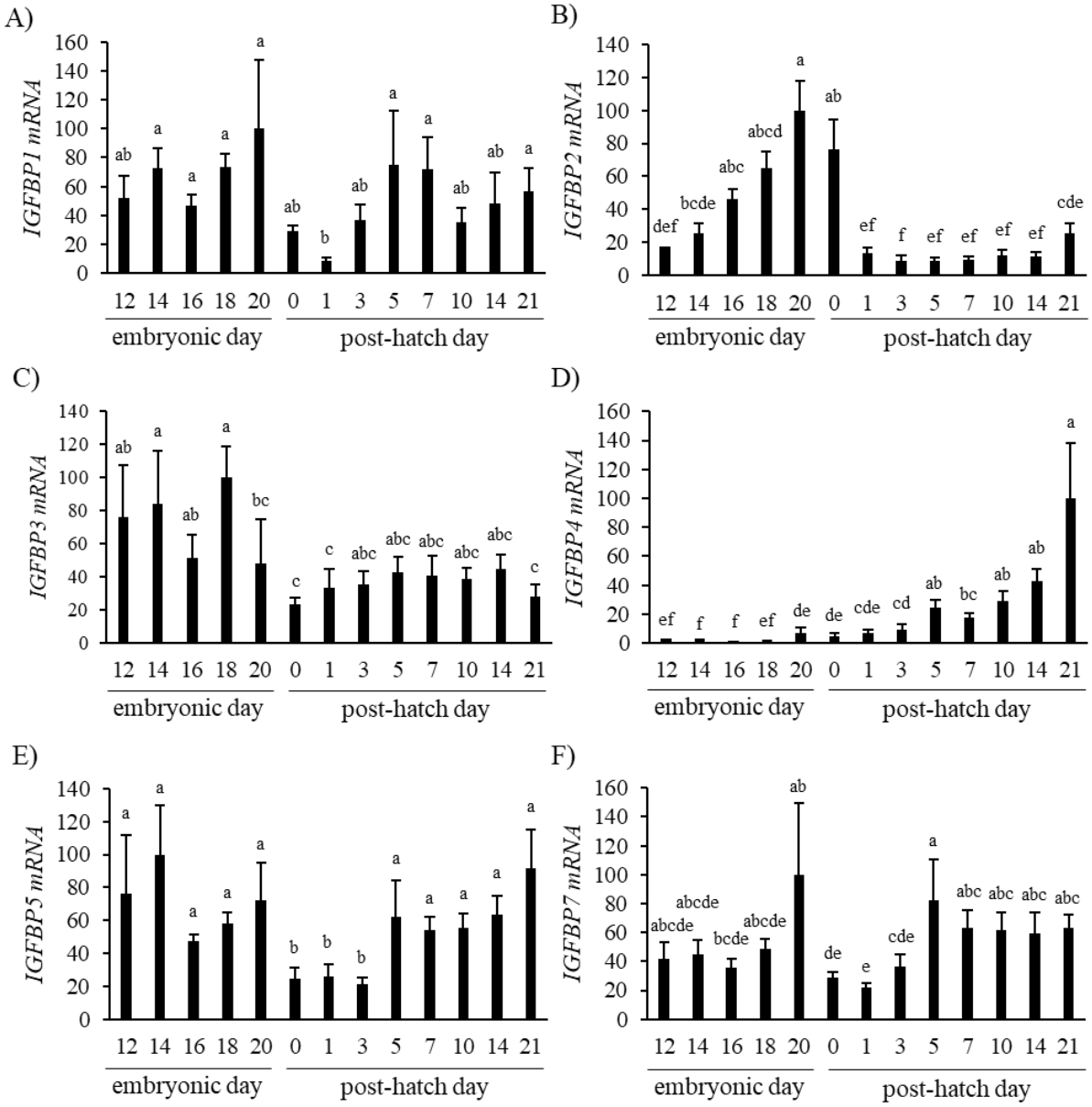

Figure 5.3. Relative mRNA expression of (A) *IGFBP2*, (B) *IGFBP3*, (C) *IGFBP4*, (D) *IGFBP5*, and (E) *IGFBP7* in liver on embryonic days (e) 12, 14, 16, 18, and 20, day of hatch (d0), and post-hatch days (d) 1, 3, 5, 7, 10, 14, and 21 in Ross 308 male broilers. Relative expression levels were measured using RT-qPCR and normalized to *18S* RNA. The data (mean + SEM) are expressed relative to the age with the highest expression level (equivalent to 100%). Data were analyzed by one-way ANOVA followed by Fisher's least significant difference test. All genes exhibited an effect of age, and values without a common letter are significantly different ($P \leq 0.05$; n = 6 replicate birds at each age).

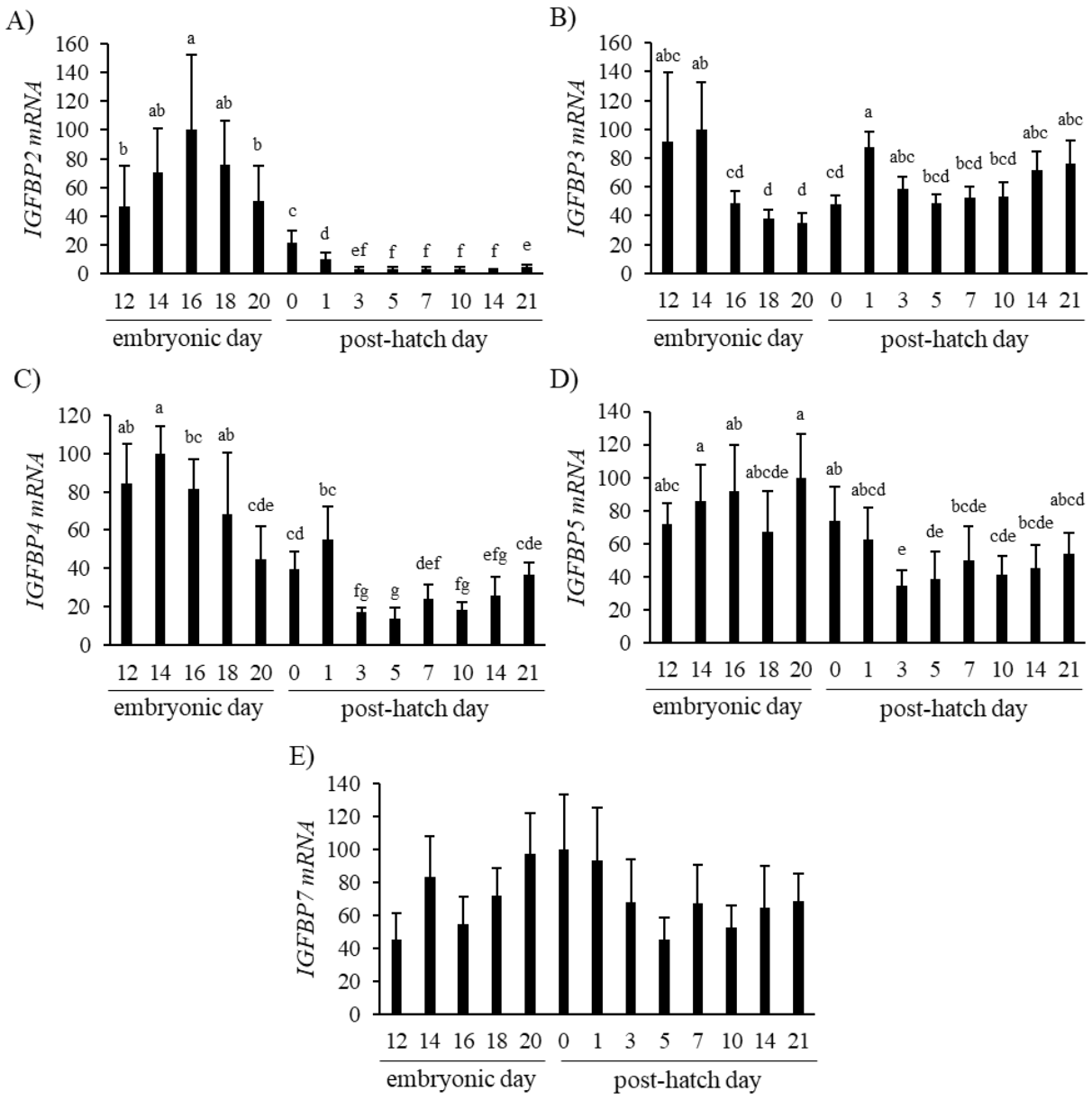

Figure 5.4. Relative mRNA expression of (A) *IGFBP2*, (B) *IGFBP3*, (C) *IGFBP4*, (D) *IGFBP5*, and (E) *IGFBP7* in breast muscle on embryonic days (e) 12, 14, 16, 18, and 20, day of hatch (d0), and post-hatch days (d) 1, 3, 5, 7, 10, 14, and 21 in Ross 308 male broilers. Relative expression levels were measured using RT-qPCR and normalized to *18S* RNA. The data (mean + SEM) are expressed relative to the age with the highest expression level (equivalent to 100%). Data were analyzed by one-way ANOVA followed by Fisher's least significant difference test. For genes demonstrating an effect of age, values without a common letter are significantly different ($P \leq 0.05$; n = 6 replicate birds at each age).

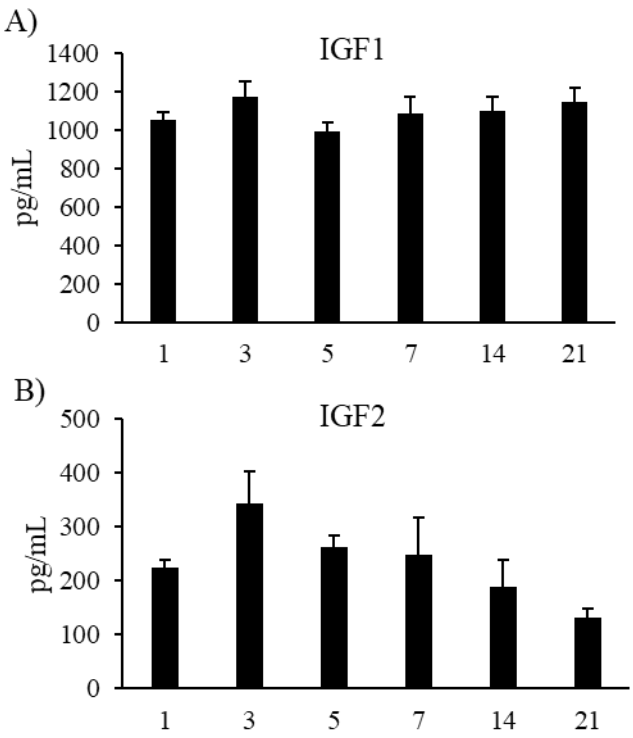

Figure 5.5. Circulating (A) IGF1 and (B) IGF2 in Ross 308 male broilers on post-hatch days (d) 1, 3, 5, 7, 14, and 21 using an IGF1 and IGF2 ELISA, respectively. Data were analyzed by one-way ANOVA followed by Fisher's least significant difference test. The data (mean + SEM) are presented as the average hormone level at each age (pg/mL). No significant age effects were observed for (A) IGF1 or (B) IGF2 ($P>0.05$; $n = 6$ replicate birds at each age).

Table 5.1. Primers used for reverse transcription-quantitative PCR.

Gene Symbol	Forward primer (5'-3')	Reverse primer (5'-3')	ID[a]	Efficiency
IGFs				
IGF1	TGAGCTGGTTGATGCTCTTC	AGCCTCCTCAGGTCACAACT	20816	0.99
IGF2	AGTCAGAGCGTGACCTCTCC	CTGCGAGCTCTTCTTCTGC	53800	1.05
Hormone receptors				
GHR	TGCTGATTTTTCCTCCTGTG	GGCTGGCTAAGATGGAGTTC	23973	1.08
IGF1R	TGGGGACCTCAAAAGTTACC	ATCCCATCAGCAATCTCTCC	74990	1.04
IGF binding proteins				
IGFBP1	CAGAGAAGTGGAGGGGACAT	CTTCTGGGGATCCAGGAAT	47713	
IGFBP2	ATCACAACCACGAGGACTCA	GAGGGAGTAGAGGTGCTCCA	18698	0.96
IGFBP3	TTGAGTCCTAGGGGTTTCCA	ATATCCAGGAAGCGGTTGTC	82156	1.02
IGFBP4	AACTTCCACCCCAAGCAG	AATCCAAGTCCCCCTTCAG	68153	0.96
IGFBP5	CTGAAGAGCAGCCAGAGGAT	TTGTCCACACACCAACACAG	38163	0.98
IGFBP7	ATGTGACAGGAGCACAGATCTACCT	TCTGGATACCATACTGTCCTCGAAT	61018	0.95
Reference genes				
GAPDH	AGCCATTCCTCCACCTTTGAT	AGTCCACAACACGGTTGCTGTAT	23323	1.00
18s[b]	AGCCTGCGGCTTAATTTGAC	CAACTAAGAACGGCCATGCA	173612	0.86

[a] Transcript identification from Ensembl chicken genome assembly GRCg6a (http://www.ensembl.org/Gallus_gallus/Info/Index) preceded by ENSGALT000000.
[b]This sequence is not on the assembled chicken genome and primers were designed based on the sequence for GenBank accession number AF173612.

CHAPTER 6

THE EFFECT OF THYROID HORMONES ON SOMATOTROPIC AND THYROTROPIC GENE EXPRESSION IN QM7 CELLS [1]

[1]Vaccaro, L.A., A. L. Smith, K. Herring, and L. E. Ellestad. 2023. To be submitted for publication to *Poultry Science*.

Abstract

The thyrotropic and somatotropic axes engage in crosstalk during their regulation of vertebrate growth. This could include modulation of the insulin-like growth factors (IGFs) and IGF binding proteins (IGFBPs) by the thyroid hormones (THs). Previously, several IGFBPs were identified to be differentially expressed in the breast muscle of modern and legacy broilers, and putative TH response elements were found in select IGFBP promoter regions. This suggests that the IGFBPs could contribute to increased body weight and growth rates associated with modern broilers, and their actions may be controlled by TH signaling. This study's objective was to use the Quail Muscle Clone 7 (QM7) myoblast cell line to determine effects of triiodothyronine (T_3), thyroxine (T_4), and cellular differentiation state on somatotropic and thyrotropic gene expression in avian muscle cells. Undifferentiated and differentiated QM7s were treated with 0, 1, 5, or 25 ng/mL T_3 or T_4 for 0.5, 6, or 24 hours, and mRNA expression was determined by RT-qPCR (n=4 replicates per hormone per state). Data were analyzed with a two-way ANOVA followed by Fisher's least significant difference test. Levels of *GHR* were greater in T_3-treated undifferentiated cells at 6 and 24 hours, as well as in T_4-treated undifferentiated cells at 0.5 and 6 hours ($P \leq 0.05$). T_3 treatment increased *IGF2* expression in both cell types ($P \leq 0.05$). *IGFBP5* has increased by all T_3 and T_4 treatments in undifferentiated cells, and T_4 caused *IGFBP3* to decrease at 6 hours in undifferentiated cells ($P \leq 0.05$). *THRB* and *DIO3* were also raised by T_3, regardless of cell type ($P \leq 0.05$). Expression levels of these genes were also directly compared in undifferentiated or differentiated cells that were not treated with THs. *GHR*, *IGFR1*, *IGF2*, *IGFBP3*, and *THRA* were greater in undifferentiated cells overall, while *IGFBP2*, *IGFBP7,* and *DIO2* were higher in differentiated cells. Together, these data suggest that the THs upregulate somatotropic gene expression to stimulate cell growth in muscle tissue, and that QM7 myoblasts and myotubes are

differentially sensitive to hormonal signaling as evidenced by levels of hormone receptors and enzyme metabolizing THs.

