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Technologies in smallholder poultry development: Characterization, utilization, conservation, and improvement

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Technologies in smallholder poultry development: Characterization, utilization, conservation, and improvement

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Editorial: Technologies in smallholder poultry development: characterization, utilization, conservation, and improvement

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Editorial on the Research Topic
Technologies in smallholder poultry development: characterization, utilization, conservation, and improvement

Smallholder poultry production accounts for about 80% of the poultry flock in low- and middle-income countries. The birds play important roles in rural livelihoods, food security, household income, culinary and traditional medicine and strengthening of social relationships (Li et al., 2020; Bamidele and Amole, 2021; OchoraKasima et al., 2023). Despite the benefits derived from Smallholder poultry production systems (SPPS), the production as well as the productivity is low. The low performance of the birds could be attributed mainly to low genetic potential, genetic erosion occasioned by indiscriminate crossbreeding, poor nutrition, health and housing and vagaries of climatic factors (Samaraweera et al., 2021; Birhanu et al., 2021; Wilson et al., 2022). This, according to Birhanu et al. (2023), necessitates poultry research and development using innovative and technological approaches in genetics, feed supply, health services, housing, and public-private partnership to deliver integrated innovation packages. The outcome will not only boost production, but ensure increased productivity and profitability of the SPSS.

This Research Topic (*Technologies in smallholder poultry development: characterization, utilization, conservation, and improvement*) centred on phenotypic and molecular characterization, quantitative and population genetics, genetic/genomic/proteomic evaluation and application of classical phenotyping methods to health, nutrition, production and reproduction including the interaction between the environment and poultry species. The ten articles contributed to this Research Topic are summarized below:

Feather pecking, which is a complex trait, and partly under genetic control, remains an important welfare and economic Research Topic in chickens. This prompted the work of Mott et al. to identify a potential key regulator for this behavioural disorder in laying hens.

The study found that the increased propensity of laying hens to perform feather pecking could be due to lack of CD4 T cells and gamma-Aminobutyric acid (GABA) receptors. Therefore, the authors propose KLF14 as a clear candidate regulator for the expression of genes associated with the pathogenic development.

Housekeeping genes (HKGs) usually are used as the reference (internal controls) to evaluate and compare abundances of mRNA expression of target genes in different cells or tissues of animals. Due to the dearth of information on chicken HKGs, Hasanpur et al. tested most of the reliably expressed genes (REGs) for stability in 16 important chicken tissues (skin, adipose, blood, brain, bursa of Fabricius, heart, liver, lung, kidney, muscle, duodenum, ileum, jejunum, ovary, spleen, and trachea) using at least three RNA-seq datasets per tissue. The authors discovered a total of 6, 13, 14, 23, and 32 validated housekeeping genes (V-HKGs) as the most stable and suitable reference genes for muscle, spleen, liver, heart, and kidney tissues, respectively. These V-HKGs could be exploited in more accurate normalization for future expression analysis of chicken genes.

The Tagray region in Ethiopia is an ancient entry route for domestic chickens' entry into Africa. The oldest African chicken bones were found in this region, dating back to around 800-400BCE. The region has a high chicken-to-human population ratio and diverse geography. Following the introduction of exotic chickens, the proportion of indigenous chickens in the region has decreased to 70%. The study by Gebru et al. used Ecological Niche Modelling to characterize the habitats of 16 indigenous village chicken populations in Tigray, identifying four main chicken agro-ecologies and potential indigenous Tigrayan chicken ecotypes. This information can guide conservation and breeding improvement initiatives for indigenous Tigrayan chickens.

The study by Kanakachari et al. investigated the difference in muscle development, egg production, and plumage colors between native and broiler chickens. The researchers conducted a microarray analysis using the 7th-day embryo and 18th-day thigh muscle of improved Assel broiler chickens, respectively. They selected 24 candidate reference genes and isolated total RNA from the chickens to study their expression profiles using real-time quantitative PCR. The study identified differentially expressed genes that regulate muscle growth, myostatin signaling and development fatty acid metabolism, and other pathways in improved Assel chickens. The findings may be used to improve muscle development, differentiation, egg production, protein synthesis, and plumage formation in native chickens and optimize growth in broiler chicken.

Improved tropically adapted birds have been reported to be suitable for backyard poultry production. In the light of this, Bamidele et al. introduced improved, dual-purpose chicken genetics into the smallholder farming households (SFH) as a helpful intervention to mitigate the impact of COVID-19 pandemic. The study was conducted in three states in Nigeria, each representing a distinct agroecological zone. The birds, which were managed under semi-scavenging production system, were evaluated for growth, survivability and profitability. The body weight of Noiler and FUNAAB Alpha chickens appeared similar. Agroecology and genetics significantly affected growth and survivability of the birds. Profitability was higher in Nasarawa state, followed by Kebbi and Imo. The study, therefore, posits that the provision of improved, dual-purpose chickens to vulnerable SFH is viable for economic growth, and resilience during emergencies in Nigeria.

Genetic and phenotypic relationships among feed efficiency, immune and production traits measured pre- (9–20 weeks of age) and post- (12 weeks from on-set of lay) maturity in indigenous chicken of Kenya were assessed by Miyumo et al. The genetic correlations obtained suggest that improved feed efficiency would be associated with high growth rates, early maturing chicken, high egg mass and reduced feed intake. Contrastingly, improved general keyhole limpet hemocyanin (KLH-IgM) and specific Newcastle disease virus (NDV-IgG) immunity would result in lower growth rates and egg mass but associated with early sexual maturation and high feed intake. This is an indication that indigenous chicken improvement programs should account for the potential genetic consequences of selective breeding for feed efficiency and immune-competence on production traits.

The study by Yussif et al. highlighted the phenotypic diversity and potential use of indigenous chicken breeds for breed improvement strategies in Uganda. Most of the chickens were raised under the scavenging as naked necks, frizzles, and polydactyls, within the Ugandan local chicken population even though some were rare. Certain alleles associated with traits such as tufted crests and rose combs were consistent with Mendelian expectations, whereas others, such as frizzles and polydactyl, had lower frequencies, implying possible feeding system in a mixed-crop livestock production system. A significant percentage of women (41%) were responsible for managing the chickens even though there were fewer (21%) female-headed households in Uganda. This was not unexpected as women are the primary keepers of smallholder poultry (Yakubu et al., 2020). The average flock size was 20, with hens laying approximately 40 eggs per year. The feather patterns, skin colours, earlobe sizes and colours, comb types, and beak shapes of indigenous chickens were all unique. The study indicated the presence of unique traits such endangerment. This study highlights the need for a careful balance between adaptability, farmer-preference and conservation of animal genetic resources in the sustainable development of local chickens in Uganda.

Performance of four distinct lines (L1, L2, L3, and L4) of Japanese quail (*Cortunix japanoica*) kept in the tropical climate of Tamil Nadu, India was investigated by Arunrao et al. The parameters measured were weekly body weight, daily feed consumption and egg production and mortality, if any, were recorded during the laying period. The weight of the eggs was measured once a week; in addition, age at sexual maturity, hen-day egg production, hen-housed egg production, livability, and feed efficiency in terms of feed per dozen eggs were calculated. It was observed on the average that Lines 3 and 4 outperformed others. The researchers, therefore suggest the selection of L3 and L4 for body weight and egg production in order to boost Japanese quail production in the tropics.

The smallholder poultry production must be maintained as an alternative source of food security and income in communities disturbed by various types of pollution in the environment. Among the different pollutants, the hydrocarbon contamination from oil spills, natural gas flaring, and organic pollutants (Ansah et al., 2022) is more prevalent and poses some major health and welfare difficulties in certain area around the word and the broad

disruption of homeostasis caused by this pollutant puts the genetic potential of the birds at risk. In this regard, Oleforuh-Okoleh et al. examined role of genes and antioxidants in enhancing poultry's tolerance to hydrocarbon toxicity. According to epidemiological research, they highlighted that the aryl hydrocarbon receptor (AhR) and nuclear factor erythroid 2p45-related factor 2 (Nrf2) genes, which regulate disease defense mechanisms, may be the cause of tolerance to hydrocarbon exposure. Different species may have different mechanisms and degrees of tolerance to hydrocarbon fragments, which could lead to differences in gene expression when exposed within members of the same species. They further emphasized that for the development of intensive, commercial, and financially viable smallholder poultry production in hydrocarbon-polluted communities, evaluation of the genetic architecture and risk assessment of diverse chicken breeds exposed to pollutants are essential.

The black-bone chicken (BBC) is an indigenous village chicken breed with unique properties that have contributed to its utilization and conservation. The fibromelanosis (Fm) locus on chromosome 20, which causes melanin hyperpigmentation, is primarily responsible for the distinct flavour and texture of BBC meat. Shinde et al. investigated the genetic basis of this trait in a variety of BBC breeds, including the Indian Kadaknath. According to the long-read sequencing data generated by the study, all BBC breeds have a complex chromosomal rearrangement at the Fm locus. The study clarified a previously debated scenario of chromosomal rearrangement and emphasised the significance of linkage in shaping genetic diversity. Compared to other BBC, the study revealed that Kadaknath had distinct genetic signatures in regions close to Fm, which could be related to immune function and disease resistance. The identification of specific genes with such genetic signatures for Kadaknath specific-phenotypes contributes to the genetic uniqueness of Kadaknath. The results provide insight into Kadaknath's distinct genetics and immune modifications, thus suggesting a link between genetic diversity, artificial selection, and chromosomal rearrangements in domesticated species.

We are highly appreciative of the robust contributions of several authors to this Research Topic. It is our sincere hope that papers in this Research Topic would be found useful, while soliciting for more articles in the second volume which is currently open for submission in Frontiers in Genetics and Frontiers in Veterinary Science (Livestock Genomics Section).

Author contributions

AY: Writing–original draft, Writing–review and editing. OB: Writing–original draft, Writing–review and editing. AT: Writing–original draft, Writing–review and editing. MO: Writing–original draft, Writing–review and editing. SÇ: Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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eQTL analysis of laying hens divergently selected for feather pecking identifies KLF14 as a potential key regulator for this behavioral disorder

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Feather pecking in chickens is a damaging behavior, seriously impacting animal welfare and leading to economic losses. Feather pecking is a complex trait, which is partly under genetic control. Different hypotheses have been proposed to explain the etiology of feather pecking and notably, several studies have identified similarities between feather pecking and human mental disorders such as obsessive-compulsive disorder and schizophrenia. This study uses transcriptomic and phenotypic data from 167 chickens to map expression quantitative trait loci and to identify regulatory genes with a significant effect on this behavioral disorder using an association weight matrix approach. From 70 of the analyzed differentially expressed genes, 11,790 genome wide significantly associated variants were detected, of which 23 showed multiple associations (≥15). These were located in proximity to a number of genes, which are transcription regulators involved in chromatin binding, nucleic acid metabolism, protein translation and putative regulatory RNAs. The association weight matrix identified 36 genes and the two transcription factors: *SP6* (synonym: *KLF14*) and *ENSGALG00000042129* (synonym: *CHTOP*) as the most significant, with an enrichment of KLF14 binding sites being detectable in 40 differentially expressed genes. This indicates that differential expression between animals showing high and low levels of feather pecking was significantly associated with a genetic variant in proximity to *KLF14*. This multiallelic variant was located 652 bp downstream of *KLF14* and is a deletion of 1-3 bp. We propose that a deletion downstream of the transcription factor *KLF14* has a negative impact on the level of T cells in the developing brain of high feather pecking chickens, which leads to developmental and behavioral abnormalities. The lack of CD4 T cells and gamma-Aminobutyric acid (GABA) receptors are important factors for the increased propensity of laying hens to perform feather pecking. As such, *KLF14* is a clear candidate regulator for the expression of genes involved in the pathogenic development. By further elucidating the regulatory pathways involved in feather pecking we hope to take significant steps forward in explaining and understanding other mental disorders, not just in chickens.

KEYWORDS

feather pecking, eQTL, AWM, genomics, transcriptomics, gene regulation, behavioral disorder

Introduction

Feather pecking (FP) behavior in chickens is a serious issue, which has an impact on animal welfare, where damage to the feathers, bodily injuries and even death can occur (Hughes and Duncan, 1972; Allen and Perry, 1975). As well as this negative impact on animal welfare, there is also a financial impact for the chicken producer, through the increased animal care costs, and ultimately with the loss of injured animals (Huber-eicher and Wechsler, 1997). FP is a trait observed in chickens that are kept in all types of housing (Appleby and Hughes, 1991). Previous attempts to manage this disorder have seen producers utilizing beak trimming, a process that is now being banned in more and more countries due to its impact on the welfare of individual animals (Duncan et al., 1989; Gentle et al., 1990).

Some studies have hypothesized that pecking at the plumage of other birds is related to foraging behavior and occurs due to the low incentive value of floors without litter, (Hoffmeyer, 1969; Blokhuis, 1986; Huber-eicher and Wechsler, 1997), while others have indicated links with behavioral traits such as general locomotor activity (GLA), and fearfulness (Vestergaard et al., 1993; Kjaer, 2017). This suggests that the reasons underpinning FP are associated with both environmental and genetic factors, with a number of studies indicating the possibility of reducing FP by means of breeding, to select for low FP (Kjaer and Sørensen, 1997; Rodenburg et al., 2003; Bennewitz et al., 2014). Quantitative trait loci (QTL) mapping studies and genome wide association studies (GWAS) confirmed the assumption that this trait is polygenically determined with some trait-associated chromosomal regions (Buitenhuis et al., 2003; Lutz et al., 2017; Falker-Gieske et al., 2020a).

There have been a number of studies undertaken to attempt to describe the underlying processes of FP, using gene expression analysis and mapping studies to link FP to immune response and multiple signaling pathways (Wysocki et al., 2010; Haas and van der Eijk, 2018), with some studies linking FP to schizophrenia and obsessive-compulsive disorders, indicating its use as a potential model for these diseases (Falker-Gieske et al., 2021). However, these studies have so far been unable to elucidate the exact mechanism for this feather pecking.

Here the transcriptomic and phenotypic data from 167 chickens was analyzed through a comprehensive strategy combining expression quantitative trait loci (eQTL) mapping and the network analysis of gene-gene interactions to predict SNPs and regulatory pathways that had significant impacts on these abnormal behavior patterns. It is also hoped that the data presented here can be used as a possible model system for both obsessive-compulsive disorder and schizophrenia, due to the similarities of the disease (Falker-Gieske et al., 2021). As such, further elucidating the pathways involved in FP could prove significant in explaining and understanding other mental disorders, not just in chickens.

Methods

Sample collection

The samples used in the following study were collected as previously described in Falker-Gieske et al. (2020a); Falker-Gieske et al., 2020b). Briefly, White Leghorn strains were selected for more than ten generations based on estimated breeding values for feather pecking (Grams et al., 2015). These lines were created and are maintained at the Hohenheim University and neither commercially obtained nor from a private source, with rearing and husbandry conditions being previously described (Bennewitz et al., 2014; Falker-Gieske et al., 2020b). A total of 492 birds from two experimental runs were separated into 7 groups of 39–42 birds (ratio of 1:1 LFP-HFP), and 6 groups of 39–42 birds (ratio of 1: 2 LFP-HFP) (Iffland et al., 2020b). At 31–33 weeks, the hens, were phenotyped according to established ethograms. In short, feather pecking [feather pecks delivered (FPD), and feather pecks received (FPR)], and aggressive behavior [aggressive pecks delivered (APD) and aggressive pecks received (APR)], were recorded and hens were marked with numbered plastic batches on their backs to aid in identification (Lutz et al., 2017; Falker-Gieske et al., 2020a; Iffland et al., 2020a). Observation of FP behavior was done in 20-min sessions on four consecutive days by a minimum of six different trained observers. To prevent FP, birds were kept under low light conditions. One bird from each full-sib pair kept under dark conditions was sacrificed, and due to time constraints, the whole brains were immediately collected for RNA isolation in order to preserve expression levels. Chickens were CO_2-stunned and sacrificed by ventral neck cutting. For light stimulation, the remaining birds were kept under increased light intensity (≥100 lux) for several hours. Upon initiation of FP behavior these birds were then sacrificed and brains were collected for RNA isolation. 48 birds were utilized for the transcriptomic analysis described previously (Falker-Gieske et al., 2020b), with 167 animals in total (the previously mentioned 48 animals plus a further 119 animals) being collected and utilized in this study.

Generation of cDNA

cDNA needed for the HT-qPCR analysis was generated from selected high feather pecking (HFP) and low feather pecking

(LFP) animals. RNA was extracted using an RNeasy kit (Qiagen, Hilden, Germany) as per the provided protocol. cDNA preparation was then performed using the Fluidigm Reverse Transcription assay kit (Fluidigm Corporation, San Francisco, CA, United States).

Quantitative gene expression analysis

In order to quantitatively analyze the gene expression in feather pecking, HT-qPCR analysis was performed for 86 differentially expressed genes (DEGs) highlighted by our previous transcriptomic study (Falker-Gieske et al., 2020b). A Fluidigm BioMark system, using a 96.96 Dynamic Array integrated fluidic circuit for gene expression (IFC; Fluidigm Corporation, San Francisco, CA, United States), and Delta Gene Assays (Fluidigm Corporation, San Francisco, CA, United States) were used, and delta Ct values were obtained by normalization against *GAPDH*. The primers utilised in this study can be found in the Supplementary Material S1

Detection of expression quantitative trait loci

Whole genome sequencing genotypes mapped to chicken reference assembly GRCg6a (GCF_000002315.5 RefSeq assembly) from our previous study (Falker-Gieske et al., 2020a) were used for expression genome wide associations studies (eGWAS). Imputation from 60k chip genotypes to whole genome sequence level was performed with Beagle 5.2 (Browning et al., 2018) to make use of the latest imputation algorithm and with the setting ne = 1000 to factor in the small effective population size. Since the default setting is ne = 1000000 we adjusted the setting according to conclusions of Pook *et al.* who showed that this improves imputation accuracy in small populations (Pook et al., 2020). eGWAS were performed with gcta v. 1.92.3 beta3 (Yang et al., 2011) with the setting maf 0.01 and hatch as covariate. Population structure was accounted for in the linear mixed model based on the GRM, which is included in the analysis.

Association weight matrix construction

An association weight matrix (AWM) utilizes the results of multiple GWAS as the basis for calculation of gene-gene or gene-variant associations. Followed by the application of network inference algorithms it generates gene networks with regulatory and functional significance. Input variants for the AWM were selected to contain only highly associated signals, either with the main phenotype (feather pecks delivered box-cox-transformed, FPD_BC) or multiple of the gene expression phenotypes. Since the AWM analysis was designed for SNP chip data, stringent pre-filtering of whole genome sequencing data is necessary to produce a compatible input dataset. Therefore, variants with a p-value $< 1 \times 10^{-5}$ for the main phenotype or variants that were associated ($p < 1 \times 10^{-5}$) with at least ten of the gene expression phenotypes were retained. This resulted in the selection of 2,753 variants, which represent 0.042% of all genome wide variants that were studied. Distances up to 100 kb to the closest genes for each variant were predicted with the Ensembl Variant Effect Predictor (Assembly: GRCg6a, accessed Jan. 13th 2022). The AWM was constructed with an established method by Reverter and Fortes (Reverter and Fortes, 2013). The analysis was performed with the following settings: the p-value thresholds for primary SNP selection and selection of non-key phenotype SNPs were set to 5×10^{-6}, the p-value threshold for secondary SNP selection was set to 1×10^{-9}. SNP based variants with a distance ≤ 2,500 bp to the closest gene were considered close and variants with a distance ≥ 20,000 bp were considered far. Associations between SNP based variants and phenotypes, the gene expression values for DEGs between high feather peckers (HFP) and low feather peckers (LFP), were analyzed with the partial correlation and information (PCIT) algorithm (Reverter and Chan, 2008) to retrieve significant associations between the closest genes to SNP based variants and DEGs. The analysis with the PCIT algorithm results in pairs of genes, which are predicted to interact. The gene-gene interaction network was visualized with Cytoscape (Shannon et al., 2003) and gene ontology classifications were assigned with PANTHER v. 16.0 (Mi et al., 2017). Genes, which were not annotated by PANTHER were manually annotated using information provided on the UniProt website (accessed March 2022) (UniProt: the universal protein knowledgebase in 2021., 2021).

Transcription factor enrichment

Transcription factor enrichment analysis was performed with CiiiDER (build 15 May 2020) (Gearing et al., 2019). Genes that reached genome wide significance (p-value $< 7.6 \times 10^{-9}$) for at least one variant associated with the transcription factor were used as input. Background genes with an absolute log_2 fold-change (abs. LFC) < 0.5 were selected from the differential expression analysis results from our previous study (Falker-Gieske et al., 2020b), which resulted in a set of 19,088 genes. The p-value threshold for gene coverage enrichment was set to 0.05, base position upstream scan limit to 1,500 bp, and base position downstream scan limit to 500 bp. To predict transcription factor binding to a target DNA sequence, a position frequency matrix (PFM) is required, which assigns nucleotide frequencies to each position in the binding motif. For the analysis of SP6 (synonym: KLF14) the PFM MA0740.1 (https://testjaspar.uio.no/matrix/MA0740.1/) was used.

Additionally the PFMs of all KLF transcriptions factors available on the Jaspar homepage have been included in the analysis, namely: KLF1 (MA0493.2), KLF10 (MA1511.2), KLF11 (MA1512.1), KLF12 (MA0742.2), KLF13 (MA0657.1), KLF15 (MA1513.1), Klf15 (MA 1963.1), KLF16 (MA0741.1), KLF2 (MA1515.1), KLF3 (MA1516.1), Klf3/8/12 (MA 1964.1), KLF4 (MA0039.4), KLF5 (MA0599.1), Klf5-like (MA 1965.1), KLF6 (MA1517.1), Klf6-7-like (MA 1966.1), KLF7 (MA 1870.1), and KLF9 (MA1107.2). Binding sites of *ENSGALG00000042129* (synonym: *CHTOP*) were screened with PFM MA1153.1 (https://jaspar.genereg.net/matrix/MA1153.1/). All PFMs were acquired in JASPAR format (Castro-Mondragon et al., 2022). The transcription factor enrichment plot was created with CiiiDER.

Results

Conducting an eGWAS on 86 genes that were differentially expressed between 167 chickens (84 HFP and 83 LFP, normalized gene expression results in Supplementary Material S2) yielded Manhattan plots with prominent peaks for the genes *AvBD4*, *BTN3A3L2*, *CHDSD*, *KIFC1*, *LOC769512*, *LOC770352*, *LOC112530399*, *LOC112531493*, and *MUC4* (Figure 1, Manhattan plots for all differentially expressed genes (DEGs) in Supplementary Material S3). QTL for these eGWAS are shown in Supplementary Material S4. We discovered 41 genome wide significant variants in proximity to (<1 kb distance to gene start or gene end) the DEGs *AvBD4*, *LOC769512*, and *MUC4* (Supplementary Material S5), which are candidates for being cis-regulatory elements. Although QTL regions were detected through the eGWAS, the use of an associated weight matrix allowed for the further recovery of associated variants from this data set. In total 11,790 genome wide significant associations (Bonferroni threshold p-value = 7.6×10^{-9}) for 70 of the analyzed DEGs were detected, of which 9,677 were unique. Stringent filtering (p-value < 1×10^{-8} in more than 15 eGWAS) was applied, which yielded 23 highly associated variants (Table 1). Due to the nature of an eQTL study one would expect to also discover trans-regulatory elements and regulators of transcription to be associated with DEGs (Gilad et al., 2008). Unsurprisingly, the PANTHER Classification System revealed *KLF14* and *CHTOP* belong to the protein class "gene-specific transcriptional regulator (PC00264)", *ASH1L* to the class "chromatin/chromatin-binding, or -regulatory protein (PC00077)", *ENSGALG00000042129* to the class "nucleic acid metabolism protein (PC00171)", and *DAP3* to the class "translational protein (PC00263)". Furthermore, as in previous studies we found putative regulatory RNAs, namely long non-coding RNAs (lncRNAs) among the top associated genes: *ENSGALG00000050482*, *ENSGALG00000048366*, *ENSGALG00000046959*, and *ENSGALG00000054815*.

To further analyze the dataset at hand we created an AWM using highly associated eGWAS signals (p-value < 1×10^{-5} for the main phenotype (FPD_BC) or p-value < 1×10^{-5} in at least 10 eGWAS) from the 86 DEGs that were analyzed in this study. Significant associations between genes that are close to variants that indicated significant association to DEGs between HFP and

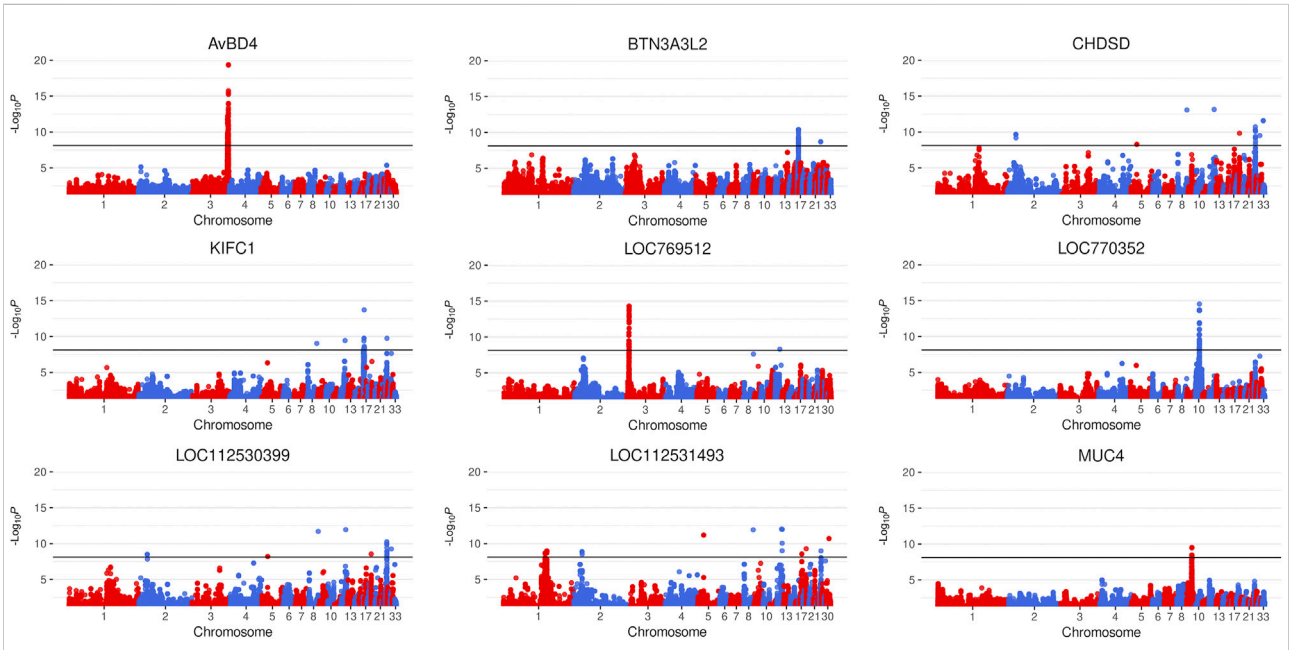

FIGURE 1
Manhattan plots of expression genome wide association studies (eGWAS) that yielded highly significant QTL, conducted on genes differentially expressed between high and low feather pecking hens (Bonferroni threshold ($\frac{number\ of\ variants}{0.05}$): p-value = 7.6×10^{-9}).

TABLE 1 Genomic variants with a p-value $< 1 \times 10^{-8}$ in more than 15 expression genome wide association studies (eGWAS). Only one variant per gene is shown since they represent the respective associated haplotype block. Closest genes up to a distance of 100 kb were considered.

Position	Variant ID	Consequence	Closest gene	Distance	Gene product	No. of associations
12: 15059746	rs318185887	downstream_gene_variant	ENSGALG00000050482	1179	lncRNA	56
8:28987015	8_28987015	upstream_gene_variant	SLC35D1	11161	UDP-glucuronic acid/UDP-N-acetylgalactosamine transporter	54
19:842041	19_842041	intron_variant	MTMR4	0	Myotubularin-related protein 4	42
25:3562220	rs1060122522	downstream_gene_variant	ASH1L	2176	Histone-lysine N-methyltransferase ASH1L	40
27:6521246	27_6521246	downstream_gene_variant	SP6 /KLF14	652	Transcription factor Sp6 /Krueppel-like factor 14	40
17:6424769	17_6424769	intron_variant	NUP214	0	Nuclear pore complex protein Nup214	33
25:3026965	rs737629884	missense_variant	ENSGALG00000042129 / CHTOP	0	Chromatin target of PRMT1	31
5:16304718	rs314040024	intron_variant	ENSGALG00000039221	0	USP6 N-terminal like	25
2:23739061	2_23739061	intron_variant	PPP1R9A	0	Neurabin-1	25
25:3312173	25_3312173	downstream_gene_variant	ENSGALG00000037599	177	S100 calcium binding protein A4	24
2:23413545	rs734790703	—	—	—		22
2:23413552	rs15074227	—	—	—		22
2:23414512	rs14152082	upstream_gene_variant	ENSGALG00000048366	10205	lncRNA	22
25:3040753	25_3040753	downstream_gene_variant	ENSGALG00000046959	276	lncRNA	19
25:3026965	25_3026965	upstream_gene_variant	ENSGALG00000053285	60	Mothers against decapentaplegic homolog 6-like	17
25:3033290	25_3033290	downstream_gene_variant	ENSGALG00000054815	2351	lncRNA	17
25:3602675	rs1058400187	intron_variant	DAP3	0	28S ribosomal protein S29, mitochondrial	16

LFP were identified with the PCIT algorithm (Reverter and Chan, 2008), which detects meaningful gene-gene associations in co-expression networks. This led to the discovery of 34 genes based on 2,753 input variants, which we visualized in a gene association interaction map, where nodes located at the center of the network indicates their centrality in the interaction cluster (Figure 2). Among these were four genes, which were associated with at least 30 DEGs (Table 1): *ASH1L*, *KLF14*, *NUP214*, and *CHTOP*, all of which are involved in gene transcription or translation.

Since *KLF14* and *CHTOP* are transcription factors, we decided to conduct transcription factor binding site enrichment analysis to clarify if DEGs, which were significantly associated with *KLF14* or *CHTOP*, are potential targets of these transcription factors. NCBI Conserved Domain Search (Marchler-Bauer et al., 2015) revealed that *CHTOP* contains an N-terminal Mad Homology 1 (MH1) domain as in the transcription factor *SMAD4*, which recognizes the palindromic DNA sequence GTCTAGAC. Transcription factor binding site enrichment analysis of putative target genes that were associated with genome wide significance (p-value < 7.6×10^{-9}) yielded no significant result for *CHTOP* with the *SMAD4* recognition site. eGWAS revealed that 40 of the DEGs (Supplementary Material S6) between HFP and LFP were significantly associated with a multiallelic variant located 652 bp downstream of *KLF14* (Chromosome: 27 Position: 6521246 bp) and which is a deletion of 1–3 bp. An enrichment of *KLF14* binding sites (Figure 3A) was detectable in those 40 genes (Figure 3B). To ensure, that this enrichment is significant in relation to other transcription factor binding sites, all available PFMs for KLF transcription factors were included in the analyses with the complete results summarized in Supplementary Material S7. Although significant enrichment of binding sites for other KLFs were detected, *KLF14* resulted in the highest log_2 enrichment value, while being statistically significant.

Out of those 40 putative *KLF14* regulated target genes 10 are involved in the immune system with the majority being leukocyte-immunoglobulin receptors (Table 2).

Discussion

To untangle the regulatory network behind feather pecking behavior we conducted an eQTL study on 86 genes that were differentially expressed between divergently selected HFP and LFP (Falker-Gieske et al., 2020b) in 167 chickens. The main result of this study is a gene-gene interaction map based on an AWM, which revealed two clusters of interacting genes (Figure 2). A

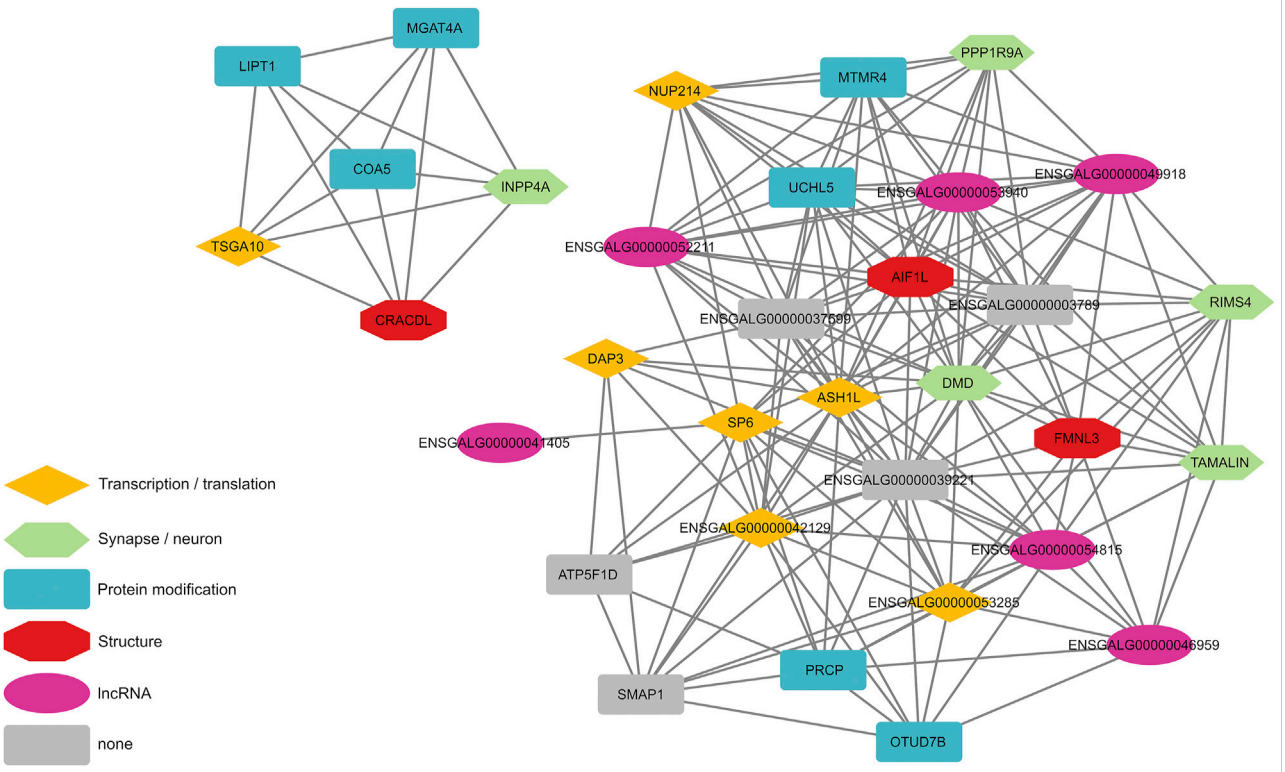

FIGURE 2
Significant gene-gene interactions, which were discovered with an association weight matrix followed by analysis with the PCIT algorithm. Input genes were derived from expression genome wide association studies with differentially expressed genes between high and low feather pecking chickens as phenotypes.

number of those genes have previously been described to be involved with brain-related functions and have generally not been detected by simple filtering based on eGWAS *p*-values (Table 1). The *DMD* gene encodes the protein dystrophin, which colocalizes with $GABA_A$ receptors in postsynaptic densities of neurons in the cerebral cortex of mice. Dystrophin deficient mice show an altered clustering of $GABA_A$ receptors (Knuesel et al., 1999). We previously reported that mutations within or close to GABA receptors as well as differential expression of *GABRA2*, *GABRB2*, *GABRE*, and *GABRG3* are associated with feather pecking (Falker-Gieske et al., 2021). The central position of *DMD* in the gene-gene interaction network provides further evidence for our theory that feather pecking is a GABAergic dysfunction disorder [already discussed here (Falker-Gieske et al., 2021)]. A lack of GABA receptor expression has been linked to schizophrenia in humans [reviewed here (Chiapponi et al., 2016)]. In previous studies we found a considerable amount of genes involved in schizophrenia to be associated with feather pecking (Falker-Gieske et al., 2020a; Falker-Gieske et al., 2020b). The results of the AWM extend this list with *PPP1R9A*, which encodes the protein Neurabin-1 and has been linked to dendritic spine loss in schizophrenia (Konopaske et al., 2015). *TAMALIN*, a trafficking molecule of Metabotropic glutamate receptor 5 (mGluR5), showed increased expression in the hippocampal region of individuals with schizophrenia (Matosin et al., 2015). Furthermore, we discovered *INPP4A*, a gene linked to schizophrenia (Föcking et al., 2015), epilepsy (Wang et al., 2012), and intellectual disability (Banihashemi et al., 2020) as well as *RIMS4*, which is involved in synaptic plasticity and the development of autism (Leblond et al., 2019). Genes that have been classified as protein modifiers in the gene-gene interaction map have also been implicated in neurological disorders. A *LIPT1* deficiency has been reported in a case of early infantile epileptic encephalopathy (Stowe et al., 2018). Low expression of *MGAT4A* in the dorsolateral prefrontal cortex was reported in cases of schizophrenia (Kippe et al., 2015). We previously proposed that chickens selected for FP behavior could serve as a model for human psychiatric disorders (Falker-Gieske et al., 2021). In this respect Johnsson *et al.* conducted a study on an intercross between domestic chickens and Red Junglefowl focusing on the suitability of chickens as a model for anxiety behavior. Here, two genes also discovered in our study *LOC770352* and *GABRB2*, were identified as top candidate genes affecting stress and anxiety

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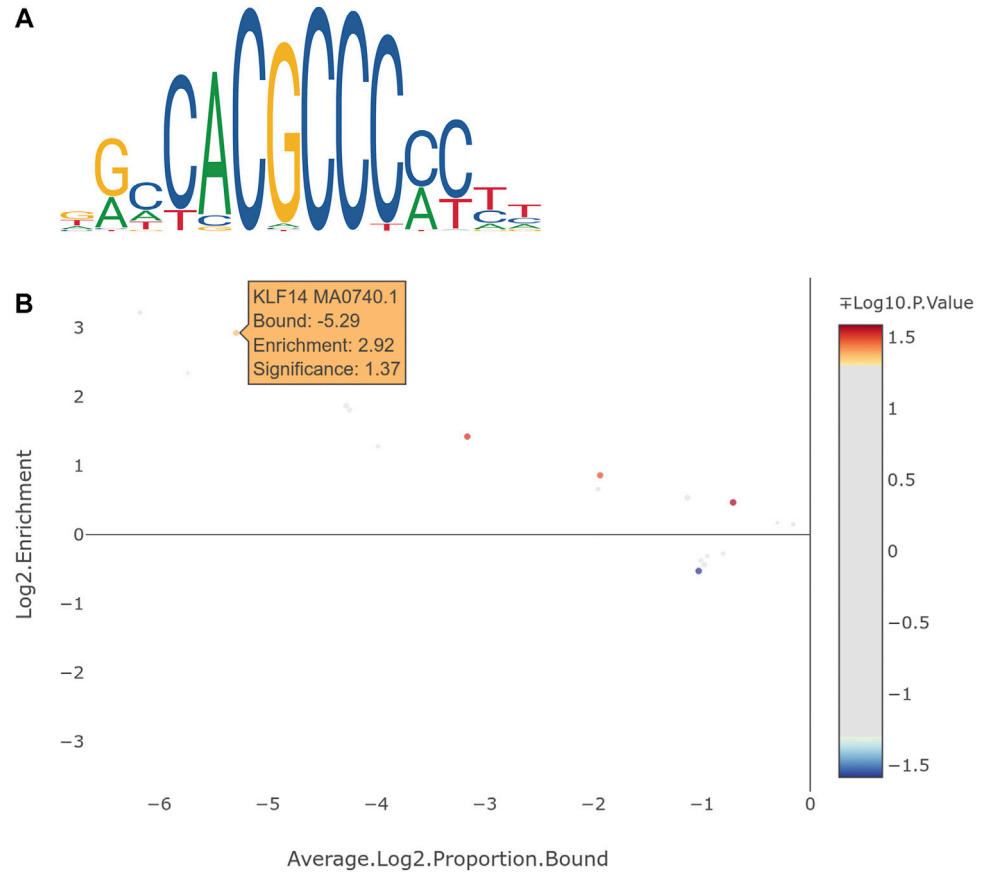

FIGURE 3
(A) Graphical representation of the position frequency matrix of the *KLF14* transcription factor. **(B)** Result of transcription factor binding site enrichment analysis for *KLF14* with genes from expression genome wide associations studies with genome wide significant association. All available KLF transcription factors were included as controls for the specificity of *KLF14* binding site enrichment.

TABLE 2 Differentially expressed genes between high and low feather peckers significantly associated with *KLF14*, which are involved in the immune system. Obsolete gene Symbols, due to the recent release of new genome assemblies, are shown in parentheses.

