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Identification of differences in digestive organ weight, bone mineral concentration, and ileal transcriptomic profiles of low and high weight broiler chicks

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Abstract

A growth monitoring study (0-7 day of age) was conducted involving 87, one-day old Ross 308 male broilers to evaluate organ weights, bone parameters and ileal transcriptomic profile of broiler chicks as influenced by day 7 bodyweight (BW) grouping. The chicks were raised in a deep-litter house under common controlled environmental conditions and commercial starter diet. Chicks were grouped on day 7 into two distinct BW, super performer (SP) and under performer (UP) with bodyweights >260g, and <200g respectively. Results revealed that the SP chicks had significantly higher bone ash, sodium (Na), phosphorus (P) and rubidium (Rb) concentrations compared to the UP chicks on D7. In contrast, the UP chicks had significantly higher tibial cadmium (Cd), caesium (Cs) and lead (Pb) compared to the SP group; the UP chicks also had proportionally heavier relative gizzard weight than the SP chicks. The ileal transcriptomic data revealed differentially expressed genes between the two groups of chicks, with 150 upregulated and 83 down-regulated genes with a fold change of ≥1.25 or ≤ 1.25 in the SP chicks relative to the UP chicks. Furthermore, functional annotation and pathway analysis revealed that some of these differentially expressed genes were involved in various pathways including calcium signaling, Wnt signaling, cytokine-cytokine receptor interaction and mucin type O-glycan biosynthesis. This study revealed that chicks of the same breed and of uniform environmental and diet management exhibited differences in digestive organ weights, tibial bone characteristics and ileal gene expression that may be related to BW.

Keywords: Transcriptomics, ileum, bodyweight, variation, bone mineral concentration

Introduction

Chicken is one of the most preferred animal protein sources globally due to its comparatively lower cost, nutritional content and perceived health values. Despite improved genetic modification and stringent management practices in broiler production, there have been reports of considerable bodyweight variation which results in varying slaughter weight (Piórkowska, et al., 2020; Lundberg, et al., 2021). There are many reasons underpinning variation in broiler growth such as broiler breeder age, incubation factors, genetics, disease, nutrient malabsorption, and poor feed intake (Tegeda, et al., 2021).

The first week of life is a critical period for the broiler, as the chicks are exposed to more varied conditions on the farm following a relatively common and controlled environment during the incubation period (Yerpes, et al., 2020). Bodyweight increases two to threefold during the first week of life and considerable changes occur in the gastrointestinal development and in muscle accretion (Jin et al., 1998; Iji, et al., 2001; Willemsen et al., 2008). These developmental changes can be categorized into morphological, functional and immunological development (Schokker, et al., 2009). The development of the chicken intestine as a digestive and absorptive system is closely related to the development of the gut-associated lymphoid tissue (Shira, et al., 2005). It has been reported that the immune organ development of the chicken occurs within the first two weeks of life (Dibner., 1998). The immune development in young chicks has also been reported to be associated with early nutrition which makes essential nutrients available for cell proliferation and differentiation. In this aspect, early feed intake stimulates many antigens involved in the development of immunoglobulin in the chicken bursa (Jeurissen, et al., 1989; Dibner et al., 1998). Research has reported that the expression of proinflammatory cytokine and chemokine (IL-1β, IL-8, K203) during the first week of life in broiler are initiated by the exposure of the hatchlings to exogenous feed and the environment (Bar-Shira et al., 2006). This unique development of the chicken intestine with a coinciding succession of microbiota and changes in microbial community during the early life can influence the host physiological and metabolic functions (Tang, et al., 2020). The small intestine plays a vital role in the regulatory, endocrine, and immune function, which can thus affect birds' health, feeding behavior and energy homeostasis (Scanes and Pierzchala-Koziec, 2014; Sugiharto, 2016 and Honda, et al., 2017). Svihus (2014) reported that the functionality of the digestive tract is pivotal to optimal performance of broiler chicks. Therefore, development and growth performance in the first week is critical and indeed day 7 BW has been reported to have a stronger correlation with important parameters such as slaughter weight and carcass composition when compared to hatch weight (Ribeiro, et al., 2004 and Tona et al., 2004b).

Mineral metabolism is an important aspect in broiler nutrition and growth as minerals play useful roles as a catalyst in most enzyme and hormone activities (Suttle, 2010). Bone mineral concentrations, especially calcium (Ca) and phosphorus (P), affect skeletal integrity (Underwood and Suttle, 1999) and determine the extent of mineralization. They are also actively involved in many physiological and metabolic roles in the body such as cell signaling and nerve impulse transmission (Underwood and Suttle, 1999). Previous studies have reported bone mineral concentration as a vital tool in assessing mineral bioavailability, utilization and storage in broiler chicks (Yair and Uni, et al., 2011), for example Ca concentration in the tibia serves as a reservoir for maintaining serum calcium levels (Weaver, et al., 2016). Therefore, evaluating bone mineral concentration in broiler chicks in early life could be a valuable biomarker to determine the mineral status of chicks post hatch. Generally, mineral absorption in broilers is uniquely governed by the activation of important pathways, for example Wnt signaling, that comprises several ligands activated by Wnt proteins, which when secreted bind to the frizzled transmembrane receptors to initiate intracellular signaling cascade that modulates gene expression (Mohammed, et al., 2016), resulting in specific mineral absorption such as Ca and P (Wang, et al., 2022).