Introduction

Vertebrate growth and metabolism are in part regulated by the highly conserved somatotropic and thyrotropic endocrine axes [8, 9, 69, 468]. The somatotropic axis induces tissue growth and has metabolic effects in muscle, bone, and adipose tissue, while the thyrotropic axis regulates basal metabolism and the growth of skeletal muscle and bone [10, 49, 50]. Previously, expression of somatotropic and thyrotropic genes, as well as circulating hormone levels, were measured in modern commercial broilers (Ross 308) and a legacy line of broilers representing birds from the 1950's, the Athens Canadian Random Bred (ACRB). Compared to the ACRB, modern broilers have greater body weights, lower feed conversion ratios (FCR), and improved muscle yield [2, 5, 6]. Levels of T_3 were reduced in Ross 308 [446], while circulating insulin-like growth factors (IGFs) did not differ between the lines [446]. The levels of several somatotropic and thyrotropic genes, however, were altered in the breast muscle of Ross 308 as compared to ACRB. Levels of *THRA* were elevated post-hatch in ACRB breast muscle. Levels of *DIO2* were lower in Ross 308 breast muscle, whereas *DIO3* was greater three-week post-hatch [449]. In Ross 308 breast muscle, *IGFBP3* was reduced during juvenile development compared to the ACRB whereas *IGFBP4* and *IGFBP5* were increased [446]. Given the known intercommunication between these systems, it is reasonable to assume that hormonal crosstalk between endocrine axes facilitates growth and development required for skeletal muscle development in modern broilers.

The IGFs represent a component of somatotropic signaling and are synthesized in the liver and skeletal muscle [20]. Broadly, the IGFs induce cell growth and anabolic effects in target tissues such as muscle, kidneys, bone, and lungs [9, 469]. Therefore, the IGFs are involved in cellular muscle growth and differentiation. During embryonic limb myogenesis, IGF1 induces muscle fiber growth by activating myofiber development from myoblasts [356]. Satellite cells

isolated from one to seven-day old chicks selected for high growth rates had greater amounts of DNA synthesis when stimulated with IGF1 compared to chicks selected for low growth rates [355]. However, satellite cells from 5-week-old female chickens did not express *IGF1* despite *IGF2* being detected [470]. Cloned turkey muscle cells exhibit similar patterns, alongside *IGF2* levels increasing prior to myofiber formation [471]. One interpretation of these data is that the effect of IGF1 on muscle hypertrophy is most critical during embryogenesis and early post-hatch. Activation of satellite cells by IGFs can also contribute to muscle repair by preventing atrophy [472]. It is inconclusive whether these effects are mediated directly by IGF concentrations, however. A dwarf phenotype has been observed in chickens with deficient hepatic IGF production, suggesting that reduced IGF availability to bind type 1 IGF receptor (IGFR1) negatively impacts growth [79-81, 91, 146].

In mammals and other vertebrates, the IGF signaling is understood to be regulated, in part, by the IGF binding proteins (IGFBPs), a highly conserved protein family [21, 96-99]. Impacts on IGF action occurs in one of three ways when an IGFBP physically binds an IGF: enhanced or reduced receptor affinity, extended hormone half-life, or altered tissue specificity [102, 103]. The effects individual IGFBPs varies by and are likewise tissue dependent. Skeletal muscle protein synthesis can be inhibited by IGFBP1, while IGFBP5 activates myoblast proliferation when bound to IGF1 but not IGF2 [119]. On the other hand, IGFBP4 has an inhibitory effect on myoblast proliferation regardless of which IGF it binds [121]. Several IGFBPs also have IGF-independent effects. For example, IGFBP5 can still bind to the surface of myoblasts without an IGF present [106]. Thus, IGFBP activity in chickens may contribute to growth and development of skeletal muscle by controlling IGF signaling or acting independently.

Both T_3 and thyroxine (T_4) are secreted by the thyroid gland. Of the two, T_4 is the gland's major product and the primary hormone found in circulation [266]. Plasma TH levels are not always indicative of TH activity, as T_4 has a lower affinity for TH receptors (THRs) and needs to be converted to T_3 at target tissues by a class of TH-metabolizing enzymes called the deiodinases (DIOs) [49, 51, 52]. The major producer of T_3 from T_4 is DIO2, but other DIOs also regulate TH signaling. Both DIO1 and DIO3 can remove an iodine from T_3, resulting in 3,5-diiodo-L-thyronine (T_2) or convert T_4 to biologically inactive reverse T_3 (rT_3) [270, 320]. All T_3 signals are relayed by nuclear transcription factors, thyroid hormone receptors alpha (THRA) and beta (THRB). The THs induce muscle differentiation during embryogenesis. Artificial hypothyroidism induced in chicken embryos from mid to late embryogenesis gradually reduced myosin heavy chain (MHC) expression in *anterior latissimus dorsi* and *posterior latissimus dorsi* [473]. Dietary T_3 supplementation improved chicken thigh muscle growth, but only before six weeks of age, after which growth was reduced [358]. The decrease in circulating T_3 previously observed in Ross 308 compared to the ACRB may allow for rapid muscle accretion b commercial broilers by increasing the number of myofibers and inducing rapid hypertrophy in those fibers after hatch. Therefore, investigating somatotropic and thyrotropic activity in muscle provides insight into the molecular mechanisms behind this crosstalk that ultimately contribute to muscle growth and development.

The somatotropic and thyrotropic axis have been observed to engage in hormonal crosstalk outside of their canonical "top-down" regulatory pathways, leading to additive, synergistic, or antagonistic effects. Typically, thyrotropin-releasing hormone (TRH) induces secretion of thyroid stimulating hormone (TSH) from the anterior pituitary [8]. It can, however, stimulate the production of growth hormone (GH), in addition to its stimulation by growth hormone releasing hormone (GHRH) [9]. The administration of TRH to broilers and dwarf chickens increased plasma

GH levels [59-61], and pituitary somatotrophs also release GH when stimulated by TRH. The THs also regulate GH secretion in birds, inhibiting GH release stimulated by TRH as part of the negative feedback loop [327]. Similarly, IGF1 can inhibit GH release triggered by TRH [59]. The transcription of hepatic GH receptor (GHR) is also upregulated by THs, and increases affects IGF1 production as a consequence [328]. This known interplay between the two axes, in tandem with the regulatory effects of the IGFBPs, suggests that the THs may control IGFBP activity and thereby modulate IGF effects indirectly in skeletal muscle tissue.

One model that allows for the study of avian muscle development *in vitro* is the Quail Muscle Clone 7 (QM7) cell line. It is an immortalized myogenic cell line isolated from *Coturnix japonica* (Japanese quail) fibrosarcoma. They can be maintained in an undifferentiated state or induced to form multinuclear myotubes when cultured with reduced serum media [67]. Japanese quail have a close genetic proximity to chickens and thus QM7 cells are suitable replacements for immortalized myoblast lines, which are unavailable in chickens [68]. The culture of primary myoblasts from chickens is also unreliable. Additionally, *in vitro* studies demonstrated the importance of T_3 signaling on retinoid-x-receptor activation in myoblast differentiation, making it an ideal candidate for determining whether THs affects IGFBP activity in avian muscle [474]. Therefore, the objective of this study was to determine effects of THs on the expression of somatotropic and thyrotropic genes in QM7 cells to determine if avian muscle cell growth and development is regulated, in part, by interactions between the THs and IGFBPs.

Materials & Methods

Bioinformatics

Putative TREs were identified using Promo Version 3.0.2. Input sequences were in the immediate 5000-bp region upstream of the start codons for *IGFBP3, IGFBP4,* and *IGFBP5* using the Ensembl

Japanese quail (Coturnix_japonica_2.0) and chicken (GRCg6a) genome assemblies. The TRE consensus sequence used by the program was TCACCTCGGA.

QM7 cell culture

Cells were maintained in T75 flasks (ThermoFisher, Waltham, MA) at 37-degrees C in an atmosphere of 5% CO_2 and 60%-to-70% relative humidity and cultured in completed medium composed of Medium 199 (M199; Gibco, New York, NY) with 10% fetal bovine serum (FBS; Gibco), 10% tryptose-phosphate broth solution (Gibco), and 1% penicillin/streptomycin (Gibco) and seeded into 12-well plates for treatment with THs. At 70% confluency, cells were rinsed with 10-mL 1X phosphate buffered saline solution (PBS; ThermoFisher), 3-mL of 0.25% trypsin (Sigma Aldrich, St. Louis, MO) and 1 mM ethylenediaminetetraacetic acid (EDTA) (Cole Parmer, Vernon Hills, IL) in 1X PBS was added directly to the cells, and they were incubated at 37-degrees C for five minutes. Cells were transferred to a 15-mL conical tube and washed via centrifugation at 310 x *g* for 5 minutes. Supernatant was removed and cells were resuspended in serum-free M199 containing 10% tryptose-phosphate broth solution, 1% penicillin/streptomycin, and 1.33% bovine serum albumen (BSA). Cells were washed again via centrifugation at 310 x *g* for 5 minutes before supernatant was removed and replaced with 5-mL serum free media, using a flame-polished glass pipette to resuspend cells. Cells were counted with a hemocytometer using a 1:1 dilution with trypan blue. One-half of the cell suspension was transferred to a new tube and both sets of cells were centrifuged at 310 x *g* for 5 minutes. Medium was removed from both tubes and cells were resuspended in enough serum-free or complete media to bring the final concentration to 240,000 cells per mL. Afterwards, 240,000 cells were seeded into each well of one 12-well plate (Greiner Bio-One, Monroe, NC) in serum-free medium for undifferentiated cells. A second 12-well plate was seeded with 240,000 cells per well in complete medium for differentiated cells. Plates were

then incubated for 24 hours, and complete medium was removed from the wells of the plate where cells were designated for differentiation and replaced with reduced serum medium composed of M199 with 0.5% FBS, 10% tryptose phosphate broth, and 1% penicillin-streptomycin. Treatments with THs began within 24 hours for undifferentiated cells, and differentiated cells were allowed to differentiate for six days prior to TH treatment. On the sixth day, reduced serum medium was removed from differentiated cells and replaced with serum-free media.

TH preparation and treatments

Briefly, T_3 powder (Sigma Aldrich) was reconstituted at 1 mg/mL in 1N sodium hydroxide (NaOH). Serum-free medium was used to create serial dilutions of T_3 until the final treatment concentrations in the wells were 1, 5, and 25 ng/mL. A vehicle control was prepared by diluting 1N NaOH with serum-free medium to a final concentration of 25 nM. T_4 powder (Sigma Aldrich) was reconstituted at 1 mg/mL in 1M dimethyl sulfoxide (DMSO) (Sigma Aldrich) and diluted with serum-free medium as described above. A vehicle control was prepared by diluting 1M DMSO with serum-free medium to a concentration of 25 nM.

TH treatment of both cell types was identical. Each experimental replicate was defined as a single flask of cells, and experiments were carried out over two weeks for each hormone such that two replicates were prepared, treated, and collected each week (n=4 replicates per hormone per state). At 0.5, 6, and 24 hours prior to collection, medium was removed from appropriate wells and replaced with 0 (vehicle only), 1, 5, or 25 ng/mL of T_3 or T_4 in serum-free media. Immediately prior to collection, the medium was removed from each well, and cells were washed in 1X PBS. Cells were collected via trypsinization using 250-µL 0.25% trypsin/1 mM EDTA solution per well. Plates were incubated at 37-degrees C for 5 minutes in the conditions described above. Afterwards, cells were removed from wells using a siliconized glass pipette and placed into microcentrifuge

tubes. All tubes were centrifuged at 300 x *g* for 5 minutes. Supernatant was removed and replaced with 1-mL 1X PBS. Tubes were inverted and centrifuged again for 5 minutes at 300 x *g*. Finally, PBS supernatant was removed, and cells were snap frozen in liquid nitrogen before storage at -80°C.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated from undifferentiated and differentiated QM7 cells using a Zymo RNA Miniprep Extraction Kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. Isolated RNA was quantified using a VICTOR3 Multilabel Plate Reader (Perkin Elmer) and run on a denaturing gel to verify integrity. Total RNA was reverse transcribed in 20 µl reactions using 500 ng total RNA, 5 µM random hexamer primers (ThermoFisher), 5 µM anchored oligo$(dT)_{20}$ primer (ThermoFisher), 200 units M-MuLV reverse transcriptase (New England Biolabs, MA, USA), 0.5 mM dNTPs, and 8 units RNaseOUT (ThermoFisher). Resulting cDNA was amplified by qPCR using intron-spanning primers (Table 1; Integrated DNA Technologies, Coralville) designed with Primer Express software (ThermoFisher). Serial dilutions of pooled quail and chicken muscle cDNA were analyzed by qPCR to determine amplification efficiency for each primer pair, which was calculated using the following equation: efficiency = $(10^{(-1/\text{slope})}-1)$ [376, 377]. Transcripts were analyzed in duplicate using qPCR reactions (10 µl) that consisted of 2 µl diluted cDNA, 5 µl 2X PowerUp SYBR Green Master Mix (ThermoFisher), and 400 nM each forward and reverse primer. Cycling was performed using a StepOne Plus Real-Time PCR System (Applied Biosystems) with the following conditions: 50°C for one minute, 10 minutes at 95°C, followed by 40 cycles of 95°C at 15 seconds, 30 seconds at 58°C, and 30 seconds at 72°C, and a post-amplification disassociation curve analysis to ensure amplification of a single product.

Transcripts were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The equation $(2^{\Delta Ct})_{target}/(2^{\Delta Ct})_{GAPDH}$, where $\Delta Ct = Ct_{no\ RT} - CT_{sample}$, was used to transform and normalize data as previously described [378-381, 449]. Data were expressed relative to the 0-ng/mL treatment at 24 hours, and main effect data are expressed relative to the 0-ng/mL treatment concentration (set to 1 in all cases). A secondary analysis of a subset of the data was performed to directly compare target gene expression levels in undifferentiated and differentiated cells not treated with THs, and these data are expressed relative to levels in undifferentiated cells (set to 1 in all cases). Another secondary analysis investigating expression of undifferentiated and differentiated cells treated with 5 ng/mL T_3 or T4 for 24 hours was conducted to directly compare responsiveness of the cells to each TH, and these data are expressed relative to levels in undifferentiated cells treated with vehicle only for 24 hours (set to 1 in all cases).