Symbol	Description	Functions or other designations
BLEC2	C-type lectin-like receptor 2	lectin-like natural killer cell surface protein
CHIR-IG1-5	immunoglobulin-like receptor CHIR-AB1-like	immunoglobulin-like receptor CHIR-Ig1-5
CSF2RB	colony stimulating factor 2 receptor beta common subunit	cytokine receptor common subunit beta
LOC100857964	platelet glycoprotein VI-like	leukocyte immunoglobulin-like receptor subfamily B member 5
LOC107049819	leukocyte immunoglobulin-like receptor subfamily B member 3	leukocyte immunoglsobulin-like receptor subfamily A member 2
LOC107050473	platelet glycoprotein VI-like	leukocyte immunoglobulin-like receptor subfamily A member 2
LOC112531100	leukocyte immunoglobulin-like receptor subfamily A member 2	leukocyte immunoglobulin-like receptor subfamily A member 2
CLEC2D2L	C-type lectin domain family 2 member D2-like	natural killer cell lectin-like receptor binding
MHCY2B3P (LOC112533562)	major histocompatibility complex Y, class II beta 3 pseudogene	leukocyte receptor cluster member 9-like
MHCY35 (HLA.F10AL3)	major histocompatibility complex Y, class I heavy chain 35	major histocompatibility complex Y, class I heavy chain 35

behavior (Johnsson et al., 2016). As this study did not use selection lines, their findings indicate that results derived from selection lines are clearly relevant in the general production populations. Furthermore, these common findings of homologous genes such as SP6/KLF14, *GABRB2* and *LOC770352* underlines the suitability of chickens as model organisms for behavioral disorders of the brain.

With *AvBD4*, *LOC769512*, and *MUC4* we discovered three potential cis-regulated DEGs in eGWAS. However, from a regulatory point of view those genes identified by the PCIT algorithm that are involved in transcription and translation are the most relevant. Variants in the *NUP214* gene cause acute febrile encephalopathy (Fichtman et al., 2019) and rare mutations in *ASH1L* are connected to numerous neurodevelopmental disorders [reviewed in (Zhang et al., 2021)]. Furthermore, among those genes were two transcription factors - *KLF14* and *CHTOP*. Whilst we found the presence of a missense mutation in *CHTOP*, leading to an alanine being replaced with a valine, no significance in the enrichment analysis was observed. Dysregulation at the cellular level of *CHTOP* has been shown however, to play an important role in the tumorigenicity of glioblastoma cells (van Dijk et al., 2010b), as well as being a critical regulator of γ-globin gene expression (van Dijk et al., 2010a). Although very little is still known about the molecular mechanism of transcriptional control that it mediates, it has also been associated with the methylosome complex containing PRMT1, PRMT5, MEP50, and ERH, which are critical for mammalian development through transcription regulation, DNA repair, RNA splicing, and signal transduction (Fanis et al., 2012; Liang et al., 2021) (Fanis et al., 2012). In fact, Hannon *et al.* identified robust psychosis associated differences derived from DNA methylation, with increased proportions of monocytes and granulocytes and decreased proportions of natural killer cells, $CD4^+$ T-cells and $CD8^+$ T-cells (Hannon et al., 2021).

Genome-wide significant associations with a novel deletion downstream of *KLF14* were detected in 40 of the 86 DEGs that we screened in the eGWAS. A number of which were associated with natural killer (NK) cells, where major dysfunctions have been linked with functioning impairment correlated with psychotic, manic, and depressive symptoms in subsequently diagnosed patients with schizophrenia and bi-polar disorder (Tarantino et al., 2021). Transcription factor binding sites for KLF14 were significantly enriched in those 40 genes (Figure 3B). Hence, we propose that *KLF14* is a candidate regulator for the expression of genes that are involved in the pathogenic development that leads to feather pecking behavior. 10 of the 40 potential KLF14 targets play a role in the immune system, more specifically in leukocytes. CD4 T cells belong to the generic term leukocytes and have recently been shown to be essential for healthy development from the fetal to the adult brain in mice and humans (Pasciuto et al., 2020). Single-cell sequencing revealed that in the absence of murine CD4 T cells, resident microglia remained suspended between the fetal and adult states. This maturation defect resulted in excess immature neuronal synapses and behavioral abnormalities. The authors proposed that CD4 T cells play a so far neglected role in the development and evolution of the neurological system. An involvement of the immune system in feather pecking behavior has been proposed in numerous studies (Parmentier et al., 2009; Mashimo et al., 2017; Gandal et al., 2018; Sneeboer et al., 2019; van der Eijk et al., 2019; Falker-Gieske et al., 2020b; Falker-Gieske et al., 2021). Interestingly van der Eijk et al. showed that a HFP line expressed significantly lower amounts of CD4, $CD4^+$ and CD8α+ T cells in comparison to an LFP line (van der Eijk et al., 2019). This would mirror results in human studies that also showed decreased proportions of natural killer cells, $CD4^+$ T-cells and $CD8^+$ T-cells in psychosis-associated patients (Hannon et al., 2021).

Although these analyses have been conducted in adult chickens it is fair to assume that lower T cell counts are also present during the embryonic development of HFP. The tissue expression of *KLF14* is by far highest in human placenta, 4-fold higher compared to skin (Fagerberg et al., 2014). As such, we submit a possible model in which a deletion downstream of the transcription factor *KLF14* has a negative impact on the level of T cells in the developing brain of HFP chickens, leading to developmental and behavioral abnormalities. As the evidence of the GABAergic systems involvement mounts, we currently believe that a lack of CD4 T cells and GABA receptors are major contributors of the propensity to perform feather pecking behavior in laying hens. Using transcriptional and computational analysis brings its own limits to the outcome of this study. Here we have put forward a solid hypothesis for the role of *KLF14*. It is however important, to understand that without further practical analysis this cannot be proven. Through further quantification of CD4 T cells and GABA receptors in HFP and LFP animals, throughout multiple stages of development, it could be possible to elucidate the role that *KLF14* plays in FP. Further analysis of other neurological disorder looking at the role *KLF14* plays there, could also open the door for the use of HFP animals as a model to study other neurological disorders.

Conclusion

We propose that a deletion downstream of the transcription factor *KLF14* has a negative impact on the level of T cells in the developing brain of FP chickens, which leads to developmental and behavioral abnormalities. This reduction of CD4 T cells and GABA receptors are a major cause for the propensity of laying hens to perform feather pecking behavior. As such, *KLF14* is a clear candidate key regulator for the expression of genes involved in the pathogenic development. By further elucidating the regulatory pathways involved in FP we hope to take significant steps forward in explaining and understanding other mental disorders, not just in chickens.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJNA664592 https://www.ebi.ac.uk/ena, PRJNA656654.

Ethics statement

The animal study was reviewed and approved by The animal treatment was approved by the German Ethical Commission of Animal Welfare of the Provincial Government of Baden-Wuerttemberg, Germany (code: HOH 35/15 PG, date of approval: 25 April 2017).

Author contributions

AM designed primers, analysed expression data, and wrote the manuscript. AM optimized primers. SP performed the Fluidigm experiments. JT and JB developed the project outline. JT developed data analysis strategies, and contributed to the manuscript. CF-G performed all bioinformatics and statistical analyses and wrote the manuscript. All authors have read and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.969752/full#supplementary-material

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Investigation of chicken housekeeping genes using next-generation sequencing data

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Accurate normalization of the gene expression assays, using housekeeping genes (HKGs), is critically necessary. To do so, selection of a proper set of HKGs for a specific experiment is of great importance. Despite many studies, there is no consensus about the suitable set of HKGs for implementing in the quantitative real-time PCR analyses of chicken tissues. A limited number of HKGs have been widely used. However, wide utilization of a little number of HKGs for all tissues is challenging. The emergence of high-throughput gene expression RNA-seq data has enabled the simultaneous comparison of the stability of multiple HKGs. Therefore, employing the average coefficient of variations of at least three datasets per tissue, we sorted all reliably expressed genes (REGs; with FPKM ≥ 1 in at least one sample) and introduced the top 10 most suitable and stable reference genes for each of the 16 chicken tissues. We evaluated the consistency of the results of five tissues using the same methodology on other datasets. Furthermore, we assessed 96 previously widely used HKGs (WU-HKGs) in order to challenge the accuracy of the previous studies. The New Tuxedo software suite was used for the main analyses. The results revealed novel, different sets of reference genes for each of the tissues with 17 common genes among the top 10 genes lists of 16 tissues. The results did disprove the suitability of WU-HKGs such as *Actb*, *Ldha*, *Scd*, *B2m*, and *Hprt1* for any of the tissues examined. On the contrary, a total of 6, 13, 14, 23, and 32 validated housekeeping genes (V-HKGs) were discovered as the most stable and suitable reference genes for muscle, spleen, liver, heart, and kidney tissues, respectively. Although we identified a few new HKGs usable for multiple tissues, the selection of suitable HKGs is required to be tissue specific. The newly introduced reference genes from the present study, despite lacking experimental validation, will be able to contribute to the more accurate normalization for future expression analysis of chicken genes.

KEYWORDS

housekeeping genes, chicken, New Tuxedo, RNA-sequencing, coefficient of variation

Introduction

Housekeeping genes (HKGs), by definition, are genes required for the maintenance of basal cellular function, irrespective of their specific roles in the tissue or organism. HKGs are expected to express stably in all tissues of an organism under different conditions, regardless of developmental stage, sex, or external stressors (e.g., heat stress, disease, and immunological challenge, among others). Full characterization of a minimal set of genes that are required to sustain the life of a tissue is of particular interest (Eisenberg and Levanon, 2013). The current trend of analyses of global gene expression data using microarray or RNA-seq technologies has enabled the simultaneous analysis of tens of thousands of genes. However, quantitative real-time PCR (qPCR) has remained the only valid, more preferred independent tool for validating the results of genome-wide gene expression analyses (VanGuilder et al., 2008). The reliability of the final quantification result of qPCR depends heavily on the utilization of one or multiple internal reference genes for the normalization of the expression of the genes of interest. Normalization to a set of HKGs is nowadays a current and crucial procedure and is preferred to the normalization to a single reference gene. Therefore, the identification of at least two proper, stable HKGs for a specified tissue is crucial (Bagés et al., 2015).

The simultaneous analysis of a large number of genes was not possible until the emergence of high-throughput next-generation sequencing data. Although the evaluation of expression stability of potential reference genes has been carried out earlier for several tissues of chicken (Yang et al., 2013; Olias et al., 2014; Oliveira et al., 2017; Hassanpour et al., 2018; Zhang et al., 2018), the methods of choice of almost all of them were merely based on the qPCR and utilization of the BestKeeper (Pfaffl et al., 2004), geNorm (Vandesompele et al., 2002), and NormFinder (Andersen et al., 2004) statistical algorithms. To our knowledge, the work on identifying suitable HKGs using high-throughput microarray or RNA-seq data is scarce and limited to only some plant species including olive and *Arabidopsis* (Zhuo et al., 2016; Carmona et al., 2017), grapevine (González-agüero et al., 2013), white campion (Zemp et al., 2014), and some insects and animals including sweet potato whitefly (Su et al., 2013), Arctic charr (Pashay Ahi et al., 2013), human (Carmona et al., 2017), and human and mouse (de Jonge et al., 2007). However, there is no comprehensive study to address the most suited HKGs in chicken using RNA-seq data. In the present work, we tested most of the reliably expressed genes (REGs) for stability in the 16 important chicken tissues using at least three RNA-seq datasets per tissue and reported 10 most stably expressed genes for each of them to be used as proper sets of HKGs in the future gene expression assays. In addition, the consistency of the results was evaluated for five tissues, namely, heart, kidney, liver, muscle, and spleen. The evaluation step was not performed for the remaining 11 tissues as a sufficient, required number of high-depth datasets were not available.

Materials and methods

Selection of the desired tissues for study

Based on the importance of the tissues in research and availability of sufficient gene expression data, we selected 16 chicken tissues, namely, adipose, blood, brain, bursa of Fabricius, duodenum, heart, ileum, jejunum, kidney, liver, lung, muscle, ovary, skin, spleen, and trachea, for the current work. There were no sufficient datasets (at least three) for other tissues in the databases.

RNA-seq data collection from databases

The required RNA-seq datasets were downloaded from NCBI (https://www.ncbi.nlm.nih.gov). At least three datasets were downloaded per tissue. Each dataset should have at least three replicated samples for case group and at least three replicated samples for control or another treatment group. The treatment of the case samples was not important as the only criterion for the comparison was to compare the expression of genes between case (treated) and control (untreated) or another treated group. For simplicity, the two mentioned groups will be called case and control, hereafter. The data were downloaded in SRA format using the SRA Toolkit and converted into fastq format using the fastq-dump tool. In Table 1, the accession numbers are shown, and in Supplementary Table S1, the meta information of the used datasets are presented.

Required conditions for the datasets to be chosen for the analyses

The main question of the current work was "Which HKGs are the most suitable?" It is obvious that genes with the lowest expression differences between the case and control groups within a specified dataset as well as with expression sustainability among all datasets of a specified tissue could be considered as the most suitable HKGs. Since at least three datasets were analyzed per tissue, genes with the highest consistency of expression across all the experiments were finally introduced as the most proper sets of HKGs. We screened the NCBI database exhaustively to download only the datasets that address the question of research. Therefore, we downloaded only

TABLE 1 Accession numbers of the used datasets for discovering the most stably expressed genes for 16 chicken tissues.

Training datasets	Adipose	SRP143406	SRP343295	SRP042257	SRP212250		
	Blood	SRP200185	SRP200118	SRP200118	SRP310357	SRP310357	
	Brain	SRP102082	SRP081121	SRP233052	SRP233052		
	Burs	ERP122030	ERP122030	SRP163233	SRP098825		
	Duodenum	SRP348148	SRP299602	SRP173587	SRP055561		
	Heart	SRP265642	SRP097223	SRP153755			
	Ileum	SRP149780	SRP200118	SRP300399	SRP126304		
	Jejunum	SRP280208	SRP140601	ERP121879	ERP121879		
	Kidney	SRP097223	SRP092600	SRP338989			
	Liver	SRP143406	SRP097223	SRP321387	SRP294224	SRP161836	SRP133195
	Lung	SRP097223	SRP265640	SRP233531	SRP238721	SRP081121	
	Muscle	SRP217060	SRP217060	SRP217060	SRP217060	SRP159467	SRP321387
	Ovary	SRR12315154	SRP143406	SRP256253	SRP256253		
	Skin	SRP343295	SRP142597	SRP126033	SRP112878		
	Spleen	SRP097223	SRP225741	SRP174144	SRP280208	SRP158365	SRP174144
	Trachea	SRP338989	SRP247563	SRP226600	SRP126851		
Evaluation datasets	Heart	SRP152925	SRP266037	SRP159467			
	Kidney	SRP338989	SRP338989	SRP338989			
	Liver	SRP111815	SRP104528	SRP233052	SRP233052	SRP081121	SRP100368
	Muscle	SRP255211	SRP104528	SRP327337	SRP327185	SRP313854	SRP226900
	Spleen	SRP254842	SRP254842	SRP223412	SRP173965	SRP174144	SRP174144

the datasets with the following conditions: 1) Illumina paired-end RNA-seq data (no single-end data used); 2) each of the tissues should have at least three datasets; 3) each dataset could be subset into only two case and control groups; and 4) each case group and control group should have at least three replicate samples.

Analyses of individual datasets

The individual datasets were analyzed separately using the New Tuxedo software suite employing the Ensembl Gallus gallus Build 6.0 reference genome (https://asia.ensembl.org/Gallus_gallus/info/index). At first, Fastqc (Andrews, 2010) and Trimmomatic (Bolger et al., 2014) software tools were employed for quality control and trimming, respectively. Datasets with insufficient quality metrics were excluded. The data were trimmed using ILLUMINACLIP, SLIDING WINDOW (window size 3–5 and Phred quality mean of 20–28), CROP (to trim 3–10 left-end nucleotides), AVGQUAL (minimum Phred quality of 20–25), and MINLEN (read length ≥ 40–45) options. Depending on the dataset, the values were varied. The Hisat2 software (Kim et al., 2019) (available at https://daehwankimlab.github.io/hisat2) was used for both indexing of the genome and mapping of the clean reads onto the indexed reference genome. The Stringtie software (Kovaka et al., 2019) (available at http://ccb.jhu.edu/software/stringtie) was used for assembly of transcripts of each sample using the -G option that forces the assembly to be limited to only the known genes. The transcripts of all samples of all experiments were assembled using the merge option of Stringtie. The Cuffdiff software (Trapnell et al., 2010) (available in http://cole-trapnell-lab.github.io/cufflinks) was used for differential expression analysis between the case and control groups with the multiread and bias correction options enabled. Genes with considerable expression differences between the two mentioned groups resulted in statistically significant differences, while genes with constant expressions between the two groups have *p*-values approximate to 1. Genes with unreliable expression (FPKM < 1) in all samples of a dataset were excluded. The expression (FPKM) values of all samples within a specified experiment were gathered, and mean, variation, and coefficient of variation (CV) statistics were calculated for each gene. Only REGs (i.e., genes passing the aforementioned filter in all datasets of a tissue) were considered for the discovery of the stably expressed genes. Then, average CV was calculated for each gene across all datasets within a specified tissue. Genes were sorted in ascending order and ranked based on the average CV, and those with the lowest average CV values were reported as the most stable and suitable housekeeping genes. The flowchart of the analyses is shown in Figure 1. This process was repeated for all 16 tissues, and the top

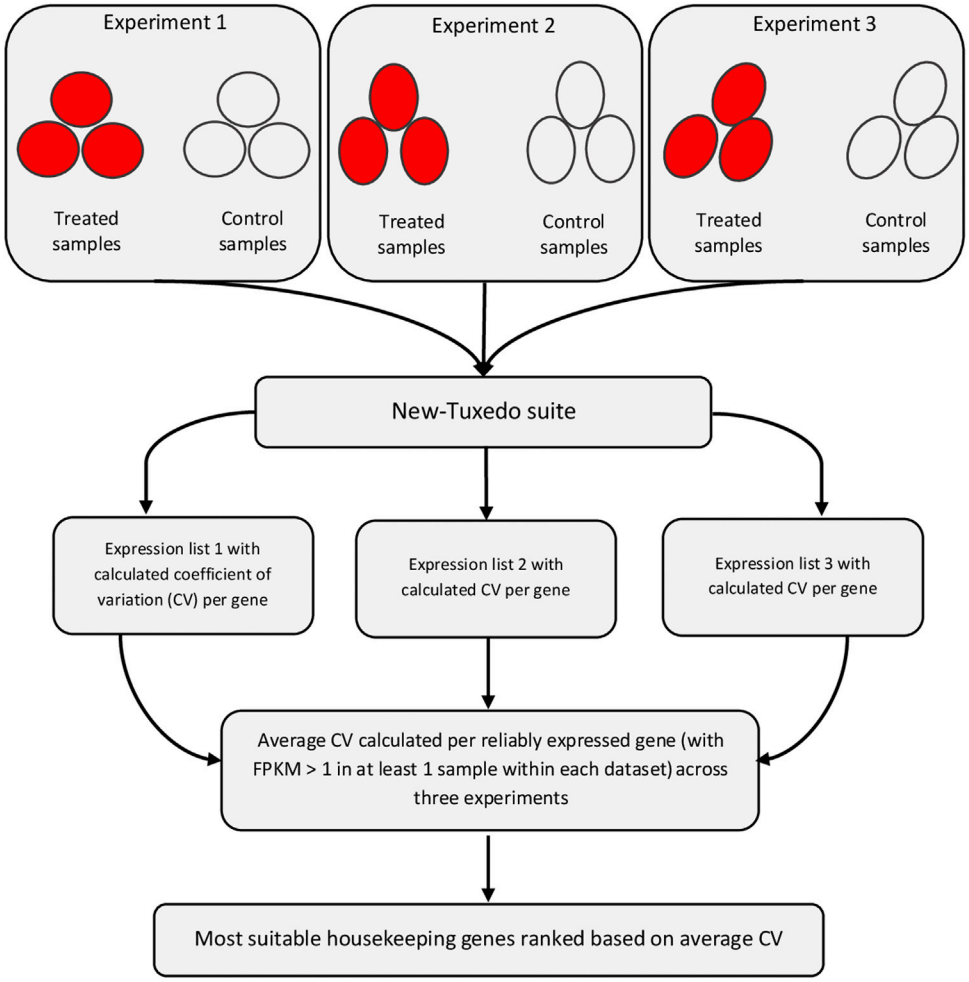

FIGURE 1
Flowchart of detection of the most suitable housekeeping genes for a specific tissue. This workflow was repeated for all tissues separately. At least three datasets were analyzed per tissue. The comparisons within each experiment were performed between the treated and control groups, each with at least three replicates. All the used datasets are publicly available data generated using Illumina paired-end RNA-Sequencing method. Software programs within the New Tuxedo suite were hisat2 (for mapping of reads onto reference genome), Stringtie (for assembly and read counting), and Cuffdiff (for differentiall expression analysis). Expression stability was monitored per gene based on the coefficient of variation (CV), and ranking of most stably expressed genes were performed based on the average CV criterion.

10 most stably expressed genes were reported for each tissue separately.

Gene ontology and pathway enrichment analyses of the most stably expressed genes

All the top 10 genes of the 16 tissues were gathered, and the duplicated genes were deduplicated. In the end, a total of 139 unique genes were submitted to gene ontology and pathway enrichment analyses using the DAVID web-based software (Jiao et al., 2012) (available at https://david.ncifcrf.gov) in order to understand the functions and to gain insight into the pathways that the less variable genes are involved in.

Evaluation of the consistency of the results

For five of the tissues, there were six or more datasets. For each of heart and kidney tissues, there were six datasets, and for each of liver, muscle, and spleen tissues, there were 12 datasets. We analyzed half of the datasets of the mentioned tissues as training and the second half for the evaluation of the consistency

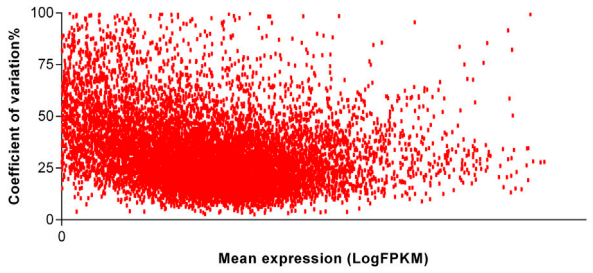

FIGURE 2
Scatterplot of the reliably expressed genes of one dataset of adipose tissue. The relationship of mean (in log10 scale) and variation of expression of genes were assessed in order to understand whether the variability of expression is increased with the mean of expression. The expression values are given as fragments per kilobase of transcript per million reads in log10 scale.

of the results of the training datasets. We named the second set as evaluation datasets. As for the training datasets, the REGs of the evaluation datasets were also ranked based on the average CV. The top 100 genes of the training datasets were compared with those of the evaluation datasets and those genes that were in common in both of the top 100 genes lists were reported as validated housekeeping genes (V-HKGs). The more the counts of matched genes between the two top 100 genes lists, the greater the accuracy and repeatability of discovering the stable genes.

Assessment of the suitability of widely used housekeeping genes

A total of 96 WU-HKGs were selected from the literature. The list of WU-HKGs and their corresponding citations are shown in Supplementary Table S2. Gene expression analysis using a real-time PCR assay was the main subject of the reviewed papers. The main objective of the current section was to challenge the accuracy of the previously conducted gene expression studies that had used nonproper HKGs.

Results and discussion

We used 94 datasets (70 for training and 24 for evaluation) sourced from 16 tissues, namely, adipose, blood, brain, bursa of Fabricius, duodenum, heart, ileum, jejunum, kidney, liver, lung, muscle, ovary, skin, spleen, and trachea. A total of 23,403 genes were analyzed in each dataset, and almost 3,000–11,000 REGs (with FPKM value ≥ 1 in at least one sample of a datasets) were assessed for the stability of expression. In contrast, nearly 50–85% of genes were filtered out because of the inconsistency of expression or because of low coverage of

TABLE 2 Top 10 most suitable reference genes for chicken tissues based on average coefficient of variations (CVs) across at least three experiments.

Adipose	Blood	Brain	Bursa	Duodenum	Heart	Ileum	Jejunum	Kidney	Liver	Lung	Muscle	Ovary	Skin	Spleen	Trachea
Abcb6	Psma1	Serbp1	Rap2c	Amot	Mrpl33	Dhx30	**Atp5b**	Itgb1bp3	Fam120a	Atl1	Adam17	**Tasor2**	**Hnrnpab**	Wdr81	**Tasor2**
Prrc2c	*Gapdh*	Nr1h3	Tmem259	**Xpo5**	**Ilf2**	Dhx38	*Rpl6*	Uck1	Xpo6	Tfip11	Slc39a3	Pcif1	Ddb1	Hdac1	**Atl1**
Ubr7[a]	*Oaz1*	Bet1l	Cnot9	**Exosc10**	Rufy3	Arnt	**Gnb2l1**	*Polr2b*	**Ubr7**	Mvb12a	**Cops7a**	Tomm22	Lonp1	Scyl3	**Ikbkb**
Eif3a	Rpl39l	Arhgef9	Wasf2	Zyx	**Cep68**	Ckap2l	**Ilf2**	Col4a1	Mrps25	Slc35a1	Xpo7	Erlin1	Tcf25	Zc3h11b	**Cep68**
Pabpc1	Tmed10	**Atp5b**	**Grb2**	Usp5	Nfyc	**Hnrnpab**	Lasp1	*Gusb*	*Ap2m1*	Pisd	Fem1b	Poll	**Grb2**	Dnajc5	Cfap92
Tmem57	*Rpl27a*	Nono	Ascc2	Cnot1	Cuedc2	Nup188	Mif4gd	**Atl1**	Pcbd1	Phc1	Ipo9	**Ctnna1**	Mtmr3	Nek9	Parp9
Thrap3	Cox7b	Sumo3	Rpl7l1	Psmd13	**Tasor2**	Stx10	**Ikbkb**	Hbp1	Rnf130	Spoutl	**Gsr**	Kbtbd4	Baz1b	Gzf1	Hspd1
Rpl4	Rpl7a	Rps3a	Man2c1	Fhl3	Slc7a3	**Eif2b5**	**Atl1**	Herc2	Adat1	**Tor1b**	Copg2	Brd8	**Exosc10**	Cdk12	Cd80
Ctnna1	Rpsap58	Uso1	Tpm3	Med1	**Gnb2l1**	Map3k14	Mkrn2	Ndufb9	Myh9	Cog5	Arcn1	Gsr	**Cops7a**	**Xpo5**	Mettl21c
Kcnh4	Eif4ebp1	Cd99l2	Chmp1a	**Eif2b5**	**Ak2**	Nol9	Aamp	**Tor1b**	Arpc2	**Cep68**	Eya3	Clpx	Yipf3	Mpp1	Mrpl40

[a]Highlighted (bold) genes are in common for at least two tissues. Underlined genes are among the widely used housekeeping genes.

sequencing in some of the datasets. It is worth mentioning that only REGs of all datasets within a tissue were allowed for the final analysis. For tissues with more available datasets (such as spleen, liver, and muscle), we only chose the datasets with sufficiently deep sequencing, because the low coverage datasets would not guarantee the possibility of the evaluation of all REGs. In contrast, for less studied tissues that had no abundant datasets available, we decided to utilize all available datasets regardless of their sequencing depths. It is obvious that the insufficient coverage of datasets will cause the number of analyzed genes to be reduced. The total number of datasets and REGs for each of the tissues are reported in Supplementary Table S3.

To identify the most stable reference genes, we first checked the relation of mean of expression and variation. We observed no relation between the mentioned coefficients, and thereby, the suggested reference genes can be used for the normalization of the interested genes irrespective of their expression levels. In Figure 2, a scatterplot demonstrating the relation of mean and CV of one experiment of adipose tissue is shown. Scatterplots of the remaining experiments were similar and therefore were not shown here.

The methodology used in the present work to find the most stable reference genes revealed interesting results. Almost all of the introduced reference genes (i.e., top 10 most stably expressed genes) were new, indicating that the previously used HKGs were not as stable as required for a gene to be considered as a proper HKG. Only eight (8.3%) of the WU-HKGs were present in the top 10 lists of five tissues including *Rpl4* for adipose; *Oaz1*, *Rpl27a*, and *Gapdh* for blood; *Rpl6* for jejunum; *Gusb* and *Polr2b* for kidney; and *Ap2m1* for liver. The top 10 most stably expressed genes introduced here for the remaining tissues were completely novel. In Table 2, the top 10 most stable reference genes are reported for the studied tissues.

As can be seen in Table 2, some of the introduced reference genes (17 genes) were in common in the top 10 lists of at least two tissues. For example, *Atl1* was identified as suitable for four tissues. *Cep68* and *Tasor2* genes were in common in the lists of three tissues, and *Ubr7*, *Ctnna1*, *Atp5b*, *Grb2*, *Xpo5*, *Exosc10*, *Eif2b5*, *Ilf2*, *Gnb2l1*, *Hnrnpab*, *Ikbkb*, *Tor1b*, *cops7a*, and *Gsr* genes were in common in the top 10 lists of two tissues. It is obvious that genes with more frequent occurrence in multiple top 10 genes lists are more likely suggested as suitable HKGs than are those with only one occurrence. Therefore, these 17 genes are strongly suggested to be used as HKGs for the mentioned tissues.

Gene ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses of the most stably expressed genes

Understanding the functionality of the most stable reference genes is of great importance. In the current work, the top 10 most stable genes of all 16 studied tissues (totally 134 unique genes) were subjected to gene ontology and KEGG pathway enrichment analyses using the DAVID web tool. The results revealed that the most stably expressed genes that enriched significantly ($p < 0.05$) in molecular function terms, biological processes terms, and KEGG pathways were generally related with the transcription and translation of proteins (e.g., *RNA* binding, protein binding, structural constituent of ribosome, translational initiation, positive regulation of transcription, cytoplasmic translation, nucleocytoplasmic transport, RNA degradation, and ribosome, among others). In other words, as compared to genes of other processes or pathways, it seems that genes associated with transcription and translation are less variable under different conditions and are more suited as HKGs. These results are in accordance with the findings of Kouadjo et al. (2007) and de Jonge et al. (2007), who reported the protein biosynthesis–related genes as the most stably expressed genes. Gene ontology terms as well as KEGG pathways enriched by the top 10 most stably expressed genes are reported in Supplementary Table S4.

Consistency of the results of the training datasets

As mentioned above, other sets of data of heart (three datasets), kidney (three datasets), liver (six datasets), muscle (six datasets), and spleen (six datasets) tissues were analyzed in the same way as the training datasets in order to evaluate the consistency of the results of the training datasets. The average CVs of the training datasets strongly correlated with that of the evaluation datasets with Pearson correlation coefficient ranging from 0.64 to 0.88 and Spearman correlation coefficients ranging from 0.67 to 0.82, indicating the relatively high accuracy of the discovering stable genes. The top 100 genes of the training datasets were compared with that of the evaluation datasets. We found that the top 100 genes of the training datasets also took place in higher ranks in the evaluation datasets. Except for muscle, we found relatively consistent results for the remaining four tissues. There were 6, 13, 14, 23, and 32 genes in common in the top 100 genes of the training and evaluation datasets of muscle, spleen, liver, heart, and kidney tissues, respectively. In total, 80% of the top 100 genes for kidney tissue and almost 50% of the top 100 genes for heart, liver, and spleen tissues were present in the top 500 genes of the evaluation datasets. It should be noted that in the case of random distribution of genes in the various ranks of the evaluation datasets, less than 10% of the top 100 genes of training datasets would be present in the top 500 genes of the evaluation datasets [(500/number of analyzed genes) × 100 = ~4.6–8%]. These findings indicate that the identification of the most stably expressed genes with the used method is, to some extent, accurate, and the repeatability of

TABLE 3 Number of genes that are in common in the top 100 genes list of the training datasets and the top 100 to top 500 genes lists of the evaluation datasets.

	Heart	Kidney	Liver	Muscle	Spleen
Top-100 genes of evaluation datasets	23	32	14	6	13
Top-200 genes of evaluation datasets	38	51	26	11	21
Top-300 genes of evaluation datasets	48	68	36	16	31
Top-400 genes of evaluation datasets	59	76	46	19	35
Top-500 genes of evaluation datasets	63	80	49	23	43

TABLE 4 Most stably expressed genes of five chicken tissues that are in common between the top 100 genes of the training dataset and the top 100 genes of the evaluation dataset.

Heart	Kidney	Liver	Muscle	Spleen
Mob1a	Ikbkb	Rp11-529k1.3	Srpra	Nfyc
Strada	Wnk1	Dpagt1	Cops7a	Hdac1
Ticam1	Aplp2	Rbm7	Hnrnpd	Cnp
Cep68	Ilf2	Npepps	Dhx38	Adam17
Phc1	**Strada**	Eif2b5	Gtpbp1	Spata5
Atl1	Ugp2	*Ap2m1*	Puf60	Grk2
Fancm	Bpnt1	Amfr		Rpn1
Abi2	**Phc1**	Psmd7		Hnrnpab
Aamp	Etfdh	Fam120a		Mtmr3
Tmem41a	Rufy3	Ctnna1		Nup188
Ak3	Oraov1	Xpo6		Tor1b
Hbp1	Slirp	Lig3		Znrf2
Casc4	**Atl1**	**Ankrd16**		**Ankrd16**
Rbl2	Hspd1	Prpf6		
Pepd	Tanc1			
Mkrn2	*Rpl5*			
Tfip11	Cp			
Pisd	Gpr18			
Spout1	Cog5			
Uck1	Vwa9			
Mif4gd	**Mkrn2**			
Ankrd16	Hvcn1			
Tasor2	**Uck1**			
	Gsn			
	Fam104a			
	Mrps7			
	Gusb			
	Rps6kb1			
	Myo19			
	Tubb2a			
	Stx17			
	Mrps16			

[a]Highlighted (bold) genes are in common for at least two tissues. Underlined genes are among the widely used housekeeping genes.

the results is considerable. In Table 3, the number of common genes in the top 100 genes of the training datasets and top 500 genes of the evaluation datasets are presented. Although the employed approach identified reliable, stable reference genes for all tissues, we recommend the utilization of the reported HKGs cautiously. In addition, we invite related researchers to further validate the reported HKGs using real-time PCR as this was not possible in the current work.

Here, we introduced only those genes that were in common between the top 100 genes of the training and evaluation datasets as V-HKGs. Six of the V-HKGs, namely, *Ankrd16*, *Strada*, *Phc1*, *Atl1*, *Mkrn2*, and *Uck1*, were observed commonly for at least two tissues. *Ankrd16* gene was identified as suitable for heart, liver, and spleen tissues. The remaining five genes were identified as the best for both heart and kidney tissues. In Table 4, the official names of the V-HKGs are reported. In addition, in Figure 3, the boxplots of the V-HKGs are illustrated. As can be seen in Figure 3, the expression variations of the V-HKGs were negligible.

Assessing the suitability of 96 widely used housekeeping genes

In Supplementary Tables S5 and S6, the average CVs and rankings of the WU-HKGs among the REGs are reported, respectively. The expression profile of most of the 96 WU-HKGs showed inconsistency in some of the chicken tissues. Eight genes (i.e., *Mb*, *Dimt1*, *Rps29*, *Stx5*, *Gys1*, *Il6*, *Rbx1*, and *Rnasek*) showed no expression in more than five tissues. It is obvious that the mentioned genes do not have merit to be suggested as proper HKGs, although some of them have been traditionally used widely for the normalization of real-time PCR assays. In contrast, only 13 genes showed consistent expression in all 16 tissues, including *Ap2m1*, *Gusb*, *Polr2b*, *Rpl6*, *Eif4a3*, *Rpl4*, *Eef1a1*, *Gapdh*, *Rpl19*, *Rpl27a*, *Rpl31*, and *Rps6*. Six out of the 13 mentioned genes (i.e., *Eef1a1*, *Gapdh, Rpl19, Rpl27a*, *Rpl31*, and *Rps6*) showed relatively consistent, stable expression and ranked among the best 100 most stably expressed genes of two tissues. Although not detected in three datasets, *Ap2m1* performed well in seven tissues, which was

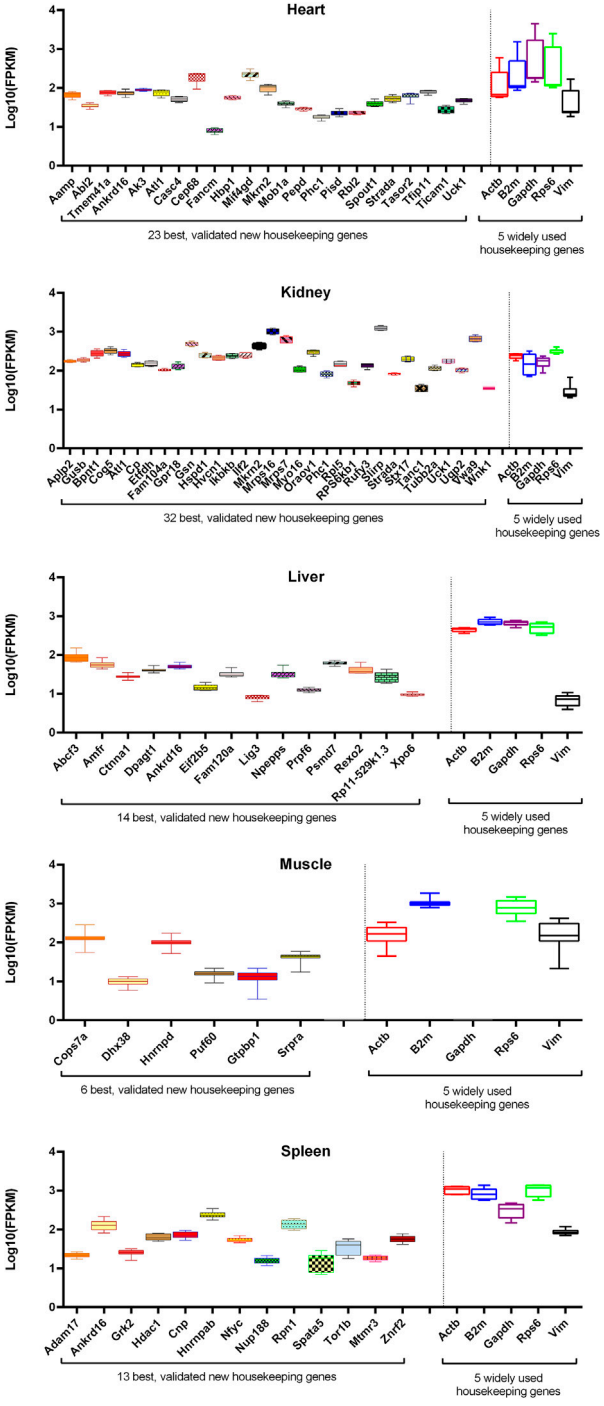

FIGURE 3
Box plots of the expression of most stable, validated housekeeping genes in comparison with that of five randomly selected widely used housekeeping genes of five chicken tissues. For each tissue, the expression variation of newly introduced housekeeping genes (left side of the vertical line) was compared with that of five old and widely used housekeeping genes (right side of the vertical line). The expression values are given as fragments per kilobase of transcript per million reads in log10 scale (whiskers: min to max).

followed by *Gusb*, *Polr2b*, *Rpl6*, *Rpl*4, and *Eif4a3*. These WU-HKGs were high-ranked in three or more tissues and seem to be suitable for further utilization.

For blood, 17 WU-HKGs were relatively suitable, which was followed by adipose (seven genes), jejunum (six genes), kidney (six genes), and liver (five genes), indicating that the WU-HKGs are more suitable for blood than for other tissues. For the remaining tissues, almost none of the WU-HKGs is suitable, and further use of them is not suggested.

The variation of expression of the 96 WU-HKGs were considerably more than that of the stable reference genes that were introduced in the present work. In Figure 3, the expression variations of five randomly selected WU-HKGs (i.e., *Gapdh*, *Actb*, *Vim*, *Rps6*, and *B2m*) are illustrated. As can be seen in Figure 3, some of the mentioned genes were not stably expressed across all experiments of 16 tissues. Two of them (i.e., *Gapdh* and *Rps6*) were among the top 100 stable genes, while the remaining three (i.e., *Vim*, *B2m*, and *Actb*) were not.

Previous studies have reported relatively high variability for the expression of WU-HKGs in a varied range of tissues and organs in livestock animals (Gromboni et al., 2020; Lozano-Villegas et al., 2021) as well as in humans (Mahoney et al., 2004; Caracausi et al., 2017; Cadenas et al., 2022), mice (Fu et al., 2020; Muñoz et al., 2021), and insects (Shi et al., 2016), among others.

For adipose tissue, only *Rpl4* gene took place within the top 10 stable genes among 10,343 REGs (Table 2). Other WU-HKGs were not even within the best 100 genes. We found it interesting that having fold changes of ~9.7 and 12.0, respectively, *Acta1* and *Scd* genes showed significant difference between the case and control groups in one experiment of adipose (q-value < 0.05). Therefore, these genes are no longer suggested for further utilization. Unlike *Rpl4* gene, which is also proposed here, *Tbp* has previously been reported elsewhere as a suitable reference gene for the normalization of gene expression data of adipose tissue (Wang et al., 2020). *Tbp* was the only WU-HKG that was reliably expressed in all 16 tissues with nonsignificant differential expression in all of the 94 studied datasets. However, *Tbp* ranked 1,092 among the 10,343 REGs of adipose tissue. Other studies have reported *Rpl32* and *B2m* genes as two suitable HKGs for abdominal fat, compared with the other three genes, namely, *Sdha*, *Tbp*, and *Ywhaz* (Bagés et al., 2015). Neither of them, however, were identified as suitable HKGs for adipose in the current work. Na et al. (2021) compared 14 chicken reference genes and reported both *Tbp* and *Hmbs* genes as the most stably expressed genes during the growth and development of abdominal adipose tissue of broilers. They also reported *Tbp* and *Rpl13* genes as the most stable during the differentiation of primary preadipocytes and *Tbp* and *Hmbs* genes in preadipocytes and mature adipocytes.