It was hypothesized that the mineral status, organ measurements and transcriptomics may be different between chicks ranked based on Day 7 bodyweight. Identifying some of those differences may be useful in developing intervention strategies for improved broiler performance. The present study therefore evaluated differences in digestive organ weight, ileal transcriptomic profile, and bone mineral concentrations of 7-day old broiler chicks.

Materials and Methods

Experimental Design and Animal Management

A total number of 87-day old male Ross 308 chicks were used for the study and all chicks were housed in the same deep litter pen with softwood shaving as bedding, and under the same common environmental and diet conditions. The chicks were reared from day 0 to day 7 and were characterized based on the day 7 bodyweight, before sample collection. Chicks were fed commercial Hygates baby chick crumbs (containing 19% crude protein, 4.5% crude fiber and 3.5% oil) that met the nutritional requirement of the Ross 306 breed.

Bodyweight of chicks was recorded individually on day 0 and day 7. Chicks were ranked and those in the first and fifth quintiles were categorized as super performers (SP) and under performers (UP) respectively. SP chicks had an average bodyweight of 260g and UP; 200g, bodyweight thresholds were selected based on the performance target outlined for male Ross 308 chicks on day 7 (Aviagen, 2019). On day 7, ten chicks from each group SP and UP (n=10/bodyweight group) were randomly selected and euthanized. Bodyweight uniformity was calculated using the formula below.

Uniformity % = Number of birds within range ±10% of mean weight ÷ Total number of birds weighed × 100

The liver, gizzard and full intestine were excised and weighed using a precision balance while the legs were collected and stored at -20^{0}C until further bone mineral analysis. The ileal segment was excised, and snap frozen immediately with dry ice before being stored at -80°C until RNA extraction.

Crude ash and mineral analysis

The legs collected were thawed and defleshed to extract the tibial bones. Care was taken to make sure all the flesh was removed and immediately stored in the freezer at -20^{0}C until drying the next day. The tibial bones were oven-dried at 105^{0}C using a Griffin oven for 24hrs and ashed at 600^{0}C overnight using Carbolite AAF 11/18 to determine the tibial ash, then the ash weight of individual tibial bone was expressed as a percentage of dry weight. The tibial bone ash was acid digested using the hot plate method following internal laboratory procedure for sample preparation. A maximum of 0.2g of each sample was digested with 10ml of nitric acid and heated for 2 hours at 95^{0}C, 50ml MilliQ water was added to each and 8ml taken from the top, transferred to 8ml tubes and samples were diluted to 1/10 and mineral concentration analyzed using an ICP-MS method (Thermo-Fisher Scientific iCAP-Q; Thermo Fisher Scientific, Bremen, Germany).

RNA extraction and microarray analysis

RNA was extracted from the ileum of 7-day old broiler chicks using the Direct-zol™ RNA MiniPrep Kit (Cambridge Bioscience, UK). RNA integrity was confirmed using an Agilent 2100

Bioanalyzer with the RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA). The RNA integrity numbers (RIN) were ≥8.7 for all samples. Whole-genome transcriptome analysis was conducted by hybridising three biological samples of total RNA per group to GeneChip^{TM} Chicken Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA). First strand cDNA was produced by reverse transcription followed by second strand synthesis. Double stranded cDNA was then used to synthesise biotinylated complementary RNA *in vitro*, which was purified and fragmented in different sizes (200-2000 bp). These fragments were hybridised onto GeneChip^{TM} Chicken Gene 1.0 ST arrays using the GeneChip System 3000 instrument platform (Affymetrix, Santa Clara, CA, USA). All steps were conducted at the Nottingham Arabidopsis Stock Centre.

Gene expression profile data was generated as CEL files and analysed using Partek Genomics Suite 6.6 (Partek Incorporated, St. Louis, MO, USA). The raw CEL files were normalised using the RMA background correction with quantile normalisation, log base 2 transformation and mean probe-set summarisation with adjustment for GC content.

Quantitative real-time polymerase chain reaction (qRT-PCR) confirmation of the microarray data

To verify the reliability of the microarray data, three immune related genes (IL20RA, IL8L1 and CCL17) and one gene related to detoxification (GSTA3) were selected for further validation using the RT-qPCR technology. The immune-related genes were selected to verify the observation from the microarray data that the SP chicks had better innate immune activation compared to the UP group. Four genes from the microarray data GAPDH, GALNS, FABP5 and FAM133B were also chosen as housekeeping genes for qRT-PCR because there was no change in their expressions between the two groups. The primer pairs used for the quantitative PCR of these genes are reported in supplementary file 1. Total RNA (250ng) was reverse transcribed using the cDNA reverse transcription kits according to the manufacturers' protocol UltraScript 2.0 cDNA synthesis kit (PCR Biosystems, London UK). The real time PCR reactions were performed using the Bio-Rad CFX Maestro, the reaction contained 1ul of cDNA as a template in a 10ul reaction, the master mix contained 0.4ul of the reverse and forward primers from a 10uM stocks, 5ul of the Syber green master mix 2X qPCRBIO SyGreen Blue Mix Hi-Rox (PCR Biosystems, London UK), and 3.6ul of RNase free water. The PCR reaction conditions were set at 95^0C for 20 seconds, followed by 40 cycles of 95^0C for 3seconds and 60^0C for 30 seconds. A melting temperature curve for every PCR reaction was determined at the end of each run for amplification specificity, and all the 4 samples were performed in triplicate. Relative expression of each mRNA was determined using the $2^{-\Delta\Delta Ct.}$ method using the Bio-Rad software.