Statistical analysis

Data were originally analyzed with a three-way analysis of variance (ANOVA) using the Fit Model Procedure of JMP Pro 16 (SAS Institute, Charlotte). Cell state (undifferentiated or differentiated), hormone concentration (0, 1, 5, or 25 ng/mL), and time (0.5, 6, or 24 hours) and their interaction were the effects included in the model. Several genes exhibited a three-way cell state-by-hormone concentration-by time significant interaction. These included *IGF2, GHR, THRA, THRB, DIO3, IGFBP2, IGFBP3,* and *IGFBP5* for the T_3 experiment, and *IGF2, GHR, IGFR1, THRA, DIO2, DIO3, IGFBP2, IGFBP3, IGFBP5,* and *IGFBP7* for the T_4 experiment. As such, data for undifferentiated and differentiated cells were analyzed separately using a two-way ANOVA included only hormone concentration and time and their interaction as model effects. When ANOVA indicated a significant hormone concentration-by-time interaction or hormone concentration main effect ($P \leq 0.05$), *post hoc* multiple means comparisons were performed using

Fisher's test of least significant difference. Main effect means were only calculated and analyzed when there was not a significant interaction (P>0.05). For the secondary analysis comparing expression in undifferentiated versus differentiated cells, a Student's T-test was performed to detect significant differences between the two cell states. For the secondary analysis comparing responsiveness of cells to T_3 or T_4, a two-way ANOVA that that included cell state (undifferentiated or differentiated), hormone (vehicle, T_3, or T_4), and their interactions was performed. When ANOVA indicated a significant cell state-by-hormone effect (P≤0.05), *post hoc* multiple means comparisons were performed using Fisher's test of least significant difference. Main effect means of hormone were only calculated and analyzed when there was not a significant interaction (P>0.05).

Results

Bioinformatics analysis and identification of TREs

Putative TREs were identified in the 5000-bp upstream region of chicken and Japanese quail *IGFBP3* (9 TREs), *IGFBP4* (7 TREs), and *IGFBP5* (4 TREs) (**Figure 6.1A**). In culture, QM7 cells can be maintained as mononuclear myoblasts (**Figure 6.1B**, left) or differentiated into multinucleated myotubes (**Figure 6.1B**, middle) in the presence of reduced serum medium. Blue staining in all three images indicates the locations of nuclei. Green staining in the left and middle images indicates the presence of myosin heavy chain (MyHC), and the formation of multinucleated tubules expressing MyHC is apparent in differentiated cells (**Figure 6.1B**, middle). The multinucleated, differentiated tubules also lose expression of the skeletal muscle satellite cell marker paired box 7 (PAX7) in their nuclei (**Figure 6.1B**, right). These cells also express *IGFR1*, THRs, and all IGFBPs except *IGFBP1*, as denoted by gel electrophoresis (**Figure 6.1C**). Collectively, these data indicate that QM7 cells are a suitable model to study the hormonal

regulation of muscle cell growth and development, as they express hormone receptors and other proteins that mediate hormonal signaling. Further, the presence of multiple TREs in several IGFBPs suggests that TH signaling could regulate expression of these genes.

Treatment of undifferentiated cells with T_3

Somatotropic gene expression

After T_3 administration, mRNA levels of *GHR* and *IGFBP5* exhibited signficant interactive hormone concentration-by-time effects (**Figure 6.2**; $P \leq 0.05$). At 0.5 hours, *GHR* expression was decreased at all treatment concentrations relative to the control but increased after longer exposure. At 6 hours, 5 ng/mL and 25 ng/mL T_3 increased *GHR* mRNA levels, while all three hormone concentrations induced expression at 24 hours (**Figure 6.2A**). Levels of *IGFBP5* were significantly higher at 6 and 24 hours with all three treatments (**Figure 6.2F**). A hormone concentration main effect for *IGF2* was significant (**Figure 6.2C**; **Table 6.2**; $P \leq 0.05$), with levels greater than the no treatment control in cells treated with 1 ng/mL and 25 ng/mL T_3. No significant interactive or hormone concentration main effects were observed for *IGFR1*, *IGFBP2*, or *IGFBP3* (**Figures 6.2B – 6.2E**; **Table 6.2**; $P > 0.05$). The main effect of hormone treatment for *IGFBP2* approached significance ($P = 0.1175$), with levels in treated cells slightly higher than those in untreated cells (**Table 6.2**). Expression of *IGF1*, *IGFBP1*, *IGFBP4*, and *IGFBP7* was not detected in this experiment, which aligns with previous observations [446].

Thyrotropic gene expression

Both *THRB* and *DIO3* demonstrated significant interactive effects of concentration-by-time in undifferentiated cells treated with T_3 (**Figure 6.3**; $P \leq 0.05$). When cells were exposed to T_3 for 6 and 24 hours, all three treatment concentrations increased *THRB* expression (**Figure 6.3B**). Similarly, expression of *DIO3* was greater for all three treatments relative to the untreated control

at 6 and 24 hours (**Figure 6.3C**). No significant interactive or hormone concentration main effects were observed for *THRA* (**Figure 6.3A**; **Table 6.2**; P>0.05), though the main effect of concentration approached significance (P=0.0622), where levels tended to be reduced by all three concentrations (**Table 6.2**). Neither *DIO1* nor *DIO2* mRNA were detected, which aligns with previous observations [449].

Treatment of differentiated cells with T_3

Somatotropic gene expression

No significant interactive effects were observed for differentiated cells treated with T_3 (**Figure 6.4**; P>0.05). However, a nearly significant interactive effect was observed for *GHR* (**Figure 6.4A**; P=0.0569), in which expression tended to be increased by all treatments at 6 and 24 hours, with the greatest induction in cells treated with 25 ng/mL for 24 hours. Significant main effects of hormone concentration were observed for *IGF2* and *GHR* (**Table 6.3**; P≤0.05), where mRNA levels of both genes were increased by all three T_3 concentrations. Levels of *IGFR1* and the IGFBPs were not affected by T_3 treatment at any concentration (**Figures 6.4B** and **6.4D – 6.4F**; **Table 6.3**.; P>0.05). As in undifferentiated QM7 cells, *IGF1*, *IGFBP1*, *IGFBP4* and *IGFBP7* were not detected in this experiment.

Thyrotropic gene expression

In differentiated QM7 cells treated with T_3, a significant hormone concentration-by-time interaction was detected for *THRB* and *DIO3* (**Figure 6.5**; P≤0.05). At 0.5 hours, 1 ng/mL and 25 ng/mL T_3 increased *THRB* expression, and greater *THRB* mRNA levels were induced by all three concentrations at 6 and 24 hours (**Figure 6.5B**). Increased *DIO3* expression was observed for all three hormone concentrations at all three timepoints, with the highest induction at the highest dose and longest treatment (**Figure 6.5C**). No significant interactive or hormone concentration main

effects were observed for *THRA* (**Figure 6.5A**; **Table 6.3**; P>0.05). As in undifferentiated QM7 cells, *DIO1* and *DIO2* were not detected (data not shown).

Treatment of undifferentiated cells with T_4

Somatotropic gene expression

Undifferentiated QM7 cells treated with T_4 exhibited significant interactive effects for *GHR, IGFBP3,* and *IGFBP5* (**Figure 6.6**; P≤0.05). At 0.5 hours, T_4 treatment increased *GHR* mRNA at all doses, and at 6 hours, 5 ng/mL and 25 ng/mL increased its expression. No effects of T_4 on *GHR* were observed at any concentration when cells were treated for 24 hours (**Figure 6.6A**). Though no effects on *IGFBP3* levels were observed when cells were treated with T_4 at any concentration for 0.5 or 24 hours, cells treated with 5 ng/mL T_4 for 6 hours exhibited decreased *IGFBP3* expression relative to the control (**Figure 6.6E**). Comparatively, all treatment concentrations elevated *IGFBP5* mRNA at 0.5 hours, 5 ng/mL and 25 ng/mL also increased mRNA levels of this gene at 6 hours, and the cells did not respond to T_4 upon longer exposure for 24 hours (**Figure 6.6F**). No interactive or hormone concentration main effects were observed for *IGFR1*, *IGF2*, *IGFBP2*, or *IGFBP7* (**Figure 6.6B – 6.6D** and **6.6G**; **Table 6.4**; P>0.05). Expression of *IGF1*, *IGFBP1*, and *IGFBP4* was not detected in these cells.

Thyrotropic gene expression

Of the four genes measured, only *THRB* exhibited an interactive effect in undifferentiated QM7 cells treated with T_4 (**Figure 6.7**; P≤0.05), where all treatment concentrations increased *THRB* expression at 0.5 hours and the two highest doses increased expression at 6 hours. There was also a dose response, and the highest levels were observed in cells treated with 25 ng/mL T_4 at both timepoints. However, under prolonged treatment for 24 hours, this responsiveness was lost (**Figure 6.7B**). Comparatively, *THRA*, *DIO2*, and *DIO3* mRNA levels were unaffected by T_4

treatment and did not exhibit significant interactive or hormone concentration main effects (**Figure 6.7A** and **6.7C – 6.7D**; **Table 6.4**; P>0.05). However, *DIO2* expression approached significance for the hormone concentration-by-time interaction P=0.0615), with expression apparently increased by 1 and 25 ng/mL at 0.5 hours, 5 and 25 ng/mL at 6 hours, and 5 ng/mL at 24 hours (**Figure 6.7C**). Changes in *DIO3* expression also approached significance for the main effect of hormone concentration (P=0.0541), and treatment with T_4 at all concentrations tended to increase expression (**Table 6.4**).

Treatment of differentiated cells with T_4

Somatotropic gene expression

Of all the genes investigated, significant interactive effects were exhibited only for *GHR* in differentiated QM7 cells treated with T_4 (**Figure 6.8**; P≤0.05). Levels rose with 1 ng/mL treatment but decreased to basal levels with higher doses at 0.5 hours. At 6 hours, treatment with 25 ng/mL T_4 reduced *GHR* expression, and all three concentrations lowered expression at 24 hours (**Figure 6.8A**). No significant interactive or main hormone concentration effects were observed for *IGFR1*, *IGF2*, *IGFBP2*, *IGFBP3*, *IGFBP5*, and *IGFBP7* (**Figure 6.8B – 6.8G**; **Table 6.5**; P>0.05). However, main effects of hormone concentration were nearly significant for *IGF2* (P=0.0882) and *IGFBP5* (P=0.0964). Levels of *IGF2* were slightly lower with the 25 ng/mL treatment, whereas *IGFBP5* expression was higher with the same concentration (**Table 6.5**).

Thyrotropic gene expression

Both *THRA* and *DIO3* mRNA levels exhibited a significant hormone concentration-by-time effect in differentiated QM7 cells treated with T_4 (**Figure 6.9**; P≤0.05). Expression of *THRA* increased with 1 ng/mL T_4 at 0.5 hours but returned to untreated levels at higher doses at the same timepoint. Similarly, it decreased with 25 ng/mL T_4 at 6 hours and with 1 ng/mL at 24 hours,

though other doses did not influence expression at these times (**Figure 6.9A**). Levels of *DIO3* mRNA were significantly higher in cells treated with 25 ng/mL T_4 at 0.5 hour ($P \leq 0.05$) but were not affected by other doses at 0.5 hour or by any concentration at other timepoints (**Figure 6.9D**; $P > 0.05$). Levels of *THRB* did not show a significant interaction (Figure 9B; $P > 0.05$) but did exhibit a main effect of hormone concentration, with levels significantly induced by treatment with 25 ng/mL T_4 (**Table 6.5**; $P \leq 0.05$). Expression of *DIO2* did not demonstrate either a significant interactive or main hormone concentration effect (**Figure 6.9C**; **Table 6.5**; $P > 0.05$),

Differential gene expression in undifferentiated and differentiated QM7 cells

In basal cells not receiving any hormone treatment, expression of *GHR*, *IGFR1*, *IGF2*, *IGFBP2*, *IGFBP3*, *IGFBP7*, *THRA*, and *DIO2* differed between undifferentiated myoblasts and differentiated myotubes (**Figure 6.10**; $P \leq 0.05$). In undifferentiated myoblasts, *GHR*, *IGFR1*, *IGF2*, *IGFBP3*, *THRA*, and *DIO2* levels were greater than those in differentiated cells. Only *IGFBP2* and *IGFBP7* were higher in differentiated cells. A difference between states approached significance for *DIO3* ($P = 0.0950$), in which mRNA levels were higher in undifferentiated cells.

Differential sensitivity of QM7 cells to T_3 and T_4 treatment

In order to test if QM7 cells exhibited different sensitivity to T_3 and T_4, and if this was influenced by differentiation state, select data from each experiment were analyzed together. Treatment of undifferentiated and differentiated cells with 5 ng/mL T_3 or T_4 for 24 hour was examined, since this concentration should be representative of physiological levels of both hormones. None of the somatotropic (**Figure 6.11**; $P > 0.05$) or thyrotropic (**Figure 6.12**; $P > 0.05$) demonstrated a hormone-by-differentiation state interaction. However, main effects of hormone were detected for select genes in each axis. In the somatotropic axis, levels of *IGFR1* and *IGFBP3* were reduced by treatment with T_4 but not T3, while *GHR* and *IGFBP5* were not affected by T_4

but increased by T_3 treatment (**Table 6.6**; $P \leq 0.05$). None of the genes in the thyrotropic axis were affected by treatment with T_4 ($P > 0.05$); however, expression of *THRA* was diminished and levels of *THRB* and *DIO3* were increased following T_3-treatment (**Table 6.6**; $P \leq 0.05$).