In the present work, as compared to those of other tissues, greater number of WU-HKGs were relatively constant across all

datasets of blood. Studying human peripheral blood, Martínez-Sánchez et al. (2019) found *Hprt* and *Tbp* as the most reliable genes. They suggested the utilization of *Gapdh*, *B2m*, and *Rpl13a* genes to be avoided. Likewise, Dheda et al. (2004) emphasized the avoidance of employment of *Gapdh*, *B2m*, and *Actb* genes (each with ~10- to 30-fold variability across conditions) for normalizing mRNA levels in human pulmonary tuberculosis. For blood, we found the following four WU-HKGs as relatively stable: *Gapdh* (rank = 2), *Rpl13* (rank = 80), *Oaz1* (rank = 3), and *Rpl27a* (rank = 6).

For heart tissue, except *Gusb* (rank = 50) and *Tbp* (rank = 57) genes, further utilization of other WU-HKGs genes is not suggested. *TBP* has been also identified as a suitable reference gene for lung and heart (Hassanpour et al., 2018) and abdominal fat (Wang et al., 2020) tissues. Because of the low coverage of the used datasets of heart tissue in the current work, only 3,334 (6,235) genes in the training (evaluation) datasets were reliably expressed. Therefore, as expected, many of the WU-HKGs with little expression were not detected in heart datasets. On that account, only 33 WU-HKGs were analyzed. Gromboni et al. (2020) assessed the suitability of eight HKGs for heart. Their studied WU-HKGs were present in neither the top 10 genes nor the list of 23 V-HKGs of heart tissue in the current study. Hassanpour et al. (2018) investigated a panel of nine HKGs and introduced *Ywhaz* and *Rpl13* genes as suitable for chicken heart (Hassanpour et al., 2018). All of their studied genes were filtered out in the current study as lacking the criteria we employed to categorize the stable reference genes.

For kidney tissue, only *Polr2b* and *Gusb* genes (rankings of 3 and 5, respectively) and *Eef1a1*, *Nelfcd*, *Rpl5*, and *Rpl6* (rankings of less than 100) outperformed other WU-HKGs within 7,527 REGs. On the contrary, *Il6*, *Scd*, and *Dimt1* were among the worst genes and identified as the most inappropriate HKGs for kidney. Other WU-HKGs were also not stable and therefore not recommended for further use.

For liver tissue, only *Ap2m1* gene appeared suitable with a ranking of 5 among 8,428 REGs. Out of 94 analyzed datasets, *Ap2m1* was reliably expressed in 80, while it was significant (q-value < 0.05) in only two datasets belonging to blood and brain tissues. Therefore, although not suggested for brain, blood, heart, and lung tissues, *Ap2m1* seems to be suggested for more than one tissue. In yellow feathered broilers, Zhang et al. (2018) reported *Rpl13* gene as the most proper HKG for liver, compared with only six other candidate genes. In another research, *Ywhaz* and *Tbp* were found more stable than *B2m*, *Rpl32*, and *Sdha* genes (Bagés et al., 2015). In the present work, being significant in one, two, two, and three experiments among 12 experiments of liver, *Actb*, *B2m*, *Gapdh*, and *Rpl13* genes, respectively, were not proved to be suitable HKGs.

For lung tissue, only 34 WU-HKGs showed reliable expression. Almost 95% of these 34 genes were not stable. Only *Gusb* (rank 30 among 3,562 REGs) was identified as relatively suitable. In an attempt, Kriegova et al. (2008) investigated 10 candidate HKGs and introduced *Rpl32* as the most suitable HKG for lung tissue. Being excluded from the analyses in the filtration steps, *Rpl32* was not identified as a suitable HKG in the current work for neither lung tissue nor other tissues. The results of Fu et al. (2020) indicated that none of the 15 WU-HKGs that they studied were sufficiently good as reference genes. However, they suggested the combination of *Grcc10* and *Ppia* genes as a proper choice for the lung tissue of mouse infected with IAV.

The datasets of muscle in the current work appeared very variable. Although we only used datasets belonging to pectoral major muscle tissue, as compared to other tissues, there were less genes in common between the training and evaluation datasets. It seems that sampling of the tissues of the different studies had been done differently and not from the same section of the pectoral major muscle. The six identified V-HKGs (i.e., *Srpra*, *Cops7a*, *Hnrnpd*, *Dhx38*, *Gtpbp1*, and *Puf60*), however, showed sufficiently less variability within the used 12 different datasets and seem to be a suitable set of reference genes for muscle. The findings of Nascimento et al. (2015) showed *Hmbs* and *Hprt1* genes as the most stable while *Tfrc* and *B2m* as the least stable reference genes for the pectoralis major muscle of chicken. Their results, also, revealed that *Hmbs* and *Hprt1* gene expression did not change owing to dietary variations and thus were recommended for accurate normalization of RT-qPCR data of chicken pectoralis major muscle. In our results, the best WU-HKG was MIF (rank = 89). *Hprt1*, although expressed nonsignificantly in all six training analyses of pectoral muscle and its fold change ranged 1.0–1.3 between the case and control groups, appeared relatively variable within the case or control groups of the evaluation datasets and ranked 1,172 among 10,745 REGs. In accordance with the findings of the current work, Barber et al. (2005) analyzed *Gapdh* expression in a panel of 72 human tissues and observed a 15-fold difference in *Gapdh* mRNA copy numbers between the skeletal muscle and the breast. Their results confirmed previous reports of the marked variability of *Gapdh* expression between tissue types. On the contrary, Mahoney et al. (2004) concluded that *B2M* and *ACTB* were the most stably expressed HKGs in human skeletal muscle following resistance exercise, while *B2m* and *Gapdh* were the most stable following endurance exercise.

For ovary, we discovered three genes (i.e., *Tasor2*, *Ctnna1*, and *Gsr*) within its top 10 genes that were also identified as the most stable for other tissues. These stable genes along with all the top 10 genes of brain, bursa of Fabricius, duodenum, ileum, jejunum, lung, skin, and trachea were completely new and, to our knowledge, are first reported in the present study. Hassanpour et al. (2019) reported *Ywhaz*, *Hprt1*, and *Hmbs* genes as most stable. They suggested the combination of *Ywhaz*, *Hprt1*, and *Hmbs* as the best set of reference genes for ovarian and uterine tissues of laying hens under control and heat stress conditions. Cadenas et al. (2022) found that the stability of all reference genes differs among ovarian cell types in humans. They identified *Actb*

as the best reference gene for oocytes and cumulus cells and *B2m* for medulla tissue and isolated follicles. They concluded that using a single validated reference gene may be sufficient when the available testing material is limited. For the ovarian cortex, depending on culture conditions, *Gapdh* or *Actb* were found to be the most stably expressed genes. Their reported stable genes were not confirmed in the current work.

Similar to the tissues discussed above, we evaluated the 96 HKGs for spleen transcriptome data as well. Having a relatively low CV and high rank (58 among 9,904 genes), only *Rpl6* proved to be relatively suitable for spleen. In a previous study, 10 HKGs were assessed and 2 genes, i.e., *Tbp* and *Ywhaz*, were identified as the most suitable HKGs for spleen tissue (Khan et al., 2017). None of them, however, was in the list of best, high-ranked HKGs in the current work. For spleen, liver, and cecum of different-aged specific-pathogen-free layer chickens and commercial turkeys, Mitra et al. (2016) suggested *Rpl13* and *Tbp* as the most stable reference genes. They also observed a stable expression of *Rpl13* and *Tfrc* genes in the mentioned tissue samples of turkey. In the current work, both *Rpl13* and *Tfrc* genes were expressed differently between the case and control groups of two experiments of evaluation datasets (q-value < 0.05). Therefore, our results did not prove the consistency of expression of *Rpl13* and *Tfrc* genes. Likewise, we could not approve the suitability of spleen HKGs introduced by Khan et al. (2017) and Borowska et al. (2016).

To our knowledge, the present work is the first comprehensive study that investigated all REGs for 16 most important chicken tissues. Most of the previous studies have compared only a handful of HKGs in which the used genes were in common (Zinzow-Kramer et al., 2014; Bagés et al., 2015; Mitra et al., 2016; Khan et al., 2017; Hassanpour et al., 2018; Zhang et al., 2018; Gromboni et al., 2020). The employed methodologies of the mentioned studies were NormFinder, GeNorm, BestKeeper, RefFinder, and delta CT (Mitra et al., 2016). Each of them has its own strengths and weaknesses. In RefFinder, PCR efficiencies are not taken into account. The NormFinder software is influenced by sample size (Spiegelaere et al., 2015). GeNorm ranking for genes is based on the highest degree of similarity in their expression profile and does not take the amount of variation into account (Andersen et al., 2004). BestKeeper utilizes Pearson correlation analysis and is just valid for normally distributed data with a homogeneous variance. In general, the ranking of HKGs is different based on the output of RefFinder, NormFinder, GeNorm, and BestKeeper, and there is little overlap (Kou et al., 2017). Owing to the dynamic and high-throughput nature of the next-generation sequencing data, the methodology that was introduced in the current work seems to overcome the weakness of the previously used methods. Moreover, utilization of different datasets that belong to different studies, instead of real-time PCR data, is the superiority of the current work over the previous research. In addition, the integration of the results of at least three datasets per tissue seems to increase the reliability of the results.

Conclusion

In the present work, we, for the first time, conducted a comprehensive genome-wide gene expression evaluation of 3,000–11,000 genes, analyzing 94 experiments in order to assess the suitability of previously known HKGs as well as to discover the most stable, new housekeeping genes for each of 16 chicken tissues. The results clearly revealed novel reference genes with more stable expressions in different experimental conditions. On the basis of the definition of ubiquitous and stable expression, our results suggest that no single gene qualifies as a real HKG. In addition, although we identified some genes that were suited for more than one tissue, most of the introduced new and validated HKGs were tissue specific. Thus, instead of one suitable HKG, we reported 10 high-ranked, stable genes for each tissue to provide future studies with more options to choose from. The identified new HKGs were predominantly involved in transcription, translation, and protein biosynthesis. There were 17 common HKGs that were suitable for more than one tissue. We strongly suggest them as well as the V-HKGs for normalization in all future qRT-PCR experiments. We believe that the results of the present work will contribute to more accurate normalization of chicken gene expression data, especially for the data of heart, liver, kidney, spleen, and muscle tissues, and that their results will be validated by analyzing additional sets of datasets.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, and further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval were not required for the animal study because we used the publicly available RNA-seq datasets and we did not collect the data ourselves.

Author contributions

KH: research concept and design, data analysis and interpretation, wrote the article, and final approval of the article. AM and SH: data analysis and wrote the article. SA: critical revision of the article and final approval of the article.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.827538/full#supplementary-material

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Ecological niche modelling as a tool to identify candidate indigenous chicken ecotypes of Tigray (Ethiopia)

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The Tigray region is an ancient entry route for the domestic chickens into Africa. The oldest African chicken bones were found in this region at Mezber, a pre-Aksumite rural farming settlement. They were dated to around 800–400 BCE. Since then, the farming communities of the region have integrated chicken into their livelihoods. The region is also recognised for its high chicken-to-human population ratio and diverse and complex geography, ranging from 500 to 4,000 m above sea level (m.a.s.l.). More than 15 agro-ecological zones have been described. Following exotic chicken introductions, the proportion of indigenous chicken is now 70% only in the region. It calls for the characterisation of indigenous Tigrayan chicken ecotypes and their habitats. This study reports an Ecological Niche Modelling using MaxEnt to characterise the habitats of 16 indigenous village chicken populations of Tigray. A total of 34 ecological and landscape variables: climatic (22), soil (eight), vegetation, and land cover (four), were included. We applied Principal Component Analysis correlation, and MaxentVariableSelection procedures to select the most contributing and uncorrelated variables. The selected variables were three climatic (bio5 = maximum temperature of the warmest month, bio8 = mean temperature of the wettest quarter, bio13 = precipitation of the wettest month), three vegetation and land cover (grassland, forest land, and cultivated land proportional areas), and one soil (clay content). Following our analysis, we identified four main chicken agro-ecologies defining four candidates indigenous Tigrayan chicken ecotypes. The study provides baseline information for phenotypic and genetic characterisation as well as conservation interventions of indigenous Tigrayan chickens.

KEYWORDS

habitat, MaxEnt, climate, agro-ecology, poultry, Tigray

Introduction

The multifaceted benefit of the chicken triggered their human-mediated transport to a wide range of environments, which led them to adapt to different agro-ecologies. The Tigray region is likely one of the first routes for the domestic chicken to Africa, with the earliest osteological evidence of chicken in the continent discovered at the Mezber site in the pre-Aksumite rural farming settlement, dated at least 800–400 BCE (Woldekiros and D'Andrea, 2017). Since then, domestic chicken has been integrated into the livelihood of all communities across Ethiopia. Still, the Tigray region is recognised for its higher chicken-to-human population ratio compared to other Ethiopian regions (e.g., 1.3, 0.9, and 0.5 for Tigray, Amhara, and the Oromia regions, respectively) (CSA, 2017, 2020). Furthermore, it has the highest chicken density per km^2, with 139, 129, and 72 chickens for the Tigray, Amhara, and Oromia regions, respectively (CSA, 2017; 2020). Tigray also comprises diverse eco-geographic areas, following large altitudinal variations, ranging between 500 and 4,000 m above sea level (m.a.s.l.) (Waterbeheer and Van Gaelen, 2011; Haftom et al., 2019). Haftom et al. (2019) have divided the region into 15 agro-ecological zones based on the region's traditional elevation-based classification and aridity. Despite such agro-ecological diversity, indigenous Tigrayan chicken populations are still grouped as a single category, indigenous chicken, with no specific ecotypes yet recognised. Describing indigenous chickens as one single group while there is such a diverse environment in the Tigray region is likely inappropriate (Vallejo-Trujillo et al., 2022).

As mentioned above, chicken husbandry is old in the region (Woldekiros and D'Andrea, 2017). Also, indigenous chickens are found across the region, where they represent a major source of income for the farmer communities. The agro-ecologies of the Tigray region are dominantly characterized by lowland (<1,500 m. a.s.l.) and midland (1,500 < altitude <2,500 m. a.s.l.) areas, covering 92% of the region (Beyene et al., 2001). The environment is typically warm, with an annual average temperature of 20°C (Hijmans et al., 2005; Fick and Hijmans, 2017). Environmental constraints and human socio-cultural preferences are believed to have shaped the diversity of the chicken. Birds typically have large appendages (to dissipate heat) and have white or light plumage (to shine sunshine). However, in high predation exposed areas, farmers prefer to select dark color chickens, which supposedly will make them less visible to predators (Terfa et al., 2019). Double comb and large frame cocks are the most preferred for breeding and fetch a high price in the market (Alem and Yayneshet, 2013; Asfaw et al., 2017). Hence, besides the environmental adaptation of the chicken, farmers' and consumers' preferences have also contributed to adapting the indigenous chicken to the local chicken production systems.

Indigenous chickens are raised under a scavenging system (free range) by the smallholder farmers with little supplementary feed input (e.g., kitchen waste). Accordingly, environmental challenges (e.g., temperature, diseases, feed, water and predation) have been major and selective factors with indigenous chicken expected to be locally adapted.

This lack of recognition of the adaptive diversity of indigenous chickens associated to relatively low productivity have contributed to the massive introduction of commercial breeds in the region. It follows the objectives of the Ethiopian livestock masterplan to increase chicken meat production by 235% and egg production by 828% (Shapiro et al., 2015, 2017), to meet the expected increased demand of 80% for meat and 356% for egg within the country, by the year 2020. Following these introductions, the proportion of exotic chicken and their crossbred is now higher in the Tigray region (30%) compared to the national average (21%), the Amhara (15%) and Oromia (22%) regions (CSA, 2021). This proportion increased by 67% in the past year (CSA, 2020; 2021), following increased consumer demand for chicken products and by-products. This calls for the rapid characterisation of indigenous Tigrayan chicken and their habitats to guide conservation and breeding improvement initiatives. Studying the habitats and defining the potential chicken ecotypes in the Tigray region will provide insight into where and what to conserve.

Ethiopia is one of the countries that has signed the international Convention on Biological Diversity (CBD) (https://www.cbd.int/), with signatory countries committed to take measures to protect biodiversity and to regularly report on the progress (Mackenzie and Jenkins, 2005). Yet, little effort has been undertaken to conserve and protect the indigenous livestock species in the country, including the Tigray region, with only one chicken improvement and conservation program started so far (Hailu et al., 2021). However, considering the low productivity of the indigenous chickens compared to the exotic breeds and their crossbreds, it may be expected that their population size will reduce considerably in the coming years. Accordingly, they may be considered as endangered.

The Ecological Niche Modelling (ENM) approach has been previously used to understand wild species' habitat distribution and conservation (Thorn et al., 2009). Different algorithms are available for ENM but a prominent method applies maximum entropy modelling - a machine learning algorithm - in MaxEnt software (Phillips et al., 2006). It has better prediction power than other methods and is increasingly becoming the method of choice for habitat characterisation since its first application in 2004 (Morales et al., 2017). The MaxEnt method has numerous advantages: it requires only presence data, it is applicable for both continuous and categorical data simultaneously, it efficiently predicts optimal probability distribution, it is amenable to analysis and it provides continuous outputs (Phillips et al., 2006). The ENM approach has been applied to different species. For examples Nagaraju et al. (2013) applied ENM to identify suitable habitats and to assess regenerating ability and

genetic diversity of the Lam tree *Myristica malabarica*. Bentlage and Shcheglovitova (2012) assessed niche similarity of *Anolis* lizard species, Suárez-Mota et al., 2015 used it for the characterisation and conservation of the habitat of *Dyscritothamnus* and *Loxothysanus* flowering plants. Roubicek et al. (2010) used it to study time frame impact of an environmental variable on plants and insects, and Pitt et al. (2016) used it to predict past potentially suitable habitats of domestic chicken across the world in comparison to the habitats of their wild ancestor, Red Junglefowl (*Gallus gallus*).

In livestock, the application of the ENM approach is still in its infancy, with only a few studies so far, primarily in chicken (Vajana et al., 2018; Lozano-Jaramillo et al., 2019; Gheyas et al., 2021; Kebede et al., 2021; Vallejo-Trujillo et al., 2022). Applying ENM on Ethiopian indigenous chickens, Gheyas et al. (2021) identified six major environmental variables. Then, the author chose the extreme environments to identify signatures of positive selection in the genome of these populations associated with the selected environmental parameters. Kebede et al. (2021) studied Ethiopian environmental gradients and using ENM classified the indigenous Ethiopian chicken's habitats into three agro-ecologies. They reported significant morphological differences between the chicken populations among these agro-ecologies, supporting them as chicken ecotypes (Kebede et al., 2021). In a recent study (Vallejo-Trujillo et al., 2022), described a framework for delineating chicken ecotypes through a detailed environmental characterisation of the population habitats using ENM, followed by the genomic characterisation of the ecotypes. None of the previous studies have fully characterised the Tigrayan indigenous chicken populations that have been adapted to the region's complex landscape and diversified agro-ecology. For example (Vallejo-Trujillo et al., 2022), study only included Tigrayan indigenous chicken populations living between 1,295 and 2,312 m. a.s.l.

This study was therefore designed to include Tigrayan chicken populations representing all the altitudinal zones of the Tigrayan regions with the aim to identify candidate Tigrayan chicken ecotypes for further phenotypic and genetic characterisation as well as for guiding conservation interventions. Here we have adapted the ENM protocol described in Vallejo-Trujillo et al. (2022) for idenifying the candidate Tigrayan chicken ecotypes and provide a detailed step-by-step desciption of the protocol.

Material and methods

Sampling sites and sample size

The study was carried out in the Tigray regional state of Ethiopia, located 556 km away from the capital city Addis Ababa. This region is laid at 12^0–150 N and 360 30′- 400,30^0 E and covers ≈54,000 sq km (Yihdego et al., 2018). It has an estimated population of 5.2 million, of which 77% live in rural areas (CSA, 2017). Agriculture is the mainstay of the people. The altitude range from 500 to 4,000 m. a.s.l. And the agro-ecologies comprised 53% lowland (<1,500 m. a.s.l.), 39% midland (1,500 to 2,300 m. a.s.l) and 8% highland (>2,300 m. a.s.l.) areas (Beyene et al., 2001). The soil, geology, vegetation cover, and topography across this region are diverse, which result in different agro-ecologies (Hadgu et al., 2013). The climate of the region is predominantly categorised as semi-arid. The main rainy season is June to mid-September, whereas the warmest season is from March to May.

A stratified sampling strategy, based on the agro-ecological zones and the presence of indigenous chicken, (Figure 1B) (Hadgu et al., 2013), was applied to select districts and villages, with a total of 16 districts with 32 villages (Table 1). For each village, latitude and longitude were taken using a geographic positioning system (GPS) GARMIN 72 with an accuracy level of fewer than 3 m (Figure 1A). The district was considered as the entry point for the ENM analysis, so the number of environmental observation for each district depended of the number of villages with 10 data points per village. It ranged from 20 (two villages) to 30 (three villages) data points (Table 1).

Generating environmental predictors

A total of 34 ecological and landscape variables (Table 2), including 22 climatic, eight soil, and four vegetation and landcover, were selected, based on their biological relevance to indigenous chicken husbandry and suitability for abiotic area classification (Préau et al., 2018). The gridded climatic data (mean values for years 1970–2000) were obtained from the WorldClim database (http://www.worldclim.org/for the variables bio1 - bio19, 'water vapor pressure' and elevation) with a spatial resolution of 1 km^2 (Hijmans et al., 2005; Fick and Hijmans, 2017). The seven soil variables that potentially determine food availability for foraging chickens were obtained from the SoilGrids 1 km v 0.5.8 database (containing global gridded soil information) (Hengl et al., 2014). The water capacity of the soil (mm water per 1 m soil depth) with a 0.5-degree grid was obtained from the Spatial Data Access Tool (SDAT; ORNL DAAC 2017) from NASA (Batjes, 2000). Vegetation and land cover variables (total cultivated land, forest land, and grass/scrub/woodland) that affect both food availability and predation were generated from the 'Harmonized World Soil Dataset - Land Use and Land Cover' with a 30 arc-second raster's (FAO/IIASA/ISRIC/ISS-CAS/JRC, 2009) (Fischer et al., 2008). The crop dominance data were accessed from the

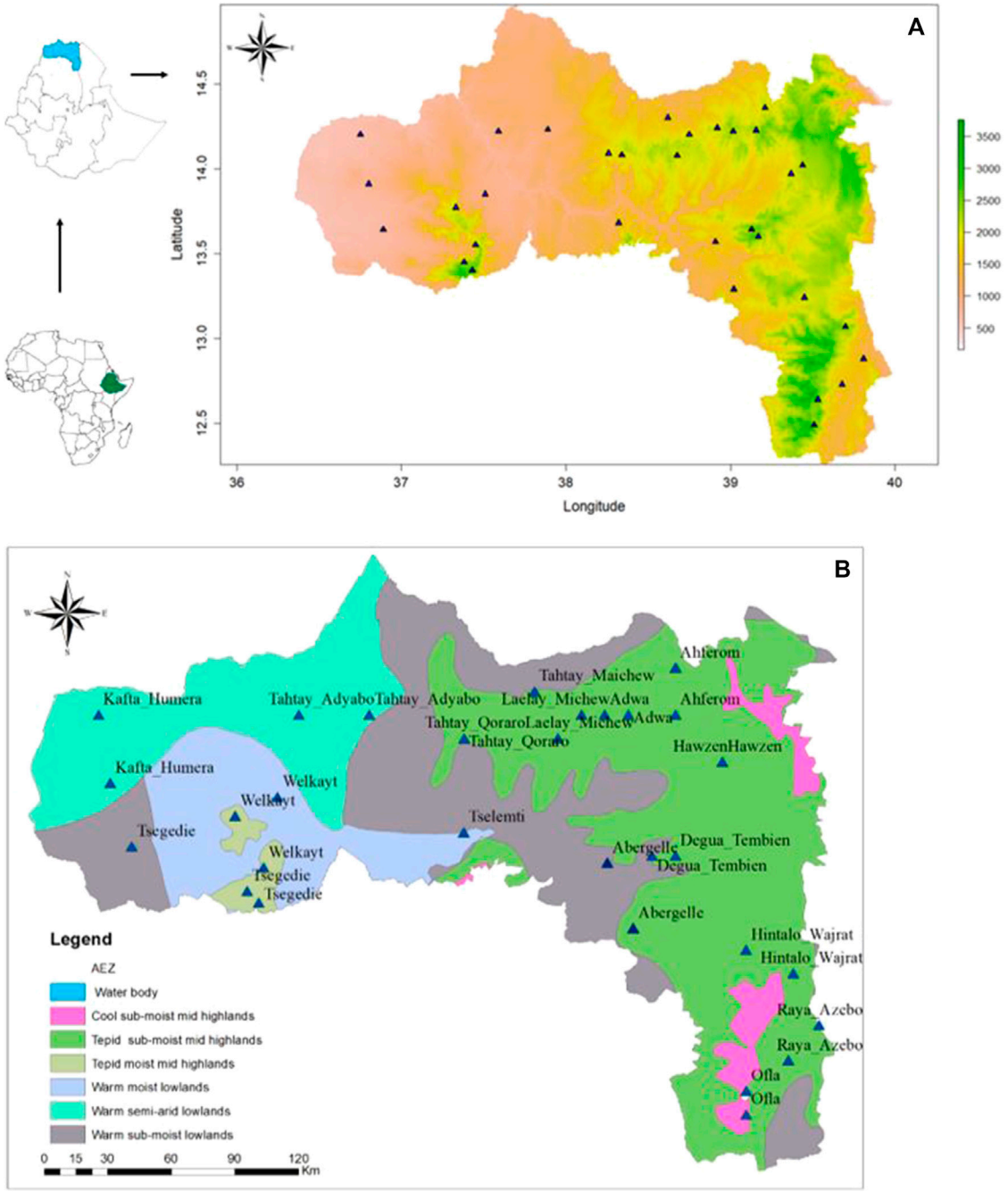


FIGURE 1
Maps showing the sampling sites of indigenous chickens from the Tigray region **(A)** based on bio-elevation, and **(B)** based on major agro-ecological zones; source (MOA, 1998).

Global Food Security Analysis-Support Data (Teluguntla et al., 2015; Oliphant et al., 2017).

R packages' rgdal', 'maptools', 'rgeos', and 'raster' were used to adjust the dimension and extension of the grid to 1 km^2 based on the earth-fixed terrestrial reference system and geodetic datum WGS84 for the agro-ecological variable 'raster' layers. Rather than representing the sampling area with a single point, we added proximate areas to enhance the probability of an accurate description of the area. Hence, each sampling population represents ten sampling points (one sampling point and nine nearby surrounding areas within 1.2 km^2

TABLE 1 Sampling areas.

Wereda (districts)	Villages	Latitude	Longitude	Altitude in meter (m.a.s.l.)	Agroecology
Abergelle	Adi_Weyane	13.55	38.94	1,699	Midland
Abergelle	Lemlem	13.28	39.06	1,598	Lowland
Hawzien	Debrebzien	14.16	39.39	2,187	Midland
Hawzien	Debrehiwot	13.96	39.38	2,106	Midland
Ofla	Selam-Bkalsi	12.65	39.38	2,809	Highland
Raya_Azebo	Genete	12.76	39.68	1,671	Midland
Raya_Azebo	Rabia-Tsigea	12.84	39.63	1924	Midland
Hintalo_wajirat	Mesano	13.24	39.44	2033	Midland
Hintalo_wajirat	Meseret	13.75	39.72	2,158	Midland
Tahtay-Adyabo	Gemhalo	14.57	37.76	1,062	Lowland
Tahtay-Adyabo	May-Kuhli	14.23	37.73	1,111	Lowland
Tahtay-Qoraro	Adi_Gidad	14.09	38.26	1888	Midland
Tahtay-Qoraro	May-Tafat	14.23	38.34	1895	Midland
Kafta_Humera	May-Kadra	14.07	36.56	626	Lowland
Kafta_Humera	Adi-Goshu	14.15	37.35	1,158	Lowland
Kafta_Humera	Adebay	14.20	36.75	665	Lowland
Welkayt	Mogue	14.05	37.49	907	Lowland
Welkayt	Adi_Remets	13.77	37.33	1970	Midland
Tselemti	May_Dagusha	13.68	38.68	1,245	Lowland
Tselemti	Dima	13.68	38.32	1,613	Lowland
Tsegedie	Enda_mariam	13.39	37.41	2,850	Highland
Tsegedie	Enda_Slassie	13.42	37.39	2,584	Highland
Ahferom	Sefo	14.36	39.25	2,175	Highland
Ahferom	May_Keyah	14.42	39.4	2,419	Highland
Adwa	Mariam_Shewito	14.23	39.05	2,227	Highland
Adwa	Bete_Yohannes	14.24	38.92	2,145	Highland
Laelay_Maichew	Dura	14.2	38.75	1943	Midland
Laelay_Maichew	Madego	14.26	38.71	1,662	Lowland
Tahtay_Maichew	Chila	14.3	38.62	1,570	Lowland
Tahtay_Maichew	Shenako	13.83	38.64	2064	Midland
Degua_Tembien	Melfa	13.64	39.13	2,495	Highland
Degua_Tembien	Seret	13.6	39.17	2,494	Highland

taken as random mid-point sample). Google Earth Pro 7.3.1.4507 (2016) was applied, to identify these points, and then the separate grids were extracted using the 'raster' R package.

Environmental variable selection procedure

The environmental variables were analysed with two investigative approaches—Spearman correlation and Principal Component Analysis (PCA), and one selection process - 'MaxentVariableSelection' (MVS). First, we examined the correlations among the environmental variables. The strengths of correlation was defined as: $r_s \geq 0.8$ very strong, $r_s \geq 0.6$ to <0.8 strong, $r_s \geq 0.4$ to <0.6 moderate, $r_s \geq 0.2$ to <0.4 weak, and $r_s \geq 0$ to <0.2 very weak. Since most of the environmental variables (except soil cation exchange) did not follow a normal distribution pattern (Shapiro-Wilk normality test output: W = 0.6–0.98; p = 2.20e-16 to 4.60e-04), correlations among the variables were tested using Spearman's rank correlation coefficients (r). The correlations were evaluated using a threshold value of 0.6 with $p < 0.0001$. The results were plotted using the R package 'corrplot' (Wei et al., 2017). Then, the PCA was performed to assess each variable's contribution and relationship in their respective group (climatic, soil, and vegetation and land cover) using the R package 'stats'. The variables' eigenvector (direction)

TABLE 2 Climatic variables analysed.

	Variables	Description	Units	Database
Climate variable	Bio1	Annual Mean Temperature	^{0}C	WorldClim - Global Climate Data http://www.worldclim.org/
	Bio2	Mean Diurnal Range (Mean of monthly max temp - mean of monthly min temp)	^{0}C(Bio2/Bio7)	
	Bio3	Isothermality (BIO2/BIO7) (* 100)	^{0}C	
	Bio4	Temperature Seasonality (standard deviation *100)	^{0}C	
	Bio5	Max Temperature of Warmest Month	^{0}C	
	Bio6	Min Temperature of Coldest Month	^{0}C	
	Bio7	Temperature Annual Range (BIO5-BIO6)	^{0}C(Bio5-Bio6)	
	Bio8	Mean Temperature of Wettest Quarter	^{0}C	
	Bio9	Mean Temperature of Driest Quarter	^{0}C	
	Bio10	Mean Temperature of Warmest Quarter	^{0}C	
	Bio11	Mean Temperature of Coldest Quarter	^{0}C	
	Bio12	Annual Precipitation	mm/m^2	
	Bio13	Precipitation of Wettest Month	mm/m^2	
	Bio14	Precipitation of Driest Month	mm/m^2	
	Bio15	Precipitation Seasonality (Coefficient of Variation)	mm/m^2 (Coefficient of variation)	
	Bio16	Precipitation of Wettest Quarter	mm/m^2	
	Bio17	Precipitation of Driest Quarter	mm/m^2	
	Bio18	Precipitation of Warmest Quarter	mm/m^2	
	Bio19	Precipitation of Coldest Quarter	mm/m^2	
	WatVapPress01	Water Vapor Pressure of the wettest month	kPa	
	WatVapPress01	Water Vapor Pressure of the driest month	kPa	
	Elevation	Meters above sea level	m.a.s.l	
Soil variable	Soil_pH	Soil pH	pH (x10 in H_2O)	Global gridded soil information https://soilgrids.org/
	Soil_CatEx_Capacity	Soil Cation Exchange Capacity	cmole/kg at depth 0.00 m	
	Soil_Bulk_D	Soil Bulk Density	kg/m3 at depth 0.00 m	
	Soil_Organic Carbon	Soil Organic Carbon	g/kg at depth 0.00 m	
	Soil_Clay	Soil Clay Content	mass fraction in % at depth 0.00 m	
	Soil_Silt	Soil Silt Content	mass fraction in % at depth 0.00 m	
	Soil_Sand	Soil Sand Content	mass fraction in % at depth 0.00 m	
	Soil_Water_Capacity	Soil total available Water Capacity	mm2/1 mt soil depth	Spatial Data Access Tool (SDAT)-NASA https://webmap.ornl.gov/ogc/dataset.jsp?ds_id=546
Vegetation variable	Forest	Forest Cover	%	Harmonized World Soil Dataset http://www.fao.org/soils-portal/soil-survey/soil-maps-and-databases/harmonized-world-soil-database-v12/en/
	Grass_Land	Grass/Shrub Land	%	
	Cult_L	Land use for agricultural purpose (Cultivated land)	%	
	Crop_Dominance	Crop Dominance (major crops)	Category	Global Food Security Analysis-Support DATA https://croplands.org/

and eigenvalue (magnitude) were assessed in the PCA-based inspection. In the final variable selection process, all variables were simultaneously evaluated using the R package MVS (Jueterbock, 2015) to select a set of uncorrelated and high contributing variables for the execution of the ENM.

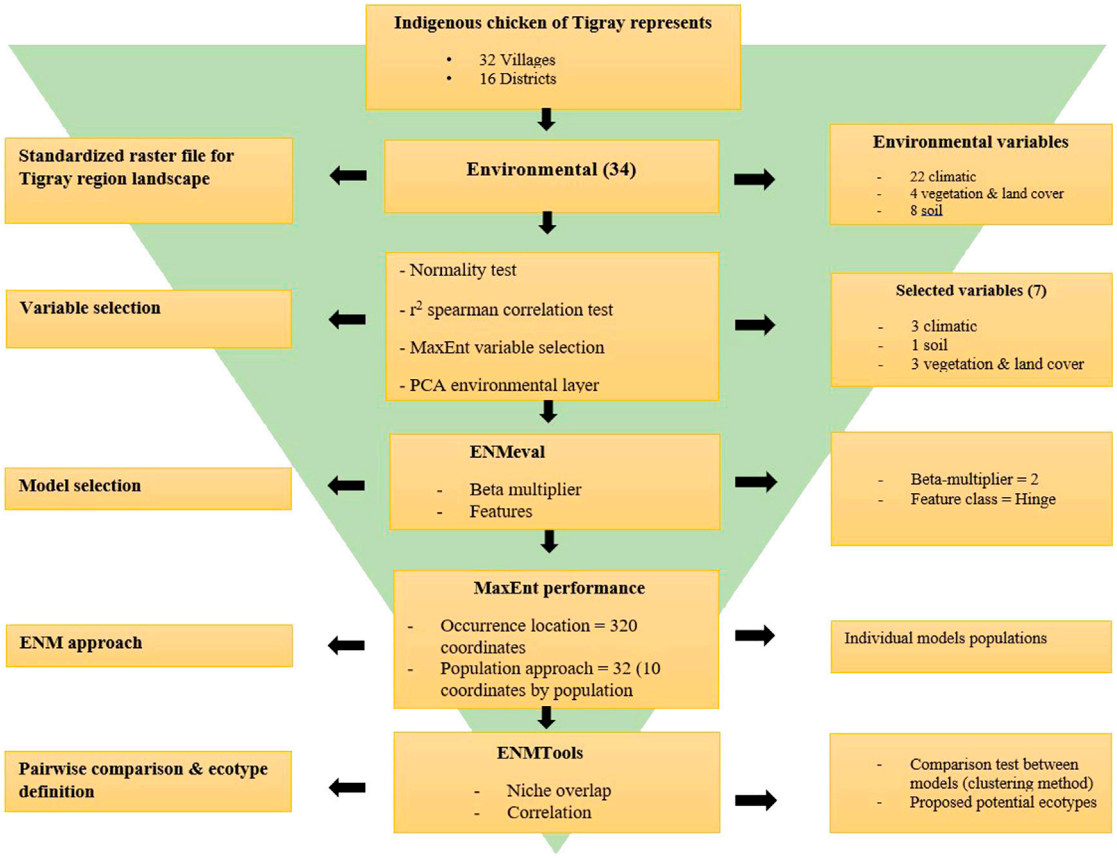


FIGURE 2
Flowchart of pipeline for Tigrayan chicken ecotype definition following the Ecological Niche Modelling (ENM) approach.

Selection of model parameters

The performance of ENM can be affected by the model parameters—feature class (FC) and beta-multiplier (BM), and the default setting may not be ideal for generating maximum entropy (Anderson and Gonzalez, 2011; Cao et al., 2013). The FC work on transforming the environmental predictors to model complex relationships (Elith et al., 2010). The BM helps to prevent over-complexity or overfitting of the model by manipulating the intensity of the nominated FC (Merow et al., 2013). ENMeval package (Kass et al., 2021) was applied to choose the best combination of FC and BM. The following FCs were tested: linear (L), quadratic (Q), product (*p*), hinge (H), categorical (C), and threshold (T), in combination with different values of BM ranging from one to twelve. The least Akaike Information Criterion, corrected for small samples (AICc) values, was considered as the point for the optimal FC and BM combination (Muscarella et al., 2014). The best FC and BM identified here were used to optimise the MaxEnt and to develop the suitability maps.

Ecological niche modelling

MaxEnt (ver. 3.4.1; https://biodiversityinformatics.amnh.org/open_source/maxent/) was used for the ENM analysis of the environments of district (Phillips et al., 2006). First, we ran the MaxEnt with all 320 points as a single entity to evaluate the model's performance, and then we run it for the individual 16 districts. In each ENM run, we withheld 25% of the occurrence data as testing, while we used the remaining 75% as training; we then applied the regionalised ten k-fold cross-validations (Vallejo-Trujillo et al., 2022). We used the logarithmic scale of logistic and cumulative outputs to develop the niches. The logistic output was used for the pairwise comparison of the models and the cumulative one was used to display the suitability habitat.

We used the Area Under the Receiver Operating Characteristics (ROC) Curve (AUC) to evaluate the model's accuracy (Phillips et al., 2006). The AUC values ranged from 0 to 1, where 1 is most suitable, and 0 is unsuitable, with values in between showing suitability in gradient. Further, the jackknife

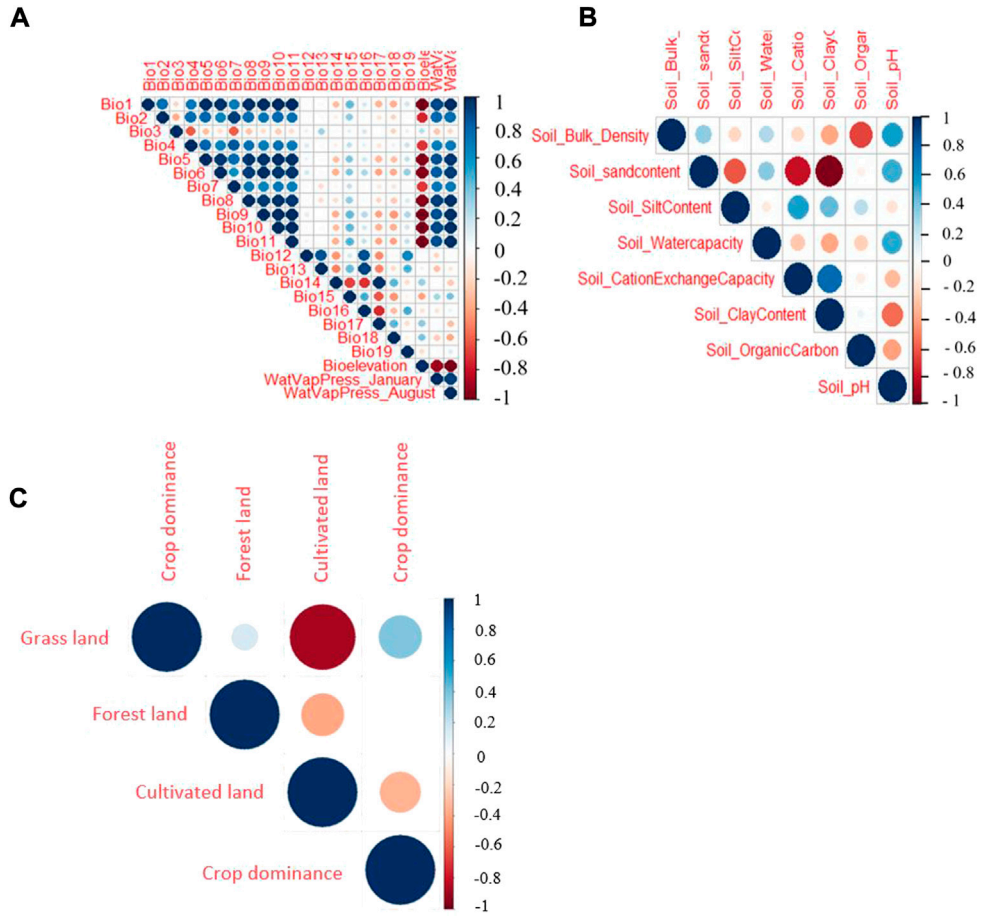


FIGURE 3
Spearman correlation test for three different groups of climatic and environmental parameters evaluated **(A)** Climatic variables, **(B)** soil variables, **(C)** vegetation and land cover variables.

was used to assess the importance and contribution of the variables.