Functional annotation and pathway analysis

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/tools.jsp) and Ingenuity Pathway Analysis (IPA) were used to determine the biological functions of the differentially expressed genes based on the *Gallus gallus* reference. Pathway analysis was carried out using the KEGG database as utilized through the DAVID online database.

Statistical Analysis

The individual chick served as the experimental unit. Bodyweight measurement, digestive organ weights and other data derived from the two experimental BW groups SP and UP were compared using the student t-test (Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com), significant differences were observed at $p<0.05$.

Differentially expressed genes (DEG) were identified by one-way ANOVA, DEG comprised genes upregulated or downregulated by at least 1.25-fold with an un-adjusted p-value ≤ 0.05. Statistical analysis for the qPCR data were performed using the ANOVA statistical package of the Bio-Rad CFX Maestro analysis software.

Results

Day 7 bodyweight and Digestive Organ Weights

The mean bodyweight of the bird population on day 7 was 231.2±34.2g, CV of 14.8% and uniformity of 56%. The organ characteristics of the chicks in the BW groups are presented in Table 1. The SP chicks had significantly heavier liver (SP = 12g; UP = 8g; $P < 0.0001$), gizzard (SP = 14g; UP = 10g; $P < 0.0001$), intestine weight (SP = 23g; UP = 15g; $P < 0.0001$) and intestinal length (SP = 110cm; UP = 94cm; $P = 0.0001$). It was noteworthy that the UP group had a proportionally heavier gizzard compared to the SP groups.

Tibia bone ash and mineral concentration

The tibial bone ash and macro mineral concentration of the UP and SP chicks on D7 is shown in table 2, while the trace mineral concentration is presented in table 3. The SP group had higher bone ash when compared with the UP group (SP = 47%; UP = 44%; $P = 0.014$). The UP group had significantly higher Cs (UP = 0.04; SP = 0.03; $P = 0.023$), Cd (UP = 0.02; SP = 0.01; $P = 0.04$) and Pb (UP = 0.34; SP = 0.20; $P = 0.014$) when compared with the SP group. While the SP chicks had significantly higher tibial Na (SP = 12.7%; UP = 11%; $P = 0.014$), P (SP = 19.57%; UP 18.62%; $P = 0.018$), and Rb (SP = 0.009, UP = 0.008; $P = 0.033$) concentrations compared to the UP group.

Ileal transcriptomic profile and differentially expressed genes.

The transcriptomic profile analysis revealed 233 genes that were differentially expressed with a $P < 0.05$ and fold change cutoff of ≥1.25 between the SP and UP groups. The biological details of the DEGs mapped in the IPA database are provided in supplementary file, while the details of the top 29 most conspicuous DEGs with fold change (≥+1.5 and ≥-1.5) are shown in table 4. All the DEGs including the up-regulated (150 genes with low stringent cutoff ≥+1.25) and down-regulated (83 genes with cutoff ≥-1.25) expressed in the ileum of 7-day old chicks of distinct bodyweight were categorized into 3 main functions of biological process, molecular function, and cellular component according to GO analysis using DAVID online tool. Each of the GO categories were further divided into subcategories, and the DEGs were all annotated in all the three GO terms as shown in figure 1. The biological process comprises of 26 terms, including prostaglandin biosynthesis, positive regulation of cell proliferation, superoxide metabolic process, tissue development, inflammatory response etc. Molecular function was divided into 12 terms, including heparin binding, frizzled binding, growth factor activity etc. The cellular component comprises of 8 terms which includes extracellular space, integral component of plasma membrane, extracellular region, photoreceptor outer segment, brush border etc. as illustrated in figure 1. Functional annotation clustering was performed using DAVID tool on the GO terms and 2 clusters were obtained. The first cluster relates to Wnt protein binding, and the second cluster relates to polymerase II core promoter proximal region sequence-specific DNA binding. The enriched pathways annotated include calcium signaling, Wnt signaling, cytokine-cytokine receptor interaction, cardiac muscle contraction, mucin type O glycan and other mucin type O glycan as shown in table 5.