Discussion

Avian growth and development are regulated by several hormonal axes, and these systems engage with and influence one another outside of their typical signaling pathways in a form of signaling "crosstalk" [29, 46, 236]. Regulatory crosstalk between two of these endocrine systems, the somatotropic and thyrotropic axes, has been previously demonstrated in the embryonic and adult chicken [59, 60, 326, 327, 475]. Injections of TRH increased circulating GH in broilers when they were four to seven weeks old [59], and a similar effect was observed in dwarf chickens between three and seven weeks of age [60]. Thus, this study sought to evaluate if these two axes also might engage in crosstalk to regulate avian muscle development and examined if somatotropic and thyrotropic genes, including hormones, receptors, binding proteins, and enzymes regulating TH bioavailability, were altered by *in vitro* treatment of undifferentiated or differentiated QM7 cells with THs. The results indicate that TH treatment altered somatotropic and thyrotropic gene expression, including that of select IGFBPs. Gene expression differences between cell states may be the result of the molecular changes myoblasts undergo during differentiation, with cells differentiating into myotubes showing unique gene expression profiles in mouse and human models [476, 477]. In both cell types, T_3 increased *GHR*, *IGF2*, *THRB*, and *DIO3*. However, *IGFBP5* was only heightened in undifferentiated cells that received T_3. Treatment with T_4 in undifferentiated cells increased *IGFR1*, *THRB*, and *IGFBP5*, but reduced *IGFBP3* at later timepoints. Differentiated cells were less affected by T_4 administration, with changes only observed in *THRA*, *THRB*, and *DIO3*. Broadly, expression for many genes appears higher in

undifferentiated cells, with *IGF2*, *GHR*, *IGFR1*, *IGFBP3*, *THRA*, and *DIO2* levels in undifferentiated, untreated control cells. Expression of *DIO3* was also greater in these cells. One interpretation of these data is that undifferentiated cells are more hormonally responsive than differentiated ones, and that these hormones could induce cell growth and proliferation in myoblasts. This may also be linked to T_3 responsiveness, as *GHR*, *THRB*, *DIO3*, and *IGFBP5* were raised while *THRA* was reduced by T_3 treatment. The increase in *DIO3* and decrease in *THRA* are likely part of the feedback loop regulating T_3 signaling. Greater *DIO3* would result in greater T_3 deactivation, while diminished levels of *THRA* would reduce hormonal signaling. Overall, these data suggest that somatotropic gene regulation, including that of *IGFBP3* and *IGFBP5*, is affected by TH signaling. However, such changes could be more readily induced by T_3, and undifferentiated cells appear more sensitive to hormonal signaling overall signaling based on higher levels of receptors being expressed.

Levels of *GHR* were raised in T_3-treated cells at later timepoints in both states, and undifferentiated cells administered T_4 treatment had increased *GHR* at 0.5 and 6 hours. This suggests that T_3 increases the sensitivity of muscle tissue to GH. Additionally, fasting has been demonstrated to lower the sensitivity of broiler chick muscle to T_3, lessening the effect it had on muscle growth, while lowering *GHR* mRNA [478]. *GHR* levels were decreased in T_4-treated differentiated cells. By 24 hours, all T_4 treatment concentrations increased *GHR* expression levels in undifferentiated cells but not differentiated cells. Directly comparing the two cell states showed that *GHR* was greater in undifferentiated myoblasts. These results indicate that T_3 and T_4 regulate *GHR* expression in QM7 cells, and thereby GH sensitivity, but also that there is a differential response to TH treatment across the two cell types.

This differential sensitivity to THs based on cell state extended to several other genes that were measured throughout the study. In addition to *GHR*, *IGFR1* and *THRA* were greater in undifferentiated, untreated control cells, suggesting that undifferentiated cells are more sensitive to somatotropic and thyrotropic signaling. Only *IGFBP2* and *IGFBP7* were greater in differentiated cells. This could indicate that they are involved in myofiber development. The THs are metabolized by the DIOs at target tissues and can be rendered active or inactive depending on the metabolizing DIO and TH metabolite [49, 51, 52, 270, 320]. Levels of *DIO3* were generally raised across undifferentiated and differentiated cells treated with T_3. This was likely a direct response to T_3 administration, as *DIO3* is the primary T_3 inactivator by converting it to T_2 with the removal of an iodine, whereas *DIO2* is more commonly involved in providing T_3 via T_4 conversion [479]. Therefore, increased *DIO3* is likely part of the negative feedback loop activated by T_3. In undifferentiated cells, *DIO2* approached significance for a hormone concentration-by-time effect ($P=0.0615$), in which expression was greater in 5 ng/mL treated cells than the control at 6 hours. No change was observed in differentiated cells, however. One conclusion to be drawn from these results is that undifferentiated cells have a greater capability to convert T_4 to T_3. This is supported by both greater levels of *DIO2* and a nearly significant increase of *DIO3* in undifferentiated cells ($P=0.0541$), where 25 ng/mL T_4 treatment raised *DIO3* expression. This concentration is on the higher end of biologically relevant values [449], so an increase in T_3 as produced by *DIO2* would require a subsequent upregulation of *DIO3* to inactivate T_3 or conversion to T_4 to rT_3. Therefore, differential expression profiles of the DIOs likely altered the cellular response of treated QM7s to THs.

Certain genes were also more responsive to T_3 than T_4. When treated with T_3, *IGF2* expression was elevated in both cell types. This suggests that *IGF2* mRNA synthesis in avian

skeletal muscle can be stimulated by T_3 signaling, not unlike the promotion of *IGF1* in mouse cardiac muscle [480]. Levels of *IGF2* were unchanged in T_4-treated cells, suggesting that T_4 must be converted to T_3 to induce *IGF2* production. Both TH receptors are expressed in skeletal muscle and can be upregulated in response to TH administration [481, 482]. However, the receptors did not exhibit similar changes in expression during this study. Levels of *THRB* were elevated in both undifferentiated and differentiated cells treated with T_3. Similar effects were also observed when cells were treated with T_4, where expression was greater in undifferentiated cells at 0.5 and 6 hours and in differentiated cells treated with the highest amount of T_4. On the other hand, *THRA* levels were unchanged by T_3 regardless of cell state, and the higher doses of T_4 decreased expression of this receptor at 6 and 24 hours in differentiated cells. One interpretation of these results is that *THRB* is a TH signaling antagonist in avian skeletal muscle, possibly as part of a negative feedback mechanism, as THRB has been observed to suppress TSH expression in mice [483, 484]. When exposed to high TH levels, THRB expression increases to control the intensity of TH signaling. The primary receptor, *THRA*, is then downregulated.

The putative TREs identified in the upstream region of *IGFBP3* and *IGFBP5* prior to this study are likely functional, as expression of both genes was altered by TH treatment. Changes in *IGFBP3* expression were short-term and did not last past six hours, whereas *IGFBP5* was altered in the short-term by T_4 and long-term by T_3. These short-term effects indicate that TH signaling at putative TREs can be altered by negative feedback loops activated by 24 hours and this may be due to differentiated THRA/THRB ratios. TH regulation of these IGFBPs is likely to influence avian skeletal muscle development but those effects are not the same in undifferentiated and differentiated cells. A lack of changes in *IGFBP4* expression suggests the putative TREs identified prior to this work are nonfunctional. Levels of *IGFBP5* mRNA in undifferentiated cells treated

with all concentrations of T_3 were greater than the untreated controls at 6 and 24 hours, but no effects were observed in differentiated cells regardless of treatment concentration or time. The same was seen in T_4-treated cells, where expression was higher at earlier times in undifferentiated cells, but this did not extend to differentiated cells. This implies that T_3 and T_4 upregulate *IGFBP5* expression, but effects are dependent on muscle cell type. A study in mammals concluded that *IGFBP5* promotes myoblast proliferation when bound to IGF1 [121]. Additionally, *IGFBP5* is upregulated in the breast muscle of fast-growing Ross 308 juvenile broilers compared to slow-growing ACRB birds [446]. A conclusion to be drawn from these expression patterns is not only that IGFBP5 acts in a growth-promotive fashion, and that these promotive effects can be induced by TH signaling in immature myoblasts. This likely occurs in a paracrine fashion, as the QM7 cells maintained during the study were not exposed to any additional IGFBP5 that would mimic its function in plasma. Differentiated myotubes do not necessarily require *IGFBP5* activity, as they are no longer actively dividing.

Levels of *IGFBP3* were only affected in undifferentiated cells treated with T_4, in which all three treatment concentrations decreased levels relative to the untreated control at 6 hours. This may indicate that *IGFBP3* has an inhibitory effect on growth in myoblasts as it was downregulated by THs that could have a growth-promotive effect. Ross 308 birds, which have greater amounts of breast muscle tissue, also had lower *IGFBP3* mRNA levels than the ACRB [446]. Therefore, reduced IGFBP3 activity in modern broilers allows for greater breast muscle growth.

Findings from this study have several broad implications. Firstly, skeletal muscle growth can be affected by crosstalk between the somatotropic and thyrotropic axes. Higher expression of *IGF2* and *GHR* after T_3 administration could result in increased muscle growth via paracrine IGF2 signaling as well as greater sensitivity to GH-induced proliferation. Secondly, certain IGFBPs are

regulated by TH signaling. The stimulation of *IGFBP5* mRNA production by T_3 and T_4 reinforces previous conclusions that *IGFBP5* has a growth-promotive effect when produced locally in skeletal muscle, as well as the novel conclusion that its levels are regulated by TH. However, *IGFBP3* remains inhibitory when signaling in a paracrine fashion, and is thus downregulated in skeletal muscle treated with THs. Finally, THs differentially influence cells when they are in the undifferentiated, proliferating state or a differentiated state. Undifferentiated myoblasts appear to be more sensitive to hormonal signaling, particularly THs, than differentiated myotubes. This suggests that the THs contribute to muscle growth and development prior to myotube formation. Taken together, these data demonstrate that crosstalk between the thyrotropic and somatotropic axes is involved in the growth of avian skeletal muscle tissue, making this an important avenue of investigation to optimize muscle accretion in commercial broiler chickens.

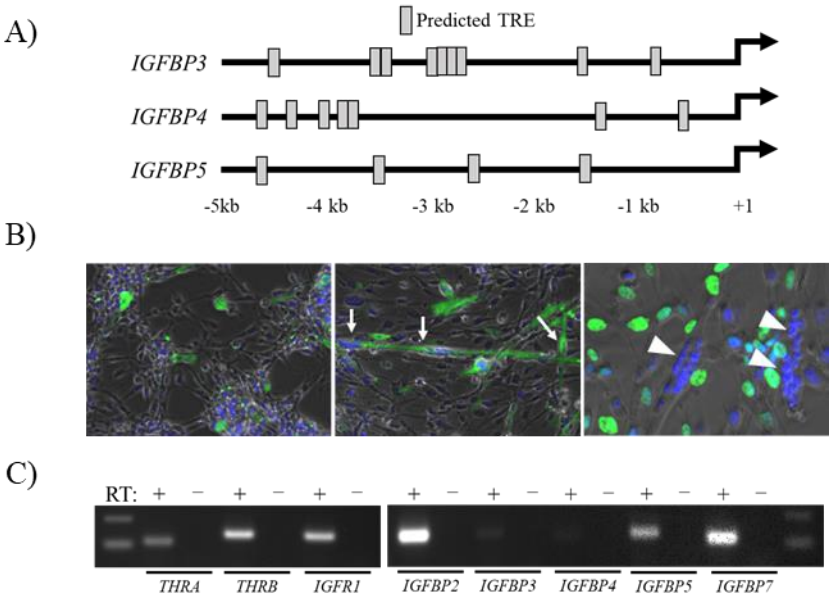

Figure 6.1. Expression of IGFBPs in QM7 cells may be regulated by THs. (A) Predicted TREs were identified in the 5' upstream regulatory region of Japanese quail *IGFBP3*, *IGFBP4*, and *IGFBP5*. Other IGFBPs did not contain TREs. (B) QM7s grown as mononuclear myoblasts (left) or differentiated into fused, multinucleated myotubes (middle and right). Nuclei are indicated with DAPI stain (blue). In the left and middle panels, green staining indicates the presence of myosin heavy chain. In the right panel, green staining indicates the presence of paired hox 7 in nuclei. The arrows in the middle panel point to a multinucleated tubule, and the arrowheads in the right panel point to the loss of PAX7 in these multinucleated tubules. (C) Gel image demonstrating the expression of hormone receptors and IGFBPs in QM7 cells. *IGFBP1* was not detected.

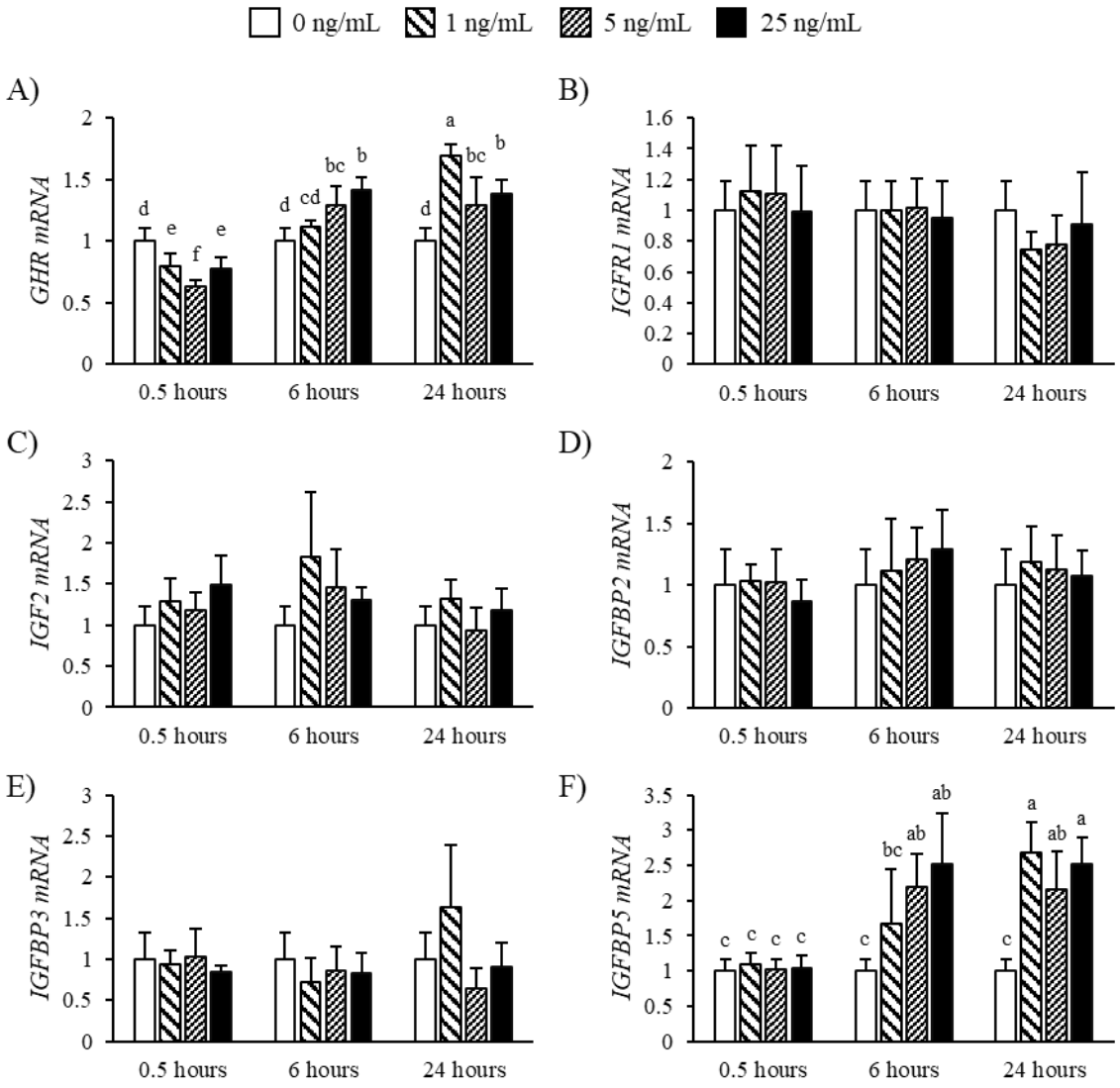

Figure 6.2. Relative mRNA expression of (A) *GHR*, (B) *IGFR1*, (C) *IGF2*, (D), *IGFBP2*, (E) *IGFBP3*, and (F) *IGFBP5* in undifferentiated QM7 cells treated with 0, 1, 5, or 25 ng/mL T_3 for 0.5, 6, or 24 hours. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* mRNA. The data (mean + SEM) are expressed relative to the 0 ng treatment (set to 1). Data were analyzed by two-way ANOVA followed by Fisher's least significant difference test. Significant hormone concentration-by-time interactions were identified for (A) *GHR* ($P<0.0001$) and (F) *IGFBP5* ($P=0.0187$), and bars without a common letter are significantly different ($P\leq0.05$). No significant concentration-by-time interactions were detected for (B) *IGFR1* ($P=0.5201$), (C) *IGF2* ($P=0.4005$), (D) *IGFBP2* ($P=0.1175$), and (E) *IGFBP3* ($P=0.2236$), and main effects of hormone concentration are shown in **Table 6.2**. Expression of *IGF1*, *IGFBP1*, *IGFBP4*, and *IGFBP7* was not detected in these cells.