Pairwise comparison of population models for ecotypes definition

The ENMTools Perl software (Warren et al. (2008); Warren and Seifert, (2011)) was applied to assess the similarity of habitats among the populations following Warren et al. (2008) approach that runs in two steps. In the first procedure, we run Pearson's pairwise correlation between the population models, with coefficient values ranging from -1 to +1 (negative values indicate negative linear correlation, positive values indicate positive linear relationship, and if it approaches zero, it indicates no linear correlation between the district's niches). The second procedure used Hellinger's distance or Niche overlap (noted with the letter 'I') with values ranging from 0 to 1 (a value close to one means the niches are closely similar, and a value close to zero, they are entirely distinct) (Warren et al., 2008). Similarity metrices from these two methods were then used to cluster the populations into candidate ecotypes as follows.

We applied 'stats' and 'cluster' packages of R to calculate the "Euclidean distance" of each dataset (Pearson's pairwise correlation and Hellinger's distance). Using these Euclidean distance values, we performed hierarchical clustering. We clustered the topologies by calculating the agglomerative coefficients of the single or minimum linkage, complete or maximum linkage, average or UPGMA, and Ward methods. We chose the Ward method due to its largest agglomerative coefficient value that explains the strength of the structuring.

Finally, for easy visualisation of the similarity between ENMs, we generated dendrograms and heatmaps for each dataset using the 'ggplot2' and 'Reshape2' R packages. This helped to classify the populations from the districts into potential ecotypes in

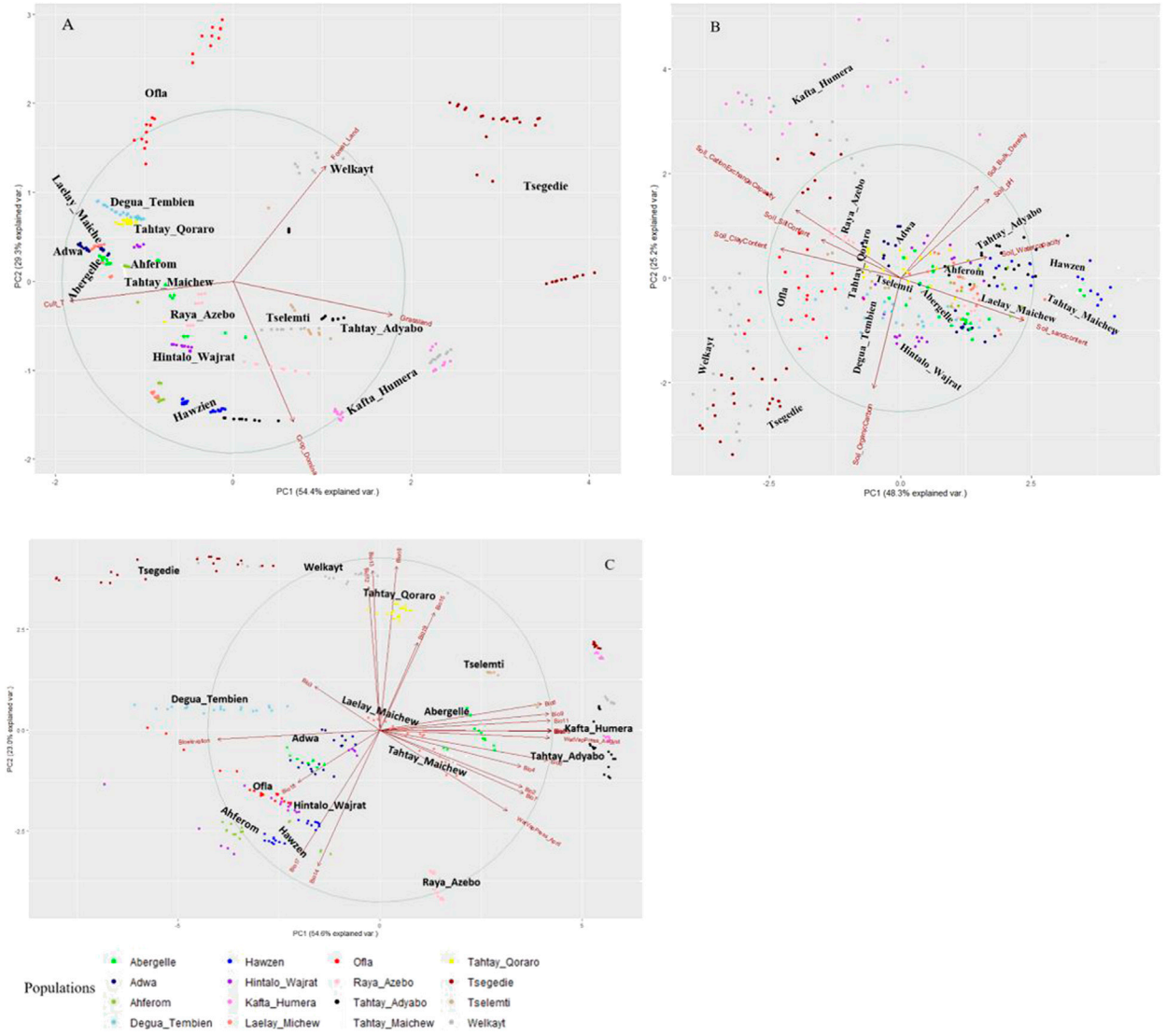


FIGURE 4
Principal component analyses of 16 Tigray chicken districts based on agro-ecological and climatic variables **(A)** vegetation and land cover variables, **(B)** soil variables, and **(C)** climatic variables.

combination with the Jackknife of AUC and percent contribution of the variables. The summary of the procedure we followed is presented in Figure 2.

Results

Correlation-based explanation of variables

Before using for ENM, the environmental variables were shortlisted by removing highly correlated variables and those with a low contribution to the model. Hence, a threshold of $r_s > 0.6$ (with a p-value of 0.001) was used to remove variables from a correlated set except for the one variable expected to be the most relevant one to the indigenous chicken biology. The Spearman's rank-order correlation results for the three groups of variables (climatic 'A', soil 'B', and vegetation and land cover 'C') are shown in Figure 3. In the soil group, there are five variables with a correlation coefficient <0.6 (the threshold for retaining variables for ENM). These are the pH, water capacity, and contents of organic matter, clay, and silt. The vegetation and land cover group (forest land, crop dominance, and grassland) are not strongly correlated. Based on the

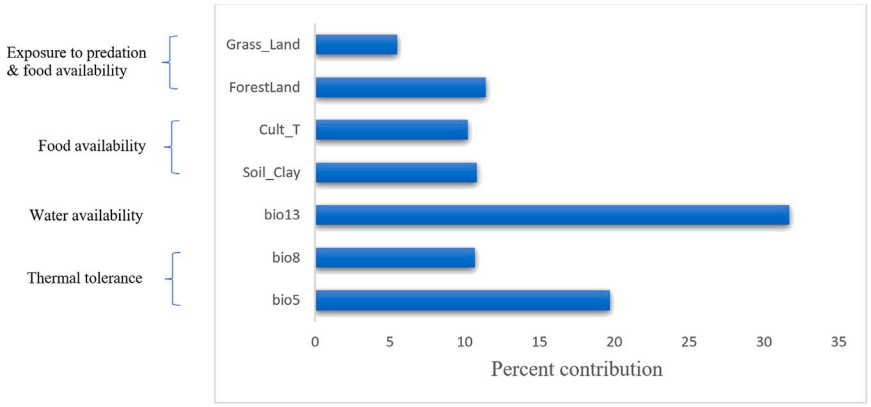


FIGURE 5
Percent contribution of the final selected seven variables using MVS. The left-hand side of the diagram shows the biological importance of these variables to environmental adaptation in chicken.

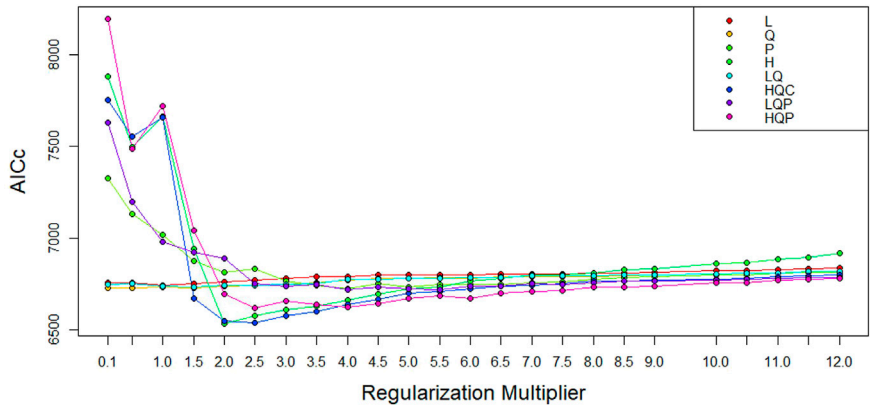


FIGURE 6
AICc values for analyzed feature class (FC) combinations using different beta-multiplier (BM) values using ENMeval.

correlation from the climatic variables, we find six variables with a correlation <0.6 out of the 22 variables, namely bio3 (isothermality), bio10 (mean temperature of the warmest quarter), bio13 (precipitation of the wettest month), bio17 (precipitation of the driest quarter), bio18 (precipitation of the warmest quarter), and bio19 (precipitation of the coldest quarter).

PCA of Tigrayan chicken populations based on agro-climatic variables

We perform the PCA-based clustering to see how the 34 environmental variables cluster the indigenous chicken population and the association of each variable with the population. The PCA plots of Tigrayan chicken samples based on environmental data (Figure 4) helped us to assess the environmental variables' contribution and association. The arrows and direction of the variables show which PC and axis (x or y) are associated with the shown variance. The arrow lengths indicate the extent of the contribution of a variable in explaining the populations' environmental structure. The first two principal components (PC1 and PC2) represent more than 73% of the variance, with the distribution of the populations widely different among the PCAs using the three defined variable clusters (vegetation and landcover 'A', Soil 'B', and climatic 'C'). All four vegetation and land cover variables show high variation (Figure 4A). For the soil variable, we see several variables showing contribution in the same direction (no variation) (e.g. soil bulk density and

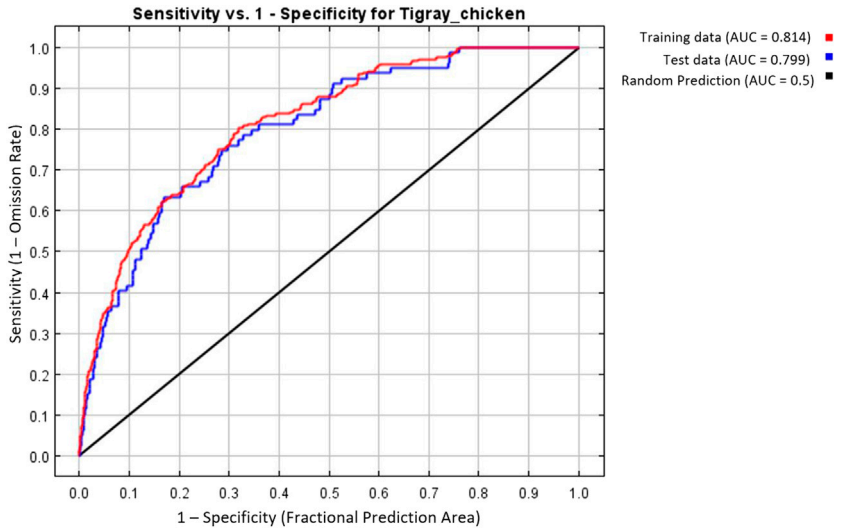


FIGURE 7
MaxEnt model based on seven selected variables and the best feature (H) and beta-multiplier value (two). Area Under Receiver Operating Curve for training and test data.

soil pH, or soil clay content and soil cation exchange capacity) (Figure 4B). Four variables, namely soil organic, sand, clay, and water capacity content, show high variation. Within the climatic variables (Figure 4C), the water vapor pressure for the hottest month (April) and bio-elevation show strong variation and no association with other variables. On the contrary, variables bio3, bio4, bio18, and bio19 show weak contributions. Despite differences between PC plots, several districts appeared repeatedly close to each other, such as Welkayt - Tsegedie, Degua Tembien—Tahtay Qoraro, Tselemti—Tahtay Adyabo, and Adwa—Laelay Maichew. It suggests a homogeneous landscape configuration for these districts.

MaxentVariableSelection (MVS) package for variable selection

While the correlation and principal component analyses described above explored the relationships among variables (soil, vegetation and land cover, and climatic), MVS was used to simultaneously analyse all variables to select the most important set of uncorrelated variables ($r < 0.6$). Accordingly, seven variables (three from climatic variables (bio5 = maximum temperature of the warmest month, bio8 = mean temperature of the wettest quarter, and bio13 = precipitation of the wettest month), three from vegetation and land cover variables (grassland, forestland, and cultivated land) and one soil variable (clay content)) were selected (Figure 5).

Selection of model parameters

To develop the optimal ENM, we selected the best combination of FC (H) and BM (=2) out of the192 model combinations evaluated using the ENMeval package (Figure 6, Supplementary Table S1). The selected FC and BM combination resulted in a better prediction potential of the suitable niches than the default values (Supplementary Figure S1).

ENM development using selected predictors and parameters

MaxEnt model was executed with the seven selected variables and the best combination of FC (Hinge) and BM (=2) to predict the suitability habitat of each indigenous chicken population and to identify environmental variables that define each habitat. To evaluate the model prediction efficiency, MaxEnt produces different outputs (Figures 7–9). The AUC values 0.814 and 0.799 for training and test data, respectively (Figure 7), showed the model's excellent prediction power. The AUC values of the individual populations range from 0.9854 to 0.9981 for the training sample and from 0.9693 to 0.9946 for the test sample. The individual variable AUC value also displayed excellent prediction power (>0.9) except for a few variables across different populations showing moderate prediction power (Supplementary Table S3).

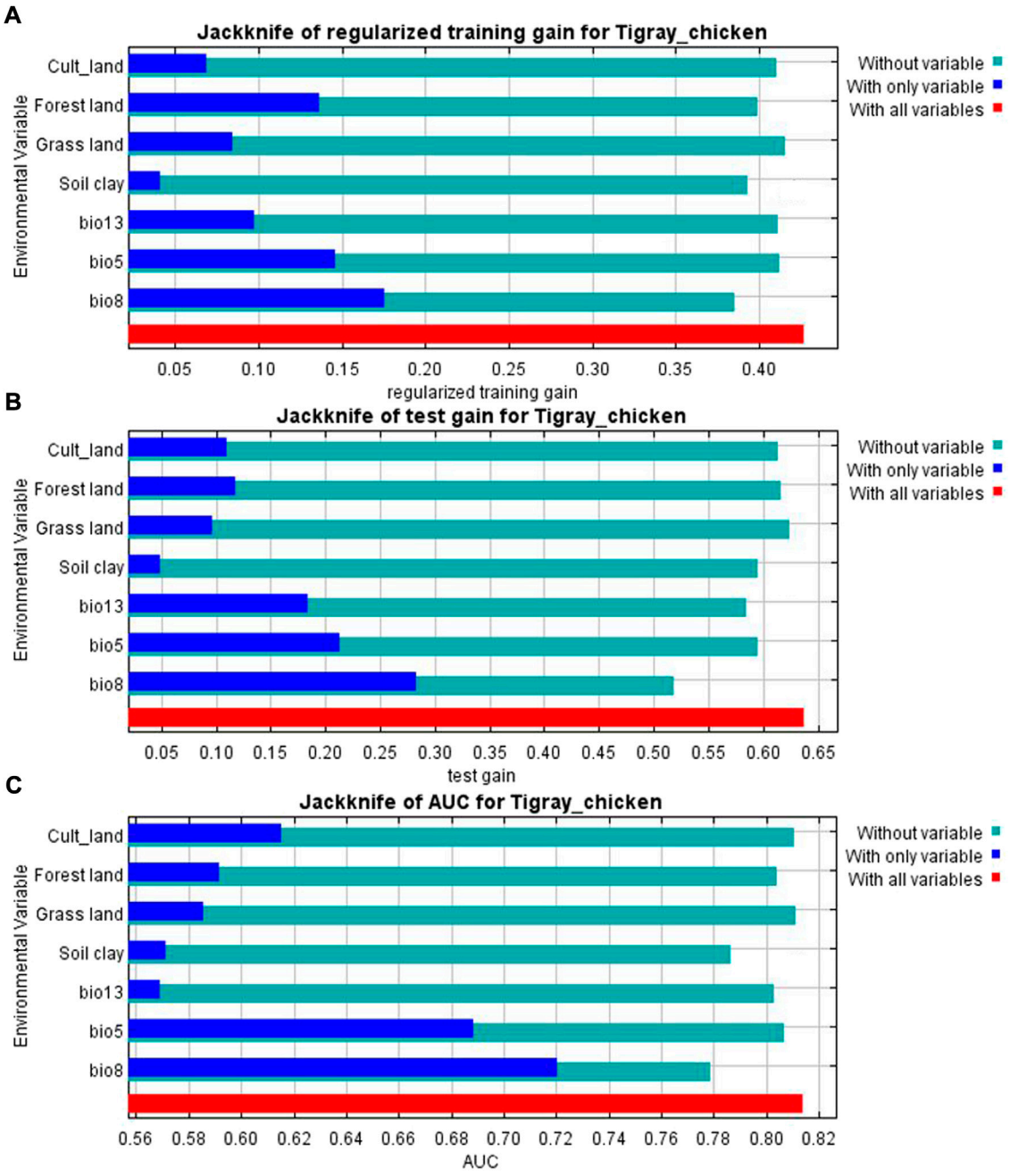


FIGURE 8
(A) Jackknife result for AUC (Area Under Receiver Operating Curve) **(B)** Jackknife of training gain and **(C)** test gain for ENM produced for the complete set of analyzed populations.

The variables' importance was also checked through the jackknife tests (Figures 8A,B) for regularized training and test gains. According to these tests, the variables that contributed most to the model were bio13, bio5, and bio8. Most of the selected variables contributed to the overall model (Figure 8C), except the soil clay content. Moreover, variable importance was not only evaluated by their contribution to the prediction power of the model but also their contribution to the model building (Çoban et al., 2020). Variables that contributed most to the model were also explained by their percentages of contribution (>6.9) and their permutation importance (>9.8 except bio13) (Supplementary Table S2) with the response curves explaining how the individual variables affect ENM prediction. The logistic prediction

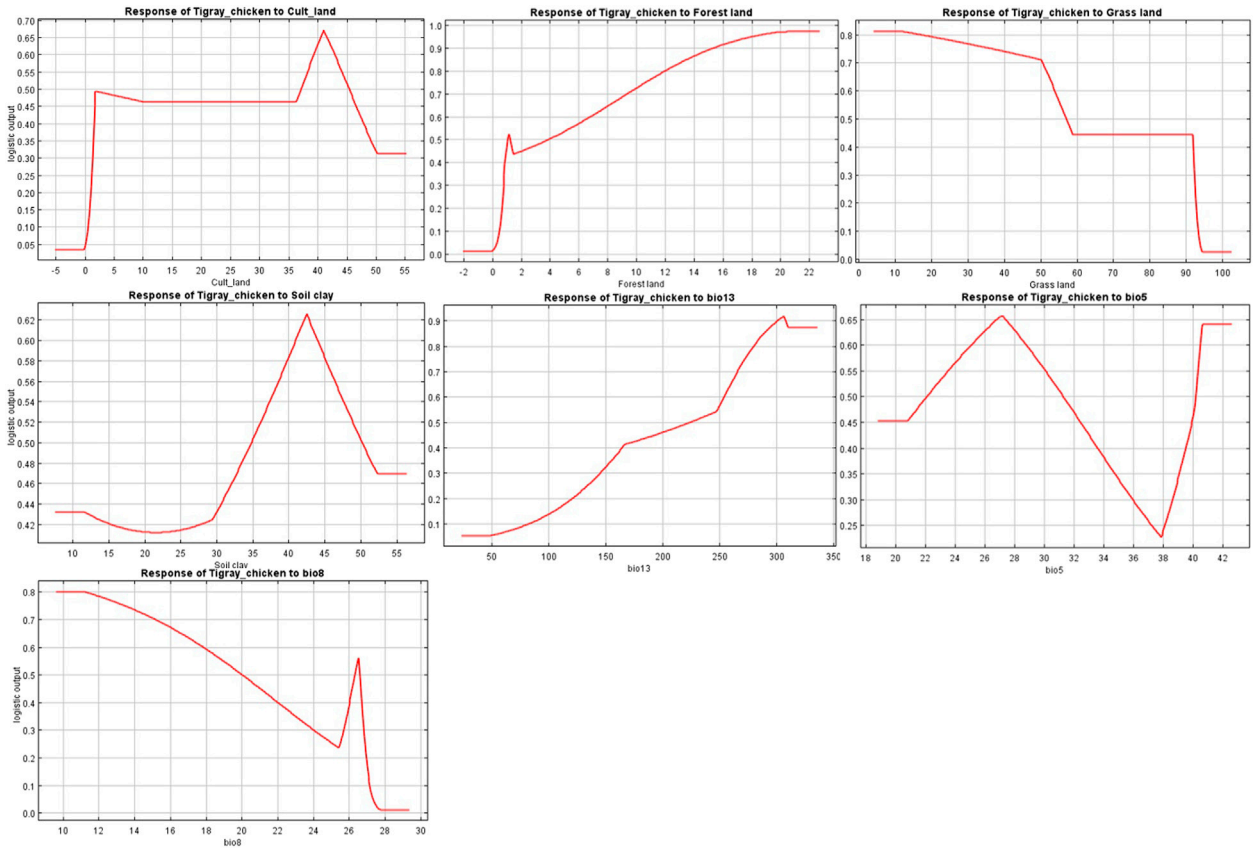


FIGURE 9
Individual response curves for seven environmental variables selected for the final Maxent model.

varied with the individual variable while the other variables were kept at their average value (Figure 9).

Pairwise comparison of population models for ecotypes definition

For the ecotype definition, we present two approaches, pairwise Pearson correlation and niche overlap (Vallejo-Trujillo et al., 2022). Both methods group the Tigray indigenous chicken habitats into four agro-ecologies. Twelve districts out of 16 showed consistent clustering in both methods. The exceptions are Tselemti, Tahtay Adyabo, Degua Tembien, and Laelay Maichew. The highest agglomerative coefficient was obtained with Ward Method of hierarchical clustering - 0.83 for niche overlap and 0.77 for the Pearson correlation (Figures 10, 11). Henceforth, it was selected for the clustering of the Tigray chicken populations.

Besides the above dendrogram and heat map categories, by considering their Jackknife of AUC and percent of the contribution, we proposed four agro-ecologies, namely: agro-ecology 1 - Tselemti, Abergelle, Tahtay Adyabo, and Raya Azebo; agro-ecology 2 - Welkayt, Tsegedie, and Kafta Humera; agro-ecology 3 - Tahtay Maichew, Hawzen, Ahferom, and Adwa; and agro-ecology 4 - Tahtay Maichew, Degua Tembien, Tahtay Qoraro, Ofla, and Hintalo Wajrat. We also identified the environmental variables that define each suitable agro-ecology (Table 3). Following the four distinct chicken agro-ecologies, we proposed four distinct indigenous chicken ecotypes (Figure 12).

Discussion

We have applied here an ecological niche modelling approach, using 34 agro-climatic variables. It allowed us to provide the first detailed environmental characterisation of Tigrayan chicken habitats, an important premise to define ecotypes and to study their adaptive diversity, with important implications for their management, conservation, phenotypic and genetic characterisation. Besides, we have also provided

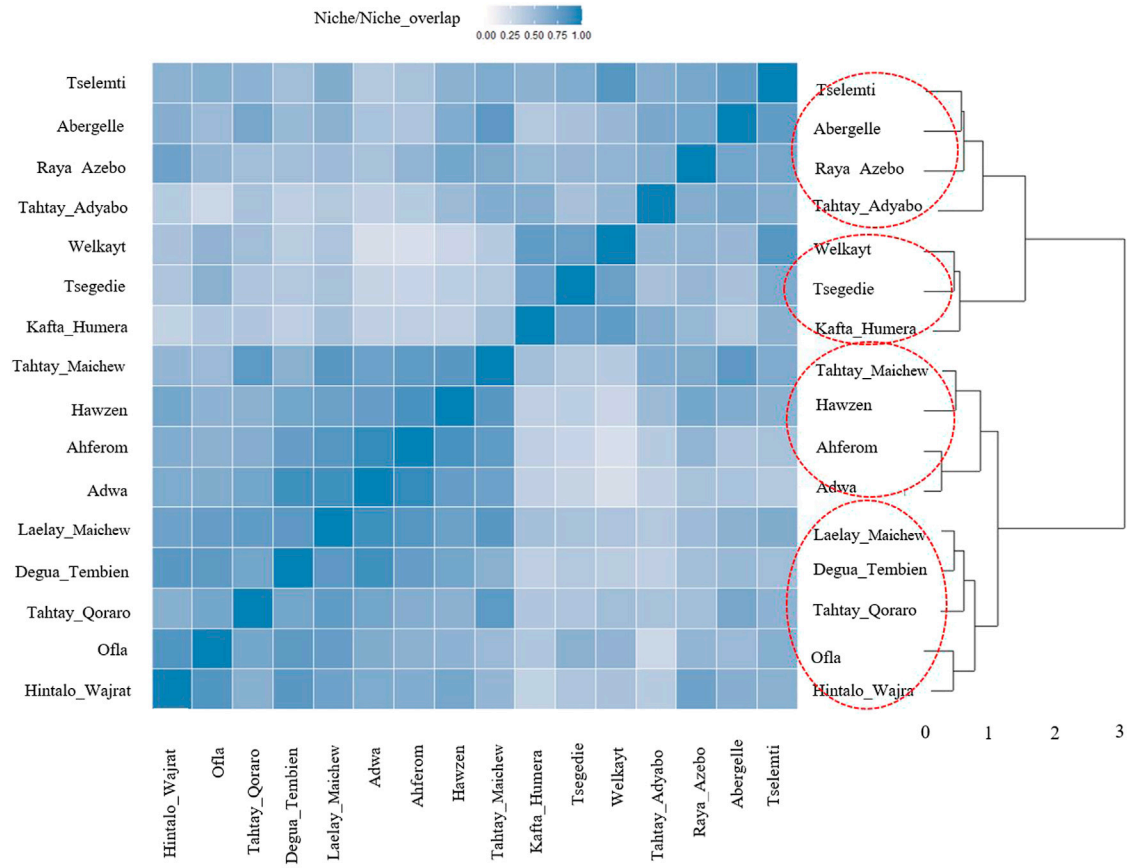


FIGURE 10
Dendrogram and heatmap of niche overlap statistics (I) between suitability maps for individual population.

here the detailed protocols for the application of ENM, that we expect will facilitate its adoption for the environmental characterization of livestock population habitats.

Although, some studies have already been undertaken to define chicken agro-ecological zones in Ethiopia, they did not represent (Kebede et al., 2021) or poorly represented (Gheyas et al., 2021; Vallejo-Trujillo et al., 2022) the Tigray region. Specifically, the studies of Gheyas et al. (2021) and Vallejo-Trujillo et al. (2022) did not include any population >2,312 m. a.s.l. And below 1,295 m. a.s.l.

Environmental variable selection and their contribution to chicken biology

ENM for suitable habitat prediction and potential ecotype definition require selecting appropriate variables to enhance the model prediction power. Prior knowledge of the species ecology is also essential in selecting the correct environmental variables (Zeng et al., 2016; Fourcade et al., 2018). Besides, the variables must be related to the life history of the species under study. Previous studies have emphasized that the variables for ENM must be selected with great care as they should be uncorrelated and with a high contribution to the biological need of the species (Reunanen, 2003; Zhu et al., 2012; Sangermano et al., 2015; Pitt et al., 2016). Removing correlated variables using PCA, correlation matrix, or any other dimension reduction methods will reduce model complexity (Merow et al., 2013). Hence, we followed a rigorous procedure, including PCA, correlation, and MVS, to select the least correlated variables. The procedures followed and our first-hand knowledge of the study area and of indigenous chicken helped us to select the most appropriate variables.

The final set of selected variables are indeed of relevance to the physiological need of the chicken; bio5 (maximum temperature of the warmest month) and bio8 (mean temperature of the wettest quarter) may be associated with bird thermotolerance - an important phenotype in the Tigray region where the temperature may fluctuate between 3 and 46°C. Bio13 (precipitation of the wettest month) is linked to water availability, equally crucial for the physiology of the

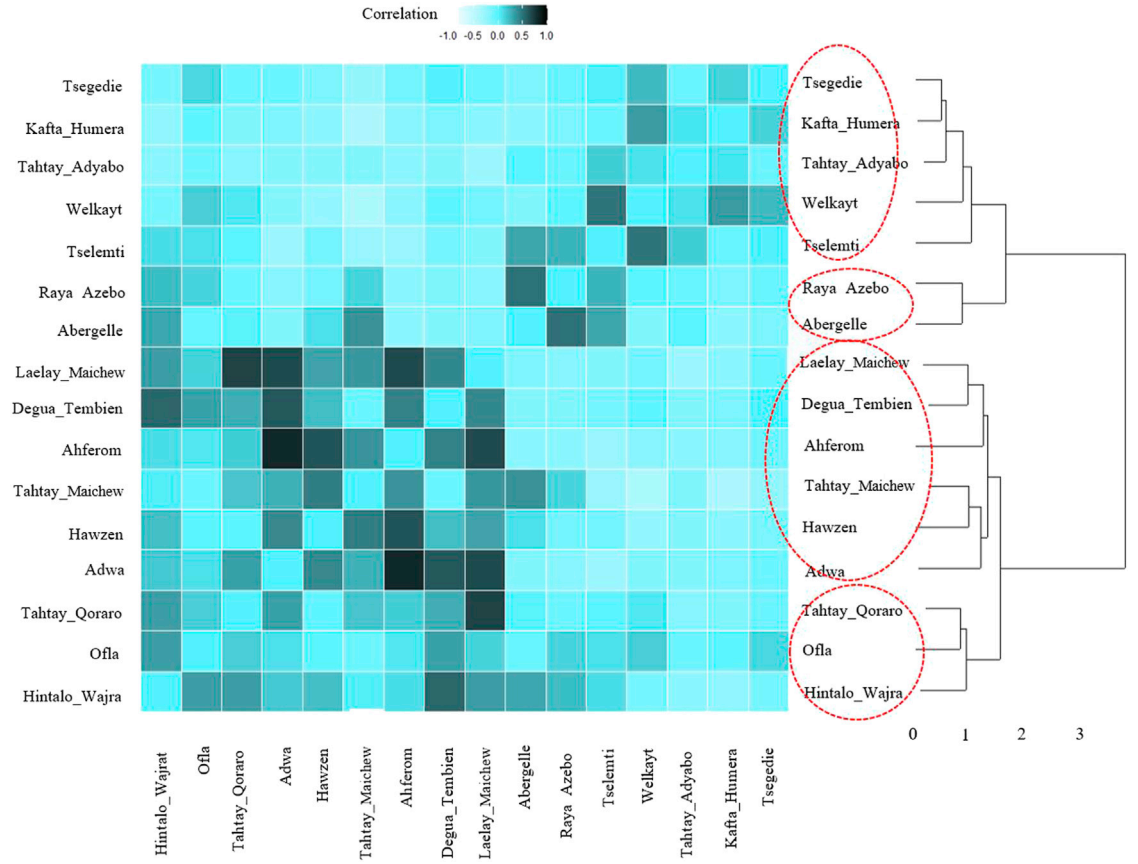


FIGURE 11
Dendrogram and heatmap of pairwise Pearson correlation coefficient between suitability maps for individual population.

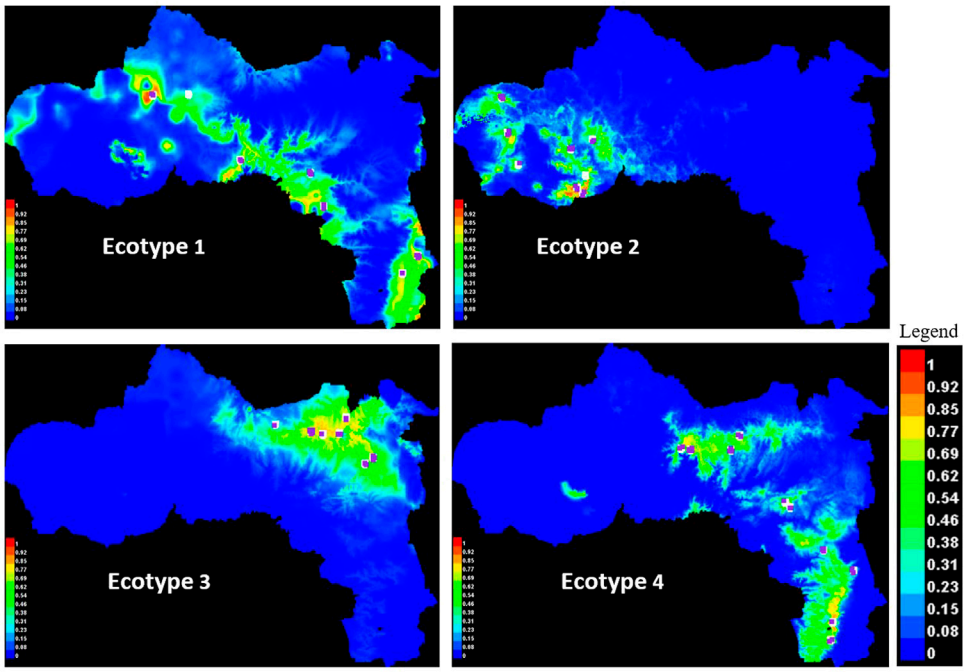
TABLE 3 Major contributing agro-ecological variables for each proposed ecotype.

Proposed ecotypes	Populations	Major contributor parameters among ecotypes (percent of contribution)
1	Abergelle, Tselemti, Raya Azebo, and Tahtay Adyabo	bio5 (39%), Forest (21.4%), Grass_Land (18.8), and Cult_L (17.2%)
2	Welkayt, Kafta Humera, and Tsegedie	bio13 (41.9%), Cult_L (26.4%), Soil_Clay (15.9%), bio5 (9%), bio8 (5.1%)
3	Tahtay Maichew, Hawzien Ahferom, and Adwa	bio5 (50.4%), Forest (40.5%), and Cult_L (8.2%)
4	Laelay Maichew, Degua Tembien, Tahtay Qoraro, Ofla, and Hintalo Wajrat	bio8 (43.4%), Grass_Land (24.8%), Soil_Clay (17.4%), and Cult_L (5.1%)

Bio5 = Maximum temperature of warmest month; bio8 = Mean temperature of the wettest quarter; bio13 = Precipitation of wettest month; Forest = Forest cover; Soil_Clay = Soil clay content; Cult_L = land use for agriculture purpose; Grass_Land = Grass/shrub cover.

chicken (Pitt et al., 2016). Cultivated land and soil clay content are associated with food availability, and grassland and forest land coverage may be related to scavenging food, predation exposure and protection (Figure 5) (Gheyas et al., 2021; Vallejo-Trujillo et al., 2022). Our selected variables (grassland cover, forest cover, cultivated land, and soil clay content) aligned with the variables (grassland cover, cultivated land, crop dominance, and soil organic content) reported previously in other studies for Ethiopian indigenous chicken (Gheyas et al., 2021; Vallejo-Trujillo et al., 2022).

The shortlisted climatic variables bio5, bio8, and bio13 based on MVS are also related to the climatic variables bio6 (minimum



Ecotype	Ecotype 1	Ecotype 2	Ecotype 3	Ecotype 4
Population	Abergelle, Tselemti, Raya Azebo, and Tahtay Adyabo	Welkayt, Kafta Humera, and Tsegedie	Tahtay Maichew, Hawzien Ahferom, and Adwa	Laelay Maichew, Degua Tembien, Tahtay Qoraro, Ofla, and Hintalo Wajrat

FIGURE 12
Suitability maps for Tigray chicken populations grouped by ecotype.

temperature of coldest month), bio15 (precipitation seasonality), bio16 (precipitation of wettest quarter), and bio17 (precipitation of driest quarter) shortlisted in previous studies (Gheyas et al., 2021; Vallejo-Trujillo et al., 2022). The variables selected in our study are also similar to variables (bio5, bio13, soil clay, grassland, forest land, and cultivated land) reported for the wild Red junglefowl (Pitt et al., 2016). Pitt et al. (2016) reported geographic areas suitable to domestic chicken on the African continent using the environmental information associated to today's geographic distribution of the Red Junglefowl. The Tigray region is one of these regions. Tigray is also a center of ancient civilization which is geographically close to Red Sea and ancient commercial maritime routes. Interestingly, it is in the Tigray region that the early osteological evidence of domestic chicken have been found on the African continent (Woldekiros and D'Andrea, 2017).

Ecological niche modelling procedures for agro-ecology classification and ecotype definition

Running MaxEnt with the default "black-box" does not guarantee an optimal model (Radosavljevic and Anderson, 2014). Instead, it may produce either over-complex or over-simple models Phillips et al. (2006); Cao et al. (2013); Elith et al. (2010); Ribeiro et al. (2016); Shcheglovitova and Anderson (2013), and overall suitability niche output can vary when applied with default and optimum model parameters, and attention should be given to the methodology when using MaxEnt for ENM (Morales et al., 2017). Hence, to get quality MaxEnt outputs, we need to be cautious in selecting the two parameters, feature classes and regularisation multiplier (Warren and Seifert, 2011; Merow et al., 2013; Morales et al., 2017). While selecting the two parameters, we should also consider the region's geographic boundaries (Merow et al., 2013).

We applied the ENMeval package to choose the best-fit settings, hinge (H) FC and BM = 2 (Figure 6) to predict suitable habitats for the studied areas. The selected H feature uses a linear function in the fitted function to transform the continuous predictors (environmental variables) to a binary output, zero below the threshold and one above the threshold (Elith et al., 2011). Using the H feature class in model development has numerous advantages: it produces smooth models, it allows complex relationships to be modelled in training data, it contributes to model improvement, it is considered as default by MaxEnt, it is

applicable to a small number of sampling sites (minimum 15), and it may replace the quadratic product, and threshold features (Phillips and Dudı, 2008; Elith et al., 2010; Merow et al., 2013). The BM (2) we chose also helped us control the model complexity by imposing a penalty.

Validation of the developed niches using statistical tests boosts the biological meaning of the model (Warren and Seifert, 2011). Therefore, we evaluate the niche similarities of the indigenous chicken populations using ENMtools by calculating the niche similarity and correlation between suitability maps (dendrogram and heat map). Besides the validation using ENMtools, we considered the Jackknife AUC and percent of contribution to further confirm the proposed four agro-ecologies.

As expected, geographically close districts generally clustered together into the same ENM agro-ecology; e.g., agro-ecology 1 Tselemti and Abergelle, agro-ecology 4 Tahtay Qoraro, and Laelay Maichew, and agro-ecology 2 and 3 for the other districts (Figure 12). However, some districts belong to different agro-ecologies despite being geographically close (Ofla and Raya Azebo; Ahferom, Adwa, and Laelay Maichew). The Ofla district belong to agro-ecology 4 and the Raya Azebo district to agro-ecology 1. Similarly, the Ahferom, Adwa, and Laelay Maichew district while geographically close displayed minimum niche overlap. They are classified in different agro-ecologies with Ahferom and Adwa district included within agro-ecology 1, and the Laelay Maichew district within agro-ecology 2. It illustrates the diversity of agro-ecologies found within the Tigray region, even within a close geographic range.

The occurrence of new habitats due to environmental change is the main reason for the formation of a "new variety" (Darwin, 2004) or, in this current context "ecotype". Darwin also emphasised that populations that adapt to a new environment may survive, resulting in the gradual formation of new species. Transpose to the evolution of livestock, introducing a population to a novel habitat may result in new phenotypes following natural selection (Schluter and Nagel, 1995; Adams and Huntingford, 2004; Lahti et al., 2009). Accordingly, we propose four potential Tigrayan chicken ecotypes that may display different chicken genotypes and phenotypes.