Discussion

Broiler chicks exhibit considerable variation in bodyweight (BW) performance despite successive selective inbreeding and stringent management practices which ultimately impacts flock uniformity. While there is an abundance of literature investigating improvement in growth performance, the basis for variation in bodyweight has received less attention. Therefore, the present study explored various physiological and transcriptomic aspects in understanding the important drivers of variation in bodyweight in the early life of the broiler chick. As expected, the SP chicks had heavier organs when compared to the UP group. Published research reported that the weight contribution of internal organs to bodyweight reflects the health condition of the animals (Smith et al., 2011). It was also reported that the size of the visceral organs may influence energy requirements for basal metabolism as it relates to feed intake (Fitzsimons et al., 2014). Thus, in the present study, the SP chicks exhibited heavier liver, and intestinal weight with longer intestines compared to the UP chicks, indicating that these observed differences in the digestive organ, are related to BW and possibly feed intake. The significant difference observed in this study in gizzard weight relative to body weight of the UP chicks disagreed with the report of Ribeiro et al. (2004), who reported no significant effect of body weight on the relative weight of the gizzard of Ross 308 chicks on day 7. The gizzard acts as a pacemaker of normal gut motility (Ravindra, et al 2021), stimulating the mixing of digesta with enzymes and nutrient digestion. In the present study, it may be suggested that the heavier relative gizzard weight observed in the UP chicks may not be necessarily related to the predicted feed intake as a function of bodyweight but could be associated with other factors related to the environment such as habitual consumption of bedding.

Bone ash has been used to assess skeletal mineralization in poultry production (Hall et al., 2003), The percentage of bone ash in poultry is a general indicator of bone mineralization (Thorp and Waddington, 1997). High bone ash and mineralization correlates to stronger bone and ability of the skeleton to withstand gravity and additional loading (Shim, et al., 2012). Ca, one of the primary bone minerals showed no significant difference between the two groups, tibial P concentration on the other hand showed a significant increase in the SP chicks compared to the UP chicks; this increase in bone P concentration in the SP chicks may be linked to the Wnt signaling pathway which was enriched in the SP relative to the UP group. Wnt signaling had been reported to be associated with both calcium and P absorption in broilers (Wang, et al., 2022). The Wnt signaling cascade had also been reported to play a central part in regulating the development of calcium signaling pathway (Lu and Carson, 2009). It is also noteworthy that the calcium signaling pathway was one of the most enriched pathways identified in the SP group relative to the UP. This may be attributed to the heavier bodyweight of the SP group with higher metabolic demand, as calcium signaling is important in stimulating metabolic process and encouraging the differentiation of adipocytes (Song, et al., 2019). Taken together, these pathways identified in the SP group could be linked to the higher concentration of bone P in the SP group.

Minerals of physiological importance including toxic metals can bioaccumulate in calcified tissues such as teeth and bones (Rasmusson and Eriksson 2001), and 80% of the bioaccumulation results from dietary intake (Baykov et al., 1996; Orzechowska et al., 2010). The UP group had significantly higher concentrations of tibial cadmium (Cd), caesium (Cs) and lead (Pb) compared to the SP group. The increase in the concentration of these minerals in the UP group, merits further mechanistic investigation. For example, the higher bone Cd concentration may be linked to the decrease in phosphorus concentration in this group, as it was reported that when cadmium accumulates in the body, it causes damage to the kidney which in turns inhibits the activity of vitamin D, thus preventing the calcination and storage of phosphorus in the bone (Youness, et al., 2012).

The exploratory ileal transcriptomic profiling of 7 Day old Ross 308 chicks was aimed at identifying the potential candidate genes and pathways associated with variability in growth performance of chicks at this life stage. The concept of the present study benefited from the sampling of chicks from the same breed population maintained under the same environmental and diet conditions. The functional annotation of the differentially expressed genes (DEGs) performed to elucidate the biological implication of these genes reported interesting observations which may be associated with the differences in the growth rate of these chicks.

In the current study, an upregulation of the IGF gene (IGF-1) in the SP group was observed relative to the UP, a gene which modulates the growth-promoting effect of growth hormones (Wang, et al., 2003). IGF-1 is among the members of the insulin-like growth factor family which regulates cell growth, and proliferation and plays a distinct role in lean meat content during the growth of dairy cattle (Mullen, et al., 2011). IGF-1 is an important gene controlling body size (Wang, et al., 2004). It has been reported that the signal transduction commenced from the binding of growth hormone (GH) to its receptor which leads to the activation of specific gene coding insulin like growth factor 1 (IGF-1) and is released into circulation to bind to its specific receptor known as the IGF type-1 receptor which then stimulates cell proliferation (Okumura and Kita, 1999). The up-regulation of the IGF-1 gene in the SP chicks relative to UP chicks could be associated with the greater bodyweight of the former, as this gene is wholly involved in growth and controlling body size (Wang, et al., 2004).

There was an up-regulation in the expression of genes acting as immune mediators including pro-inflammatory cytokines and chemokines such as Interleukin 8 like 1 (IL8L1) in the SP compared to the UP group. Interleukin 8 Like 1 (IL8L1) has been reported to be involved in the recruitment of heterophils to the site of infection in the chicken intestine (Kogut., 1994 & 2002) and these heterophils are pivotal in activating the innate immune response (Genovese, 2000). Based on the reported literature (Swaggerty, et al., 2005., Bar-Shira, and Fridman., 2006., Terada, et al., 2018), it may be speculated that the upregulations of these proinflammatory and chemokine genes in the ileum of the experimental chicks may play distinct roles in innate host defense triggered by exposure to feed and microorganism during the first week of life. It has been reported that young hatchlings respond to environmental stimuli by gradual development of pro inflammatory functions (Withanage, et al., 2004; Bar-Shira and Friedman, 2006). The immune protection of hatchlings could emanate from maternal antibodies which are active systemically and in the gut cavity and innate effector mechanisms which are active alongside all mucosa linings (Bar-Shira and Fridman, 2006).