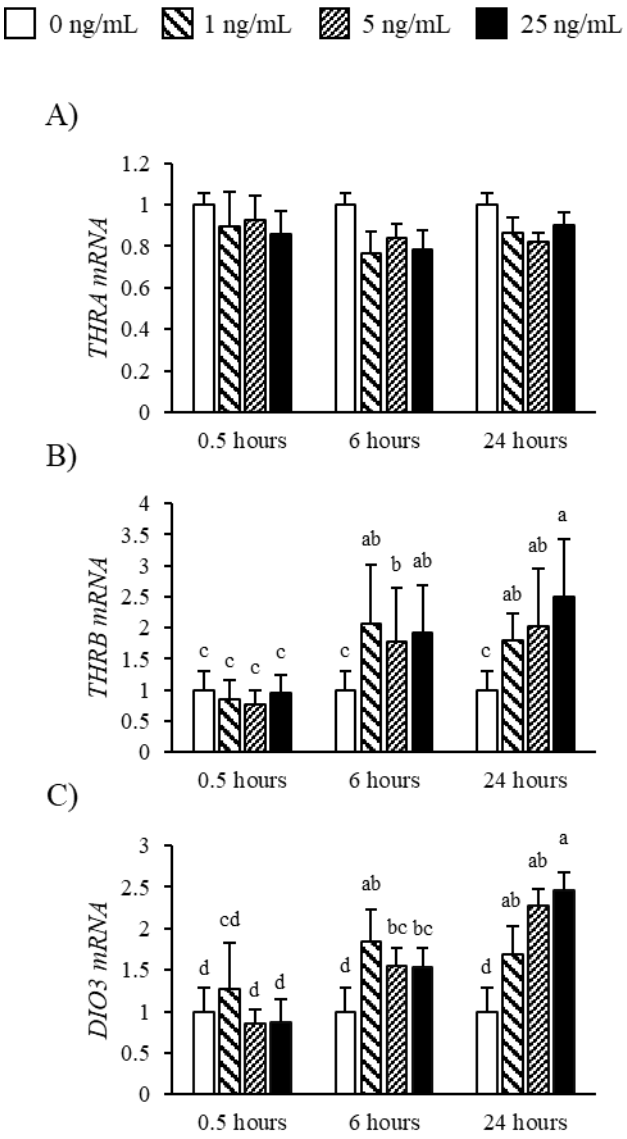

Figure 6.3. Relative mRNA expression of (A) *THRA*, (B) *THRB*, and (C) *DIO3* in undifferentiated QM7 cells treated with 0, 1, 5, or 25 ng/mL T_3 for 0.5, 6, or 24 hours. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* mRNA. The data (mean + SEM) are expressed relative to the 0 ng treatment (set to 1). Data were analyzed by two-way ANOVA followed by Fisher's least significant difference test. Significant hormone concentration-by-time interactions were identified for (B) *THRB* (P=0.0230) and (C) *DIO3* (P=0.0147), and bars without a common letter are significantly different (P≤0.05). A significant hormone concentration-by-time interaction was not detected for (A) *THRA* (P=0.0622), and the main effect of hormone concentration is shown in **Table 6.2**. Expression of *DIO1* and *DIO2* was not detected in these cells.

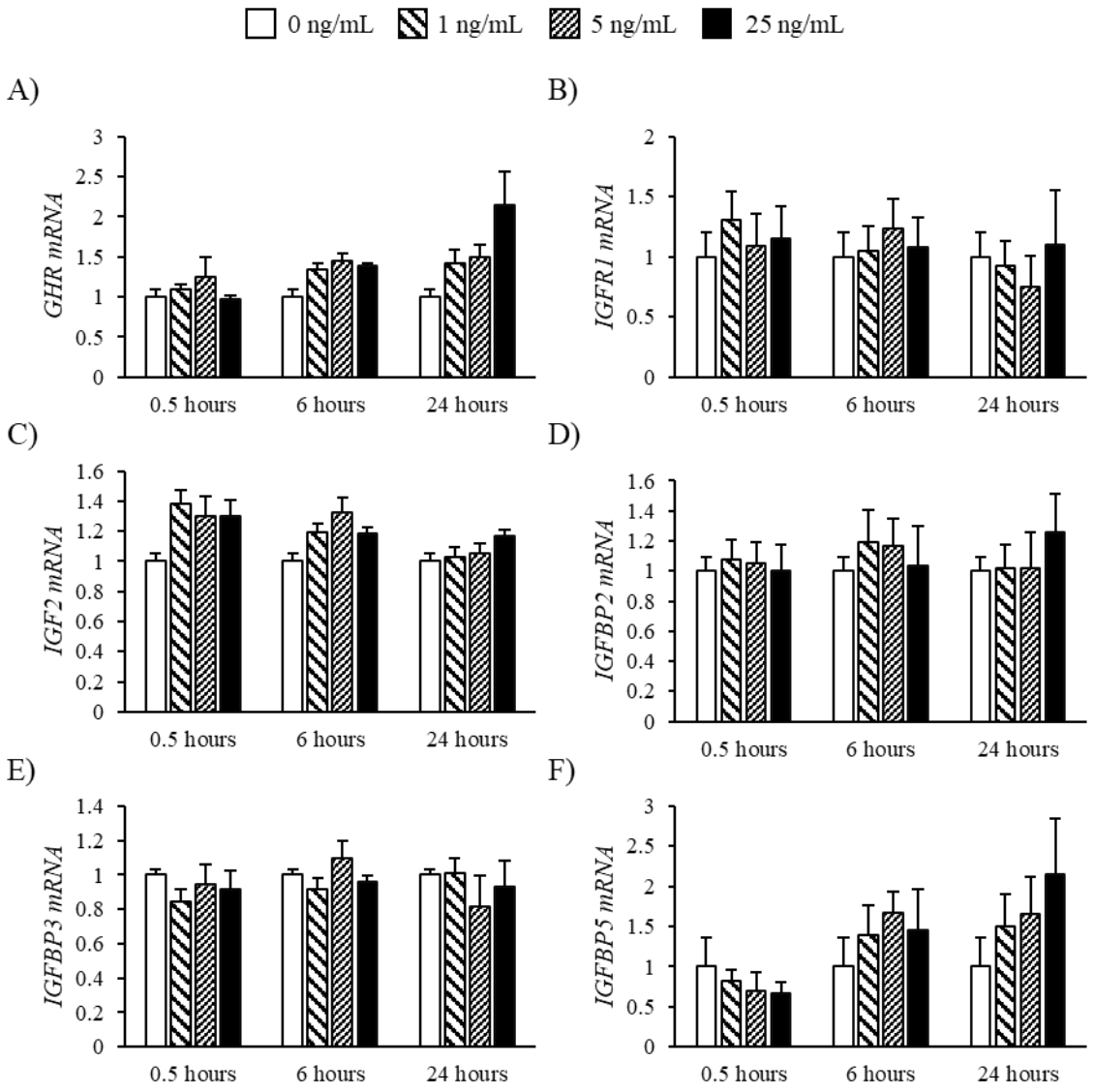

Figure 6.4. Relative mRNA expression of (A) *GHR*, (B) *IGFR1*, (C) *IGF2*, (D), *IGFBP2*, (E) *IGFBP3*, and (F) *IGFBP5* in differentiated QM7 cells treated with 0, 1, 5, or 25 ng/mL T_3 for 0.5, 6, or 24 hours. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* mRNA. The data (mean + SEM) are expressed relative to the 0 ng treatment (set to 1). Data were analyzed by two-way ANOVA followed by Fisher's least significant difference test. No significant hormone concentration-by-time interactions were detected for any genes [(A) *GHR*, P=0.0569; (B) *IGFR1*, P=0.3219; (C) *IGF2*, P=0.0983; (D) *IGFBP2*, P=0.4896; (E) *IGFBP3*, P=0.2960; (F) *IGFBP5*, P=0.4041], and main effects of hormone concentration are shown in **Table 6.3**. Expression of *IGF1*, *IGFBP1*, *IGFBP4*, and *IGFBP7* was not detected in these cells.

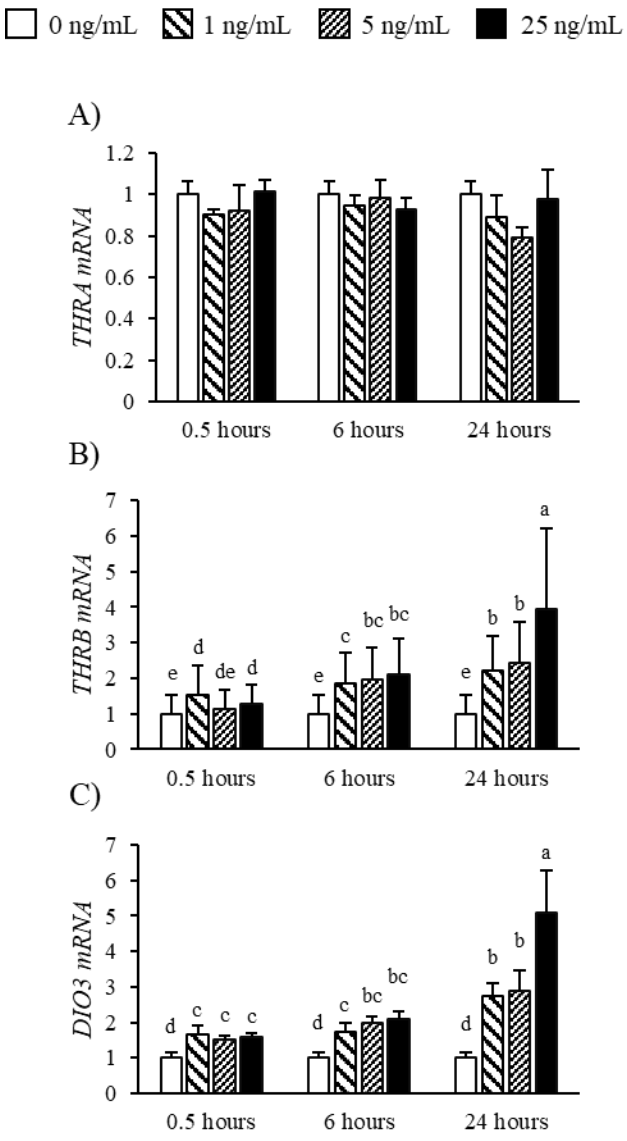

Figure 6.5. Relative mRNA expression of (A) *THRA*, (B) *THRB*, and (C) *DIO3* in differentiated QM7 cells treated with 0, 1, 5, or 25 ng/mL T_3 for 0.5, 6, or 24 hours. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* mRNA. The data (mean + SEM) are expressed relative to the 0 ng treatment (set to 1). Data were analyzed by two-way ANOVA followed by Fisher's least significant difference test. Significant hormone concentration-by-time interactions were identified for (B) *THRB* ($P<0.0001$) and (C) *DIO3* ($P=0.0079$), and bars without a common letter are significantly different ($P\leq0.05$). A significant hormone concentration-by-time interaction was not detected for (A) *THRA* ($P=0.7644$), and the main effect of hormone concentration is shown in **Table 6.3**. Expression of *DIO1* and *DIO2* was not detected in these cells.