The identification of these four ecotypes may further guide both genetic improvement and conservation efforts, maintaining the unique adaptation of the indigenous populations. Different strategies may be envisaged here. Within ecotypes productivity improvement may be envisaged at poultry stations, as it has been the case in Ethiopia for the Horro chicken (Dana et al., 2011), or following an open nucleus breeding scheme *in-situ* at village level (Gondwe et al., 2001; Okeno et al., 2013). Alternatively, crossbreeding may be envisaged, for example, crossing of improved local cocks lines with a commercial hen (Kgwatalala et al., 2015). The later may results in immediate productivity improvement, while keeping local environmental adaptation. A medium to long term, it will be important to conserve the unique environmental characteristics of the ecotypes protecting them, for examples, from the local impact of extreme climatic events or political instability (e.g., war). It is now possible to conserve *ex-situ* in biobank male and female poultry primordial germ cells (Hu et al., 2022). Ecotype identification will allow prioritizing the populations to be conserved by providing entry points for the establishment of poultry biobanks.

Conclusion

The environment-based characterization of chicken habitats presented here allowed us to propose four potential indigenous chicken ecotypes associated with different environmental variables. Beyond the current objective, the outcome of this study will guide the conservation of the endangered indigenous chicken ecotypes while providing a standardised framework for new studies on the environmental characterisation of livestock populations, and its link to phenotypes and genotypes. Also, the ecological niche modelling approach describe here may be used to predict future environmental challenges and distributional shift that indigenous chicken ecotypes may be facing owing to climate changes.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Author contributions

GG, AV-T, AG, and OH conceived the research, GG and OH collected the data, and GG, AV-T, AG, and OH analysed the data. GG, GB, AV-T, TD, AG, and OH wrote the paper and all authors approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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Embryonic transcriptome unravels mechanisms and pathways underlying embryonic development with respect to muscle growth, egg production, and plumage formation in native and broiler chickens

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Background: Muscle development, egg production, and plumage colors are different between native and broiler chickens. The study was designed to investigate why improved Aseel (PD4) is colorful, stronger, and grew slowly compared with the control broiler (CB).

Methods: A microarray was conducted using the 7th-day embryo (7EB) and 18th-day thigh muscle (18TM) of improved Aseel and broiler, respectively. Also, we have selected 24 *Gallus gallus* candidate reference genes from NCBI, and total RNA was isolated from the broiler, improved Aseel embryo tissues, and their expression profiles were studied by real-time quantitative PCR (qPCR). Furthermore, microarray data were validated with qPCR using improved Aseel and broiler embryo tissues.

Results: In the differential transcripts screening, all the transcripts obtained by microarray of slow and fast growth groups were screened by fold change ≥ 1 and false discovery rate (FDR) ≤ 0.05. In total, 8,069 transcripts were differentially expressed between the 7EB and 18TM of PD4 compared to the CB. A further analysis showed that a high number of transcripts are differentially regulated in the 7EB of PD4 (6,896) and fewer transcripts are differentially regulated (1,173) in the 18TM of PD4 compared to the CB. On the 7th^{-} and 18th-day PD4 embryos, 3,890, 3,006, 745, and 428 transcripts were up- and downregulated, respectively. The commonly up- and downregulated transcripts are 91 and 44 between the 7th- and 18th-day of embryos. In addition, the best housekeeping gene was identified. Furthermore, we validated the differentially expressed genes (DEGs) related to muscle growth, myostatin signaling and development, and fatty acid metabolism genes in PD4 and CB embryo tissues by qPCR, and the results correlated with microarray expression data.

Conclusion: Our study identified DEGs that regulate the myostatin signaling and differentiation pathway; glycolysis and gluconeogenesis; fatty acid metabolism; Jak-STAT, mTOR, and TGF-β signaling pathways; tryptophan metabolism; and PI3K-Akt signaling pathways in PD4. The results revealed that the gene expression architecture is present in the improved Aseel exhibiting embryo growth that will help improve muscle development, differentiation, egg production, protein synthesis, and plumage formation in PD4 native chickens. Our findings may be used as a model for improving the growth in Aseel as well as optimizing the growth in the broiler.

KEYWORDS

fast and slow growth chicken, 7th- and 18th-day embryo tissues, microarray, reference gene, quantitative real-time PCR

Introduction

Animal agriculture production is essential for supplying protein nutrition to the increasing global human population. The broiler chickens are genetically selected with highly improved production efficiency through rapid growth and high feed efficiency compared to improved Aseel native chicken birds. Therefore, understanding mechanisms regulating rapid muscle growth and high feed efficiency between control broiler and improved Aseel may improve the quality of improved Aseel animal production systems (Niemann et al., 2011).

In high and low production efficiency breast muscle phenotypes, male pedigree broiler breeder chickens were used for a global gene expression cDNA microarray study (Kong et al., 2011; Bottje et al., 2012; Bottje and Kong, 2013). Also, RNAseq global gene expression studies have been performed with breast muscle and duodenal tissue in commercial broilers and low and high residual feed intake broilers, respectively (Lee et al., 2015; Zhou et al., 2015). Global gene expression studies mostly showed that production ability could also be related to different cellular mechanisms such as mitochondrial oxidative stress, inflammatory response, protein degradation, stress responses, growth hormone signaling, cell cycle, apoptosis, and fatty acid transportation. A recent transcriptome study reported that differentially expressed genes are enriched in myogenic growth and differentiate on the 6th and 21st day of breast muscle in modern pedigree broiler chickens compared with legacy chicken lines (Davis et al., 2015). A transcriptome analysis was performed with the pectoralis major muscles of slow- and fast-growing chickens (n = 8) to understand the myopathies related to structural changes and molecular pathways using an 8 × 60 K Agilent chicken microarray histological study. For fast-growing breast meat yield, a functional analysis revealed the favoring of metabolic shifts toward alternative catabolic pathways, oxidative stress, inflammation, regeneration, fibrosis processes, cellular defense, and remodeling (Pampouille et al., 2019). A transcriptome profiling analysis was performed in two chicken lines, that is, high (pHu+) and low (pHu-), using an Agilent custom chicken 8 × 60 K microarray. Between these two lines, 1,436 differentially expressed (DE) genes were found, and they were related to biological processes for muscle development and remodeling and carbohydrate and energy metabolism (Beauclercq et al., 2017). A genome-wide association study (GWAS) was conducted to assess body weight in an F2 chicken population, and a microarray expression study was conducted with the liver of high and low-weight chickens. Also, we identified miR-16 as a critical regulator that will suppress chicken embryo cell proliferation and cellular growth. The mutated miR-16 by inserting 54bp showed a significant increase in body weight, bone size, and muscle mass (Jia et al., 2016). The comparative transcriptome analysis of grouper fish muscle in the hybrid and its parents showed up-regulation of genes related to glycolysis, calcium signaling, and troponin pathways that enhanced muscle growth in the hybrid grouper (Sun et al., 2016). In addition, insulin-like growth factor 1 (IGF1) and a cascade of intracellular components (protein kinase B, mTOR, GSK3β, and FoxO) play a significant role in the regulation of skeletal muscle growth during development and regeneration (Schiaffino and Mammucari, 2011).

Muscle growth contains complex network combinations, that is, cell proliferation, differentiation, and metabolism (Fuentes et al., 2013). In mammals, during the embryonic period, the total skeletal muscle fiber number is initiated, and after birth, only muscle hypertrophy occurs (Rowe and Goldspink, 1969; Timson and Dudenhoeffer, 1990; Rehfeldt et al., 2000). In teleosts, hypertrophic and hyperplastic muscle growth can happen simultaneously during the entire life (Stickland, 1983; Weatherley et al., 1988). However, chickens' total skeletal muscle fiber number is initiated and fixed during the embryonic period (Bhattacharya et al., 2015; Bhattacharya et al., 2019). As a consequence, chicken muscle mass accounts for a better proportion of body weight, and it is an excellent experimental model for studying fundamental growth regulatory mechanisms in vertebrates (Weatherley and Gill, 1985). Therefore, for controlling muscle growth, understanding the mechanisms in chicken is necessary to

optimize poultry. In America, during the mid-19th century, dual-purpose chicken, that is, Barred Plymouth Rock (BPR), a foundational or heritage breed of the modern commercial broilers, was developed by crosses with Black Java, Black Cochin, and Dominique breed with alternating white and black bars of feather pigmentation (Lopez et al., 2007; Dorshorst and Ashwell, 2009).

For egg production, multiple gene interactions in various organs regulate energy metabolism, protein synthesis, and storage (Silversides and Villeneuve, 1999). Previous genomic and transcriptomic reports identified genes associated with reproduction traits (Ciacciariello and Gous, 2005). In total, 26 differentially expressed genes (DEGs) were identified in ovaries between pre-laying and egg-laying periods (Kang et al., 2009). The 12 genes identified were related to reproduction regulation pathways such as GnRH, G protein-coupled receptor, calcium-signaling pathways, biosynthesis of steroid hormones, oocyte meiosis, and progesterone-mediated oocyte maturation (Luan et al., 2014). In chickens, nine transcripts related to high egg production were identified in the hypothalamus and the pituitary gland (Shiue et al., 2006). Recently, a comparative transcriptome study was conducted between the hypothalamus and the pituitary gland in Chinese dagu chickens (Wang and Ma, 2019). However, no studies reported how to regulate the genes in embryos for oogenesis and egg development in chickens.

The genetic and developmental foundation of morphological complexity is one of the most significant questions in evolutionary biology, and because avian feathers come in a variety of shapes, they make a great model system for research on the evolution and development of unique morphological features (Losos et al., 2013; Chen B. et al., 2015). Feathers are an excellent model to study the molecular basis of phenotypic variation of an important structure in a single species because they have evolved to have different forms in color, morphology, and mechanical properties not only among different bird species but also in different body regions of an individual bird. The structure and shape of a body feather vary dynamically from the distal end to the proximal end, with the distal end forming before the proximal end. A body feather's barbs change from being mostly pennaceous at the proximal end to plumulaceous at the distal end (Ng et al., 2015). A great example of exaptation is the feather, which may have originally been developed to regulate body temperature but was later appropriated for show and flight. These and other evolutionary innovations most likely resulted from altered feather-related gene expression patterns. The morphological novelties of feathers are the result of the origin and evolution of plesiomorphic molecular signaling modules (Prum, 2005). A novel technological platform made possible by systems biology research can disclose the molecular expression profiles connected to various morphological processes. The identification of genes linked to changes in feather and scale will be aided by transcriptome investigations and bioinformatic analysis (Ozsolak and Milos, 2011; Chang et al., 2015). A transcriptomic study was conducted on two feather types at

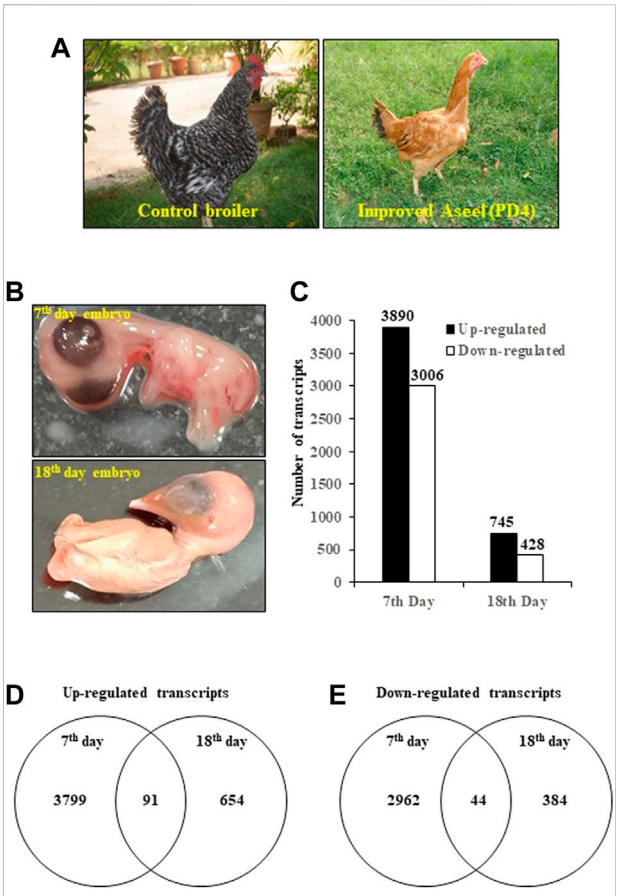

FIGURE 1
Differentially expressed transcripts/genes (DEGs) in improved Aseel (PD4) during embryo development stages (7th- and 18th-day embryos). **(A)** Fast- (control broiler) and slow-growth (improved Aseel, PD4) birds are used in this study. **(B)** Embryo tissues (complete embryos from the 7th day and thigh muscles from the 18th day) are used for the transcriptome analysis. **(C)** Number of up- and downregulated transcripts (DETs) (*p* value ≤0.05 and fold change ≥1) in Aseel as compared to control broiler samples. **(D)** and **(E)** Number of common and differentially expressed transcripts among the initial (7EB) and developed (18TM) embryo stages.

different times during their regeneration after plucking and then compared the gene expression patterns in different types of feathers and different portions of a feather and identified morphotype-specific gene expression patterns (Ng et al., 2015). Recently, transcriptome data from yellow and white feather follicles from 7- to 11-week-old F3 chickens were generated to screen for genes involved in the production of pheomelanin particles (Zheng et al., 2020). However, it is still completely unknown what causes feather variance genetically, particularly during embryo development. Understanding the molecular dynamics of embryonic development concerning the process of feather growth can help us better comprehend how different feather shapes have evolved through time.

Thus, the objectives of the present study are to explore mechanisms and pathways underlying embryonic development

with respect to muscle growth, egg production, and plumage formation in slow-growing native and fast-growing broiler chickens.

Materials and methods

Animals

The study was conducted on the fast-growing broiler pure line (developed from the synthetic population) and improved Aseel (PD4) chicken lines maintained at the institute farm, ICAR-Directorate of Poultry Research, Hyderabad, India (Figure 1A). The improved Aseel (PD-4) has been developed from the Indian native Aseel breed of chicken by imposing selection for body weights at 8 weeks of age for the last 10 generations. The body weight of these birds at 8 weeks during the S-10 generation was 551.0 ± 3.60 g. The control broiler birds are random-bred broilers, and there is no selection imposed on this population. The body weight of the control broiler line at 6 weeks was 951.0 ± 1.20 g. The birds of both populations were maintained under an intensive management system. A total of 60 fertile eggs were kept for hatching (30 for each group) in the incubator (Global Incubators, Hyderabad, India) at 100.3°F temperature and 79.2°F humidity. After the 7 and 18 days of incubation, eggs were harvested (15 for each group), and embryos were collected and stored at −80°C up to total RNA isolation.

RNA extraction and evaluation

For RNA isolation, the complete embryo from the 7th-day and thigh muscle from the 18th-day embryo were used from the control broiler and PD4 lines. The tissue samples were collected from three independent embryos during each time point to isolate RNA and consider each replicate as one biological replicate during each period. Total RNA was isolated using Trizin RNA extraction reagent (GCC Biotech (India) Pvt. Ltd.), according to the manufacturer's protocol, and RNA was puried by DNase treatment (DNase I solution, HiMedia, India) to remove a trace amount of DNA. The purity and quantity were monitored on 1.2% denatured agarose gels and the NanoDrop 1,000 Spectrophotometer (Thermo Scientic, United States). The quality of total RNA was assessed by checking 200–300 ng of total RNA on an RNA nano chip using an Agilent Bioanalyzer 2100 (Agilent Technologies, United States), according to the manufacturer's instructions.

RNA labeling, amplification, and hybridization

The Agilent Quick Amp Kit (Part number: 5190–0442) was used for sample labeling. In addition, 500 ng of total RNA was reverse transcribed using an oligo dT primer tagged to a T7 promoter sequence, and in the same reaction, the cDNA thus obtained was converted to double-stranded cDNA. Labeled cRNA preparation and hybridization on GeneChip and scanning were done following Affymetrix protocols (http://www.affymetrix.com). In the *in vitro* transcription step, the cDNA was converted to cRNA using the T7 RNA polymerase enzyme, Cy3 dye was added to the reaction mix and incorporated into the newly synthesized strands, and the obtained cRNA was cleaned up using Qiagen RNeasy columns (Qiagen, Cat No: 74106). The concentration and amount of dye incorporated were determined using a NanoDrop 1,000 Spectrophotometer (Thermo Fisher Scientic, United States). The QC-passed samples for specific activities were taken for hybridization. Then 600 ng of labeled cRNA was hybridized on the array using the Gene Expression Hybridization Kit (Part Number 5190–0404; Agilent Technologies, United States) in Sure Hybridization Chambers (Agilent technologies, United States) at 65°C for 16 h. Agilent Gene Expression Wash Buffers (Part No: 5188–5327) were used for washing the hybridized slides, and then the slides were scanned on a G2505C scanner (Agilent Technologies, United States).

Microarray data analysis

After scanning, DAT, CEL, CHP, XML, and JPEG image were generated for each array with Feature Extraction Software (Version-10.7, Agilent Technologies, United States). The CELles containing estimated probe intensity values were further analyzed with GeneSpring GX-11.0 software (Agilent Technologies, United States). Normalization of the data was performed in GeneSpring GX using the 75th percentile shift, and this normalization takes each column in an experiment independently and computes the n^{th} percentile of the expression values for this array across all spots; fold change was calculated concerning specific control samples. Genes were up- and down-regulated showing one-fold and above within the samples concerning the control sample were identified, and for the replicates, a Student's *t*-test *p*-value was calculated. The expression data obtained have been submitted to the Gene Expression Omnibus database (GEO, http://www.ncbi.nlm.nih.gov/geo) at the National Center for Biotechnology Information with the accession numbers GSE62443-GSE62445.

Hierarchical clustering analysis

The differentially expressed genes between the 7th- and 18th-day of the embryo were subjected to a hierarchical cluster analysis using the Cluster 3.0 program (Eisen et al., 1998). We imported the matrix with as many columns as stages and rows as genes, where each cell contains the log2 transformed fold change value for the gene and individual into the Cluster 3.0 program, normalizing on rows. To demonstrate the

expression pattern and tree diagram of DETs, we applied rows and columns to the cluster 3.0 software and carried out hierarchical clustering using the complete linkage approach with the Euclidean distance. The resulting dendrogram was then exported as an image file.

Functional characterization

Biological data and analysis tools are combined in the Database for Annotation Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov/), which provides systematic functional annotation for a large number of genes and proteins. GO annotation linked with biological processes, molecular function, and KEGG pathway enrichment analyses were carried out using the online DAVID tool version 6.7 (11,12) to examine the possible functions of discovered DEGs (Huang et al., 2009a,b). The FDR p-value of < 0.05 and fold change of > 1 were considered to be significant.

Pathway analyses

Ingenuity Pathway Analysis (IPA; Qiagen, Valencia, CA; http://www.ingenuity.com) software was used for functional annotation, canonical pathway analysis, upstream analysis, and network discovery. The chicken DEGs data set functionalities are primarily based on mammalian biological mechanisms because IPA is based on human bioinformatics. We have attempted to draw possible conclusions based on avian-based literature, but biomedical research biases the functional annotations toward human disease.

Selection of candidate reference genes

A total of 24 candidate reference genes were chosen based on their previous use/study in chicken or other avian species; the sequences were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/), and the CDS (coding DNA sequence) region was identified by using the ExPASy translation tool (https://web.expasy.org/translate/) (Supplementary Table S1).

Real-time quantitative PCR analysis

Microarray expression data were validated using two-step real-time quantitative PCR (qPCR) for specific confirmation of the differentially expressed genes. First-strand cDNA was synthesized from 2 µg of total RNA using the Thermo Fisher Scientific Verso cDNA Synthesis Kit (Thermo Scientific, United States). Gene speciac qPCR primers were designed for 24 housekeeping genes and 83 DEGs using PrimerQuest software (http://eu.idtdna.com; Supplementary Table S1; Table 1). The qPCR was performed using the BrightGreen 2X qPCR MasterMix-No Dye (Applied Biological Materials Inc. Canada) in the Insta Q96™ Real-Time Machine (HiMedia Laboratories, India) detection system. The PCR was performed under the following program: 5 min at 95°C followed by 40 cycles of amplication with 15 s of denaturation at 95°C and 60 s of annealing/extension at 60°C. A total of three biological replicates were used. A melt curve analysis was performed to check the special city of the amplied products. The $2^{-\Delta\Delta Ct}$ calculated the relative expression level of each gene, and Transferrin (TFRC; Accession No: X55348.1) from *G. gallus* was used as a housekeeping gene to normalize the amount of template cDNA added in each reaction.

The statistical analysis

To assess the expression variation of the candidate reference genes, all the samples were divided into three broad categories: the combination of 7EB and 18TM samples of control broiler and improved Aseel, 7EB and 18TM samples of control broiler alone, and 7EB and 18TM samples of improved Aseel alone. The qRT-PCR machine–generated Ct values for each of the cDNA samples were then used to determine the degree of data variability between the samples. The stability level of the 24 candidate reference genes from the 7EB and 18TM of control broiler and improved Aseel was determined using five statistical algorithms: geNorm, NormFinder, BestKeeper, Delta CT, and RefFinder (Vandesompele et al., 2002; Andersen et al., 2004; Pfaffl et al., 2004; Silver et al., 2006; Xie et al., 2012). GeNorm and NormFinder, which use relative expression values as input data and convert Ct values to linear scale expression quantities using the $2^{-\Delta Ct}$ method, as well as BestKeeper, which uses the raw Ct value directly and the comparative Ct method, were used to determine the expression stability level. RefFinder, a program that combines the most important computational tools currently available (geNorm, Normfinder, BestKeeper, and the comparative $2^{-\Delta Ct}$ method) to compare and rank the stability of candidate reference genes with the geometric mean of individual gene appropriate weight, was used to determine the overall final ranking of reference genes across all samples. Comparison of mean expression values for qRT-PCR between the control broiler and PD4 improved Aseel groups were made using the Student's t-test and $p \leq 0.05$ was considered statistically significant.

Results

Source, selection, primer design, and verification of candidate reference genes

In the present study, to identify the suitable reference genes for the 7th- and 18th-day embryos of the control broiler and improved Aseel, 24 candidate reference genes with a wide range

TABLE 1 List of primers used for qPCR to validate microarray data

S. No	Gene Name	Accession No.	Primer Sequence	Tm	GC %	Amplicon Size (bp)
1	Destrobrevin alpha (D alpha)	CR733292.1	5′-CAACCCTTTGTGGAGGAAAGA-3′	62	47.6	114
			5′-GAACCTCCCGCAGAAACAA-3′	62	52.6	
2	Uncharacterized protein (UP5)	ES605836.1	5′-GAACCAAATGCTGGCAGAAG-3′	62	50	112
			5′-AAATACTCTCTGGGTGAACAGG-3′	62	45.5	
3	Toll-interacting protein (TOLLIP)	NM_001006471.1	5′-GTGTAACGAAGAGGACCTGAAA-3′	62	45.5	95
			5′-TGTTCCCTCTCTGAGCTTCTA-3′	62	47.6	
4	Asw	CN225783.1	5′-GGCAACACGTGAAATCCATTC-3′	62	47.6	119
			5′-GCGCACGTCTCTGTATTCT-3′	62	52.6	
5	Chain A, fibrinogen alpha subunit (Chain A FAS)	BX935039.1	5′-TGACGACACAGACCAGAATTAC-3′	62	45.5	106
			5′-GGTTTCCACAATTACCCGATTG-3′	62	45.5	
6	Hypothetical protein (HP29)	AW198329.1	5′-CCCAGATGACAGAAGAACAAATAAAG-3′	62	38.5	106
			5′-CCCTCTTCTCCAAAGCATGTAT-3′	62	45.5	
7	Fibrinogen gamma chain precursor (FGCP)	BG642009.1	5′-CTGGTCACCTCAATGGACAATA-3′	62	45.5	106
			5′-CATCGGTCACGCCATGTT-3′	62	55.6	
8	Apolipoprotein B precursor (ALPBP)	NM_001044633.1	5′-CTTGAGGCCAACTCCAAAGTA-3′	62	47.6	102
			5′-GTGCTCCCAGACTGCATAAA-3′	62	50	
9	Maestro heat-like repeat–containing protein family member 2B (MHCRCP2B)	CR406681.1	5′-CTGGAACACACCACAGACTT-3′	62	50	130
			5′-CCCGATAGATGTCCTTTCCATAC-3′	62	47.8	
10	Activin A receptor, type IB (AARIB)	XM_001231300	5′-GCACGGATCTCTCTTTGACTAC-3′	62	50	120
			5′-TGAGTACCCACGATCTCCAT-3′	62	50	
11	cAMP responsive element modulator (CREM)	NM_204387	5′-CAAGAGAGAGCTGCGACTTATG-3′	62	50	102
			5′-AGCACAGCCACACGATTT-3′	62	50	
12	Caveolin 1, caveolae protein, 22kDa (CAV1)	NM_001105664	5′-CATTCCCATGGCACTCATCT-3′	62	50	106
			5′-GCACTGGATC′CAATCAGGTAG-3′	62	50	
13	Caveolin 2 (CAV2)	NM_001007086	5′-TGCTGTACAAGCTGCTGAG-3′	62	52.6	140
			5′-CACTGAAGGCAAGACCATGA-3′	62	50	
14	Follistatin-like 1 (FSTL1)	NM_204638	5′-CGATGACATGTGAAGGGAAGA-3′	62	47.6	105
			5′-TCTGCAGCTCCTGAACATATC-3′	62	47.6	

(Continued on following page)

TABLE 1 (*Continued*) List of primers used for qPCR to validate microarray data

S. No	Gene Name	Accession No.	Primer Sequence	Tm	GC %	Amplicon Size (bp)
15	WAP, follistatin/kazal, immunoglobulin, kunitz, and netrin domain containing 1 (WAPFK)	NP9672441	5′-GAGGGCAACAACAAC AACTTC	62	47.6	109
			5′-TCAGCACCATCTTGCTCT TC-3′	62	50	
16	Glucose-6-phosphate isomerase (GPI)	NM_001006128	5′-CACTTCTGCCCTATGACC AATA-3′	62	45.5	110
			5′-GTAGTCCACACGAGATCC TTTC-3′	62	50	
17	Solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3)	NM_205511	5′-GTGGTACACAGGATGTAT CTCAAG-3′	62	45.8	112
			5′-CGATAGTTTGGAGAGCGG AATAG-3′	62	47.8	
18	Heat shock 60kDa protein 1 (chaperonin) (HSPD1), nuclear gene encoding mitochondrial protein	NM_001012916	5′-GGTGAGAAGGCTCAGATT GAA-3′	62	47.6	122
			5′-GCTACTCCGTCAGATAGT TTGG-3′	62	50	
19	Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa) (HSPA5)	NM_205491	5′-TGAGACAGTTGGAGGTGT AATG-3′	62	45.5	103
			5′-AGTGGGCTGATTGTCAGA AG-3′	62	50	
20	Heat shock 70kDa protein 8 (HSPA8)	NM_205003	5′-AGTTTGAGCTGACCGGTA TTC-3′	62	47.6	122
			5′-CTCCTTGCCAGTGCTCTT ATC-3′	62	52.4	
21	Heat shock factor binding protein 1 (HSBP1)	NM_001112809	5′-ATGCAGGACAAATTTCAA ACCA-3′	62	36.4	118
			5′-CTACTCCCGCTTGTGTCA TC-3′	62	55	
22	Heat shock transcription factor 1 (HSTF 1)	BM440477	5′-GCAGCAGAAGGTGGT CAATA-3′	62	50	146
			5′-AGTACTGGCGGCTGTATT TC-3′	62	50	
23	Partial mRNA for heat shock protein 70 (hsp70 gene)	AJ301880	5′-CCCAGTAAGTGCGGGTCA TAA-3′	64	52.4	85
			5′-CGCTCCGCCAGTCAC TT-3′	64	64.7	
24	Homeobox C9 (HBC9)	BX950823	5′-AGATGTCCGTACACAAAG TATCA-3′	62	39.1	105
			5′-GTTTAGGACTCGGGCTAC TTC-3′	62	52.4	
25	Insulin-like growth factor 1 receptor (IGF1R)	NM_205032	5′-TGTGTACGTTCCAGACGA ATG-3′	62	47.6	104
			5′-CCTTGGCTATTCCCTCAT ACAC-3′	62	50	
26	Insulin-like growth factor binding protein 1 (IGFBP1)	NM_001001294	5′-CAGGACCAGATGCTGAAC TATC-3′	62	50	134
			5′-CCCTGTTCTTTCCATTTC TTGTG-3′	62	43.5	
27	Mitogen-activated protein kinase 8 interacting protein 3 (MAPK8IP3)	XM_424591	5′-GTGATGACAACAGCGACA AATC-3′	62	45.5	118
			5′-CCAGGCACAGAGACA AAGAA-3′	62	50	
28	Mitogen-activated protein kinase kinase kinase 4 (MAPKKK4)	CR523470	5′-AGTGGATGAACTACGTGC TAAC-3′	62	45.5	120
			5′-CCGGGAGAGCCGAAA TAAAT-3′	62	50	

(Continued on following page)

TABLE 1 (*Continued*) List of primers used for qPCR to validate microarray data

S. No	Gene Name	Accession No.	Primer Sequence	Tm	GC %	Amplicon Size (bp)
29	Mitogen-activated protein kinase-activated protein kinase 3 (MAPKAPK3)	XM_414262	5′-CTGAAGACTGACCCAACAGAG-3′	62	52.4	139
			5′-CTTCATCCCAGTGGTCCTTATC-3′	62	50	
30	Myozenin 2 (MZ2)	BX930590	5′-GAAACAACAAGCATCAGCCATTA-3′	62	39.1	121
			5′-GCTGAGTGTTGATAGTTCCTCTAC-3′	62	45.8	
31	Angiotensin II receptor, type 1 (AGTR1)	NM_205157	5′-TTCCTGGATTCCTCATCAAGTG-3′	62	45.5	103
			5′-GGGCATAGCTGTATCCACAATA-3′	62	45.5	
32	Angiotensin II receptor–associated protein (AGTRAP)	BX930324	5′-CTTCAACATAGGTCTCAACCGT-3′	62	45.5	106
			5′-CTGAGCTGCCTTGCTTGA-3′	62	55.6	
33	CD9 molecule (CD9)	NM_204762	5′-TACTACAATGCCATGCCCTAAA-3′	62	40.9	134
			5′-TAGCACAGCAAAGAACCATACT-3′	62	40.9	
34	Dickkopf homolog 2 (DKK2)	XM_420494	5′-CGCAACAAGAAGAACAGTCATTAT-3′	62	37.5	105
			5′-GGGATCACCTTCATGTCCTTTA-3′	62	45.5	
35	Glycoprotein M6A (GPM6A)	NM_001012579	5′-GGATCTTCGCCAGTATGGTATT-3′	62	45.5	97
			5′-TAGCTCATTCGAGTCACACATC-3′	62	45.5	
36	Glycoprotein M6B (GPM6B)	NM_001012545	5′-GAACATCTGCAACACGAATGAG-3′	62	45.5	124
			5′-GGCCCAGTTAGAAGACAGTATC-3′	62	50	
37	Janus kinase 1 (JAK1)	NM_204870	5′-CAAGGAACTAGCTGACCTGATG-3′	62	50	98
			5′-CCTCCAGTTTGTTGATGTCTCT-3′	62	45.5	
38	Janus kinase 2 (JAK2)	NM_001030538	5′-GATGGATGCCCTGATGAGATT-3′	62	47.6	92
			5′-CGCTGAGCAAGATCCCTAAA-3′	62	50	
39	Janus kinase and microtubule interacting protein 2 (JAKMIP2)	CR390426	5′-GACTGCATCAGTTCATCATTTCTC-3’	62	41.7	130
			5’-ACAGGAACACATTGCTGGT-3′	62	47.4	
40	Janus kinase and microtubule interacting protein 3 (JAKMIP3)	XM_426548	5′-TATCAACTTCCACCACGTTCC-3′	62	47.6	100
			5′-CATCAGCTCTGCCACTACTATG-3′	62	50	
41	Leiomodin 3 (fetal) (LMOD3)	BX935813	5′-GAGAATGACTGCAGAGGAGATG-3′	62	50	97
			5′-TTTGTAGTGCCGCTCCTTC-3′	62	52.6	
42	Musculoskeletal, embryonic nuclear protein 1 (MUSTN1)	NM_213580	5′-CCAAGTCATGAAGCAGTGTGA-3′	63	47.6	94
			5′-TGACTTCTCAAAGACCGTTTCG-3′	63	45.5	

(Continued on following page)

TABLE 1 (*Continued*) List of primers used for qPCR to validate microarray data

S. No	Gene Name	Accession No.	Primer Sequence	Tm	GC %	Amplicon Size (bp)
43	Myosin binding protein C, fast type (MYBPC2)	NM_001044659	5′-CTGATGGAGCGCAAG AAGAA-3′	62	50	105
			5′-GAAGACGCCCTCGATCAT TT-3′	62	50	
44	Myosin binding protein C, slow type (MYBPC1)	BX935207	5′-CCTGAAACGTAGGGAGGT TAAA-3′	62	45.5	131
			5′-TGCCTCTCAGGTCAGTGA TA-3′	62	50	
45	Perilipin 1 (PLIN1)	NM_001127439	5′-CCAGAAGAGGAGGAG GAAGAT-3′	62	52.4	100
			5′-TAGCACTGTGAGCCCTGT A-3′	62	52.6	
46	Phospholamban (PLN)	NM_205410	5′-CGATAGCAGGGTTTCCAT ACTT-3′	62	45.5	117
			5′-TGTCAGCTCTCTCCAGTA GAA-3′	62	47.6	
47	RCD1 required for cell differentiation1 homolog (S. pombe) (*RS. pombe*	001006521	5′-TGATTGGAGCCTTGGTGA AA-3′	62	45	105
			5′-GTTCACTGCCAGACTCCA TAAT-3′	62	45.5	
48	Slow muscle troponin T (TNNT1)	NM_205114	5′-CCCTCCACATTGAGCACA T-3′	62	52.6	104
			5′-CTCCATCAGGTCGAACTT CTC-3′	62	52.4	
49	Troponin T type 3 (skeletal, fast) (TNNT3)	NM_204922	5′-GAAGCAAACAGCTAG AGAGACA-3′	62	45.5	125
			5′-GGTATAACCAGTCCCACA GTTC-3′	62	50	
50	Troponin I type 1 (skeletal, slow) (TNNI1)	BX931462	5′-TCTCTTCGTCCACAATCT CAAC-3′	62	45.5	128
			5′-ACAGTCGGAGAAGGA GAGATAC-3′	62	50	
51	Myostatin (MSTN)	NM_001001461.1	5′-GGATGGGACTGGATTATA GCAC-3′	62	50	97
			5′-GGTGAGTGTGCGGGTATT T-3′	62	52.6	
52	Follistatin (FST)	NM_205200.1	5′-ACAACTTATCCGAGCGAG TG-3′	62	50	112
			5′-CTTCCTCTGGGTCTTCGT TAAT-3′	62	45.5	
53	Activin A receptor type 2A (ACVR2A)	NM_205367.1	5′-GCAAGAATGTGCTGCTGA AA-3′	62	45	109
			5′-CCAACCTGTCCATGTGTA TCT-3′	62	47.6	
54	Activin A receptor type 2B (ACVR2B)	NM_204317.1	5′-GAAGTGTTAGAGGGA GCAATCA-3′	62	47.5	118
			5′-CTGGACCATCAACTGCTC TAC-3′	62	52.4	
55	SMAD family member 2-Z (SMAD2Z)	NM_204561.1	5′-GGGAGTGCGTCTCTATTA CATC-3′	62	50	110
			5′-CAGGATGCCAGCCATATC TT-3′	62	50	
56	Activin A receptor type 1B (ACVR1B)	XM_015300267.2	5′-GCACGGATCTCTCTTTGA CTAC-3′	62	50	120
			5′-TGAGTACCCACGATCTCC AT-3′	62	50	

(Continued on following page)

TABLE 1 (*Continued*) List of primers used for qPCR to validate microarray data

S. No	Gene Name	Accession No.	Primer Sequence	Tm	GC %	Amplicon Size (bp)
57	Transforming growth factor beta receptor 1 (TGFBR1)	NM_204246.1	5′-TCGTGTGCCAAGTGAAGAAG-3′	62	50	102
			5′-CCAGAGCCTGAAGTTGTCATATC-3′	63	47.8	
58	Myogenin (MYOG)	NM_204184.1	5’-GGCTGAAGAAGGTGAACGAA-3’	62	50	116
			5’-GCGCTCGATGTACTGGATG-3’	62	57.9	
59	Mitogen-activated protein kinase kinase 6 (MAP2K6)	XM_003642348.2	5′-CTCAGCAGAG′TCGTCGATTT-3′	62	47.6	101
			5′-GCAGGGTGAAGAAAGGATGT-3′	62	50	
60	Mitogen-activated protein kinase kinase kinase 7 (MAP3K7)	XM_015284683.2	5′-CAGCCCTTGTTTCAGGAGAAG-3′	63	52.4	101
			5′-GCCTCGTTTAGGCTTGGAATAG-3′	63	50	
61	Caveolin 3 (CAV3)	NM_204370.2	5′-GCTTTGATGGTGTCTGGAAAG-3′	61	47.6	142
			5′-ATGTGGCAGAAGGAGATGAG-3′	61	50	
62	Protein kinase AMP-activated catalytic subunit alpha 1 (PRKAA1)	NM_001039603.1	5′-CTTGACGATCACCATCTGTCTC-3′	62	50	140
			5′-TGCCACTTCGCTCTTCTTAC-3′	62	50	
63	Protein kinase AMP-activated catalytic subunit alpha 2 (PRKAA2)	NM_001039605.1	5′-GGAGGTCTGTGAGAAGTTTGAG-3′	62	50	124
			5′-GTTCATGATCCTCCGGTTGT-3′	62	50	
64	Creatine kinase, M-type (CKM)	NM_205507.1	5′-CGACCACTTCCTGTTCGATAA-3′	62	47.6	109
			5′-GAACGTCTTGTTGTCGTTGTG-3′	62	47.6	
65	Mechanistic target of rapamycin (serine/threonine kinase) (MTOR)	XM_417614.5	5′-AAGGTTTCTTCCGGTCCATATC-3′	62	45.5	98
			5′-ATCAGGCCAGTGACCATAATC-3′	62	47.6	
66	Ribosomal protein S6 kinase A1 (RPS6KA1)	NM_001109771.2	5′-GGAACCCAGCCAACAGATTA-3′	62	50	104
			5′-TTCCCTTCGGTACAGCTTATTC-3′	62	45.5	
67	Carnitine palmitoyltransferase I (CPT1)	DQ314726.1	5′-GCCTTCGTGCGCAGTAT-3′	62	58.8	146
			5′-ACGTAGAGGCAGAAGAGGT-3′	62	52.6	
68	Acyl-CoA synthetase long-chain family member 1 (ACSL1)	NM_001012578.1	5′-GCCAGTACGTAGGCATCTTT-3′	62	50	116
			5′-TGCTTCAGTTCCCAGTGTATC-3′	62	47.6	
69	Enoyl-CoA hydratase, short chain 1 (ECHS1)	NM_001277395.1	5′-CAGGTGGGAGCTATTGTCATC-3′	62	52.4	97
			5′-CATAGCACTCCTGGAAGGTTT-3′	62	47.6	
70	Hydroxyacyl-CoA dehydrogenase (HADH)	NM_001277897.1	5′-GCTATCCCATGGGTCCATTT-3′	62	50	100
			5′-AGAGGATTGTTGGGCTCTATTG-3′	62	45.5	

(Continued on following page)

TABLE 1 (*Continued*) List of primers used for qPCR to validate microarray data

S. No	Gene Name	Accession No.	Primer Sequence	Tm	GC %	Amplicon Size (bp)
71	Acyl-CoA oxidase 2 (ACOX2)	XM_015293306.2	5′-TGCCACCATCTGTCACTTATC-3′	62	47.6	141
			5′-TAGCTGCTGTGCTGCTTATC-3′	62	50	
72	Sterol regulatory element binding protein 1 (SREBP1)	AJ310768.1	5′-CATGGAGGTGGCGAAGG-3′	62	64.7	134
			5′-TGTCAGGCTCGGAGTCA-3′	62	58.8	
73	Fibroblast growth factor 2 (FGF2)	NM_205433.1	5′-TTCGAGCGCTTGGAATCTAATA-3′	62	40.9	94
			5′-GCTTGTACTGTCCAGTCCTTT-3′	62	47.6	
74	Fibroblast growth factor receptor 1 (FGFR1)	NM_205510.1	5′-CGTCACCAAAGTGGCTGTA-3′	62	52.6	98
			5′-TGCCGATCATCTTCATCATCTC-3′	62	45.5	
75	DNA methyltransferase 3 alpha (DNMT3A)	NM_001024832.1	5′-CCTTCTTCTGGCTCTTTGAGAA-3′	62	45.5	111
			5′-CAGACACCTCTTTGGCATCA-3′	62	50	
76	Forkhead box O3 (FOXO3)	MK861853.1	5′-CTCTCAGGCTCCTCTTTGTATTC-3′	62	47.8	109
			5′-CACACTCCAAGCTCCCATT-3′	62	52.6	
77	Peroxisome proliferator–activated receptor gamma (PPARγ)	AF163811.1	5′-CCCAAGTTTGAGTTTGCTGTG-3′	62	47.6	99
			5′-TGGGCGATCTCCACTTAGTA-3′	62	50	
78	Myogenic factor 6 (MYF6)	FJ882409.1	5′-GCTGGATCAGCAGGACAAA-3′	62	52.6	100
			5′-GCAGGTGCTCAGGAAGTC-3′	62	61.1	
79	Acetyl-CoA carboxylase α(ACACA)	NM_205505.1	5′-CAGATTTGTTGTCATGGTGAC-3′	60	42.9	162
			5′-ACAGCCTGCACTGGAATGC-3′	60	57.9	
80	Acetyl-CoA carboxylase β(ACACB)	XM_025155692.1	5′-GCTCCTGCTGCCCATATATTA-3′	60	47.6	94
			5′-GTCCGTGATGACACCTTTCT-3′	60	50	
81	Fatty acid synthase (FASN)	NM_205155.3	5′-GTTCTCTGTACAGAGAATGTG-3′	60	42.9	168
			5′-CCATGTTTGACTTGGTTGATC-3′	60	42.9	

of biological functions were selected based on previous studies of various avian and non-avian species. These are 18S rRNA, ALB, B2MG, β-ACT, EEF1A1, GAPDH, GUSB, HMBS, HSP10, HSP70, L-LDBC, MRPS27, MRPS30, PGK2, PPP2CB, RPL5, RPL13, RPL14, RPL19, RPL23, SDHA, TBP, TFRC, and DNAJC24. The chicken orthologous genes were obtained from NCBI, and the CDS region was found and amplified with gene-specific primers (Supplementary Table S1). For all the primer pairs, the melting curve analysis was performed to confirm the specific amplification for each reference gene, a single peak with no visible primer-dimer formation and genomic DNA contamination was observed, and no signals were detected in the non-template controls.