Another interesting cytokine that was upregulated in the SP chicks in the present study is Interleukin 26 (IL26). Interleukin 26 is a member of the IL-10 cytokine family which plays a role in the local mechanism of mucosal immunity and induces the expression of IL8 (Ouyang and O’Garra, et al., 2019). It has also been reported that the IL26 gene activates the immune-related pathways such as JAK/STAT, NF-kB, and MAPK signalling pathways; crosstalk between these pathways may modulate the expression of chemokines and cytokines in chicken cell lines (Truong, et al., 2017). Also, the JAK/STAT pathway is crucial to T cell differentiation, B cell maturation, and development, secretion of SIgA, mucus, and antibody production which are pivotal to maintaining antiviral and anti-bacterial defense at the mucosal surface (Heneghan, et al.,2013).

Based on this report, the up regulation of IL26 and chemokine (IL8L1), may suggest that the SP chicks could be more advantaged in terms of innate preparedness of the gut for development and strong defense against enteric pathogens.

In addition to the increased expression of important pro-inflammatory cytokines genes involved in immune response, in the SP group, we observed an increase in the expression of glutathione S-transferase alpha (GSTA3), which is an antioxidant enzyme specifically involved in the clearance of various peroxidation products (Anyia and Imaizumi, 2011). The increase in the expression of the GSTs (GSTA3) and their activities in the SP chicks compared to UP chicks may positively affect glutathione metabolism and metabolism of xenobiotics by cytochrome P450. The chicken intestine is known to be the primary site of exposure to dietary xenobiotics, which are potential toxins and may promote the proliferation of cellular free radicals (Wang, et al., 2019). Thus, it may be speculated that the observed increase in expression of the GSTs genes in the SP group may play a strong role in the detoxification of xenobiotic toxins and reduction in oxidative stress compared to the UP chicks. This may also be attributed to the speculated higher feed intake in the SP chicks, as a result, SP group may be exposed to a higher intake rate of xenobiotics, thus higher expression of the GST genes to combat this.

It is also noteworthy that in the present study there was upregulation of microRNAs (MiRNAs) such as MiRNA 23, 25, 27 and 7 (Mir-23, Mir-25, Mir-27, and Mir-7), in the SP relative to UP group. MiRNAs are a class of endogenous non-coding RNA, comprising about 22 nucleotides (Bartel, 2004) which are known to play a crucial role in the regulation of gene expression at the post-transcriptional level. They act by binding complementary sequences on messenger RNA target genes, thereby causing cleavage or repressing translation (Bartel, 2004). Mir-27 is known to regulate the expression of NFE2L2 (a transcriptional factor that modulates gene transcription of antioxidant response element), and an increase in the expression level of NFE2L2 is associated with oxidative stress (Zaccaria, et al., 2017). An increase in the expression level of Mir-27 has been reported to downregulate mRNAs coding for NFE2L2 and in turn reduce oxidative stress markers in an in-vitro study involving Human keratinocyte cell lines (HaCat cells) (Zaccaria, et al 2017). There was an upregulation of Mir-27 and downregulation of the NFE2L2 gene in the SP group relative to the UP group, this may agree with the study of Zaccaria, et al. (2017), who reported an increased expression level of Mir-27 which consequently led to a decrease in the expression level of NFE2L2 in an in-vitro experiment.

The enriched pathways annotated by DAVID from the DEGs reported in the SP and UP chicks revealed 6 pathways that could be associated with the differences in bodyweight performance of these chicks, and they involved calcium signalling, Wnt signalling, cytokine-cytokine receptor interaction, cardiac muscle contraction, mucin-type O-glycan biosynthesis, and other O-glycan biosynthesis. Genes involved in the calcium signalling pathway were mostly upregulated in the SP chicks which include HTR2A, ADCY1, CACNA1C, CCKAR, and NOS2. Calcium signalling has been noted to be one of the highly versatile intracellular signals that participates in cell signalling for a wide range of cell processes such as apoptosis, cell cycle, division, migration, invasion, metabolism, differentiation, transcription etc. (Pratt, et al., 2020). The Ca ion governs intracellular signalling pathways and contributes to long term physiological response regulation such as muscle contraction, neurotransmission, and metabolic regulation (Pratt, et al., 2020). This important pathway enriched in the SP chicks may be playing a vital role in growth and contributing to the differences observed in the SP and UP groups. Importantly, further studies may be merited to understand if circulatory levels of calcium serve as a better biomarker in assessing differences in growth rates in broiler chicks.