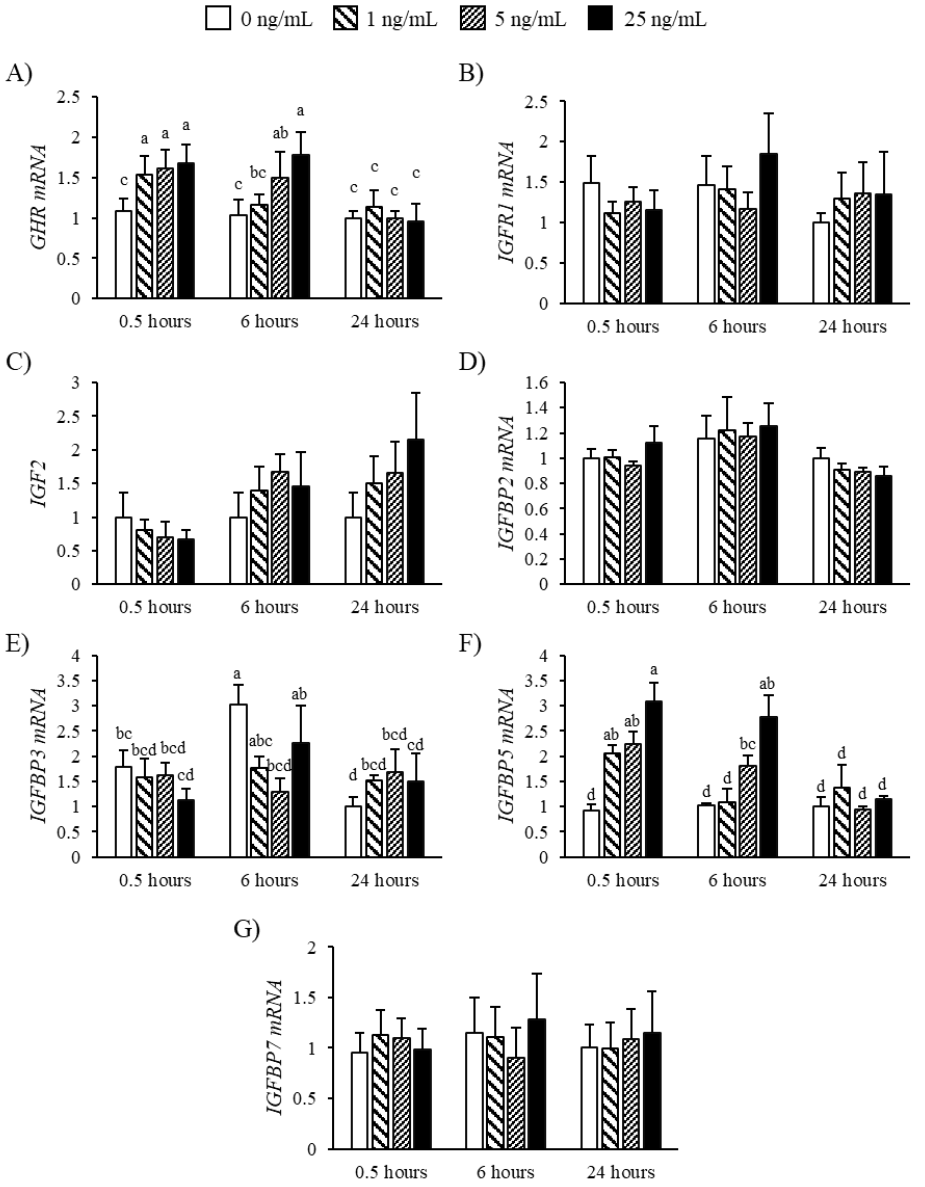

Figure 6.6. Relative mRNA expression of (A) *GHR*, (B) *IGFR1*, (C) *IGF2*, (D), *IGFBP2*, (E) *IGFBP3*, (F) *IGFBP5*, and (G) *IGFBP7* in undifferentiated QM7 cells treated with 0, 1, 5, or 25 ng/mL T_4 for 0.5, 6, or 24 hours. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* mRNA. The data (mean + SEM) are expressed relative to the 0 ng treatment (set to 1). Data were analyzed by two-way ANOVA followed by Fisher's least significant difference test. Significant hormone concentration-by-time interactions were identified for (A) *GHR* (P=0.0243), (E) *IGFBP3* (P=0.0323), and (F) *IGFBP5* (P=0.0102), and bars without a common letter are significantly different (P≤0.05). No significant hormone concentration-by-time interactions were detected for (B) *IGFR1* (P=0.3261), (C) *IGF2* (P=0.8108), (D) *IGFBP2* (P=0.9367), and (F) *IGFBP7* (P=0.2837), and main effects of hormone concentration are shown in **Table 6.4**. Expression of *IGF1*, *IGFBP1*, and *IGFBP4* was not detected in these cells.

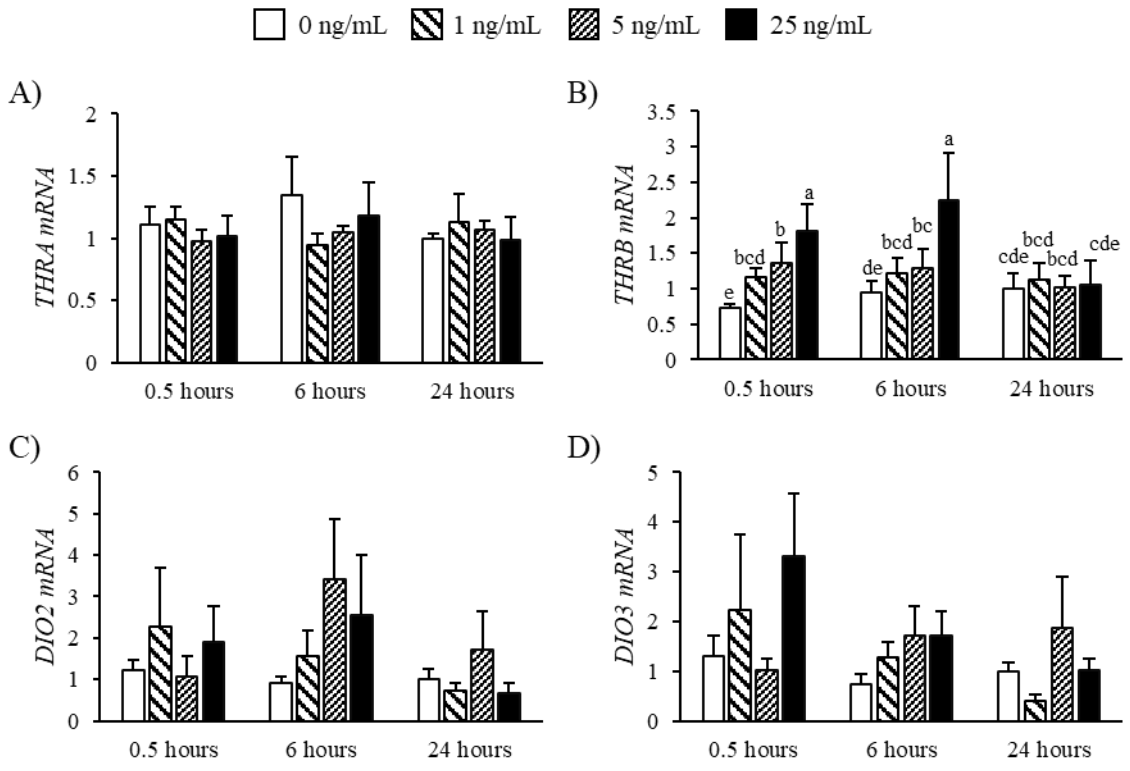

Figure 6.7. Relative mRNA expression of (A) *THRA*, (B) *THRB*, (C) *DIO2*, and (D) *DIO3* in undifferentiated QM7 cells treated with 0, 1, 5, or 25 ng/mL T_4 for 0.5, 6, or 24 hours. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* mRNA. The data (mean + SEM) are expressed relative to the 0 ng treatment (set to 1). Data were analyzed by two-way ANOVA followed by Fisher's least significant difference test. A significant hormone concentration-by-time interaction was identified for (B) *THRB* (P=0.0036), and bars without a common letter are significantly different (P≤0.05). No significant hormone concentration-by-time interactions were observed for (A) *THRA* (P=0.5532), (C) *DIO2* (P=0.0615), and (D) *DIO3* (P=0.5342), and main effects of hormone concentration are shown in **Table 6.4**.

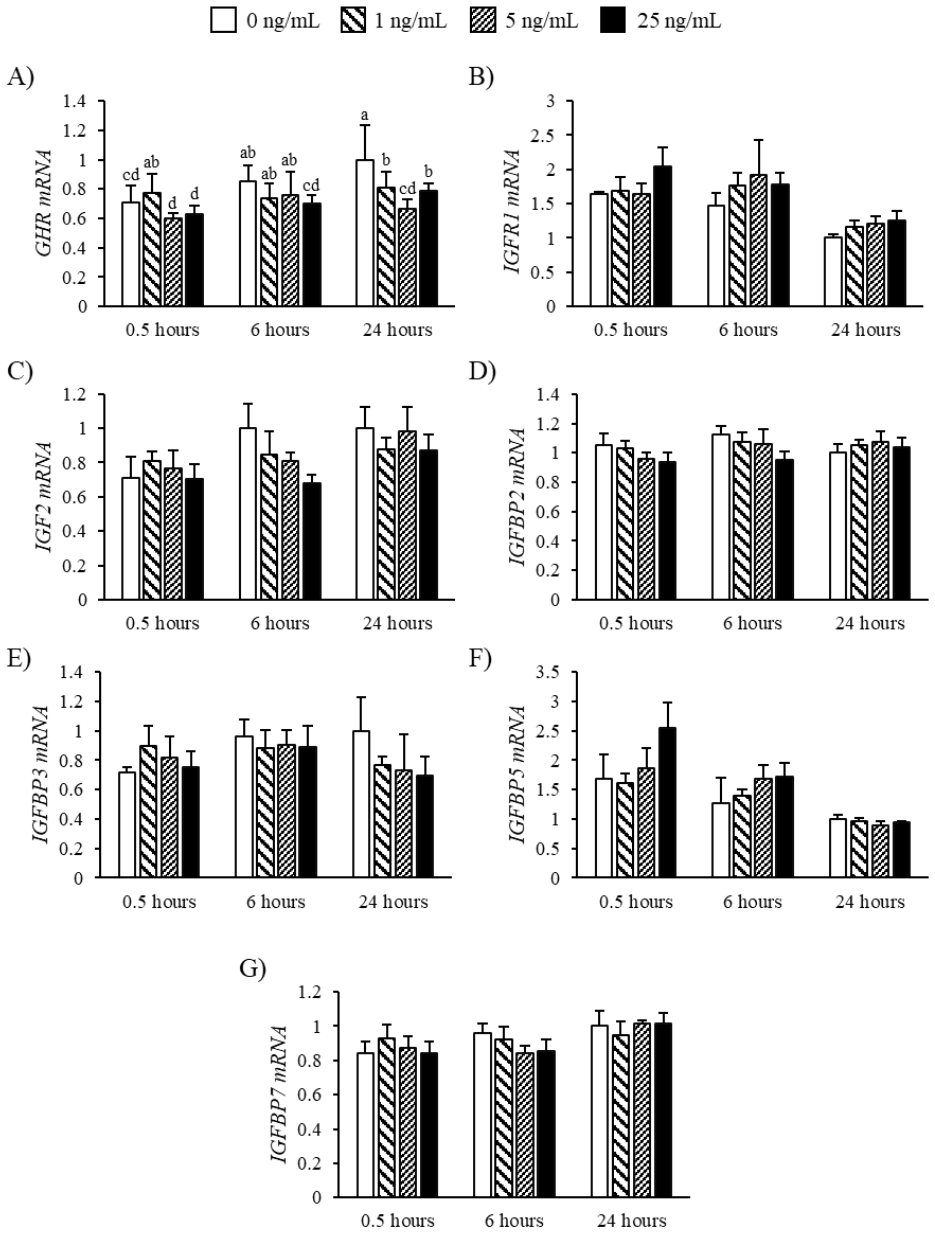

Figure 6.8. Relative mRNA expression of (A) *GHR*, (B) *IGFR1*, (C) *IGF2*, (D), *IGFBP2*, (E) *IGFBP3*, (F) *IGFBP5*, and (G) *IGFBP7* in differentiated QM7 cells treated with 0, 1, 5, or 25 ng/mL T_4 for 0.5, 6, or 24 hours. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* mRNA. The data (mean + SEM) are expressed relative to the 0 ng treatment (set to 1). Data were analyzed by two-way ANOVA followed by Fisher's least significant difference test. A significant hormone concentration-by-time interaction was identified for (A) *GHR* (P=0.0408), and bars without a common letter are significantly different (P≤0.05). No significant hormone concentration-by-time interactions were detected for (B) *IGFR1* (P=0.8391), (C) *IGF2* (P=0.2603), (D) *IGFBP2* (P=0.2219), (E) *IGFBP3* (P=0.6496), (F) *IGFBP5* (P=0.3746), and (G) *IGFBP7* (P=0.8615) and main effects of concentration are shown in **Table 6.5**.

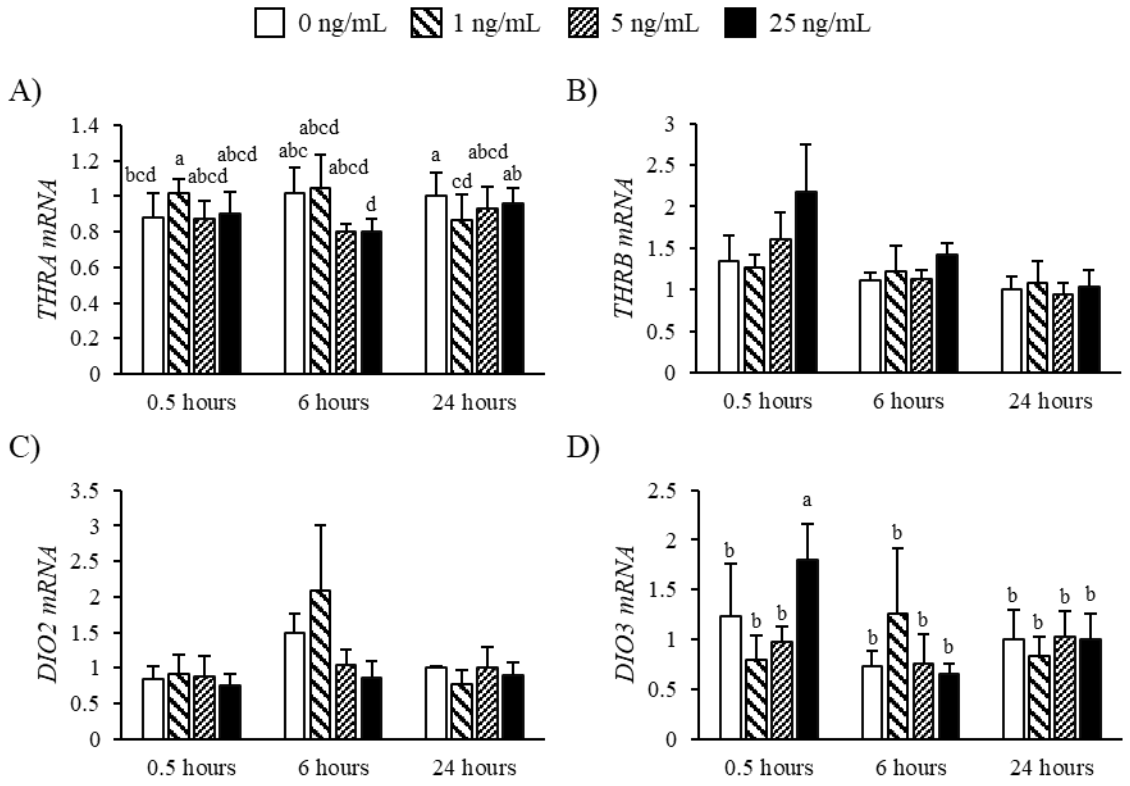

Figure 6.9. Relative mRNA expression of (A) *THRA*, (B) *THRB*, (C) *DIO2*, and (D) *DIO3* in differentiated QM7 cells treated with 0, 1, 5, or 25 ng/mL T_4 for 0.5, 6, or 24 hours. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* mRNA. The data (mean + SEM) are expressed relative to the 0 ng treatment (set to 1). Data were analyzed by two-way ANOVA followed by Fisher's least significant difference test. Significant hormone concentration-by-time interactions were identified for (A) *THRA* (P=0.0207) and (D) *DIO3* (P=0.0408), and bars without a common letter are significantly different (P≤0.05). No significant treatment concentration-by-time interactions were detected for (B) *THRB* (P=0.2912) and (C) *DIO2* (P=0.1793), and main effects of hormone concentration are shown in **Table 6.5**.