Expression stability and ranking of candidate reference genes

Expression levels of all candidate reference genes were measured in the samples collected from the 7EB and 18TM of the control broiler and improved Aseel. Each reference gene had different expression ranges across all sample sets, and the 18S rRNA and DNAJC24 genes showed the most (Ct = 12.34) and the least (Ct = 33.88) abundant transcripts, respectively. In the combined analysis, we observed that not all selected reference genes were expressed uniformly across 7EB and 18TM of the control broiler and improved Aseel. The genes with the lowest global variability were GUSB, PP2CB, and HSP70 (Figure 2A). The results show that the GUSB reference gene had the least variation in expression, with mean Ct values ranging from 17.78 to 22.52, whereas the RPL5 gene showed a much higher expression variation, with mean Ct values ranging from 13.07 to 32.89 across all sample sets (Figure 2A). In control broiler samples, PP2CB, ALB, and GUSB were the top three genes with the lowest variation (Figure 3B); whereas HSP70, GUSB, and β-2MG showed little variation in improved Aseel (Figure 3C). It is important to note that there was a wide range of variation among selected reference genes, and it shows that not a single reference gene was expressed constantly across the 7EB and 18TM of control broiler and improved Aseel in the present study. Therefore, it is essential to choose the most reliable reference gene for expression profiling gene/s in different embryos of the control broiler and improved Aseel. The most popular statistical tools geNorm, NormFinder, BestKeeper, Delta CT, and RefFinder were used for the analysis to choose the best and most trustworthy reference gene and rank all the potential reference genes according to their stability values for accurate gene expression (Table 2). The variation among the reference genes determined by geNorm is stability measure (M value) and pairwise comparison expression ratio and provides an optimal number of genes in a given experiment. NormFinder measures the reference gene stability by overall expression variation and across samples variation to reduce sensitivity toward co-regulation. BestKeeper calculates the gene expression variation based on Ct values, calculates the Pearson correlation coefficient by pairwise correlation analysis for all reference genes, and finds the stable genes. The Delta CT method directly used the raw Ct values and found the best stable genes. RefFinder is conclusive of the calculations using the aforementioned algorithms and suggested stable genes.

Differentially expressed transcripts during embryo development stages

To study the effect of muscle development, genome-wide expression analysis was carried out at muscle initiation (7th-day embryo) and muscle development (18th-day thigh muscle) stages (Figures 1A,B). Labeled RNA was hybridized to the Affymetrix GeneChip™ Chicken Genome Array. After statistical data analysis, transcripts with an FDR-adjusted p-value ≤ 0.05 and a fold change ≥ 1 were considered as differentially expressed transcripts (DETs) (Figure 1C). The complete list of the DETs in improved Aseel during embryo development stages as compared to their respective control broiler samples is presented in Supplementary Material S1. In total, 8,069 transcripts, which accounted for approximately 24% of the total transcripts present on the GeneChip™ Chicken Genome Array, showed differential expression in improved Aseel at various stages analyzed. The maximum number of transcripts (6,896, 21% of total DETs) showed differential expression on the 7th day of the embryo, and the least number of transcripts (1,173, 3.5% of total DETs) showed differential expression on the 18th day of the embryo. Commonly up- and downregulated muscle-responsive transcripts were identied among the embryo development stages to nd out the degree of overlap (Figures 1D,E). The maximum unique number of upregulated (3,799) and downregulated transcripts (2962) was observed in a 7th-day embryo. A small number of upregulated (654) and downregulated (384) transcripts were uniquely differentially expressed on the 18th-day of the thigh muscle. The commonly differentially expressed (91 upregulated and 44 downregulated) transcripts were identified among the embryo development stages, respectively (Figures 1D,E; Supplementary Material S1).

Cluster analysis of differentially expressed transcripts

To profile the gene expression patterns in response to muscle slow growth and egg production during embryo development, the 8,069 DETs were classied using hierarchical clustering. The expression patterns were separated into eight major clusters (I–VIII) based on tree branching (Figure 3). Transcripts and involved pathways present in each stage within each cluster are presented in Supplementary Material S2. Among the eight major clusters, upregulated transcripts were enriched in clusters I, VI, and VII, and downregulated transcripts were enriched in clusters II, III, and IV.

GO annotation and pathway enrichment analysis of differentially expressed genes

DAVID 6.7 was used to annotate and enrich the DEGs related to GO annotations and pathways between the 7th-day up and the 18th-day down and *vice versa*. The results showed that coenzyme metabolism, cell division and

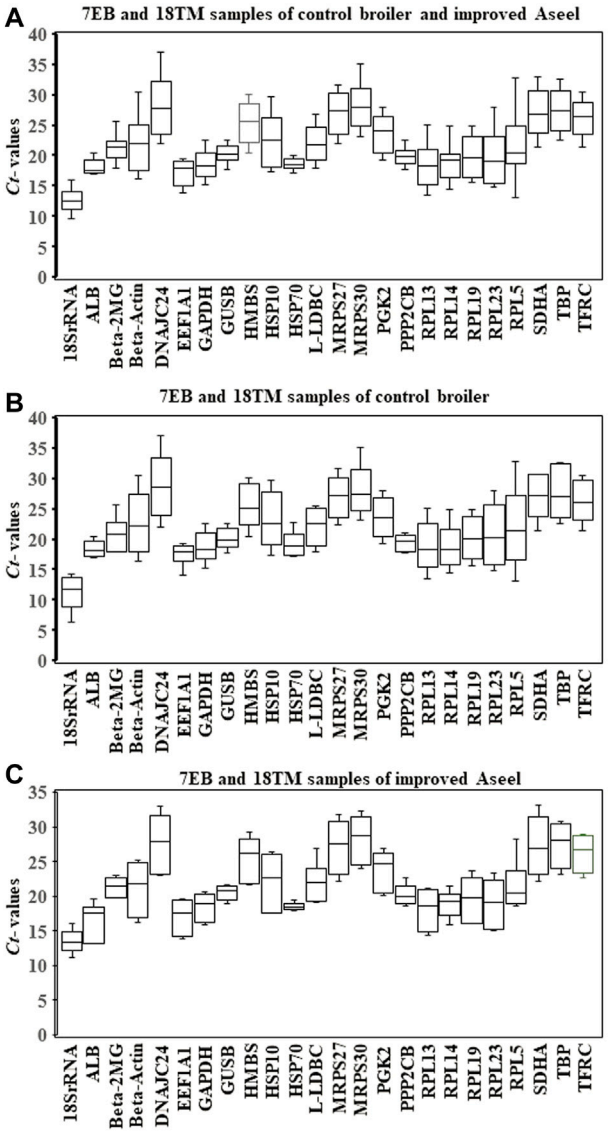

FIGURE 2
Relative level of mRNA expression of the 24 candidate reference genes in the 7EB and 18TM of both control broiler and improved Aseel. The data are presented as mean cycle threshold (Ct) values and shown as box and whisker plots. The boxes represent the interquartile range of the mean Ct values, whereas the middle, up, and lower bars represent the mid-hinge, maximum, and minimum Ct values, respectively. The *X*-axis represents the gene names, and *Y*-axis represents the Ct values of all the tissues. **(A)** 7EB and 18TM samples of control broiler and improved Aseel; **(B)** 7EB and 18TM samples of control broiler; **(C)** 7EB and 18TM samples of PD4.

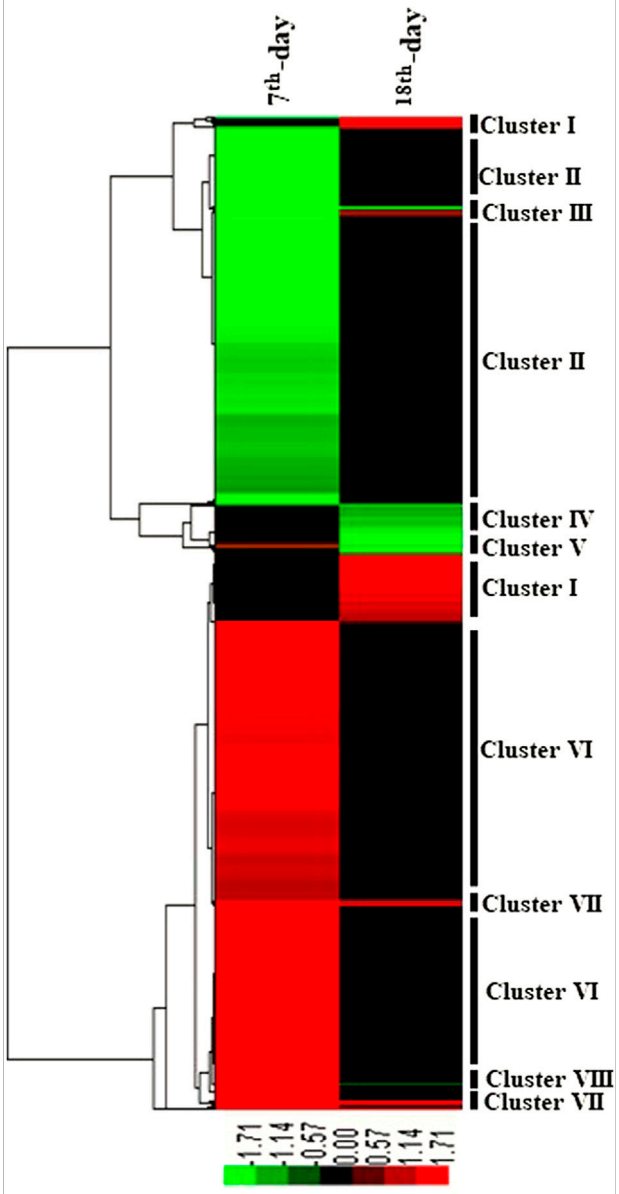

FIGURE 3
Hierarchical clustering of DEGs on the 7EB and 18TM of the embryo (Aseel vs. control broiler). Hierarchical clustering of differentially expressed genes led to the formation of eight distinct clusters: I, II, III, IV, V, VI, VII, and VIII, which include genes up- and downregulated, defining the specific molecular regulation of Aseel growth. Each row represents the expression pattern of a single gene, and each column corresponds to a single sample: column 1, 7EB; column 2, 18TM. The expression levels are represented by a color chat (p value ≤0.05 and fold change ≥1), with red representing upregulation, green representing downregulation, and black representing the missing values or no change.

chromosome partitioning, outer membrane, and transcription/cell division and chromosome partitioning functions were upregulated in the 7th-day embryo and downregulated in the 18th-day thigh muscle, whereas chaperones, lipid metabolism, outer membrane/ carbohydrate transport and metabolism, protein turnover, and posttranslational modification functions were downregulated in the 7th-day embryo and upregulated in

TABLE 2 Ranking of the candidate reference genes according to their stability value per indicated software

Gene Name	geNorm						NormFinder						BestKeeper						ΔCT						Comprctensive					
	7EB & 18TM of CB & PD4 combined analysis		7EB & 18TM of CB analysis		7EB & 18TM of PD4 analysis		7EB & 18TM of CB & PD4 combined analysis		7EB & 18TM of CB analysis		7EB & 18TM of PD4 analysis		7EB & 18TM of CB & PD4 combined analysis		7EB & 18TM of CB analysis		7EB & 18TM of PD4 analysis		7EB & 18TM of CB & PD4 combined analysis		7EB & 18TM of CB analysis		7EB & 18TM of PD4 analysis		7EB & 18TM of CB & PD4 combined analysis		7EB & 18TM of CB analysis		7EB & 18TM of PD4 analysis	
	M	R	M	R	M	R	SV	R	SV	R	SV	R	SD	R	SD	R	SD	R	SD	R	SD	R	SD	R	GM	R	GM	R	GM	R
18S rRNA	2.05	18	2.27	18	1.70	18	3.02	19	3.25	18	2.26	19	1.83	6	2.13	7	1.35	5	3.75	20	4.09	19	2.96	21	14.43	18	14.6	18	13.95	18
ALB	3.00	23	2.48	19	2.60	23	5.99	24	3.22	17	7.95	24	2.54	10	1.13	**2**	4.56	24	6.32	24	3.86	18	8.08	24	19.28	23	10.52	12	24	24
B2MG	2.70	22	3.27	23	1.82	19	4.02	23	5.48	24	2.27	20	1.66	4	2.25	8	0.99	**3**	4.4	23	5.63	24	2.93	20	14.85	19	18.24	22	12.45	16
βActin	1.48	13	1.64	14	1.04	11	2.38	16	2.74	16	2.01	16	4.12	23	4.52	22	3.72	22	2.88	16	3.18	16	2.44	14	16.94	21	17.05	21	15.59	20
DNAJC24	1.60	15	1.72	15	1.16	13	2.71	17	3.25	19	2.28	21	4.37	24	4.98	23	3.77	23	3.13	17	3.59	17	2.63	17	18.25	22	18.57	23	18.41	22
EEF1A1	1.54	14	1.83	16	0.48	**2**	0.87	4	1.25	6	0.33	**2**	1.73	5	1.39	4	2.08	8	2.43	9	2.85	13	1.86	**3**	7.21	6	8.53	7	3.46	**3**
GAPDH	1.18	8	1.23	8	1.10	12	0.37	**1**	0.06	**1**	0.57	4	1.91	7	1.97	6	1.85	7	2.28	5	2.52	4	2.01	7	4.21	**3**	3.83	**3**	7.1	6
GUSB	2.38	20	2.86	21	1.99	21	3.07	20	3.83	21	2.40	22	1.14	**3**	1.29	**3**	0.89	**2**	3.68	19	4.28	21	3.02	22	12.44	15	13.06	16	12.08	15
HMBS	0.67	**2**	0.54	**2**	0.91	8	1.02	5	0.87	4	1.20	9	3.01	14	3.21	14	2.82	16	2.24	**3**	2.37	**3**	2.08	10	5.01	4	4.74	4	10.67	12
HSP10	1.01	6	0.97	5	0.99	10	1.86	14	2.0	14	1.81	14	3.79	21	4	20	3.58	21	2.54	12	2.73	10	2.29	13	12.54	16	11.38	13	14.32	19
HSP70	2.54	21	3.06	22	1.91	20	3.55	21	4.76	23	2.23	18	1.08	**1**	1.62	5	0.42	**1**	4.04	22	5.01	23	2.92	19	10.04	10	15.71	19	9.21	8
L-LDBC	1.32	10	1.42	10	1.28	15	1.09	6	1.31	7	0.95	7	2.45	9	2.74	9	2.16	9	2.45	10	2.72	8	2.19	12	8.78	8	8.63	8	10.49	11
MRPS27	1.10	7	1.32	9	0.95	9	1.36	10	1.21	5	1.57	13	3.08	16	2.94	13	3.22	19	2.43	8	2.62	6	2.18	11	10.06	11	7.9	6	12.84	17
MRPS30	0.92	5	1.03	6	0.77	5	1.48	12	1.80	13	1.20	8	3.26	18	3.43	17	3.10	18	2.42	7	2.74	11	1.96	6	9.76	9	11.42	14	8.49	7
PGK2	0.38	**1**	0.35	**1**	0.41	**1**	0.52	**3**	0.51	**3**	0.48	**3**	2.59	11	2.8	10	2.38	11	2.13	**2**	2.35	**2**	1.82	**2**	2.85	**2**	2.78	**2**	2.85	**2**
PPP2CB	2.23	19	2.69	20	1.56	17	2.91	18	3.80	20	1.86	15	1.11	**2**	1.12	**1**	1.1	4	3.55	18	4.25	20	2.67	18	10.67	13	9.57	11	11.81	14
RPL13	0.83	4	0.75	**3**	0.82	6	1.15	7	1.47	9	0.82	5	3.06	15	3.39	16	2.72	14	2.26	4	2.54	5	1.89	4	6.77	5	7.33	5	6.65	5
RPL14	1.66	16	1.13	7	1.41	16	1.45	11	1.46	8	1.54	12	2.15	8	2.86	11	1.42	6	2.66	13	2.72	9	2.55	15	11.81	14	8.92	9	11.64	13
RPL19	1.25	9	1.53	12	0.62	**3**	1.33	8	1.76	12	0.87	6	2.92	13	3.24	15	2.60	13	2.46	11	2.9	14	1.91	5	10.34	12	13.45	17	6.28	4
RPL23	1.43	12	1.59	13	0.70	4	2.09	15	2.60	15	1.22	11	3.64	20	4.46	21	2.82	15	2.77	15	3.09	15	2.04	9	15.55	20	16.04	20	9.28	9
RPL5	1.83	17	2.03	17	2.10	22	3.59	22	4.52	22	2.83	23	3.93	22	5.32	24	2.54	12	4.04	21	4.75	22	3.43	23	20.68	24	21.38	24	19.55	23
SDHA	1.37	11	1.48	11	1.22	14	1.84	13	1.68	11	2.11	17	3.45	19	3.43	18	3.47	20	2.7	14	2.82	12	2.60	16	14.27	17	12.99	15	16.90	21

(Continued on following page)

TABLE 2 (*Continued*) Ranking of the candidate reference genes according to their stability value per indicated software

Gene Name	geNorm					NormFinder					BestKeeper					ΔCT					Comprctensive				
	7EB & 18TM of CB & PD4 combined analysis		7EB & 18TM of CB analysis		7EB & 18TM of PD4 analysis		7EB & 18TM of CB & PD4 combined analysis		7EB & 18TM of CB analysis	7EB & 18TM of PD4 analysis		7EB & 18TM of CB & PD4 combined analysis		7EB & 18TM of CB analysis	7EB & 18TM of PD4 analysis		7EB & 18TM of CB & PD4 combined analysis		7EB & 18TM of CB analysis	7EB & 18TM of PD4 analysis		7EB & 18TM of CB & PD4 combined analysis		7EB & 18TM of CB analysis	7EB & 18TM of PD4 analysis
	M	R	M	R	M	R	SV	R	SV	SV	R	SD	R	SD	R	SD	R	SD	R	SD	R	GM	R	GM	R
TBP	0.78	3	0.88	4	0.87	7	1.35	9	1.61	1.21	10	3.23	17		19		17	2.35	6	2.66		2.03		7.78	7	9.03	10	10.21	10
														3.57		2.89					7		8					
TFRC	0.38	1	0.35	1	0.41	1	0.4	2	0.49	0.27	1	2.59	12		12		10	2.1	1	2.33		1.79		2.21	1	2.21	1	1.78	1
														2.88		2.31					1		1					

M, gene expression stability measure; SD, standard deviation value; SV, stability value; GM, geomean value; and R, ranking

the 18th-day thigh muscle. Cell envelope biogenesis, cytoskeleton, and inorganic ion transport and metabolism functions were differentially regulated between the 7th-day embryo and 18th-day thigh muscle compared to the respective controls (Supplementary Material S3). The KEGG pathway enrichment analysis of DEGs was performed using the IPA tool. The results showed that differential regulation of pathways between 7th-day embryo and 18th-day thigh muscle of PD4 compared to their respective controls, that is, Cell cycle, Cell adhesion molecules (CAMs), SNARE interactions in vesicular transport, Oocyte meiosis, Endocytosis, Apoptosis, ABC transporters, Calcium signaling pathway, MAPK signaling pathway, Wnt signaling pathway, Jak-STAT signaling pathway, Toll-like receptor signaling pathway, TGF-βsignaling pathway, cytokine–cytokine receptor interaction, Basal transcription factors, Focal adhesion, Tight junction, Regulation of actin cytoskeleton, Cardiac muscle contraction, Vascular smooth muscle contraction, Insulin signaling pathway, Oxidative phosphorylation, Glutathione metabolism, Glycolysis/Gluconeogenesis, Citrate cycle (TCA cycle), Pentose phosphate pathway, Pyruvate metabolism, Fatty acid biosynthesis, Fatty acid metabolism, Glycerophospholipid metabolism, Heparan sulfate biosynthesis, N-Glycan biosynthesis, Purine metabolism, Pyrimidine metabolism, Tryptophan metabolism, Serine and threonine metabolism, and Valine, leucine and isoleucine degradation (Supplementary Materials S2, S3).

Validation of DEGs by qPCR

In this study, the expression of DEGs between the fast (CB) and slow growth (PD4) chickens of the 7th-day embryo and 18th-day thigh muscle was verified by qPCR (Supplementary Table S2). The verified transcripts were divided into four groups: i. muscle development, myostatin signaling, muscle metabolism, and protein synthesis (Figure 4), ii. Embryo development (Figure 5), iii. Fatty acid metabolism (Figure 6), and iv. Cell signaling and egg production (Figure 7). The results showed that the expression trend of the DEGs between the fast and slow-growing chickens is consistent in qPCR results, and this attests to the reliability of the microarray data.

Discussion

Globally, chicken is one of the most protein-rich meat sources. Muscle development and egg production are essential genetic traits in commercially grown chickens. However, not much information is available on genes involved in muscle development and egg production in slow and fast-growing chickens. In this study, we selected fast (CB) and slow-growth (PD4) chickens to determine the expression of genes related to embryo initiation and developmental stages. Microarray was conducted with the 7th-day embryo (7EB) and 18th-day thigh

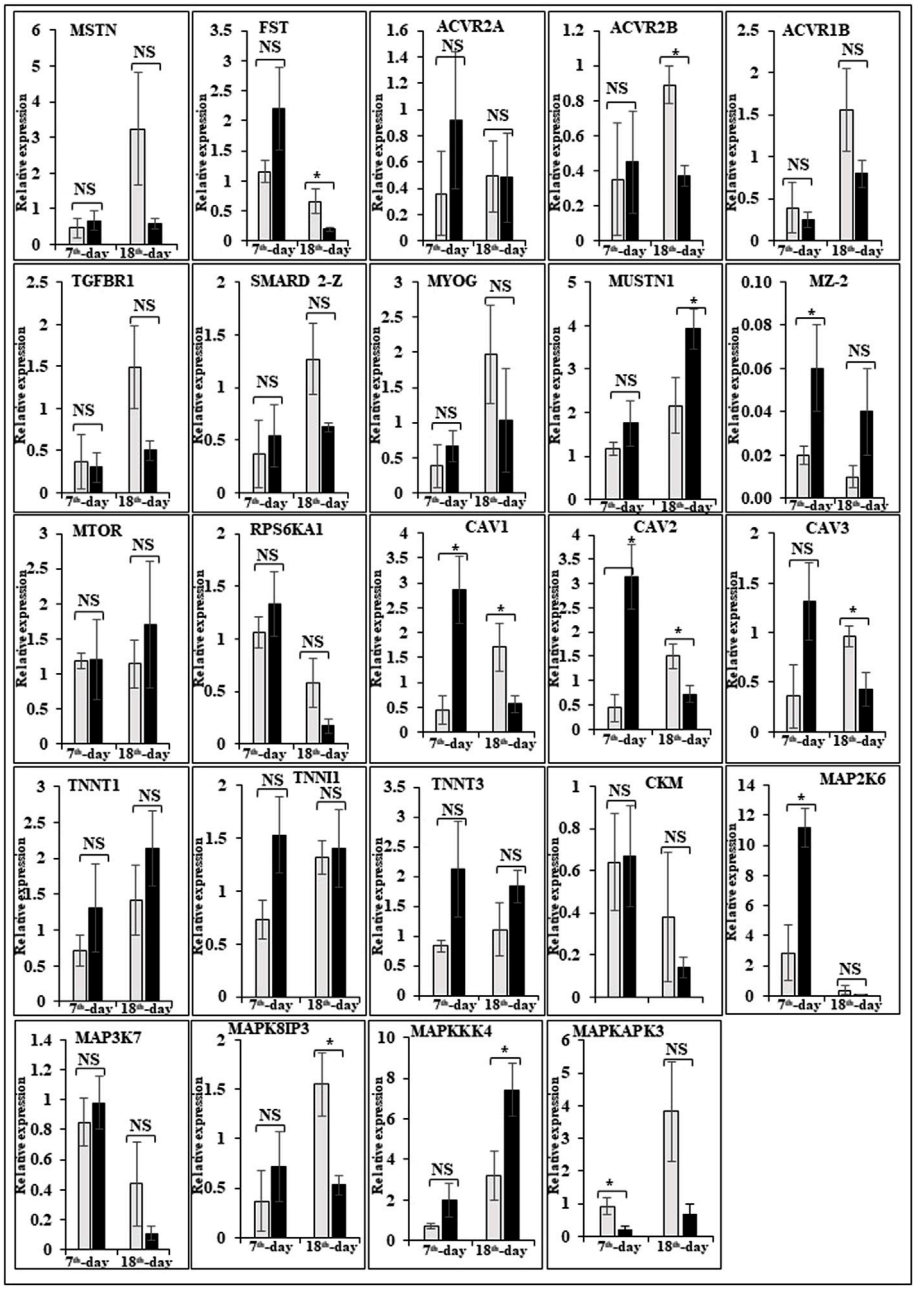

FIGURE 4
Relative expression of DEGs that are involved in muscle development, myostatin signaling, muscle metabolism (energy sensing and storage), and protein synthesis. The Y-axis represents relative mRNA expression level, and the X-axis represents tissue samples used for the qPCR study (Control broiler, PD4). The *p* values have been stated on the comparative bars; * indicates the $p \leq 0.0\ 5$, and NS indicates the non-significant. Standard error was used for error bars.

muscle (18TM) of PD4 and CB, respectively. According to the MIQE guidelines, selecting suitable reference genes may vary for different species, varieties, experimental conditions, and tissues and has to be validated before gene expression study (Bustin et al., 2009). Previous and recent studies also described different expression patterns of reference genes and focused on validating reference genes applied to different avian tissues (Olias et al., 2014; Bages et al., 2015; Nascimento et al., 2015). However, so far, validation of genes for their stable expression patterns in different embryo tissues, such as 7th and 18th-day embryos of control broiler and improved Aseel has not been performed.

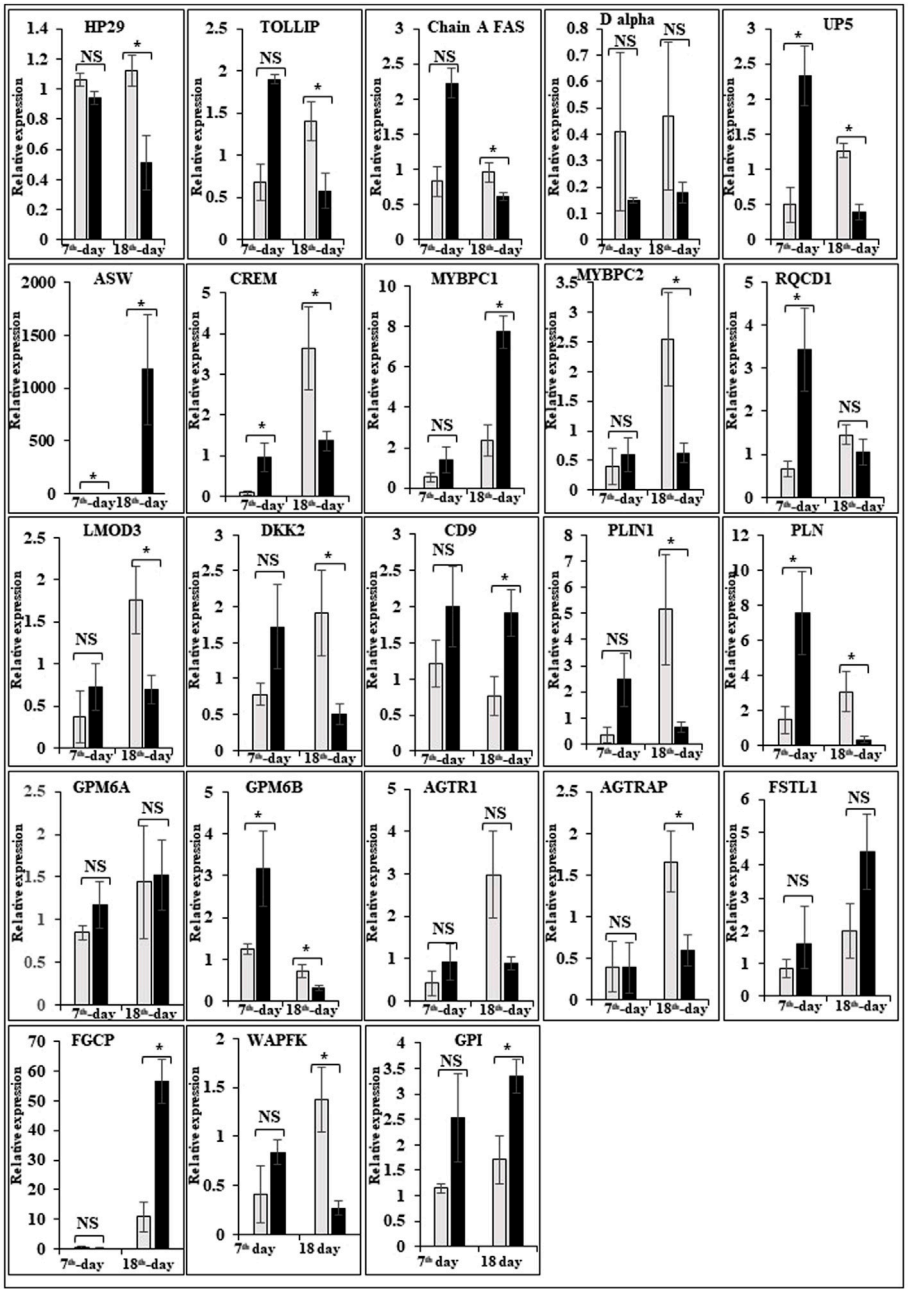

FIGURE 5
Relative expression of DEGs that are involved in embryo development. The Y-axis represents relative mRNA expression level, and the X-axis represents tissue samples used for the qPCR study (Control broiler, PD4). The *p* values have been stated on the comparative bars; * indicates the $p \leq 0.05$, and NS indicates the non-significant. Standard error was used for error bars.

Candidate reference genes validation

For accurate gene expression, to select the best and most reliable reference gene and rank all the candidate reference genes according to their stability value, the most commonly used statistical programs were used, that is, geNorm, NormFinder, BestKeeper, Delta CT, and RefFinder (De Boever et al., 2008; Yue et al., 2010; Yang F. et al., 2013; Olias et al., 2014; Bages et al., 2015; Nascimento et al., 2015; Borowska et al., 2016; Mitra et al., 2016; Khan et al., 2017; Zhang et al., 2018; Mogilicherla et al., 2022). These algorithms showed some differences in the stability ranking of stable

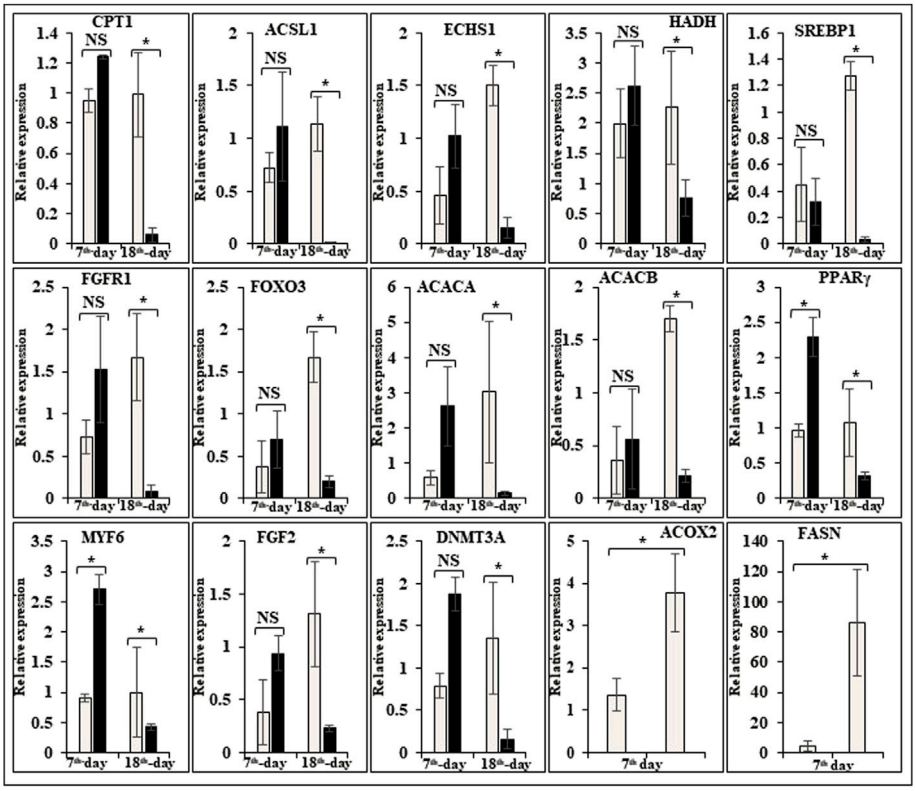

FIGURE 6
Relative expression of DEGs that are involved in fatty acid metabolism. The Y-axis represents relative mRNA expression level, and the X-axis represents tissue samples used for the qPCR study (Control broiler, PD4). The *p* values have been stated on the comparative bars; * indicates the $p \leq 0.05$, and NS indicates the non-significant. Standard error was used for error bars.

reference genes, which may be due to the differences in each statistical program (Table 2). Ranking the stability of 24 genes is crucial, as is confirming the number of reference genes required for precise gene expression profiling in various embryonic tissues. For each gene, geNorm generates a stability measure (M value), allowing for ranking based on expression stability (with the lower value indicating increased gene stability across samples). To assess the value of including more references, it additionally provides a pairwise stability measure for the normalization (Exposito-Rodriguez et al., 2008; Paolacci et al., 2009). According to the geNorm stability criteria, the most stable genes in various analyses are: control broiler alone [TFRC (0.35), PGK2 (0.38), HMBS (0.54), and RPL13 (0.75)]; improved Aseel alone [TFRC (0.41), PGK2 (0.41), EEF1A1 (0.48), and RPL19 (0.62)]; and combined analysis [TFRC (0.38), PGK2 (0.38), HMBS (0.67), and TBP (0.78)], respectively were within the M value ≤ 1 threshold range, demonstrating a trustworthy stability (Table 2). To enable a direct estimate of expression variation, including ranking genes according to their stability using a model-based approach, NormFinder provides a stability measure and groups samples (Hellemans et al., 2007; Mitra et al., 2016). The results of an analysis using NormFinder revealed the relative rankings of the genes in various combinations, including control broiler alone [GAPDH (0.06), TFRC (0.49), and PGK2 (0.51)]; improved Aseel alone [TFRC (0.27), EEF1A1 (0.33), and PGK2 (0.48)]; and combined analysis [GAPDH (0.37), TFRC (0.4), and PGK2 (0.52)], respectively (Table 2). Based on Ct values, BestKeeper determines the variation in gene expression for each housekeeping gene. By using pairwise correlation analysis, BestKeeper calculates the Pearson correlation coefficient, estimates the inter-gene relationships, and identifies the stable genes in all combinations: control broiler alone [PPP2CB (1.12), ALB (1.13), GUSB (1.29)]; improved Aseel alone [HSP70 (0.42), GUSB (0.89), B2MG (0.99)]; combined analysis [HSP70 (1.08), PPP2CB (1.11), GUSB (1.14)], respectively (Table 2). The Delta CT results supported the findings of geNorm and NormFinder, and they revealed the best stable genes in all combinations: control broiler alone [TFRC (2.33), PGK2 (2.35), HMBS (2.37)]; improved Aseel alone [TFRC (1.79), PGK2 (1.82), EEF1A1 (1.86)]; combined analysis [TFRC (2.1), PGK2 (2.13), HMBS (2.24)], respectively (Table 2). RefFinder integrated the results from each of the aforementioned algorithms and suggested stable genes for all

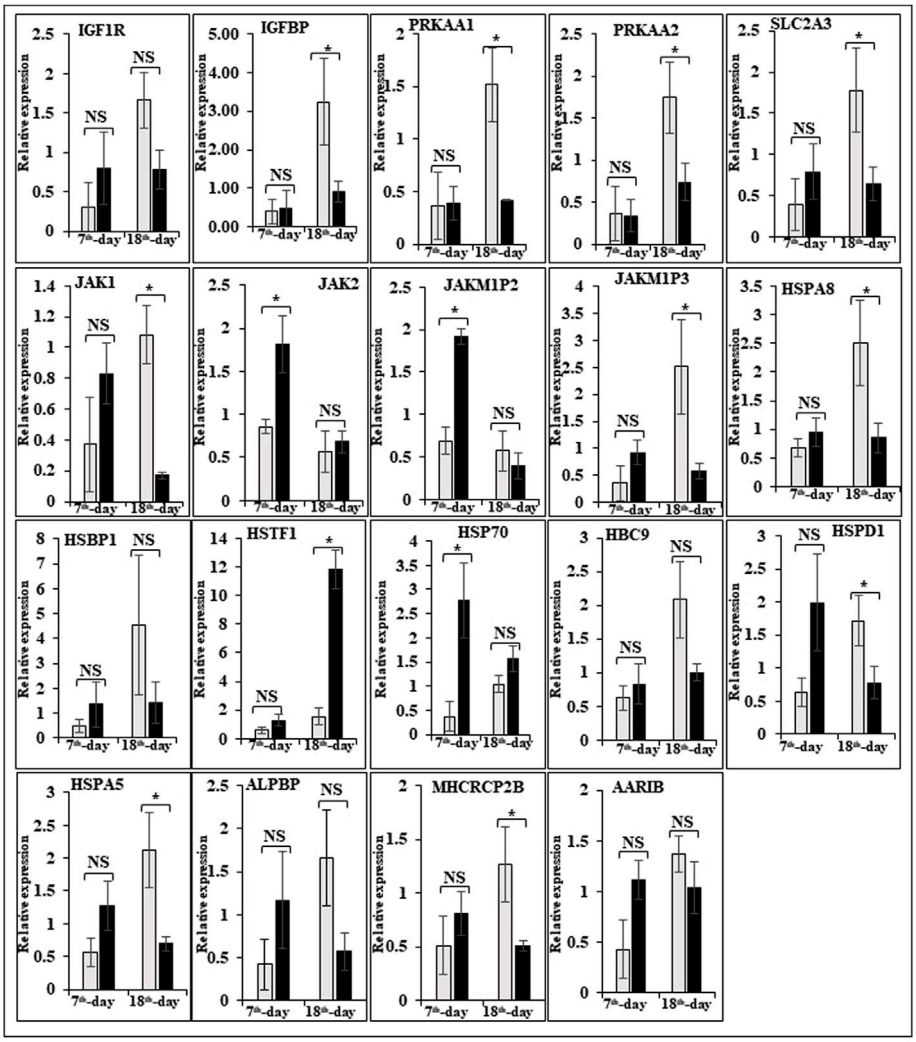

FIGURE 7
Relative expression of DEGs that are involved in cell signaling and egg production. The Y-axis represents relative mRNA expression level, and the X-axis represents tissue samples used for the qPCR study (Control broiler, PD4). The *p* values have been stated on the comparative bars; * indicates the $p \leq 0.05$, and NS indicates the non-significant. Standard error was used for error bars.

combinations: control broiler alone [TFRC (2.21), PGK2 (2.78), GAPDH (3.83)]; improved Aseel alone [TFRC (1.78), PGK2 (2.85), EEF1A1 (3.46)]; combined analysis [TFRC (2.21), PGK2 (2.85), GAPDH (4.21)], respectively (Table 2).

To overcome different software program limitations, the stability of candidate reference genes was determined based on the consensus ranking for gene expression normalization in 7th and 18th-day embryos of the control broiler and improved Aseel. Our study identified the most stable genes and indicated that TFRC and PGK2 for the 7th and 18th-day embryos of the control broiler improved Aseel. Our observations further strengthen the necessity to analyze the stability of candidate reference genes as suitable references.