The second most enriched pathway reported in this study was the Wnt signalling pathway. This pathway has been reported to play a vital role in self-renewal of most tissue in mammals, particularly the development and renewal of small intestinal epithelial tissue and stimulates the differentiation of crypts and Paneth cells (Liu, et al., 2022). It is also reported to be linked to liver development, haematopoietic system development and osteoblast maturation (Clevers, 2006: Perugorria, et al., 2019). Wnt signalling also facilitates Ca and P metabolism in broilers (Wang, et al., 2022), thus the enrichment of the Wnt pathway in the SP group in this study may be linked to the increase in the concentration of bone P in the SP compared to the UP group, as higher concentration of minerals in animal tissues are a valuable biomarker of its bioavailability (Wang, 2007). The significance of the Wnt signalling and its implication in the SP chicks in the present study may provide insight into the underlying factors contributing to growth and body size differences in these groups of chicks studied.

Most of the genes involved in Wnt signalling, cytokine-cytokine receptor interaction, and mucin-type O-glycan biosynthesis was up-regulated in the SP chicks' group. Notably, all genes related to mucin-type O-glycan biosynthesis were upregulated in the SP group, which includes ST3GAL1, GALNT15, and WBSCR17. It has been demonstrated that mucin-type O-glycans are pivotal in establishing whether host diseases will be averted or promoted concerning interactions with microbes present in the environment (Bergstrom and Xia, 2013). Mucins are the main component of mucus which are secreted by the goblet cells and form a protective homeostatic barrier between resident microbiota and the underlying immune cells (Johansson, et al., 2008., Struwe, et al., 2015). It has been reported that homeostasis of gut bacteria in chicken can be implicated by mucin types, O-glycan composition, i.e., the extent of glycosylation and oligomerization of mucin and mucus layer characteristics (Derrien, et al., 2010). Having the mucin type O-glycan pathway activated in the SP group may suggest implications which include, a higher level of mucin glycosylation which may enable mucins to function as a protective barrier. Mucus production is very important in young chicks for gut protection as they still have developing immune system (Duangnumsawang, et al., 2021), and for assimilation of metal ions in its available form in the intestine (Powell, et al., 1999).

An important consideration which may be influencing the aforementioned changes in DEG are that the SP chicks, ranked on the basis of BW on Day 7, exhibited greater bodyweight at day 1 when compared to the UP chicks. Bodyweight has been reported to be highly correlated to feed intake in Ross 308 broiler chicks (Mohammadrezaei, et al., 2011). The SP group likely consumed more feed post-hatch compared to the UP group, driving the development of the intestinal epithelium including enterocytes and goblet cells which drove gut barrier function, as suggested by the enriched pathways implicated in the SP group. Immediate access to feed by hatchlings has been reported to support intestinal epithelium development including goblet cells and enterocytes for more efficient barrier function (Duangnumsawang, et al., 2021). In the present study, 7day old chicks in the SP group exhibited superior bodyweight from day 1 compared to the UP group. Thus, this may affect the ability of the chicks in the groups to access feed due to hierarchy, thereby affecting growth performance especially in the UP group.

Conclusion

The present study revealed differences in the digestive organ weights, bone ash and mineral concentrations in 7-day old Ross 308 chicks with distinct bodyweights. The present study collected data from chicks raised in one pen which may be a potential source of limitation in the study, replication is recommended in further research to get more detailed knowledge of the wider population. The SP chicks had higher bone ash and bone P concentration which may be linked to the enriched Wnt signalling pathway in this group relative to the UP group. The increase in bone Cd, Pb and Cs in the UP group merits further mechanistic investigation, to ascertain the possible drivers of the accumulation. The transcriptomic profile revealed differentially expressed genes in the ileum of 7days old Ross 308 broiler chicks with distinct body weight. We observed the up regulation of cytokines and chemokine genes, GSTs, and Mir genes, together with Ca signalling and Wnt signalling pathways in the SP group relative to the UP group, which may be involved in the difference between the bodyweight groups.

Authors' contributions. This study was conceived by COS. COS and CLE designed the experiment, CLE conducted the experiment, CLE, COS, BB, and MC analysed data, CLE wrote the original manuscript draft, CLE, BB, GW, EB, MC and COS reviewed and edited the manuscript.

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Competing interest. Cormac J. O'Shea is a member of the Editorial board of the Journal of Agricultural Science, therefore in other to mitigate this potential conflict of interest, he was blinded from the review process.

Ethical Standards. All experimental protocols used in the study were approved by the University of Nottingham Animal Ethics Committee (approval reference number 223). The UK national NC3R ARRIVE guidelines for care, use and reporting of animals in research (Kilkenny, et al., 2010) were followed during the study.