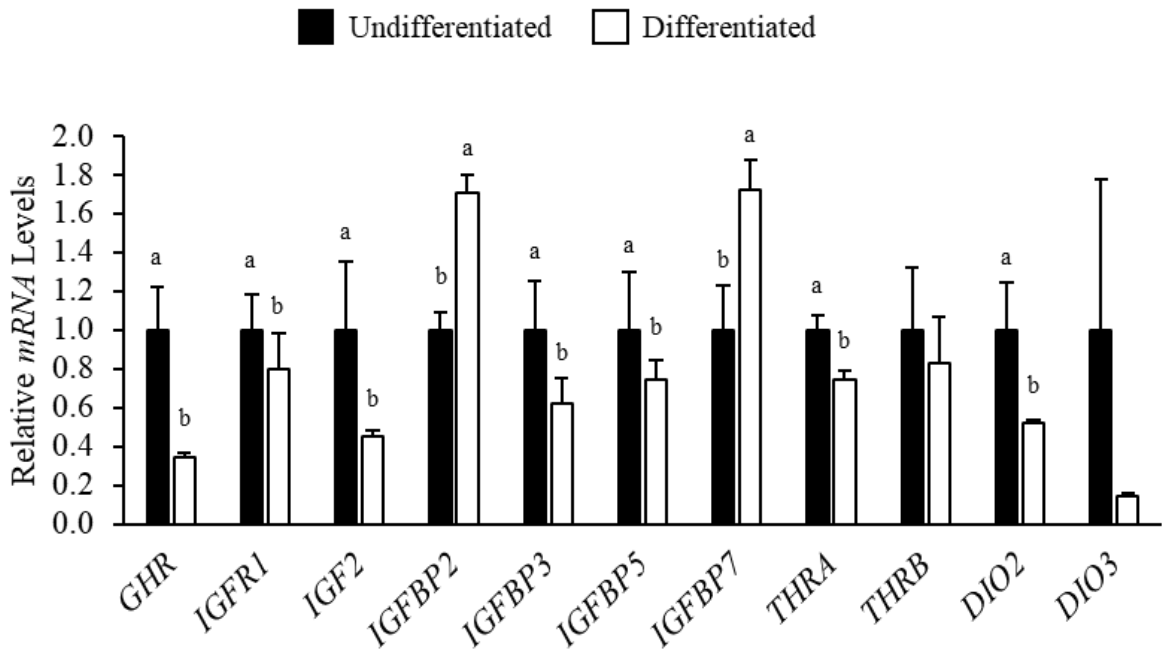

Figure 6.10. Relative mRNA expression of somatotropic and thyrotropic genes in undifferentiated and differentiated QM7 cells cultured under basal conditions without thyroid hormone treatment for 24 hours. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* mRNA. The data (mean + SEM) are expressed relative to levels in undifferentiated cells (set to 1) for each gene. Data were analyzed by one-way ANOVA followed by Fisher's least significant difference test. Bars without a common letter (per gene) are significantly different.

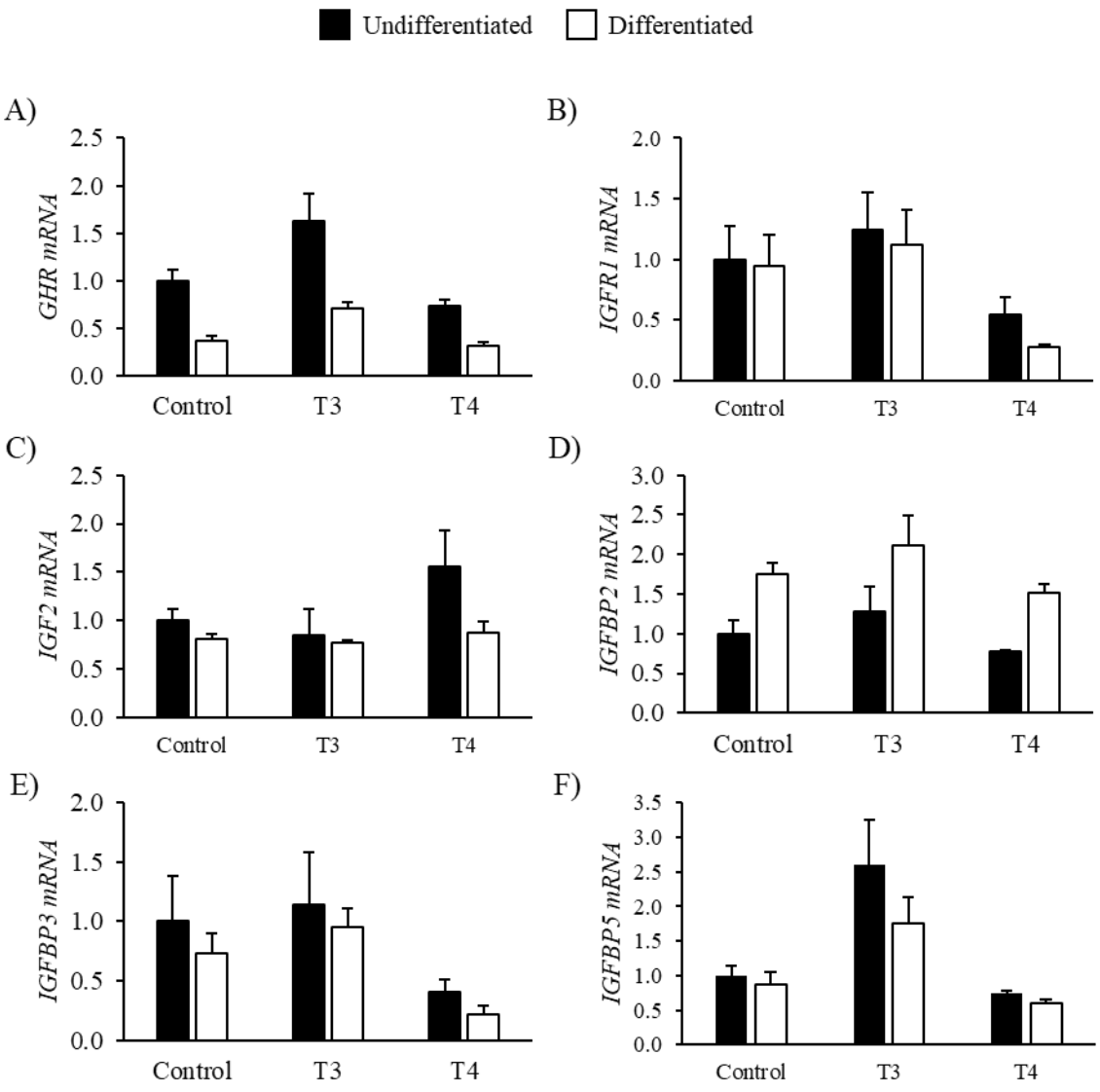

Figure 6.11. Relative mRNA expression of (A) *GHR*, (B) *IGFR1*, (C) *IGF2*, (D), *IGFBP2*, (E) *IGFBP3*, and (F) *IGFBP5* in undifferentiated and differentiated QM7 cells treated with 0 or 5 ng/mL T_3 or T_4 for 24 hours. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* mRNA. The data (mean + SEM) are expressed relative to untreated undifferentiated cells (set to 1). No significant interactive hormone-by-differentiation state interactions were detected [(A) *GHR*, P=0.696; (B) *IGFR1*, P=0.6353; (C) *IGF2*, P=0.3305; (D) *IGFBP2*, P=0.8933; (E) *IGFBP3*, P=0.6167; (F) *IGFBP5*, P=0.8221], and main effects of hormone treatment are shown in **Table 6.6**.

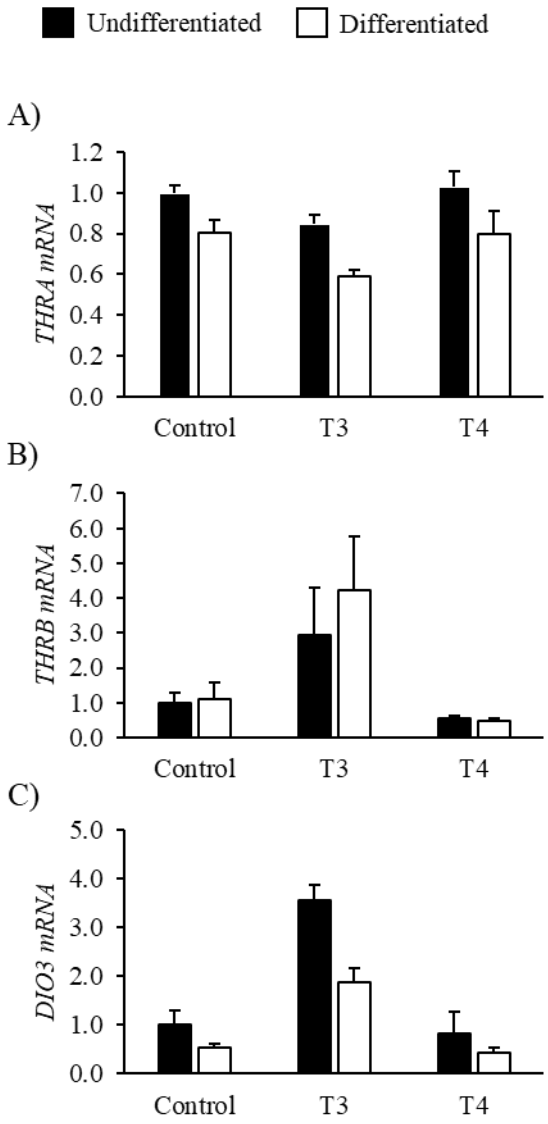

Figure 6.12. Relative mRNA expression of (A) *THRA*, (B) *THRB*, and (C) *DIO3* in undifferentiated and differentiated QM7 cells treated with 0 or 5 ng/mL T_3 or T_4 for 24 hours. Relative expression levels were measured using RT-qPCR and normalized to *GAPDH* mRNA. The data (mean + SEM) are expressed relative to untreated undifferentiated cells (set to 1). No significant interactive hormone-by-differentiation state interactions were detected ([(A) *THRA*, P=0.6612; (B) *THRB*, P=0.7220; (C) *DIO3*, P=0.7428;]), and main effects of hormone are shown in **Table 6.6**.

Table 6.1. Primers used for reverse transcription-quantitative PCR.

Gene Symbol	Forward primer (5'-3')	Reverse primer (5'-3')	ID[a]
Somatotropic axis			
GHR[b]	TGCTGATTTTTCCTCCTGTG	GGCTGGCTAAGATGGAGTTC	5023973
IGFR1[a]	AACCAGACAACTGCCCTGAT	AGACCTCTTTGAACGCTGGA	35974
IGF1[a]	TAACCAGTTCTGCTGCTGCT	TGCTGGATCCATACCCTGTA	35358
IGF2[a]	CAAGTCCGTCAAGTCAGAGC	GCCACACGTTGTACTTGGAG	32094
IGFBP1[a]	TGGAGGGGACGTTTACAAAT	ATAGACGCACCAGCACTCTG	33144
IGFBP2[b]	ATCACAACCACGAGGACTCA	GAGGGAGTAGAGGTGCTCCA	5018698
IGFBP3[b]	TTGAGTCCTAGGGGTTTCCA	ATATCCAGGAAGCGGTTGTC	5082156
IGFBP4[b]	AACTTCCACCCCAAGCAG	AATCCAAGTCCCCCTTCAG	5068153
IGFBP5[b]	CTGAAGAGCAGCCAGAGGAT	TTGTCCACACACCAACACAG	5038163
IGFBP7[a]	GGCCCTGAGAAACATGAAGT	GAAGCTGTTGCCTCTCCTTT	18096
Thyrotropic axis			
THRA[b]	CTTCAACCTGGACGACACC	ACGTCTCCTGGCACTTCTCT	40017
THRB[b]	ACCTGGGCATGTCTCTTTCT	CAGGAGGAAACCCTCTTGAC	5000966
DIO1[a]	CACAATGGAAAACCTGAGCA	CAAGACGGTGCGTATTTCCT	20915
DIO2[a]	TCAAAATTGAGGAGTTCTCTGG	TCTTCCTGATTCCTGTGCTTC	33459
DIO3[b]	CCTCATCCTCAACTTCGG	GATGTACACCAGCAGGAA	5018081
Reference genes			
GAPDH[a]	AGAGGGTAGTGAAGGCTGCT	GCATCAAAGGTGGAAGAATG	28688

[a]Transcript identification from Ensembl Japanese quail genome assembly (https://useast.ensembl.org/Coturnix_japonica/Info/Index) preceded by ENSCJPG000050.
[b]Transcript identification from Ensembl chicken genome assembly GRCg6a (http://www.ensembl.org/Gallus_gallus/Info/Index) preceded by ENSGALG0001. These primers exactly match.

Table 6.2. Means[1] (±SEM) and ANOVA P-values of the main effect of hormone concentration for somatotropic and thyrotropic gene expression in undifferentiated QM7 cells treated with T_3.

	0 ng	1 ng	5 ng	25 ng	P-value
Somatotropic Genes					
IGFR1	1.00±0.19	0.97±0.13	0.97±0.13	0.95±0.15	0.5201
IGF2	1.00±0.22^{c}	1.45±0.23^{a}	1.19±0.19^{bc}	1.32±0.15^{ab}	0.0026
IGFBP2	1.00±0.29	1.11±0.15	1.12±0.14	1.08±0.14	0.1175
IGFBP3	1.00±0.32	1.13±0.29	0.85±0.16	0.86±0.12	0.1693
Thyrotropic Genes					
THRA	1.00±0.06	0.85±0.07	0.86±0.04	0.85±0.05	0.0622

[1]Means are only presented for genes where a significant hormone concentration-by-time interaction was not present.
[2]Data within each gene are expressed relative to 0 ng treatment (equal to 1).

Table 6.3. Means[1] (±SEM) and ANOVA P-values of the main effect of hormone concentration for somatotropic and thyrotropic gene expression in differentiated QM7 cells treated with T_3.

	0 ng	1 ng	5 ng	25 ng	P-value
Somatotropic Genes					
GHR	1.00±0.09[b]	1.28±0.07[a]	1.40±0.10[a]	1.50±0.19[a]	0.0010
IGFR1	1.00±0.20	1.09±0.12	1.03±0.15	1.11±0.18	0.7460
IGF2	1.00±0.05[b]	1.20±0.06[a]	1.23±0.06[a]	1.22±0.04[a]	0.0002
IGFBP2	1.00±0.09	1.10±0.09	1.08±0.10	1.10±0.13	0.8874
IGFBP3	1.00±0.03	0.92±0.04	0.95±0.08	0.94±0.06	0.5497
IGFBP5	1.00±0.36	1.24±0.19	1.34±0.22	1.43±0.32	0.7011
Thyrotropic Genes					
THRA	1.00±0.06	0.91±0.04	0.90±0.05	0.97±0.05	0.2373

[1]Means are only presented for data where a significant hormone concentration-by-time interaction was not present.
[2]Data within each gene are expressed relative to 0 ng treatment (equal to 1).
[abc]Values that do not share a common letter are significantly different ($P \leq 0.05$).