Expression of muscle-related genes

The difference between fast and slow growth generally depends on the combination of environmental and genetic factors. The embryos collected in this experiment are under the same growth environment. In this experiment, the DEGs related to the main causes of growth and development differences mainly included muscle system processes, muscle tissue morphogenesis, muscle organ morphogenesis, etc. (Figure 4). The genes enriched by these entries are mostly muscle-related genes such as TNNC1, TNNT2, MYL3, MYH7, and FBXO32. The contraction of skeletal muscle-related genes is TNNC1, TNNT2, MYL3, and MYH7. In animals, skeletal muscle

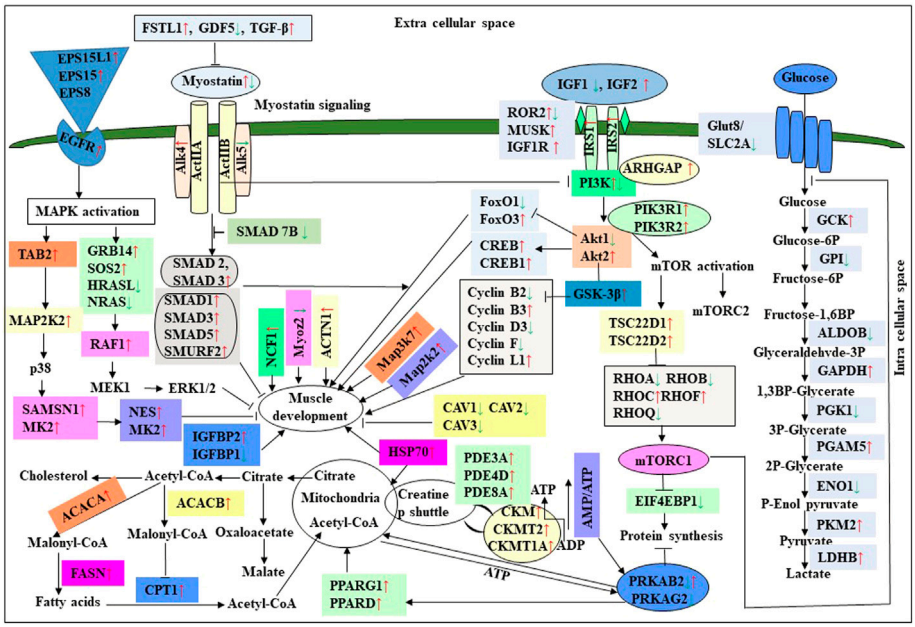

FIGURE 8
Diagrammatic representation of DEGs analysis in the 7th-day embryo and 18th-day thigh muscle of Aseel as compared to respective controls of the control broiler. The red and green arrows represent the up and downregulated genes ($p < 0.05$), respectively. → the activation of the process, ⊣ inhibition of the process. 1. Canonical pathway of Smad activation. Myostatin binds to ActRIIB and induces its assembly with activin type I receptor, subsequent phosphorylation of Smad2/3 leads to its binding with Smad4 and translocation of the complex to the nucleus where it blocks the transcription of genes responsible for the myogenesis. Smad6 and Smad7 compete for the binding with activin type I receptor, and Smad7 can also prevent the formation of the Smad2/3 and Smad4 complex. 2. MAPK activation. The activation of MAPKs is mediated *via* myostatin using different pathways: TAK-1/MAPKK for p38 MAPK or Ras/Raf/MEK1 for ERK1/2. It leads to the blockade of genes responsible for myogenesis. 3. Inhibition of Akt signaling. Akt phosphorylation occurs in the response to insulin and IGF-1. In a normal case, active Akt induces an mTOR signal leading to protein synthesis while inhibiting FoxO by phosphorylation. In pathological conditions, dephosphorylated Akt does not inhibit FoxO. It leads to the accumulation of FoxO in the nucleus where it binds to the DNA and induces the transcription of E3 ubiquitin ligases MURF-1 and Atrogin-1. Smad3 and Smad4 possibly participate in FoxO signaling.

contraction affects growth traits like muscle protein, muscle fiber diameter, and muscle fiber density (Chen, 2012; Zhang, 2014). In the corpus callosum, the muscle is the most crucial component, and the sarcomere is composed of thick and thin muscles. Myosin molecules are mainly involved in thick filament formation, and the thin filament is a complex containing troponin, actin, and tropomyosin (Dong, 2017). The skeletal muscle contraction is mainly based on the relative sliding of thick and thin filaments (Li and Ren, 2007). For controlling the poultry muscle, according to previous reports, the troponin family gene is an important candidate gene (Chen, 2012; Chen L. et al, 2015; Zhao et al., 2018). In this experiment, the DEGs obtained related to the troponin family and essential components of skeletal muscle are troponin T type 3 (TNNT3), slow muscle troponin T (TNNT1), and troponin I type 1 (TNNI1). The myosin superfamily is a highly conserved family of proteins composed of a heavy chain, an alkaline light chain, and a light chain that are widely present in eukaryotic cells (Liu, 2013). In striated muscle, smooth muscle, and non-muscle, myosin is involved in myofilaments as a class II. The MYH gene family is a crucial subunit in the myosin class II molecule and encodes the myosin heavy chain (MYHC) (He and Gu, 2017). The studies revealed that MYH7 gene mutations could cause skeletal muscle disease or skeletal muscle disease with cardiomyopathy (Yuceyar et al., 2015; Feinstein-Linial et al., 2016). In the chicken breast muscle, the MYH7 gene expression is high, and it is a hypothesis that it has a regulatory role in muscle tissue growth and development (Zhang Z. et al., 2016). The MYL3 gene is primarily expressed in slow muscle and is a member of the myosin light chain (MYL) gene family (Zhang C. et al., 2016). Inhibition of myosin light chain gene expression in cell lines showed myoblast proliferation (Zhang et al., 2009). The chicken embryonic leg muscle proteome analysis found that MYL3 protein is closely related to the regulation of muscle contraction and the long non-coding RNAs Inc00037615 and Inc00037619 together regulate muscle development (Ouyang et al., 2017). In this experiment, the DEGs obtained from the myosin family are MYH7 and MYL3. In this study, five genes related to muscle contraction were screened, that is, TNNT3, TNNT1, TNNI1, MYL3, and MYH7. A transcriptome study showed that the expression of these five genes is high in improved Aseel slow growth compared with control broiler

fast growth. Also, these genes are simultaneously present in two significantly enriched pathways, that is, adrenergic signaling in cardiomyocytes and cardiac muscle contraction. Perhaps as a result of the upregulation of these genes and the downregulation of Adrenergic signaling in cardiomyocytes and cardiac muscle contraction, the improved Aseel will grow more slowly than the control broiler. Previous studies showed that in animals under fasting, FBXO32 gene expression was significantly increased, muscles were degraded due to lack of food, and maybe it is associated with muscle atrophy (Bodine et al., 2001; Cleveland and Evenhuis, 2010). The FBXO32 gene expression was found in the chicken's leg muscles, heart, and chest muscles and played an essential role in the 7th-week growth of chickens (Chen C. F. et al., 2015). In results of this transcriptome data showed that the expression of FBXO32 and FBXO7 genes was significantly lower in slow-growth improved Aseel than in the fast-growth control broiler, consistent with previous studies. The DEGs KEGG pathway enrichment analysis revealed significantly enriched adrenergic signaling in cardiomyocytes, cardiac muscle contraction, and tight junction signaling pathways. In cell junctions, the tight junction is an essential component, and it acts as a barrier for cells to pass ions and molecules, plasma membrane apical movement regulation, and basal proteins and lipids (Liu et al., 2012; Zhang et al., 2015). In all eukaryotic cells, a tight junction is recognized as the actin cytoskeleton, and it is involved in cell division, adhesion, movement, and phagocytosis and is also found in the chicken leg muscle transcriptome (Hartsock and Nelson, 2008; Xue et al., 2017). The muscle growth epigenetic transcriptional regulators are differentially regulated in 7EB, such as the protein arginine N-methyl transferase family (PRMT1 and PRMT3), histone lysine N-methyl transferases (EHMT1, and SETDB1), and SWI/SNF chromatin-remodeling enzymes (SmarcB1 and SmarcaA4).

Muscle development and myostatin signaling

The muscle development and differentiation-related genes such as MYOD1, MYF6, MYF5, Myoz2, MAP2K6, MAP3K7, CAV1, CAV2, CAV3, HSP70, and NCF2 were differentially regulated in slow-growing improved Aseel then compared to fast-growing control broiler (Figure 4). In muscle differentiation, MYOD1 promotes muscle-specific gene expression and function together with MYF5 and MYOG (Akizawa et al., 2013). MYOD1, combined with transient placeholder protein, prevents the binding of other transcription factors to DNA and retains the inactive conformation of the DNA (Sartorelli et al., 1997). One of the critical functions of MYOD is to stop differentiated myocyte proliferation by enhancing the transcription of p21 and myogenin to remove cells from the cell cycle (Milewska et al., 2014). Altogether, upregulation of MYOD1 is involved in skeletal muscle phenotype establishment by regulation of precursor cell proliferation and promoting irreversible cell cycle arrest, facilitating differentiation and sarcomere assembly by activating sarcomeric and muscle-specific genes (Buckingham and Rigby, 2014). For this reason, transcriptome data shows downregulation of MYOD1 on the 7th day of an improved Aseel embryo; it may be due to this reason that muscle-specific gene proliferation is slow in improved Aseel. Myozenin is an α-actinin- and γ-filamin-binding protein of Z-line skeletal muscle that binds to calcineurin and is involved in skeletal muscle myocyte differentiation (Frey et al., 2000; Takada et al., 2001). Therefore, the upregulation of MYOZ2 and MYOZ3 genes in muscle tissues suggests that they are involved in muscle growth and development and directly influence meat quality. In addition, MYOZ1 and MYOZ2 genes expressed in mice significantly reduced calcineurin gene expression (Frey et al., 2004, 2008; Schulz and Yutzey, 2004). Our transcriptome study shows less expression of the MYOZ2 gene and high expression of the regulator of calcineurin one gene on the 7th day of improved Aseel embryo; maybe this is the region where muscle development is slow in improved Aseel. Mitogen-activated protein kinases are major components of pathways controlling embryogenesis, cell differentiation, cell proliferation, cell death, muscle development, and response to environmental stress (Errede et al., 1995; Gustin et al., 1998; Lewis et al., 1998; Davis, 2000). MAP2K6 and MAP3K7 activate MAP kinase and nuclear factor-kappa β (NFκB) and play an important role in its signal transduction pathway (Raingeaud et al., 1996; Ninomiya-Tsuji et al., 1999). A proteomic study predicted the expression of MAP2K1, MAP2K2, and MAP2K4, MAP4K4 genes, which may inhibit the low feed efficiency phenotype (Kong et al., 2016). In our transcriptome data, the mitogen-activated protein kinase family genes are upregulated in the 7th-day embryo of improved Aseel. Our results correlate with these results, which may be why the improved Aseel has less feed efficiency and slower muscle growth.

In this study, the expression of caveolin family genes like CAV1, CAV2, and CAV3 was downregulated in the 7th-day embryo of improved Aseel. In cell signaling, the caveolin genes act as sub-cellular structures by assisting the attribute of hormonal signals after binding hormones to the target receptor on the cell surface. CAV3 acts as a muscle-specific isoform for the caveolin protein, and mutations or different expressions of CAV3 can result in muscle myopathy (Biederer et al., 1998; Woodman et al., 2004). In pigs, CAV3 expression was upregulated during muscle hyperplasia, and it may be used as a genetic marker for meat production in swine (Zhu et al., 2006). In mice, the high or low expression of CAV3 made muscle cells more susceptible to oxidative stress and reduced survival through PI(3)K/Akt signaling (Smythe and Rando, 2006). In the low FE PedM broiler phenotype, the higher expression of CAV3 contributed to higher oxidative stress and enhanced muscle development (Bottje and Carstens, 2009). In high FE

breast muscle, the CAV1 protein is involved in insulin signaling (Kong et al., 2016). Based on previous reports, downregulation of caveolin family genes in the 7th-day embryo of improved Aseel may reduce the improved Aseel muscle development. In high FE breast muscle, the up-regulation of HSP70 maintains muscle fiber integrity and enhances muscle regeneration and recovery from damage (Senf et al., 2013). HSP70 is also responsible for the correct folding and assembly of nuclear-encoded proteins, an essential chaperone for mitochondrial DNA-encoded proteins as components of the mitochondrial electron transport chain targeted for import into the mitochondria (Herrmann et al., 1994; Truscott et al., 2003). In low and high FE phenotypes, higher expression of HSP90 and HSPB2 was in response to oxidative stress, respectively (Bottje et al., 2012; Kong et al., 2016). Our transcriptome study correlated with previous studies, and we observed up-regulation of HSP70 and HSPB1 on the 7th day and downregulation of HSP90 on the 18th-day of improved Aseel embryo. The improved Aseel muscle may grow in this region more slowly compared to the control broiler. In NADPH oxidase 2 (NOX2), the NADPH/NADH oxidase is a critical component and is encoded by neutrophil cytosolic factor 2 (NCF2) (Ferreira and Laitano, 2016). In muscle, superoxide was generated by NOX2 in the sarcoplasmic reticulum, a major source of oxidative stress (Dikalov, 2011; Ferreira and Laitano, 2016). In neutrophils, NADPH generates superoxide during phagocytosis. The nuclear factor erythroid 2-like 2 (NFE2L2) is a downstream target for NOX2 and activates genes that contain an antioxidant response element in their promoter regions (Kobayashi et al., 2004, 2006). In high FE animals, it is predicted that NFE2L2 expression should be upregulated (Zhou et al., 2015; Kong et al., 2016). In a high FE commercial broiler, the up-regulation of NCF2 was associated with muscle remodeling and hypertrophy (Zhou et al., 2015). In our transcriptome data, NCF1, NOX1, and NOX3 were upregulated on the 7th-day, and NCF2 was downregulated on the 18th-day of the improved Aseel embryo. Maybe due to the differential expression of these genes, the improved Aseel is more resistant to oxidative stress, low FE, and slow growth.

Myostatin is a member of the tumor growth factor β (TGF-β) family and is known as growth/differentiation factor 8 (GDF-8) (McPherron et al., 1997; Lee and McPherron, 2001). In the myostatin (MSTN) signaling pathway, MSTN binds to its receptors ActIIA/ActIIB and activates ALK4 and ALK5 that phosphorylate Smad2/3, leading to its binding with Smad4 and translocation of the complex to the nucleus, and where it blocks the transcription of genes responsible for myogenesis (Lee et al., 2005; Elkina et al., 2011; Han and Mitch, 2011; Lee and Glass, 2011; Lassiter et al., 2019). Myostatin is solely expressed in skeletal muscle during embryogenesis to control the differentiation and proliferation of the myoblasts (McPherron et al., 1997). However, in the adult stage, it is expressed not only in skeletal muscle but also in other tissues like the heart, adipose tissue, and mammary gland (McPherron et al., 1997; Ji et al., 1998; Sharma et al., 1999; Morissette et al., 2006; Shyu et al., 2006; Allen et al., 2008). In turkey satellite cells, MSTN is a strong negative regulator for skeletal muscle growth, differentiation, and proliferation (McPherron et al., 1997; McFarland et al., 2006). The relation between MSTN and growth performance studies in broilers shows that MSTN is a polymeric gene in which different alleles can affect performance (Gu et al., 2004; Ye et al., 2007; Bhattacharya and Chatterjee, 2013). In the PedM broiler, the FE differences may be due to different haplotypes of the MSTN gene (Lassiter et al., 2019). Myostatin knockdown by RNAi shows muscle growth enhancement in transgenic sheep and chickens (Tripathi et al., 2012; Hu et al., 2013; Bhattacharya et al., 2019). In the present study, MSTN was differentially regulated in the 7th-day improved Aseel embryo. Maybe differential regulation of myostatin is needed for myoblast's differentiation and proliferation in initial embryogenesis. Follistatin (FSTN) regulates the MSTN by inhibiting or limiting its activity. Follistatin-like 1 (FSTL1) is a glycoprotein and rich in cysteine (SPARC) family and comprises a secretion signal, a Follistatin and a Kazal-like domain, two EF-hand domains, and a von Willebrand factor type C domain (Sylva et al., 2013) (http://www.uniprot.org/uniprot/Q12841). In mice, FSTL1 is broadly expressed throughout the entire embryo and restricted to most of the tissues at the end of gestation, but in the adult mouse, it is highly expressed in the heart, lung, and subcutaneous white adipose tissue (Adams et al., 2007; Wu et al., 2010). In this study, FSTL1 was upregulated and follistatin/kazal downregulated on the 7th and 18th-day of improved Aseel embryo, respectively. Initial upregulation and later downregulation of FSTL1 may initiate muscle proliferation in the 7th-day and 18th-day embryo, slowing muscle development in improved Aseel. In humans, activin receptor type-1B (ACVR1B) or ALK4 is a protein that acts as a transducer of activin or activin-like ligand signals (Ten Dijke et al., 1993). ACVR1B forms a complex with ACVR2A/ACVR2B and goes on to recruit SMAD2/SMAD3 (Inman et al., 2002). In addition, ACVR1B transduces nodal, GDF-1, and Vg1 signals combined with other coreceptor molecules like protein cripto (Harrison et al., 2003). Transforming Growth Factor-β (TGFβ) is a key player in cell proliferation, differentiation, and apoptosis and TGFβ receptors are single-pass serine/threonine kinase receptors and can be eminent by their structural and functional properties (Dore et al., 1998). Due to their similar ligand-binding affinities, the transforming growth factor beta receptor I (TGFβR1)/ALK5 and TGFβR2 can be distinguished from each other by peptide mapping only. In mice, the TGFβ1 mRNA/protein has been present in cartilage, endochondral, membrane bone, and skin and plays a role in the growth and differentiation of these tissues (Dickinson et al., 1990). In the present study, activin A receptor type IB (ALK4) and transforming growth factor beta receptor II (TGFBR2) were upregulated, and transforming growth factor beta receptor I (TGFBR1/ALK5) was downregulated in the 7th-

day embryo of improved Aseel. The differential expression of ALK4 and ALK5 may control the myostatin signaling pathway. The SMADs are important for regulating cell development, and growth and they have structurally similar proteins and are the main signal transducers for TGFβ receptors. The eight SMAD genes are distributed into three sub-types of SMADs; they are R-SMADs, Co-SMADs, and I-SMADs (Derynck et al., 1998; Massague et al., 2005). The R-SMADs consist of Smad1, Smad2, Smad3, Smad5, and Smad8/9 and are involved in direct signaling from the TGFβ receptor (Wu et al., 2001; Massague, 2012). The Co-SMADs consist of only SMAD4 and work jointly with R-SMADs to recruit co-regulators to the complex (Shi et al., 1997). R/Co-SMADs are primarily located in the cytoplasm, following TGFβ signaling, and later accumulate in the nucleus, where they can bind to DNA and regulate transcription. I-SMADs consist of SMAD6 and SMAD7 and are predominantly found in the nucleus, where they can act as direct transcriptional regulators. SMAD6 is specifically associated with BMP signaling and SMAD7 is a TGFβ signal inhibitor and suppresses the activity of R-SMADs (Itoh et al., 2001; Macias et al., 2015; Yan et al., 2016). In the present transcriptome study, the SMAD family member 1 (SMAD1), SMAD specific E3 ubiquitin protein ligase 2, SMAD family member 3 (SMAD3), SMAD family member 5 (SMAD5), and TGF-βsignal pathway antagonist Smad7 (SMAD7B) upregulated on the 7th day of the improved Aseel embryo, and they may control the myostatin signaling pathway in the improved Aseel embryonic stage. Summarizes the initial steps in the MSTN signaling pathway in the present study that would potentially exert a negative effect on muscle differentiation and proliferation in the slow-growing improved Aseel.

Energy sensing, fatty acid metabolism, and embryo development

In humans and animals, the adenosine monophosphate-activated protein kinase (AMPK) gene regulates diverse biological functions (Hardie et al., 1998). The mammalian 5′ AMPK gene has two α subunits that is, AMPKα1 and AMPKα2 that are encoded by Prkaa1 and Prkaa2 genes, respectively. The knockout mouse clearly demonstrated that AMPKα2 controls homeostasis in skeletal muscle (Viollet et al., 2003a,b). Also observed was a reduction in fiber numbers (~25%) and sizes (~20%) in the soleus muscle of AMPKα1 knockout mice (Fu et al., 2013). However, in AMPKα2 knockout mice, both fiber size and muscle mass were significantly increased, while the muscle fiber number remained similar to WT animals. The muscle mass reduced and increased differentially expressed alternative polyadenylation sites (DE-APSs) were downregulated in AMPKα1 knockout mice but upregulated in AMPKα2 knockout mice, respectively (Zhang et al., 2018). The five genes, that is, carbonic anhydrase 3 (Car3), myosin light chain kinase family, member 4 (Mylk4), nebulin (Neb), obscurin (Obscn), and phosphofructokinase, muscle (Pfm) are utilized by different APSs and show potential effects on muscle function (Zhang et al., 2018). The high FE phenotype birds show up-regulation of both AMPKα1 and AMPKα2 (Bottje et al., 2012). In low energy level conditions, AMPK gene expression increases ATP production by inhibiting the ATP-consuming pathways like fatty acid synthesis, protein synthesis, and gluconeogenesis and stimulating the ATP-producing pathways like mitochondrial biogenesis and oxidative phosphorylation, glycolysis, and lipolysis (Zhou et al., 2001; Hardie et al., 2003; Carling, 2004). In the present study, the AMPK genes like 5′-AMP-activated protein kinase gamma-1 non-catalytic subunit variant 1 (PRKAG1), protein kinase cAMP-dependent regulatory type I alpha (tissue specific extinguisher 1) (PRKAR1A), protein kinase AMP-activated beta 2 non-catalytic subunit (PRKAB2), protein kinase AMP-activated gamma 2 non-catalytic subunit (PRKAG2), carbonic anhydrase XIII, myosin light chain kinase (MYLK), atrial/embryonic alkali myosin light chain, are downregulated in the 7th-day of improved Aseel embryo, may be due to the downregulation of energy-producing pathways, the improved Aseel muscle will grow slowly compared to control broiler. Curiously, creatine kinase (muscle isoform, CK-M) and creatine kinase (brain isoform, CK-B) were upregulated in high and low FE phenotypes, respectively (Kong et al., 2016; Bottje et al., 2017a,b). The reason for this discrepancy may be that the high FE phenotype of broiler breast muscle has enhanced capabilities for mitochondrial oxidative phosphorylation as well as creatine and phosphorylated creatine shuttle in and out of mitochondria (Bottje et al., 2017b). In the present study, the creatine kinase muscle (CKM), creatine kinase mitochondrial 1A (CKMT1A), creatine kinase brain (CKB), and creatine kinase mitochondrial 2 (sarcomeric) (CKMT2) genes were upregulated in the 7th-day embryo of improved Aseel. In skeletal muscle, nitric oxide is synthesized by nitric oxide synthases, and it is regulated by key homeostatic mechanisms like mitochondrial bioenergetics, network remodeling, mitochondrial unfolded protein response (UPRmt), and autophagy (Figures 4, 5). In mice, nitric oxide synthase deficiency inhibits the Akt-mammalian target of the rapamycin pathway and dysregulates the Akt-FoxO3-mitochondrial E3 ubiquitin-protein ligase 1 (Mul-1) axis (De Palma et al., 2014). Thus, mitochondrial biogenesis and body energy balance were controlled by the nitric oxide-cGMP-dependent pathway (Nisoli et al., 2003). In detail, the inhibition of nNOS/NO/cGMP-dependent protein kinases enhanced the FoxO3 transcriptional activity and increased the Mul-1 expression. The absence of the nitric oxide synthases significantly impaired muscle fiber growth with muscle force, decreased resistance to fatigue, and degeneration/damage post-exercise. In our study, nitric oxide synthase 2 was upregulated, and cGMP-dependent protein kinase type I and FOXO1 were

downregulated on the 7th-day of the improved Aseel embryo, maybe that this is the region where the improved Aseel muscle strength was high, and they are more energetic compared to the control broiler.

The comparative muscle transcriptome analysis between high and low pH chickens showed that most of the glycolysis pathway genes are upregulated in the lower pH chicken (Marziano et al., 2017). The previous study shows that Aseel and broiler chicken's meat do not have any significant pH variation, but the heavier bird's meat had a significantly higher pH (Rajkumar et al., 2016). In this study, glycolysis metabolism-related genes are differentially regulated on the 7th-day of an improved Aseel embryo. The glucose-6-phosphate isomerase, fructose bisphosphate aldolase, phosphoglycerate kinase, and enolase were downregulated, and GAPDH, phosphoglycerate mutase, and pyruvate kinase were upregulated in the 7th-day embryo of improved Aseel. Fructose bisphosphate aldolase is a key enzyme in glycolysis as well as gluconeogenesis and is involved in the stress-response pathway during hypoxia (Beauclercq et al., 2017). The high pH chickens have increased oxidative stress, maybe the higher expression of fructose bisphosphate aldolase is linked to its function in the stress-response pathway rather than to its role in ATP biosynthesis (Beauclercq et al., 2016). In our study, downregulation of fructose bisphosphate aldolase enhanced the ATP synthesis, maybe this is the region where improved Aseel birds have more energy than control broilers. Noteworthy, the up-regulation of glycolysis pathway genes increases the pyruvate levels and enters the citric acid cycle, and thus, higher levels of ATP are produced in improved Aseel. The protein phosphatase-1 regulatory subunit 3A (PPP1R3A) binds glycogen with high affinity, activates glycogen synthase (GYS), and inhibits glycogen phosphorylase kinase (PHK) by dephosphorylation through the protein phosphatase-1 catalytic (PPP1C) subunit. In this study, the glycogen metabolism genes that is, protein phosphatase-1 regulatory subunit 2 (PPP1R2), glycogenin 1, glycogen phosphorylase, and protein phosphatase-1 catalytic subunit beta (PPP1CB) were downregulated in the 7th-day embryo of improved Aseel, maybe this is the region that the improved Aseel muscle has more glycogen. The AMP-activated protein kinase (AMPK) complex is another key regulator of glycogen turnover, and it consists of one α catalytic and two non-catalytic subunits, β, and γ. The β subunit binds to glycogen along with α and γ subunits and forms a heterotrimeric AMPK complex. In the muscle cell, the γ subunits of the AMPK complex act as energy sensors and bind to AMP and ATP (Cheung et al., 2000). In our study, AMP-activated protein kinase beta 2 non-catalytic subunit (PRKAB2), cAMP-dependent protein kinase regulatory type I alpha (tissue-specific extinguisher 1) (PRKAR1A), AMP-activated protein kinase gamma 2 non-catalytic subunit (PRKAG2) were downregulated, and 5′-AMP-activated protein kinase gamma-1 non-catalytic subunit variant 1 (PRKAG1), AMP-activated protein kinase gamma three non-catalytic subunit (PRKAG3), AMP-activated protein kinase alpha 2 catalytic subunit (PRKAA2) were upregulated. The downregulation of β subunits and up-regulation of α and γ subunits may balance the glycogen accumulation and increase the ATP molecules for energy production in the improved Aseel muscle, maybe this is the region where the improved Aseel is stronger than the control broiler. Apart from these, several other genes indirectly influence glycogen storage in muscle. The phosphodiesterase 3B (PDE3B) gene is activated by insulin and induces antiglycogenolytic effects, and the mitochondrial creatine kinase (CKMT2) transfers the high-energy phosphate from mitochondria to creatine. In our study, phosphodiesterase 3A (PDE3A), phosphodiesterase 4D, phosphodiesterase 8A, mitochondrial creatine kinase 2 (CKMT2), mitochondrial creatine kinase 1A (CKMT1A), and mitochondrial creatine muscle (CKM) were upregulated in the 7th-day of embryo improved Aseel, maybe this is the region where the higher expression of these genes in muscle means improved Aseel birds are more energetic compared to control broiler. To produce energy and compensate for the lack of energy due to carbohydrates and glycolysis, the high pH chicken's muscle asks for more intense oxidative pathways, such as lipid β-oxidation and ketogenic amino acid degradation (Beauclercq et al., 2016). In the high pH muscle line, the 3-hydroxymethyl-3-methylglutaryl-CoA lyase (HMGCL) catalysis the final step of leucine metabolism and ketone metabolism, acetyl-CoA acetyltransferase-2 (ACAT2) involved in β-oxidation or degradation of ketogenic amino acids, and nudix hydrolases (NUDT7, NUDT12, NUDT19) hydrolyse a nucleoside di and triphosphates, dinucleoside and diphosphoinositol polyphosphates, nucleotide sugars and RNA caps, were upregulated. In the present study, 3-hydroxymethyl-3-methylglutaryl-CoA lyase like-1 (HMGCLL1), acetyl-CoA acetyltransferase 2 (ACAT2), nudix type motif 7 (NUDT7), and nudix type motif 21 (NUDT21) were downregulated, and carnitine/palmitoyl-transferase 1 (CPT1) was upregulated in the 7th-day embryo of improved Aseel (Figure 6). They may regulate the β-oxidation in peroxisomes as well as mitochondria; this is the region may be fats required for initial embryo development and excess fats involved in β-oxidation and finally provide the energy for embryo development.

Protein synthesis

To promote cell growth, the mTORC1 complex increases protein synthesis, lipid metabolism, and autophagy inhibition and regulates the transcription of several genes (Laplante and Sabatini, 2013). In the high FE phenotype, the cDNA microarray data shows a higher expression of mTORC1 (Schiaffino et al., 2013; Bottje et al., 2014). The mTORC1 complex has two major components, that is, mTOR and RAPTOR (Kim et al., 2002).

RAPTOR and mTOR were up and downregulated in high and low FE birds, respectively, and the up-regulation of RAPTOR could have a positive effect on protein synthesis (Kim et al., 2002). In the low FE phenotype, PRKAR1A and GLUT-8 were upregulated. p70S6k and eukaryotic translation initiation factor 4E (EIF4E) are the key downstream targets for mTORC1 and are involved in enhancing protein synthesis. In low FE birds, the expression of p70S6k was higher (Bigot et al., 2003). The muscle tissue of RNAseq transcriptomic data showed higher expression of eukaryotic initiation, elongation, and translation genes in high FE compared to the low FE PedM phenotype (Bottje et al., 2017a,c). In the present study, the late endosomal/lysosomal adaptor MAPK and MTOR activator 3 (LAMTOR3), protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1) (PRKAR1A), solute carrier family 2 (facilitated glucose transporter) member 8 (SLC2A8)/GLUT8/GLUTX1, solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3), ribosomal protein S6 kinase, 90kDa, polypeptide 1 (RPS6KA1), ribosomal protein S6 kinase, 90kDa, polypeptide 3, ribosomal protein S6 kinase, 52kDa, polypeptide 1, ribosomal protein S6 kinase, 90kDa, polypeptide 6, ribosomal protein S6 kinase-like 1, KIAA1328, KIAA1324, and eukaryotic translation initiation factor 4E binding protein one gene were downregulated in the 7th-day embryo of improved Aseel compared to control broiler (Figure 4). Protein synthesis for muscle growth may be lower in improved Aseel compared to control broiler due to downregulation of mTORC1 complex and ribosomal machinery genes.

Insulin signaling

In chickens, SHC1 is only activated by nutritional changes, suggesting that insulin signaling in chickens has a tissue-specific manner (Dupont et al., 1998a,b). When insulin binds to the insulin receptor, both IRS-1 and SHC1 are activated by a phosphoinositide-3 kinase (PI3K) mediated tyrosine phosphorylation activity (Bottje et al., 2012). The skeletal myoblast is mainly differentiated by two key modulators, that is, insulin-like growth factor 1 (IGF1) and fibroblast growth factor 2 (FGF2) (Florini and Magri, 1989; Florini et al., 1991a). In L6 and C2C12 myoblasts, a high concentration of IGFs reduces their differentiation, whereas a low concentration enhances their differentiation (Florini et al., 1991b, 1986). The IGF binding proteins (IGFBP-1 to IGFBP-6) have highly conserved regions and bind with high affinity to IGF-1 and IGF-2. In the extracellular matrix, IGFBP3 may regulate the interaction of IGFs and it is present in rat soleus muscle (type I muscle fiber) (Stewart and Rotwein, 1996; Spangenburg et al., 2003). In humans, IGFBP3 also plays a role in myoblast differentiation (Foulstone et al., 2003). The fibroblast growth factor 2 (FGF2), transforming growth factor beta (TGFb), and oncogenic Ras also inhibit skeletal myoblast differentiation (Florini and Magri, 1989). The 23A2 myoblast cell lines show the inhibition of 23A2 myoblasts differentiation by IGF1 and FGF2 by stimulating the signaling through mitogen-activated protein kinase (MAPK) kinase (MEK) to MAPK (Weyman and Wolfman, 1998). In our study, IGF2, FGF2, IGFBP2, IGF2BP1, IRS-1, IRS-2, INSIG1, PIK3CA, PIK3CD, TGFb, and Ras oncogene family genes are upregulated in the 7th-day embryo of improved Aseel (Figure 7). Maybe this is also one of the regions for improved Aseel muscle which differentiates slowly when compared to the control broiler.

Expression of plumage development genes

In vertebrate coloration, melanin pigmentation is an important component and is regulated by strong genetic control (Roulin, 2004; Plonka et al., 2009). In chickens, plumage coloration development is extremely complex and can be classified as structural or pigment-based (Lee et al., 2016). For animal coloration, melanin is a common component, synthesized in melanocytes and deposited in various organs as granules (Muroya et al., 2000). Different pigment patterns are formed based on the presence of melanocytes modulating, arranging, or differentiation and associated with a series of functional genes (Lin et al., 2013; Yu et al., 2018). The melanogenesis genes such as HOX, CHAC1, GPX3, BMP5, PITX2, RGN, MITF, TYR, KIT, OCA2, ASIP, MCIR, KITLG, IRF4, SLC24A4, SLC45A2, EDN, TYRP1, and TYRP2 are involved in melanin pigmentation (Zhang et al., 2015a,b; Duffy et al., 2010; Nan et al., 2009; Sulem et al., 2007). The homeobox (HOX) genes are transcription factors and involved in skin appendage development, including hair follicles and feathers (Chuong et al., 1990; Kanzler et al., 1994; Stelnicki et al., 1998; Godwin and Capecchi, 1999; Packer et al., 2000; Awgulewitsch, 2003). In black-bone chickens, four HOX genes, that is, HOXB9, HOXC8, HOXA9, and HOXC9, were identified for melanin pigmentation (Yu et al., 2018). Wnt signaling is essential for skin organogenesis and its appendages like hairs, feathers, and scales, melanocyte development, and differentiation (Yamaguchi et al., 2004, 2007; Widelitz, 2008; Cho et al., 2009). HOXB9 is identified as a target gene for Wnt signaling and HOXC8 is expressed in the first stage of feather morphogenesis like dorsal dermal and epidermal cells (Kanzler et al., 1997; Nguyen et al., 2009). In this study, HOXA2, HOXA9, HOXB3, HOXB5, HOXB7, HOXB8, HOXB9, HOXC11, HOXD1, and HOXD3 are upregulated in the 7th-day improved Aseel embryo, maybe this is the region the where improved Aseel plumage has multiple colors.

In animals, melanogenesis is regulated by GSH and it is closely associated with melanin deposition in the skin of humans and other mammals (Halprin and Ohkawara, 1966; Meister, 1983; Ito et al., 1985; Meyskens et al., 2001; Galvan and Alonso-Alvarez, 2008). The low and high levels of GSH indicate eumelanin-type pigmentation

and phaeomelanin-producing melanocytes found in the skin, respectively (Benedetto et al., 1981). Two feather melanin pigmentation genes were identified in black-bone chickens, such as ChaC glutathione-specific gamma-glutamylcyclotransferase 1 (CHAC1) and glutathione peroxidase 3 (GPX3) (Yu et al., 2018). The CHAC1 cleavage of GSH into 5-oxoproline and Cys-Gly dipeptide and GSH over-expressed mammalian cells causes GSH depletion (Kumar et al., 2012; Crawford et al., 2015). Hence, CHAC1 expression is associated with GSH metabolism and plays an important role in the melanogenesis process. In eumelanin and phaeomelanin synthesis, the hydrogen peroxide is reduced by GSH-dependent peroxidase and GPX3 belongs to the GSH peroxidase family and catalyzes the GSH to glutathione disulphide (GSSG) (Benathan, 1997; Meyskens et al., 2001). The melanoma cell's pigmentation is regulated by GSH levels, glutathione peroxidase, and glutathione reductase, suggesting that GSH-mediated redox processes play an important role in melanogenesis regulation (Benathan et al., 1999). Hence, the expression of GPX3 plays an active role in chicken feather melanogenesis. In this study, CHAC1, CHAC2, gamma-glutamylcyclotransferase (GGCT), and GPX8 were downregulated on the 7th-day of the improved Aseel embryo, maybe this is the region the improved Aseel has multiple colors on their feathers. For plumage melanogenesis in black-bone chickens, two pathways were identified: that is, the TGF-β signaling pathway, and ascorbate and aldarate metabolism (Yu et al., 2018). TGF-β regulates the proliferation and synthesis of melanin in chicken retinal pigment epithelial cells (Kishi et al., 2001). The BMP5 and PITX2 genes are involved in the TGF-β signaling pathway and play a role in the synthesis of chicken melanin, and BMP5 and PITX2 were found to be highly expressed in white and black feather bulbs, respectively (Kishi et al., 2001). The BMP3 gene was highly expressed in embryonic and post-embryonic stages of the control layer when compared to broiler chicken, and the BMP4 gene was differentially expressed in juvenile stages of broiler and layer chicken, respectively (Divya et al., 2018a,b). The regucalcin (RGN) is a calcium-binding protein involved in the ascorbate and aldarate metabolism pathways and plays a crucial role in intracellular calcium homeostasis maintenance (Moisa et al., 2013). In this study, transforming growth factor beta receptor II (TGFBR2), BMP1, BMP1A, BMP4, BMP7, BMPR1A, BMPR2, and PITX3 were upregulated and RGN was downregulated on the 7th-day embryo of improved Aseel, maybe this is the region the improved Aseel plumage has multiple colors.

melanin synthesis, TYR is a rate-limiting enzyme and is involved in different oxidative steps (Parvez et al., 2006; Olivares and Solano, 2009). In black vs. white skin chickens, the TYR is highly expressed and it is consistent with sheep coat color studies (Norris and Whan, 2008; Fan et al., 2013; Zhang J. et al., 2015). In black-coated vs. white-coated sheep, the TYRP1 gene was highly expressed (Fan et al., 2013). KIT is a receptor tyrosine kinase, the mutation in KIT showed piebaldism and auburn hair color in humans, and it plays an important role in UVB-induced melanogenesis in the *epidermis*, and inhibition of KIT expression may result in the lightening of human skin color (Yang Y. J. et al., 2013; Yamada et al., 2013). In black-skinned chickens, KIT is highly expressed, and black skin color is due to increased melanin compared to white skin color (Zhang J. et al., 2015). In melanocyte development, microphthalmia-associated transcription factor (MITF) plays a role, and mutations in the MITF gene are responsible for Japanese quail and chicken plumage color and it is supported by alternative splicing of the MITF gene in the skin of sheep (Minvielle et al., 2010; Saravanperumal et al., 2014). In ducks, TYR and MITF expression may involve the formation of black and white plumage (Li et al., 2012). Melanocortin-1 receptor (MC1R) binds to melanocyte stimulating hormone (MSH) to initiate the melanogenesis cascade and regulates mammalian skin pigmentation and hair color (Roberts et al., 2006; Schaffler et al., 2006; Solano et al., 2006; Lalueza-Fox et al., 2007). The agouti signaling protein (ASIP) is responsible for the skin color of both white and black-coated sheep, and mutations in ASIP cause black and tan pigmentation phenotypes in pigs (Drogemuller et al., 2006; Norris and Whan, 2008). The ASIP binds to MC1R and reduces the MITF and TYR gene expression, and finally, the phaeomelanin will be reduced in epidermal tissues. In black-skinned chickens, the expression of ASIP is higher than compared to white-skinned chickens, and it can suppress the MC1R gene expression in black-skinned chickens (Zhang J. et al., 2015). Oculocutaneous albinism type 2 (OCA2) is a common skin pigmentation disorder caused by a mutation in the OCA gene. In black chickens, OCA2 was upregulated and it may be related to black skin color (Zhang J. et al., 2015). In chickens, the endothelins (EDN1, EDN2, and EDN3) and their receptors (EDNRA, EDNRB, and EDNRB2) are involved in the regulation of pigmentation and plumage (Liu et al., 2019). The expression of EDNRB2 was significantly different between adult black and non-black chickens (Dorshorst et al., 2011). In this study, TYRP1, KITLG, MITF, MC1R, AGRP, YRK, and P56LCK were upregulated and EDNRA and EDN1 were downregulated on the 7th-day of the improved Aseel embryo, and this is the region where the improved Aseel plumage has multiple colors.