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Table 1: Digestive tract and ancillary organ weight of chicks at 7 days of age (*n* = 10 per BW group)

Parameters	SP	UP	SEM	*P*-value
D0 BW (g)	61	52	±2.3	0.001
D7 BW (g)	276	174	±6.4	≤ 0.001
Liver wt (g)	12	8	±0.7	≤ 0.001
Relative Liver (g/kg)	44	43	±0.30	0.921
Gizzard wt (g)	14	10	±0.6	≤ 0.001
Relative gizzard wt	52	58	±0.2	0.015
Intestinal wt (g)	23	15	±1.1	≤ 0.001
Relative intestinal wt (g/kg)	86	83	±0.4	0.463
Intestinal length (cm)	110	94	±4.5	0.003

UP denotes Under-performers, and SP- Super-performers chicks, D0 BW – Day 0 bodyweight, D7 BW Day 7 body weight, ADWG- Average daily weight gain, wt - weight

Table 2: Tibial ash and macro mineral concentrations of the UP and SP chicks at D7 of age, (n = 10 chicks per BW group)

Ash and mineral concentrations (g/kg)	SP	UP	SEM	*P*-value
Ash	470	440	±1.2	0.014
Ca	363	352	±7.0	0.143
P	195	186	±3.5	0.018
Na	12	11	±0.56	0.014
S	4	3	±0.31	0.066
K	9	10	±0.49	0.215
Mg	8	7	±0.26	0.506

UP denotes Under performers group, SP denotes Super performers group; Minerals are expressed on a crude ash basis. (n = 10 per BW group)

Table 3: Trace mineral concentrations of the UP and SP chicks at D7 of age (n = 10 chicks per BW group)

Trace mineral concentrations (mg/kg)	SP	UP	SEM	*P*-value
Cd	0.02	0.23	±0.0020	0.048
Cs	0.02	0.03	±0.0040	0.023
Rb	0.01	0.01	±0.0070	0.034
Pb	0.2	0.3	±0.04	0.014
Mn	14	16	±1.06	0.097
Se	0.2	0.2	±0.02	0.765
Sr	225	208	±8.9	0.062
Cr	1.2	1.0	±0.19	0.230
Fe	308	318	±38.0	0.789
Cu	3.2	3.1	±0.20	0.709
Zn	466	467	±19.4	0.970

UP denotes Under performers group, SP denotes Super performers group. (n= 10 per BW group)

Table 4: Most conspicuous differentially expressed genes (foldchange from +1.50 or -1.50) in the ileum of 7-day old Ross 308 male chicks in SP group compared to the UP group.

Gene symbol	Entrez Gene Name	Location	Type of molecule	Expr Fold Change	*P*-value
IL22RA2	interleukin 22 receptor subunit alpha 2	Plasma Membrane	transmembrane receptor	+2.77	0.010
CDHR1	cadherin related family member 1	Plasma Membrane	other	+2.34	0.029
TTLL2	tubulin tyrosine ligase like 2	Other	other	+2.16	0.039
ATP8B1	ATPase phospholipid transporting 8B1	Plasma Membrane	transporter	+2.12	≤ 0.001
IL20RA	interleukin 20 receptor subunit alpha	Plasma Membrane	transmembrane receptor	+1.92	0.034
ODF2L	outer dense fiber of sperm tails 2 like	Cytoplasm	other	+1.86	0.036
NOXO1	NADPH oxidase organizer 1	Plasma Membrane	other	+1.85	0.023
mir-27	microRNA 27a	Cytoplasm	microRNA	+1.81	0.004
IL26	interleukin 26	Extracellular Space	cytokine	+1.77	0.019
ITGBL1	integrin subunit beta like 1	Extracellular Space	other	+1.74	0.042
mir-23	microRNA 23a	Cytoplasm	microRNA	+1.69	0.029
ME1	malic enzyme 1	Cytoplasm	enzyme	+1.65	0.008
CCL17	C-C motif chemokine ligand 17	Extracellular Space	cytokine	+1.63	0.026
PCNX2	Pecanex 2	Other	other	+1.63	0.002
ZPLD1	zona pellucida like domain containing 1	Other	other	+1.59	0.022
SMOC2	SPARC related modular calcium binding 2	Extracellular Space	other	+1.58	0.015

MFAP5	microfibril associated protein 5	Extracellular Space	other	+1.58	0.039
HPGDS	hematopoietic prostaglandin D synthase	Cytoplasm	enzyme	+1.54	0.026
SHISAL1	shisa like 1	Other	other	+1.54	0.016
SLC38A4	solute carrier family 38-member 4	Plasma Membrane	transporter	+1.52	0.017
GSTA3	glutathione S-transferase alpha 3	Cytoplasm	enzyme	+1.51	0.002
WNT7B	Wnt family member 7B	Extracellular Space	other	+1.50	0.036
DDX60	DExD/H-box helicase 60	Cytoplasm	enzyme	-1.57	0.040
COL17A1	collagen type XVII alpha 1 chain	Extracellular Space	other	-1.65	0.044
WASF1	WASP family member 1	Nucleus	other	-1.88	0.003
LRFN5	leucine rich repeat and fibronectin type III domain containing 5	Nucleus	other	-1.92	0.006
CPO	carboxypeptidase O	Plasma Membrane	enzyme	-2.13	0.024
CA7	carbonic anhydrase 7	Cytoplasm	enzyme	-2.42	0.047
SLC34A2	solute carrier family 34-member 2	Plasma Membrane	transporter	-3.62	0.002

Table 5: Enriched Pathway implicated by bodyweight differences in SP and UP chicks.