Table 6.4. Means[1] (±SEM) and ANOVA P-values of the main effect of hormone concentration for somatotropic and thyrotropic gene expression in undifferentiated QM7 cells treated with T_4.

	0 ng	1 ng	5 ng	25 ng	P-value
Somatotropic genes					
IGFR1	1.00±0.07	1.23±0.11	1.31±0.14	1.46±0.16	0.9941
IGF2	1.00±0.11	1.16±0.17	1.31±0.28	1.31±0.28	0.6877
IGFBP2	1.00±0.05	1.05±0.09	1.00±0.05	1.10±0.09	0.9608
IGFBP7	1.00±0.14	1.04±0.14	0.99±0.14	1.10±0.19	0.8426
Thyrotropic genes					
THRA	1.00±0.10	0.93±0.07	0.89±0.04	0.93±0.10	0.7391
DIO2	1.00±0.12	1.51±0.51	1.95±0.58	1.70±0.57	0.5847
DIO3	1.00±0.17	1.34±0.54	1.48±0.37	2.03±0.52	0.0541

[1]Means are only presented for data where a significant hormone concentration-by-time interaction was not present.
[2]Data within each gene are expressed relative to 0 ng treatment (equal to 1).

Table 6.5. Means[1] (±SEM) and ANOVA P-values of the main effect of hormone concentration for somatotropic and thyrotropic gene expression in differentiated QM7 cells treated with T_4.

	0 ng	1 ng	5 ng	25 ng	P-value
Deiodinases and TH Receptors					
DIO2	1.00±0.12	1.13±0.31	0.88±0.12	0.75±0.09	0.2353
THRB	1.00±0.10[b]	1.03±0.12[b]	1.07±0.12[b]	1.34±0.20[a]	0.0193
Somatotropic genes					
IGFR1	1.00±0.11	0.91±0.07	0.79±0.07	0.83±0.04	0.7017
IGF2	1.00±0.09	0.93±0.05	0.94±0.07	0.83±0.05	0.0882
IGFR1	1.00±0.11	0.91±0.07	0.79±0.07	0.83±0.04	0.7017
IGFBP2	1.00±0.03	0.99±0.03	0.97±0.04	0.92±0.03	0.2219
IGFBP3	1.00±0.10	0.95±0.07	0.91±0.11	0.87±0.08	0.6496
IGFBP5	1.00±0.15	1.00±0.08	1.12±0.14	1.32±0.19	0.0964
IGFBP7	1.00±0.04	1.00±0.04	0.97±0.04	0.97±0.04	0.8615
Thyrotropic Genes					
THRB	1.00±0.10[b]	1.03±0.12[b]	1.07±0.12[b]	1.34±0.20[a]	0.0193
DIO2	1.00±0.12	1.13±0.31	0.88±0.12	0.75±0.09	0.2353

[1]Means are only presented for data where a significant concentration-by-time interaction was not present.
[2]Data within each gene are expressed relative to the 0 ng treatment (equal to 1).
[abc]Values that do not share a common letter are significantly different ($P \leq 0.05$).

Table 6.6. Means[1] (±SEM) and ANOVA P-values of the hormone main effect for somatotropic and thyrotropic gene expression in QM7 cells treated with 0 or 5-ng/mL T_3 or T_4 for 24 hours.

	No Treatment	T_3	T_4	P-value
Somatotropic genes				
GHR	1.00±0.15[b]	1.71±0.32[a]	0.77±0.13[b]	<0.0001
IGFR1	1.00±0.18[a]	1.21±0.23[a]	0.42±0.09[b]	0.0029
IGF2	1.00±0.08	0.90±0.14	1.34±0.25	0.1266
IGFBP2	1.00±0.11	1.24±0.23	0.83±0.11	0.2392
IGFBP3	1.00±0.24[a]	1.21±0.26[a]	0.36±0.08[b]	0.0740
IGFBP5	1.00±0.12[b]	2.34±0.44[a]	0.72±0.05[b]	0.0001
Thyrotropic genes				
THRA	1.00±0.05[a]	0.80±0.06[b]	1.01±0.08[a]	0.0060
THRB	1.00±0.25[b]	3.39±1.08[a]	0.48±0.05[b]	<0.0001
DIO3	1.00±0.21[b]	3.54±0.50[a]	0.81±0.30[b]	<0.0001

[1]Means are only presented for data where a significant interaction was not present.
[2]Data within each gene are expressed relative to cells receiving no hormone treatment (equal to 1).
[abc]Values that do not share a common letter are significantly different ($P \leq 0.05$).

CHAPTER 7

GENERAL DISCUSSION

Modern broilers are an affordable source of high-quality animal protein because of their high body weights, fast growth rates, and low FCR produced by intensive commercial genetic selection [1-6]. The hormonal signaling systems altered by selection and their molecular mechanisms have yet to be elucidated. Growth and development are understood to be regulated by several conserved hormonal axes [7, 8, 69]. Therefore, commercial genetic selection has potentially altered the function of these axes in modern broiler chickens. This makes the investigation of hormonal activity and signaling in broilers a means to developing strategies that improve and maintain broiler performance as antibiotic growth promoters are no longer in use. Such strategies could include identification of novel targets for genetic selection or finding ways to manipulate endocrine axes by managerial or nutritional approaches.

Three hormonal axes known to regulate mammalian growth and development are the somatotropic axis, the adrenocorticotropic axis, and the thyrotropic axis [7, 8]. The somatotropic axis promotes growth and metabolism via IGF signaling [9, 10]. These hormones, in turn, are regulated by IGFBP activity [20, 21]. Actions of the adrenocorticotropic axis are induced when CORT, typically secreted under stressful conditions, binds NR3C1 to increase feed consumption while decreasing growth and metabolic efficiency, as energy utilization is prioritized to respond to the stressful situation [35-38]. The action of the thyrotropic axis, whose functions include regulation of basal metabolic rate, long bone growth, and skeletal muscle growth and development, is induced via TH action facilitated by their metabolic conversion to active or inactive states at the

tissue level [49, 50]. The DIOs regulate TH signaling by converting T_4 to bioactive T_3 or inactive rT_3 and inactivating T_3 via conversion to T_2 [51, 52]. These three axes also engage in hormonal crosstalk to regulate various biological processes in additive, synergistic, or antagonistic manners [59-61, 65]. Therefore, any investigation into the effects of genetic selection on broiler performance must consider potential altered axis crosstalk, as well.

The ACRB legacy broiler line was utilized to determine changes in endocrine axis activity induced by genetic selection. The ACRB functioned as a genetic control line, as it represents broilers from the mid-1950's prior to the advent of intensive selection. The line grows more slowly, exhibits higher FCR, and has less muscle than modern broilers. Additionally, performance metrics of modern broilers cannot be restored in the ACRB by administering a modern commercial diet, indicating the above differences are physiological. This makes the ACRBs ideal to investigate the effect of genetic selection on hormonal signaling in the context of broiler growth and development [2, 5, 66].

Our findings suggest that genetic selection altered circulating adrenocorticotropic and thyrotropic hormones, as well as expression of genes associated with those axes, in such a manner to promote growth in modern broilers. Plasma levels of CORT and T_3 were reduced in Ross 308 broilers compared to ACRBs. This indicates a diminished stress response and a reduced metabolic rate in modern broilers. As such, this could lessen the negative effects of environmental stressors on broiler growth performance, as well as cause more efficient feed utilization as less energy would be lost to heat production. Levels of *THRA* were elevated post-hatch in ACRB breast muscle, potentially resulting in greater energy expenditure by this tissue for heat production rather than growth, consequently increasing FCR. Hepatic *THRB* during embryogenesis was diminished in Ross 308 birds, which may either dampen THR-regulated gene expression or weaken negative

feedback responses to maintain TH production. Expression of *DIO2* in Ross 308 liver decreased at d20 alongside circulating T_3, indicating that less circulating T_4 was being converted to T_3 by DIO2 in the liver and causing lower circulating T_3 as a result. Hepatic *DIO3* was greater in ACRB liver post-hatch, potentially as a mechanism to manage higher circulating concentrations of T_3. These results imply that these genes regulate broiler metabolism by controlling tissue specific T_3 availability. In tandem with differences in circulating T_3, these results indicate that genetic selection has altered the activity of the thyrotropic axis, causing greater muscle yield and improved efficiency of dietary nutrients for growth.

This study also found that circulating IGFs and hepatic *IGF* mRNA expression was not different between Ross 308 and ACRB broilers, indicating that growth regulated by IGF signaling must be controlled in another manner. The levels of mRNA for both *IGF*s were greater, however, in Ross 308 juvenile breast muscle. One interpretation of these data is that IGFs produced in breast muscle have local growth-promotive effects, contributing to the rapid accretion of this tissue in modern broilers. Expression of the IGFBPs varied depending on tissue type, line, and age. For example, *IGFBP3* was greater in Ross 308 liver during late embryogenesis and early juvenile development but reduced in breast muscle, suggesting endocrine IGFBP3 produced by the liver promotes growth when in circulation but restricts muscle growth by acting at a local level. These data holistically indicate that IGF effects are not entirely dependent on circulating IGF levels in plasma but rather, in part, by tissue specific IGF production or by control of IGF signaling by mediating receptor access through interactions with IGFBPs.

The previous study demonstrated that somatotropic gene expression was altered by commercial genetic selection of broiler chickens, but the function of these genes during distinct developmental stages was unclear. Thus, somatotropic gene activity from mid-embryonic through

early post-hatch development was investigated to determine the axis' functions during these distinct developmental periods. These effects were predicted to be linked to traits observed in modern commercial broilers, such as rapid growth rate and muscle accretion and improved feed efficiency. Ontogenic somatotropic gene expression was examined in Ross 308 broilers from mid-embryogenesis to three weeks post-hatch, and circulating IGF concentrations were determined in the post-hatch juvenile period. Plasma IGF levels remained consistent throughout three weeks post-hatch, strengthening the argument that endocrine IGF activity is regulated by the IGFBPs and local IGF signaling might play an important role in regulating muscle growth. In support of this, *IGF1* in breast muscle increased after embryogenesis. The expression levels of several IGFBPs also decreased during the peri-hatch period in the liver and the breast muscle and were either restored to embryonic levels or remained depressed following hatch. For example, liver and breast muscle *IGFBP2* levels were diminished during the peri-hatch period compared to embryogenesis, and they were not restored post-hatch. This implies that *IGFBP2* has a growth-inhibitive effect during juvenile development, with decreased expression allowing for rapid growth. Comparatively, hepatic *IGFBP4* during embryogenesis was low but increased greatly after hatch. The opposite was observed in breast muscle, in which expression levels decreased after embryogenesis and did not recover post-hatch. These data suggest that IGFBP4 promotes growth when secreted into the plasma but inhibits growth when produced locally in muscle tissue. Levels of *IGFBP5* decreased during the peri-hatch period in both the liver and breast muscle, but hepatic *IGFBP5* levels recovered more quickly than those in muscle tissue. Like *IGFBP4*, these results suggest that IGFBP5 has a growth promotive effect when secreted from the liver. If it has a growth-inhibitory effect in the muscle, this is only most important during the first week post-hatch. As the somatotropic axis does not fully mature until early post-hatch, this may also indicate that IGFBPs

function independently of IGF signaling during embryogenesis. Embryonic levels of *IGF1* in the liver were low compared to those post-hatch, in which they greatly increased. This is despite hepatic *IGF2* remaining consistent between developmental stages and higher levels of several hepatic IGFBPs during embryogenesis. Circulating IGF1 and IGFBPs are primarily produced in the liver. Although *IGF1* was expressed in embryonic chick muscle, a lack *IGF1* in the liver at the same time suggests that IGFBP function could be to inhibit hepatic IGF1 during this period. Expression of several IGFBPs was also lower one week after hatch in both tissue types. During this period, chicks switch from deriving energy from lipoproteins stored in the yolk to carbohydrates in corn-based feed [454]. Lower IGFBP activity during early post-hatch development could be to allow for rapid growth as the chick switches energy sources.

Studying the effects of hormonal signaling on chicken growth is important to determine how endocrine systems impact the development of economically important tissues such as muscle. Immortalized chicken muscle cell lines are unavailable, however, and the culture of primary myoblasts is unreliable. Therefore, QM7 cells were used to study the potential effect of the THs on IGFBP activity *in vitro*. This cell line served as a useful model to study molecular mechanisms in the chicken due to the genetic proximity between chickens and Japanese quail and ease of maintenance *in vitro* [67, 68]. These cells can be maintained as mononuclear myoblasts or, under reduced-serum conditions, can be induced to differentiate into multinuclear myotubes [67]. An *in vitro* model was used to study TH effects in an isolated manner, away from the influences of other endocrine systems that would be present *in vivo.* This research gave insight into the effect of TH signaling on somatotropic activity in muscle, an economically valuable tissue in chickens.

Our findings suggest that muscle cell growth and development are mediated indirectly by the THs through IGFBP activity, indicating a mechanism for somatotropic and thyrotropic

crosstalk in the context of avian muscle development. Certain IGFBPs, such as *IGFBP5*, were higher in the presence of T_3 and T_4, but only in undifferentiated cells. Several genes were also differentially expressed between cell states when no treatment was administered. Levels of *GHR*, *IGFR1*, *IGF2*, *IGFBP3*, *IGFBP5*, *THRA*, and *DIO2* were greater in undifferentiated cells, whereas *IGFBP2* and *IGFBP7* levels were higher in differentiated cells. This indicates that myoblasts and myotubes have differential somatotropic and thyrotropic gene expression profiles, and undifferentiated cells appear more responsive to hormonal signaling than differentiated cells, as evidenced by increased levels of hormone receptors and factors modulating hormonal signaling. Effects of T_3 and T_4 also differed, with T_3 inducing more effects. Levels of *GHR*, *IGFBP5*, *THRA*, *THRB*, and *DIO3* were altered in cells treated with T_3 compared to those treated with T_4 and untreated controls; *IGFR1* and *IGFBP3* were changed by T_4 treatment. Therefore, QM7 cells exhibit greater changes to expression when exposed to T_3, which is likely more effective at regulating somatotropic and thyrotropic genes because it is more bioactive.

These findings collectively suggest that the growth of modern commercial broilers is regulated, in part, by the somatotropic, adrenocorticotropic, and thyrotropic axes, and that these systems have been altered by intensive commercial genetic selection to facilitate efficient growth. Additionally, the differences in body, growth rate, and FCR between modern and legacy broilers are not directly caused by circulating IGF concentrations in plasma, but potentially by IGFBP activity that regulates IGF signaling in both endocrine and paracrine manners. These IGFBPs are, in turn, possibly regulated by TH activity in skeletal muscle in a form of hormonal crosstalk that impacts development of this tissue. This work provides insight into the molecular mechanisms of hormonal signaling regulating broiler growth and development that can be applied by the broiler industry to develop novel strategies to improve production efficiency.

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