Pathways	No of genes	%	*P*- value	DEGs involved
Calcium signalling pathway	9	4.6	0.006	HTR2A, ADCY1, CACNA1C, CCKAR, GDNF, NOS2, PPIF, RET, TACR2
Wnt Signalling pathway	6	3.1	0.036	CTBP2, WNT7B, FZD1, ROR2, SFRP1, SERPINF1
Cytokine-cytokine receptor interaction	7	3.6	0.015	LOC418668, IL1RAP, IL20RA, IL4R, IL8L1, TNFRSF1B
Cardiac muscle contraction	4	2.1	0.045	CACNB4, CACNA1C, SLC9A7, UQCR10
Mucin type O-Glycan biosynthesis	3	1.5	0.060	ST3GAL1, GALNT15, WBSCR17
Other types of O-glycan biosynthesis	3	1.5	0.100	WBSCR17, GALNT15, POGLUT1

SP: Super performers, UP: Under performers, DEG: Differentially expressed genes.

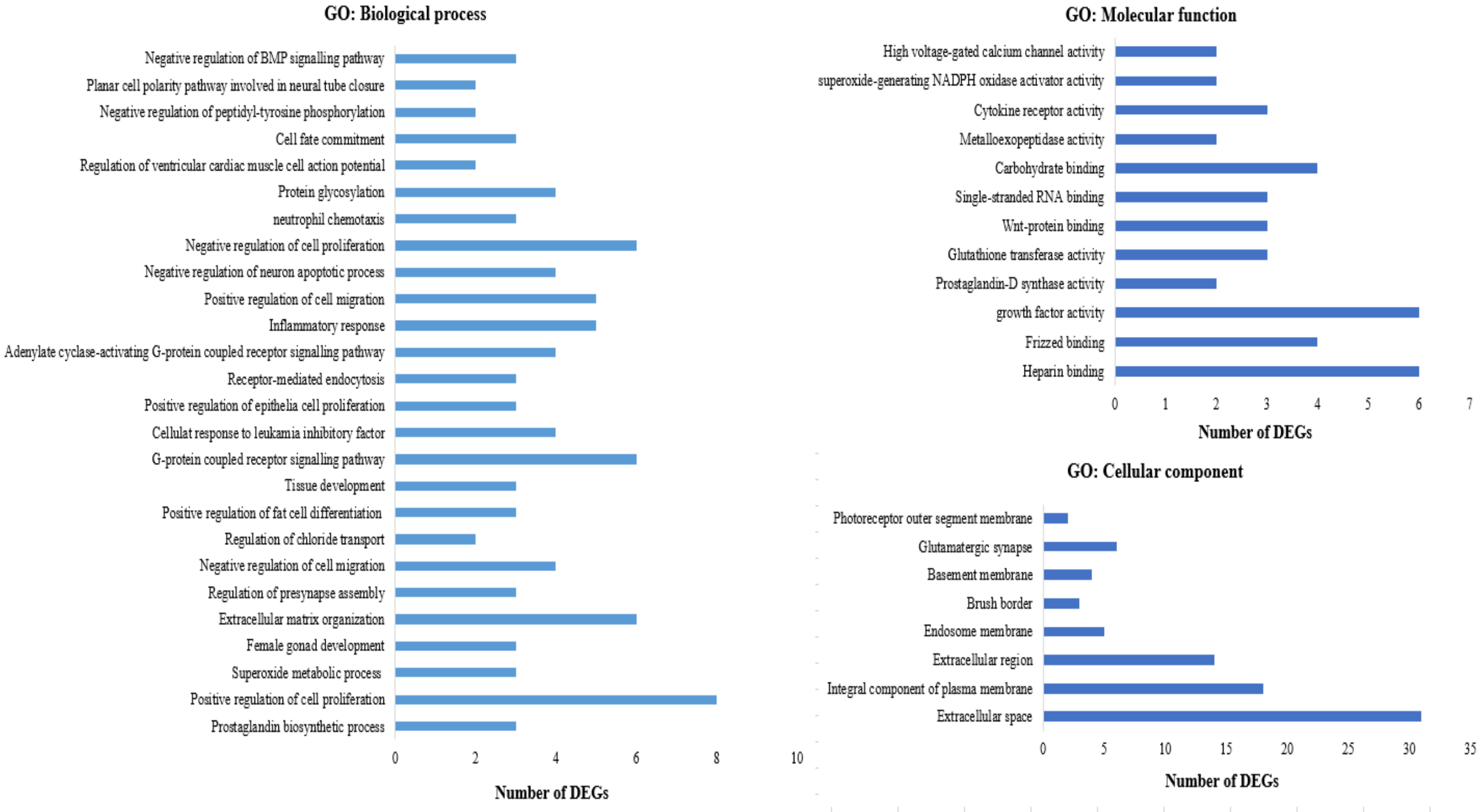

Figure 1: Functional annotation of the ileal DEGs in 7day old Ross 308 chicks (SP relative to UP), SP denotes Super performer and UP denotes Under performers. The higher the number of DEGs in each process, the more implicated will the process be in the SP group relative to the UP group.