Expression of genes related to egg production

In chickens, the reproductive system is regulated by hypothalamic–pituitary–ovarian (HPO) axis hormones, while ovulation, the GnRH-I triggers the pituitary gland to release FSH and LH, and stimulates the secretion of estradiol and progesterone in the ovary (Bain et al., 2016). Several reproductive hormone regulation genes were identified between high and low egg production chickens, such as

HADH, HMGCR, RAB11FIP1, and FAM3D (Mishra et al., 2020). HMGCR, HMGCS1, NFKB1, VAV3, SOS1, IL1R1, MEF2C, and STK3 were highly expressed in the pituitary gland, as were lipid metabolic processes, prolactin, and MAPK signaling pathway genes. In chickens, the anterior pituitary gland synthesizes and releases prolactin and is involved in reproduction, laying eggs, metabolism, development, and hypothalamic–pituitary–gonadal axis regulation (Talbot et al., 1991; Reddy et al., 2002). In chicken, the HMGCR gene variants (G-789-A, C-937-G, and A-2316-C) and high and low concentrations of VLDL showed higher and lower egg production, respectively (Han et al., 2014). In laying chickens, the APOB is a primary organizing protein for chylomicrons and VLDL and is responsible for the transport of lipoprotein, circulating in the plasma and stored in the oocytes to form an egg yolk (Deeley et al., 1985; Nys and Guyot, 2011). In our study, GNRHR, HADHB, HMGCS1, HMGCS2, RAB11FIP2, RAB11FIP3, RAB11FIP4, NFKB2, VAV2, SOS2, MEF2D, STK3, PRL, PRLR, PRLH, and PRLHR2 genes were upregulated, and FSHR, VAV3, IL1RL1, and IL1RAPL2 genes were downregulated, and family with sequence similarity genes and apolipoprotein B were differentially regulated in the 7th-day embryo of improved Aseel. Maybe this is the region where the improved Aseel has less egg production than the commercial chicken. In avian species, the genes SPP1, BPIFB3, and EDIL3 are mainly involved in egg and oviposition, development of the reproduction system, and vesicle-mediated eggshell calcication, respectively (Jeong et al., 2012; Dong et al., 2019; Stapane et al., 2019; Yang et al., 2019). In this study, secreted phosphoprotein 1 (SPP1) and secreted phosphoprotein 2 (SPP2) genes are downregulated, and EDIL3 is upregulated on the 7th-day of an improved Aseel embryo, due to this, egg and oviposition are less, and eggshell calcication is more in improved Aseel. In nandan-yao chickens, FN1, FGF7, SOX2, ALDOB, and HSPA2 genes are expressed in the ovary, and UQCRH, COX5A, FN1, TGFB, and ACTN1 genes are expressed in the uterus and involved in egg production (Sun et al., 2021). In this study, FN1, FNDC3A, FGFR1, FGFR3, FRS3, FRS2, FGFR2, FGFRL1, FGF8, FGF18, FGF3, FGF12, SOX2, SOX3, SOX4, SOX5, SOX7, SOX8, SOX9, SOX11, SOX17, HSPA2, HSPA4, COX1, COX2, COX3, TGFB4, and ACTN1 were upregulated and ALDOB, HSPA5, HSPA8, HSPA9, HSP12A, UQCRFS1, UQCRB, were downregulated in the 7th-day improved Aseel embryo. The differential expression of the ovary and uterus-related genes is differentially expressed on the 7th-day of an improved Aseel embryo, due to this region, the egg production is less in improved Aseel. The DEGs related to the pituitary gland in high and low egg production chickens are mainly involved in mTOR and Jak-STAT signaling pathways (Mishra et al., 2020). In mice, the mTOR signaling pathway will regulate granulosa cell proliferation and differentiation (Yaba and Demir, 2012). In this study, the mTOR and Jak-STAT signaling pathways were upregulated in the 7th-day improved Aseel embryo.

In high egg production chickens, several embryonic development genes are upregulated, such as GDNF, HOXD9, MEF2C, STK3, CLRN1, IRX5, LBX1, CSNK1A1, LGR5, PRDM15, and DAB2IP (Mishra et al., 2020). In this study, the GDNF, HOXA2, HOXB3, HOXB5, HOXB7, HOXB8, MEF2D, STK3, STK16, STK25, STK32B, IRX1, IRX2, IRX5, IRX6, LBX1, LBX3, PACSIN2, RGR, PRDM4, PRDM8, and DAB2IP genes are upregulated on the 7th-day of the embryo, these genes are involved in embryo development. In the ovary, the tryptophan metabolism and PI3K-Akt signaling pathways were enriched, and they are important for egg production (Mishra et al., 2020). In stressful conditions, peripheral and brain tryptophan levels can be altered by stimulating the immune system and activating the hypothalamic–pituitary–adrenal axis (Miura et al., 2008; Birkl et al., 2019). In this study, tryptophan metabolism was downregulated on the 7th-day of the embryo and upregulated on the 18th-day thigh muscle, this is the region where the improved Aseel has less egg production. In high egg production, the hypothalamus genes are highly expressed, such as EXFABP, SNRNP25, FAM114A1, and SIX1 (Mishra et al., 2020). In the hypothalamus, nerve growth factor response, lipid metabolism, and canonical Wnt signaling pathway genes were highly expressed, that is, SIX1, RPS15, and IGFBP7, thus playing a role in chicken egg production. In laying hens, the dietary corticosterone treatment shows low levels of extracellular fatty acid-binding protein (EXFABP) and suggests that the egg white protein's synthesis and secretion may be affected by environmental stress (Kim and Choi, 2014). Many studies have found that ovarian follicular development is stimulated by IGFBPs and plays a role in the ovary's FSH action (Zhou et al., 1997; Mazerbourg et al., 2003). In chicken adipose tissue, the lipid metabolism gene like insulin-like growth factor binding protein 7 (IGFBP7) was highly expressed and it was correlated with egg production (Nagaraja et al., 2000; Wang et al., 2007). In this study, FAM114A, FAM116A, FAM116B, FAM117A, FAM117B, SIX1, SIX2, RPS13, RPS24, IGFBP2, IGFBP3, and IGFBP5 genes are upregulated and FABP1, FABP2, FABP3, FABP5, SNRPB, SNRPB2, IGFBP1, and IGFBP7 genes are downregulated in the 7th-day embryo of improved Aseel, these differentially expressed genes may cause less egg production in improved Aseel (Figure 7). The cuticle or organic matrix of the eggshell-related genes that is, MEPE, BPIFB3, RARRES1, and WAP are highly expressed in oviposition (Mann et al., 2006; Rose-Martel et al., 2012; Bain et al., 2013). In this study, RARA, RARB, POSTN, CDH4, CDH13, CDH8, CDH11, and CDH20 were upregulated, and RARRES1, and CDH1, were downregulated

in the 7th-day improved Aseel embryo. Due to the differential expression of these genes, maybe the improved Aseel eggshell thickness is more than commercial laying eggs.

The mitochondrial oxidative phosphorylation, active transport, and energy metabolism related genes such as NADH dehydrogenase, ND4, ND1, ND2, ND5, ACTB, GAPDH, ATP6, and ATP1A are required for a large amount of energy and active secretion of proteins and minerals (Bar, 2009). A recent report shows differential expression of these genes like MEPE, COX1, COX3, COX2, BPIFB3, Cytochrome b, ATP6, ND5, ATP1A1, ND4, ND2, EIF4A2, UBB, Novel mitochondrial gene, IGLL1, HSPA8, RASD1 in the GNRH1 vs. AVT study (Pertinez et al., 2020). In this study, ND1, ND2, ND3, ND4, ND4L, ND5, ND6, GAPDH, ATP6, ATP1A2, COX1, COX2, COX3, and CYTB were upregulated, and NDUFAF1, NDUFA4, NDUFA5, NDUFA9, NDUFA10, NDUFAF4, ATP1A1, EIF4A2, COX15, COX19, COX20, CYB5A, CYB5B, and CYB5R2 were downregulated in the 7th-day of the improved Aseel embryo. Most of the energy metabolism genes are upregulated and maybe the region improved Aseel is stronger than commercial birds.

Conclusion

The comparative transcriptome study between slow-growth improved Aseel and fast-growth control broiler revealed the DEGs and their significantly enriched pathways in slow-growth improved Aseel, which inferred that they play an important role in regulating the growth and development of improved Aseel. The transcriptome data provides a theoretical basis for improving the performance of the slow growth improved Aseel as well as how to control the growth performance of fast grown control broiler chickens and provides reference data for revealing the molecular mechanism of slow growth improved Aseel as well as fast growth control broiler chickens. In this study, the mechanistic picture of gene expression data (Figure 8) shows the embryo development, muscle development, egg production, plumage development, and energy production in improved Aseel would be fostered by a combination of 1) differential regulation of MSTN, activin-like kinasesn and upregulation of SMADs, expect SMAD7 in the myostatin signaling pathway, combined with downregulation of caveolin's (CAV1, CAV2, and CAV3) and differential regulation of insulin-like growth factor binding proteins; 2) upregulation of HSP70, NCF1, and Map2k2 and downregulation of MYOD1 and MYOZ2; 3) upregulation of fatty acid synthesis and β-oxidation genes (ACACA, ACACB, FASN, and CPT1); 4) differential MAPK signaling pathway genes (MAP2K2, MKA, NES, SAMSN1, SOS2, and TAB2); 5) differential regulation of Jak-STAT, mTOR, and TGF-β signaling pathway genes (IGF1, IGF2, IRS1, IRS2, PI3K, Akt1, Akt2, FoxO1, FoxO3, TSC22D1, TSC22D2, RHOA, RHOB, RHOC, RHOF, RHOQ, and EIF4EBP1); 6) differential regulation of mitochondrial genes (ND1, ND2, ND3, ND4, ND4L, ND5, ND6, ATP6, ATP1A2, COX1, COX2, COX3, CYTB NDUFAF1, NDUFA4, NDUFA5, NDUFA9, NDUFA10, NDUFAF4, ATP1A1, EIF4A2, COX15, COX19, COX20, CYB5A, and CYB5B); 7) differential regulation of glycolysis/gluconeogenesis genes (GCK, GPI, ALDOB, GAPDH, PGK1, PGAM5, ENO1, PKM2, and LDHB).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ Supplementary Material.

Ethics statement

The animal study was reviewed and approved by the Institute Animal Ethics Committee, ICAR-Directorate of Poultry Research, Rajendranagar, Hyderabad, India.

Author contributions

MK conducted the wet lab experiment, data analysis, and prepared the first draft; RA performed the wet lab experiment; RC performed data analysis and prepared tables; TB developed the idea, designed and planned the research work, performed the wet lab experiment, and edited the draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.990849/full#supplementary-material

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Growth performance, survivability and profitability of improved smallholder chicken genetics in Nigeria: A COVID-19 intervention study

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The impact of COVID-19 pandemic on smallholder farming households (SFH) includes increased poverty, and loss of livelihoods. Provision of livestock to SFH is a helpful intervention to mitigate this impact. This study provided a total of 150 smallholder poultry farmers, randomly selected from three states (Kebbi, Nasarawa, and Imo) in Nigeria, with ten 5-week-old chickens (mixed sexes) each, of either FUNAAB Alpha or Noiler chicken genetics. The improved, dual-purpose chickens were evaluated for growth performance (GP), survivability and profitability. The birds were managed under semi-scavenging production system. Body weight, mortality, and cost of production (COP) were recorded every 4 weeks until 21 weeks of age. Profitability was a function of the COP, and the selling price for live-birds (cocks). Body weight of Noiler (1,927 g) birds was not significantly ($p > .05$) higher than FUNAAB Alpha (1,792 g) at 21 weeks. Agroecology and genetics had significant ($p < .05$) effects on GP and survivability. Survivability of FUNAAB Alpha was higher ($p < .05$) than Noiler, with Nasarawa (81%–96%), having the highest ($p < .0001$) survival rate compared to Imo (62%–81%), and Kebbi (58%–75%). At 21 weeks, the number of cocks and hens differed significantly ($p < .05$) within the states (Imo: 2.4 ± .2 and 5.4 ± .3; Kebbi: 2.6 ± .2 and 5.5 ± .3; and Nasarawa: 2.9 ± .2 and 5.8 ± .3). Nasarawa (NGN 7,808; USD 19) ranked best for profitability, followed by Kebbi (NGN 6,545; USD 16) and Imo (NGN 5,875; USD 14). Overall, this study demonstrates that provision of improved chickens to vulnerable SFH in Nigeria holds great potential for economic growth, and resilience during emergencies, such as the COVID-19 pandemic.

KEYWORDS

smallholder poultry, livelihoods, food security, COVID-19, Nigeria, improved chicken genetics

1 Introduction

The COVID-19 pandemic has resulted in economic hardships to smallholder farming households in low-to-middle income countries (LMIC). It increased the risk of poverty among these farming populations who were already vulnerable to food insecurity and loss of livelihoods due to other environmental, and climate-related risks. In Nigeria, during the pandemic, there was a 31% decrease in average monthly income of smallholder poultry farmers (SPF) which resulted in a 28% increase in the number of SPF living in poverty (Bamidele and Amole, 2021). In a recent study, we showed that about half (49%) of the total number of SPF, living above the international poverty line prior to the pandemic, had been plunged into poverty within a 15-month period after the onset of COVID-19 (Bamidele and Amole, 2021). The changes to household income, food security, and poverty index occasioned by the pandemic highlight the significance of livestock, especially chickens to the socio-economic status of SPF.

Several measures have been proposed as interventions to support resource-poor and vulnerable smallholder livestock farmers in LMIC, some of which include: improved access to animal health services and markets, provision of feeds and water supplies, availability of livestock re-stocking options, and targeted cash transfers (Catley, 2020; FSC, 2020). These interventions were proposed to mitigate the impact of the pandemic on household livelihoods and food security as well as prevent the adoption of negative coping mechanisms by farmers through indiscriminate sale of livestock, use of inefficient restocking options, abuse of veterinary and human-labeled medicines (i.e., antibiotics), reduced consumption of animal-sourced foods, and depletion of emergency savings (Sitko et al., 2022; FSC, 2020). In LMIC, interventions involving the use of sustainable smallholder poultry are central to livelihoods' sustenance, social and economic development (Attia et al., 2022). The introduction of improved dual-purpose chickens has been found to be suitable for backyard poultry production (Torres et al., 2019; Guni et al., 2021a). Birhanu et al. (2022) reported that the use of improved tropically adapted chicken breeds increased the production and productivity of birds in smallholder flocks in sub-Saharan Africa. This eventually paved way for the generation of more income, while contributing to food security, social and ecological resilience (Dumas et al., 2016; Kassa et al., 2021). These improved chicken breeds have also been reported to be more preferred than the indigenous (native, unimproved) chickens by smallholder farmers in terms of market-oriented performance indices (Yakubu et al., 2020; Birhanu et al., 2022).

In the current study, our intervention focused on the provision of two improved, dual-purpose (meat and eggs) chickens to SPF for re-stocking purposes, and as a source of food and income in Nigeria. The two chickens, FUNAAB Alpha and Noiler, before the advent of COVID-19 pandemic have been tested, both under on-station, and on-farm (scavenging and semi-scavenging) conditions, and identified as low-input-high-output, farmer-preferred genetics for dual-purpose functions (Ajayi et al., 2020; Bamidele et al., 2020; Yakubu et al., 2020). Also, the potential of these chickens for improving household livelihoods and food security have been reported (Alabi et al., 2020). Therefore, the objective of this study was to evaluate the growth performance, survivability, and profitability of the two chickens as intervention measures for SPF during the recovery phase of the COVID-19 pandemic in Nigeria.

2 Materials and methods

2.1 Description of study area

The study was conducted between June and November 2021 in three states of Nigeria: Kebbi (Sudan savanna/northern Guinea savanna), Nasarawa (southern Guinea savanna/derived savanna) and Imo (lowland rainforest/swamp). Each state represented a distinct agroecological zone with its features as described by Yakubu et al. (2020), and the locations of the three states within the respective agroecologies have been highlighted on the map of Nigeria by Bamidele and Amole (2021). The states were selected for the intervention study based on a previous impact assessment of COVID-19 on smallholder poultry households, in both the northern and southern regions of Nigeria (Bamidele and Amole, 2021).

2.2 Sampling procedure

A total of 150 farmers were selected for the intervention. In each of the three states, two local government areas (LGA) were purposively selected from the list of LGAs that participated in the COVID-19 impact assessment study (baseline) (Bamidele and Amole, 2021). The selection of farmers within the LGAs was conducted at the village level. One village per LGA was randomly selected from the villages previously sampled during the baseline. Geolocations of the study sites are available at https://www.mapcustomizer.com/map/Nigeria_COVID-19_Intervention_study. From each village, 25 farmers were then selected, randomly, among the farmers who had been recruited into the baseline study. In total, 50 farmers were selected per state. The selection of farmers within the households was based on the persons (adults) primarily responsible for keeping the chickens.

Also, all the farmers had not received any form of COVID-19 palliative from the Federal Government of Nigeria (FGN).

2.3 Animal distribution and husbandry practice

Ten pre-vaccinated 5-week-old chickens, of either FUNAAB Alpha or Noiler, were given to each of the farmers. During brooding, the chicks were vaccinated against Marek's, Newcastle and Fowl pox diseases. The ten chickens were equivalent to 50% of NGN 20,000 COVID-19 cash transfer payments to the poorest of the poor by FGN (Channels tv, 2020; Erezi, 2020). The two chickens were distributed to the farmers using a simple random sampling technique as described by Ajayi et al. (2020). In each village, 10 farmers received FUNAAB Alpha chickens while 15 farmers received Noiler chickens. Prior to bird distribution, the chicks were tagged at the wing. The genetic composition of the two chicken groups have been described by Adebambo et al. (2018), and Sanda et al. (2022). During the period of study, the farmers practiced semi-scavenging system of production with daily feed supplementation, and night-time shelter. The daily feed supplementation included household kitchen-waste (based on food patterns), agricultural by-products and plant parts that were locally available to the farmers. The husbandry practice included Newcastle disease vaccination (booster doses), and treatment of common poultry diseases by the farmer either through ethnoveterinary medicines or synthetic (pharmaceuticals) antibiotics. Consequently, based on the use or non-usage of synthetic antibiotics, the farmers were further classified into two groups.

2.4 Data collection

Data collection tool was designed using the web-based Google Forms App (docs.google.com/forms). All the field officers were trained on the use of the tool, and data was entered using smartphones. Each village had an assigned field officer who visited each of the households to provide technical support to the farmer on smallholder poultry husbandry, monitor the birds, and collect data. The household visits to the farmers were from the time the chicks were distributed, at 5 weeks up to 21 weeks of age. Data on growth performance, mortality, and cost of production (feed and drugs) were recorded every 4 weeks. The protocol for data collection was as described by Ajayi et al. (2020). Body weight (g) was taken using a digital weighing scale, and mortality was recorded by actual count of dead birds. At week 21, profitability was determined based on the total cost of production and the expected selling price for live-birds. Each farmer determined the appropriate selling price as guided by the prevailing market price. The decision to either sell the birds or slaughter for meat consumption was made by the farmers. During data collection, all COVID-19 safety protocols were adhered to by the field officers and farmers.

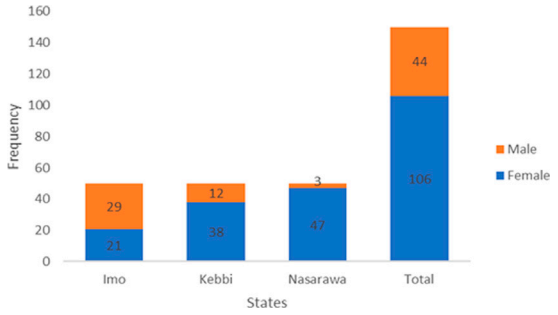

FIGURE 1
Gender distribution of smallholder poultry farmers in the study.

2.5 Statistical analysis

The collected data was assessed as spreadsheet (Microsoft Excel) through the Google Workspace and imported into R version 3.5.1 software (R Core Team, 2018) using the xlsx package (0.6.5 version). Imported data was wrangled by modifying the formats of some variables (e.g., Use of antibiotics, cost price, etc.) and removing errors such as "NaN," and the letter 'O' in place of zero. Also, prior to statistical analyses, the data was visualized using boxplot, and all outliers were removed. Growth performance data were analyzed using unbalanced type-III three-way Analysis of variance (ANOVA) implemented in R car (version 3.0-2) package (Fox and Weisberg, 2011) to test the fixed effect of genetics, sex, antibiotics usage, agro-ecology as well as their interactions on production performance of birds. Significant differences were separated using Tukey test ($\alpha = 0.05$) for multiple comparisons through R least square means (version 2.30-0) (Length, 2016), and R multcomp (version 1.4-10) (Hothorn et al., 2008) packages. The Cox proportional hazards regression analysis using R survival (version 2.42-3) (Therneau, 2015) and survminer (version 0.4.4) (Kassambara and Kosinski, 2016) packages were also used to investigate the effects of genetics, sex, antibiotics usage, and agro-ecology on the survival of birds. Significance of these factors was tested using Kaplan–Meier and log-rank tests. Hazard ratios were derived from Cox models. Proportional hazards assumed a non-significant relationship between scaled Schoenfeld residuals and time. All statistical analyses were performed in R version 3.5.1 (R Core Team, 2018). The dollar (USD) to naira (NGN) exchange rate used for the profitability analysis was USD 1 to NGN = 410.66 as listed by the Central Bank of Nigeria 2021 (https://www.cbn.gov.ng/rates/ExchRateByCurrency.asp).

TABLE 1 Effect of genetics on body weight, weight gain, and mortality (LSM ± SE).

Week	Genetics	*N*	Body weight	CV (%)	Bodyweight gain	CV (%)	Mortality (%)
5	FUNAAB Alpha	600	414.17 ± 29.51	63.29	—	—	
	Noiler	900	471.72 ± 25.89	77.13	—	—	
9	FUNAAB Alpha	512	629.39 ± 16.54	32.08	215.12 ± 29.82	13.86	18.42 ± 5.23
	Noiler	744	626.16 ± 14.55	29.62	154.75 ± 26.23	16.95	25.19 ± 4.93
13	FUNAAB Alpha	477	960.82 ± 36.16	27.97	331.42 ± 32.31	70.94	8.14 ± 2.5
	Noiler	669	999.68 ± 31.81	46.04	373.52 ± 28.43	7.61	11.48 ± 2.5
17	FUNAAB Alpha	444	1,240.71 ± 48.71	32.78	279.93 ± 41.48	97.89	12.93 ± 4.2
	Noiler	629	1,322.11 ± 42.96	44.37	322.43 ± 36.58	11.35	6.99 ± 4.2
21	FUNAAB Alpha	418	1,792.38 ± 73.25	37.02	552.10 ± 49.98	90.32	5.76 ± 4.45
	Noiler	584	1,927.02 ± 65.48	54.72	604.98 ± 44.68	7.44	15.61 ± 4.2

N, number of birds; LSM ± SE, least-square means ± standard error; CV, coefficient of variation.

2.6 Ethical standard

The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of International Livestock Research Institute (ILRI) (ILRI COVID-19 Project 03/2021). All the farmers provided informed consent prior to the start of the study.

3 Results

3.1 Sociodemographic of smallholder poultry farmers

Figure 1 shows the gender distribution of farmers in this current study. Majority of the farmers were women (106, 70.7%), with Nasarawa (47, 94%) having the highest percentage of female farmers compared to Kebbi (76%), and Imo (42%) states. The average household size varied significantly ($p < .05$) between male and female smallholder poultry farmers in Imo (Male: 6.55 ± 1.73, female: 5.76 ± 1.66), Kebbi (Male: 10.80 ± 6.47, female: 6.47 ± 2.76) and Nasarawa (Male: 7.83 ± 1.69, female: 5.75 ± 1.95) states. The average household size was higher in Kebbi (8) than in Imo (6) and Nasarawa (6) states.

3.2 Growth performance and mortality of the improved chickens

Table 1 shows the effect of genetics on body weight, weight gain and mortality of the chickens. Genetic effect was similar ($p > .05$) for body weight, weight gain and mortality across the weeks. The results showed consistent increase in body weight from week 5–21. Noiler birds were heavier and gained more body weight than FUNAAB Alpha birds, at weeks 5, 13, 17, and 21, although these differences were not statistically significant ($p > .05$). At 9 weeks, bodyweight was lower ($p > .05$) in Noiler (626.16 g) compared to FUNAAB Alpha (629.39 g), and FUNAAB Alpha had a higher ($p > .05$) body weight gain (212.12 g) than Noiler (154.75 g). The mortality rate in FUNAAB Alpha ranged from 6%–18%, compared to 7%–25% in Noiler birds.

Body weights of the birds were significantly ($p < .05$) different across the agro-ecological zones, with Nasarawa State consistently having higher body weights compared to Kebbi and Imo states all through week 5–21 (Table 2). Agro-ecological zones had no significant ($p > .05$) effect on bird mortality, except at week 9 where mortality rate was significantly ($p < .05$) higher and similar in both Imo (26.7%) and Kebbi (35.4%), compared to Nasarawa (4.7%). At week 9 and 21, body weight gain was significantly different ($p < .05$) across the agro-ecological zones. At 9 weeks, birds in Imo (282.70 g) and Nasarawa (187.70 g) had 134.83 g and 39.83 g more body weight gain than Kebbi (147.87 g), respectively. On the other hand, at week 21, birds in Nasarawa significantly ($p < .05$) had the highest body weight (1,829.45 g) and body weight gain (447.36 g), compared to Imo (1,427.88 and 246.08 g) and Kebbi (1,588.28 and 206.75 g).

As shown in Supplementary Table S1, sexual dimorphism ($p < .05$) existed in both genetics for body weight as males were heavier than the female birds from week 9 through week 21. The coefficient of variations (CV) for bodyweight in female birds, ranged from 31% to 82% while CV for male birds ranged from 30% to 64%. Body weight gain were similar ($p > .05$) across the ages with the exception of week 17, where males (380.1 g) had over 70% increase ($p < .05$) in body weight gain than females (222.1 g). Across weeks 9–21, mortality was similar ($p > .05$) in both sexes, and ranged from 8.5% to 26.0% and 8.2%–18.0% for males and females, respectively.

TABLE 2 Effect of agro-ecological zones on body weight, weight gain, and mortality (LSM ± SE).

Week	State	*N*	Body weight	CV (%)	Bodyweight gain	CV (%)	Mortality (%)
5	Imo	500	261.43 ± 38.45^{b}	20.66			
	Kebbi	500	524.14 ± 38.45^{a}	49.92			
	Nasarawa	500	569.06 ± 38.45^{a}	78.85			
9	Imo	404	544.05 ± 38.84^{c}	35.70	282.70 ± 22.34^{a}	65.69	26.7 ± 5.21^{b}
	Kebbi	373	672.01 ± 42.72^{b}	29.43	147.87 ± 24.57^{b}	28.06	35.43 ± 4.76^{b}
	Nasarawa	479	756.74 ± 41.96^{a}	18.32	187.70 ± 24.13^{ab}	19.90	4.66 ± 4.76^{a}
13	Imo	356	839.12 ± 39.87^{c}	41.65	295.69 ± 22.94	11.47	15.2 ± 2.92
	Kebbi	349	960.39 ± 40.31^{b}	28.55	288.83 ± 23.18	67.83	7.75 ± 2.92
	Nasarawa	441	1,083.93 ± 39.04^{a}	41.04	327.26 ± 22.46	9.38	6.49 ± 2.92
17	Imo	335	1,181.80 ± 40.53^{b}	49.41	342.961 ± 23.31	13.65	16.17 ± 5.11
	Kebbi	331	1,381.63 ± 39.25^{ab}	39.97	421.84 ± 22.69	13.72	4.89 ± 5.11
	Nasarawa	407	1,382.32 ± 39.25^{a}	31.57	298.48 ± 22.57	19.85	8.82 ± 5.11
21	Imo	310	1,427.88 ± 41.70^{b}	48.83	246.08 ± 23.99^{b}	21.03	16.39 ± 5.43
	Kebbi	288	1,588.28 ± 41.46^{b}	40.26	206.75 ± 23.85^{b}	16.75	16.37 ± 4.96
	Nasarawa	404	1829.45 ± 61.57^{a}	7.10	447.36 ± 35.42^{a}	66.42	1.07 ± 4.96

^{abc}means within column sharing no common superscript were significantly different ($p < .05$).
N, number of birds; LSM ± SE, least-square means ± standard error; CV, coefficient of variation.

Significant ($p < .05$) interaction effect of location, genetics and sex was evident on body weight at different ages (Table 3), although Noiler and FUNAAB Alpha birds in Nasarawa and Kebbi were superior ($p < .05$), compared to those in Imo. On the average, male birds of both genotypes were heavier ($p < .05$) than their female counterparts across the three different agro-ecological zones. With respect to body weight gain (Supplementary Table S2), FUNAAB Alpha and Noiler birds in Imo State had the highest ($p < .05$) body weight gain at 5–9 weeks of age and recorded the lowest gain in body weight at 17–21 weeks, compared to the other states. Male and female birds of both genotypes gained ($p < .05$) more body weight in Nasarawa than Imo and Kebbi at 17–21 weeks. Location, genetics and sex had no significant ($p > .05$) effect on body weight gain of TADP chickens at 9–13 weeks and 13–17 weeks of age, although male and female birds of both genotypes gained ($p > .05$) more body weight in Nasarawa than Imo and Kebbi.

At 9 weeks (Supplementary Table S3), higher mortality was evident for FUNAAB Alpha and Noiler birds across location, genetics and sex in Kebbi and Imo states compared to Nasarawa state. However, Mortality rate was similar ($p > .05$) at 13, 17, and 21 weeks, though Imo recorded the highest mortality rate, followed by Kebbi and Nasarawa.

As shown in Supplementary Table S4, antibiotics use was only significant ($p < .05$) on body weight and body weight gain at weeks 9 and 13, respectively. The mortality rate ranged from 7.6%–20.3%, and 4.5%–25.2%, for birds administered antibiotics and those not administered antibiotics, respectively. Birds with antibiotics usage had an incremental body weight gain from weeks 9–21 as against those not reared with antibiotics.

3.3 Survivability potential of the two chickens

Genetics had a significant effect ($p < .05$) on the survival performance of birds and survivability decreases as the age of birds increases (Table 4). FUNAAB Alpha showed more survivability potential than Noiler birds by 3%, 7%, 6% and 7% at 9, 13, 17 and 21 weeks of age (Figure 2; Supplementary Figure S1), respectively. Noiler showed higher cumulative risk (.19–.43) of survival than FUNAAB Alpha (.16–.36) birds. The survival probability of the chickens was significantly ($p < .001$) influenced by agroecology (Table 5), with Nasarawa showing the lowest cumulative hazard (.04–.21) compared to Imo (.21–.48), and Kebbi (.29–.55). Birds reared in Kebbi showed the highest cumulative risk of survival (Figure 3; Supplementary Figure S2). The survival rate of the birds was highest in Nasarawa State (81%–96%), followed by Imo (62%–81%), and Kebbi (58%–75%) states.

Antibiotics usage had a significant ($p < .01$) effect on the survivability of the birds (Table 6). As shown in Figure 4, birds administered antibiotics showed higher propensity to survive (69%–86%) than those without antibiotics (61%–77%), consequently, a high cumulative hazard or risk of survival was observed in birds reared without antibiotics (Supplementary Figure S3).

TABLE 3 Effects of location, genetics and sex on body weight (g) of the chickens (LSM ± SE).

Location	Genetics	Sex	*N*	5 weeks	*N*	9 weeks	*N*	13 weeks	*N*	17 weeks	*N*	21 weeks
Imo	FUNAAB Alpha	F	103	206.1 ± 40.68^{c}	80	501.93 ± 23.41^{ij}	71	751.52 ± 49.99^{e}	70	988.39 ± 67.36^{i}	66	1,151.18 ± 101.64^{j}
		M	97	247.11 ± 40.68^{c}	73	578.93 ± 23.41^{fgh}	69	849.13 ± 49.99^{cde}	63	1,244.19 ± 67.7^{bcdefgh}	60	1,525.77 ± 101.75^{efghi}
	Noiler	F	155	264.16 ± 37.47^{c}	136	502.83 ± 21.56^{hj}	117	789.08 ± 46.04^{de}	113	1,068.91 ± 62.33^{ghi}	103	1,281.5 ± 93.69^{hij}
		M	145	305.17 ± 37.47^{bc}	115	579.83 ± 21.72^{egi}	99	886.7 ± 46.38^{cde}	89	1,324.7 ± 63.47^{abcdef}	81	1,656.09 ± 95.72^{efg}
Kebbi	FUNAAB Alpha	F	91	468.8 ± 40.68^{ab}	80	606.04 ± 23.39^{fgh}	77	899.99 ± 49.94^{cde}	73	1,122.65 ± 67.43^{fhi}	69	1,354.49 ± 101.43^{gij}
		M	109	509.81 ± 40.68^{a}	86	683.04 ± 23.47^{de}	82	997.6 ± 50.13^{abcd}	78	1,378.44 ± 67.51^{abcdeg}	65	1,729.08 ± 102.37^{efh}
	Noiler	F	156	526.86 ± 37.47^{a}	111	606.94 ± 21.62^{egi}	100	937.56 ± 46.16^{bcde}	93	1,203.16 ± 62.17^{defghi}	84	1,484.81 ± 94.21^{fghij}
		M	144	567.87 ± 37.47^{a}	96	683.94 ± 21.87^{df}	90	1,035.17 ± 46.7^{abc}	87	1,458.95 ± 63.02^{abc}	70	1859.4 ± 97.1^{de}
Nasarawa	FUNAAB Alpha	F	104	513.73 ± 40.68^{ab}	100	733.16 ± 23.43^{cd}	92	1,063.04 ± 50.04^{abc}	82	1,211.76 ± 67.56^{cefghi}	81	2,243.66 ± 101.96^{cd}
		M	96	554.74 ± 40.68^{a}	93	810.16 ± 23.34^{ab}	86	1,160.65 ± 49.85^{ab}	78	1,467.55 ± 67.13^{abd}	77	2,618.25 ± 100.89^{ab}
	Noiler	F	157	571.78 ± 37.47^{a}	150	734.06 ± 21.5^{bd}	136	1,100.6 ± 45.93^{abc}	128	1,292.28 ± 61.85^{bcdefgh}	127	2,373.98 ± 92.97^{bc}
		M	143	612.79 ± 37.47^{a}	136	811.06 ± 21.57^{ac}	127	1,198.22 ± 46.06^{a}	119	1,548.07 ± 62.16^{a}	119	2,748.57 ± 93.74^{a}
Coefficient of variation				73.07		30.58		40.05		40.46		48.93
Source of variation (***p < .001,**p < .01, *p < .05)												
Location				***		***		***		***		***
Genetics				NS		NS		NS		NS		NS
Sex				NS		***		*		***		***
Interaction				***		***		***		***		***

^{abcdefghij}means within column sharing no common superscript were significantly different (p < .05).
N, number of birds; LSM ± SE, least-square means ± standard error; NS, not significant.

3.4 Profitability of the smallholder chicken intervention

The effect of genetics on profitability of chickens is shown in Table 7. Profit per bird from the sales of FUNAAB Alpha (NGN 2,532; USD 6.2) and Noiler (NGN 2,388; USD 5.8) were not significantly different (p > .05). The same applies to cost of feed per household and cost of feed and drugs per bird. Also, the expected total profit was not significantly (p > 0.05) different between the two chickens.

Farmers in Imo and Nasarawa spent more (p < .001) on the total cost of feed and drugs per household, and cost of feed and drugs per bird. However, profit per bird was highest (p < .05) in Nasarawa and lowest in Imo and Kebbi (Table 8). Expected total profit from sale of male (cock) birds was highest in Nasarawa (NGN 780; USD 19.0), followed by Kebbi (NGN 6,544; USD 15.9) and Imo (NGN 5,875; USD 14.3), although these were not significantly (p > .05) different. At week 21, there were no significant differences (p > .05) in the average number of male (2.63 ± .15) and female (5.57 ± .26) birds between the agroecologies, however the number of cocks and hens differed significantly (p > .05) within the states (Imo: 2.41 ± .2 and 5.39 ± .29; Kebbi: 2.63 ± .19 and 5.53 ± .28; and Nasarawa: 2.86 ± .21 and 5.80 ± .31).

Farmers who reared their birds without antibiotics had the lowest (p < .05) cost of feed and drugs (Total and per bird) compared to those who administered antibiotics (Supplementary Table S5). The expected profit per bird, though higher in birds

TABLE 4 Effect of genetics on survival performance of birds (5–21 weeks).

Genetics	Week	IN	FN	NM	Surv. prob.± S.E	Cum. hazard ± S.E	Log-rank (*p*-value)
FUNAAB Alpha	9	600	512	88	.853 ± .017	.159 ± .017	.049
	13	512	477	35	.795 ± .021	.229 ± .021	
	17	477	444	33	.74 ± .024	.301 ± .024	
	21	444	418	26	.697 ± .027	.361 ± .027	
Noiler	9	900	744	156	.827 ± .015	.19 ± .015	
	13	744	669	75	.743 ± .02	.297 ± .02	
	17	669	629	40	.699 ± .022	.358 ± .022	
	21	629	584	45	.649 ± .025	.432 ± .025	

IN and FN, initial and final number of birds; NM, number of mortality; Surv. prob., survival probability; Cum. hazard, Cumulative hazard; S.E, standard error; Log-rank, Test of homogeneity for differences in survival.

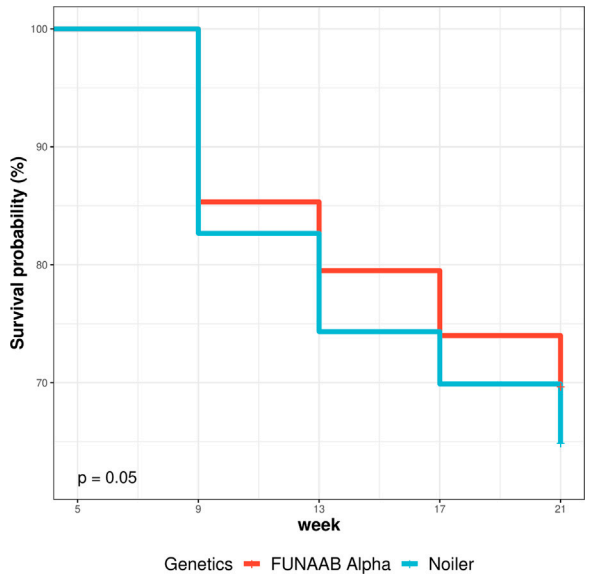

FIGURE 2
Effect of genetics on survival performance of birds (5–21 weeks).

reared without antibiotics (NGN 2,577; USD 6.3) compared to those administered antibiotics (NGN 2,344; USD 5.7) was not significantly ($p < .05$) different in the two treatment groups. Supplementary Table S6 revealed that farmers in Imo, Kebbi and Nasarawa who reared FUNAAB Alpha and Noiler birds using antibiotics spent more ($p < .05$) on the cost of feed and drugs (Total and per bird). However, profit made from the sale of birds did not differ ($p > .05$) across genetics and agro-ecological zones in flocks with and without antibiotics usage.

Expected profit per bird, and sale per bird were the only variables significantly ($p < .05$) influenced by the interaction between gender and agro-ecological zone (Table 9). Although male and female farmers in Imo made more profit per bird ($p < .05$) than their Nasarawa and Kebbi counterparts, it did not reflect ($p > .05$) in the overall profit made. At 21 weeks, there were no significant differences ($p > .05$) in the average number of birds between the male and female farmers in Imo (5.54 ± .42 and 6.00 ± .22), Kebbi (6.40 ± .35 and 5.60 ± .17) and Nasarawa (5.67 ± .27 and 6.40 ± .23) states.

4 Discussion

4.1 Sociodemographic characteristics of the farmers

The sociodemographic characteristics of farmers in this study are similar to that previously reported, before and during the COVID-19 pandemic, by Bamidele and Amole (2021). Majority of the households had women as the primary keepers of the flock. This is in consonance with several studies on the role of women in smallholder poultry production in sub-Saharan Africa, and in particular Nigeria (Birhanu et al., 2021; Alabi et al., 2020; Gueye, 2000; Wong et al., 2017; Alemayehu et al., 2018) and in sub-Saharan Africa.

4.2 Growth performance and mortality of birds

Studies on growth performance in indigenous breeds and varieties of chicken are increasingly receiving attention (González Ariza et al., 2021). Knowledge of the growth of animals is useful to improve management as well as feeding practices (Nguyen Hoang et al., 2021). Available reports in literature have shown that the genetic make-up of various animal breeds is an influential factor, which dominantly affect phenotypic characters (Buzala and Janicki, 2016; Nematbakhsh et al., 2021). In chickens, it has also been

TABLE 5 Effect of agro-ecological zone on survival performance of birds (5–21 weeks).

State	Week	IN	FN	NM	Surv. prob.± S.E	Cum. hazard ± S.E	Log-rank (*p*-value)
Imo	9	500	404	96	.808 ± .022	.213 ± .022	<.0001
	13	404	356	48	.712 ± .028	.34 ± .028	
	17	356	335	21	.67 ± .031	.4 ± .031	
	21	335	310	25	.62 ± .035	.478 ± .035	
Kebbi	9	500	373	127	.746 ± .026	.293 ± .026	
	13	373	349	24	.698 ± .029	.36 ± .029	
	17	349	331	18	.662 ± .032	.412 ± .032	
	21	331	288	43	.576 ± .038	.552 ± .038	
Nasarawa	9	500	479	21	.958 ± .009	.043 ± .009	
	13	479	441	38	.882 ± .016	.126 ± .016	
	17	441	407	34	.814 ± .021	.206 ± .021	
	21	407	404	3	.808 ± .022	.213 ± .022	

IN and FN, initial and final number of birds; NM, number of mortality; Surv. prob., survival probability; Cum. hazard, Cumulative hazard; S.E, standard error; Log-rank, Test of homogeneity for differences in survival.

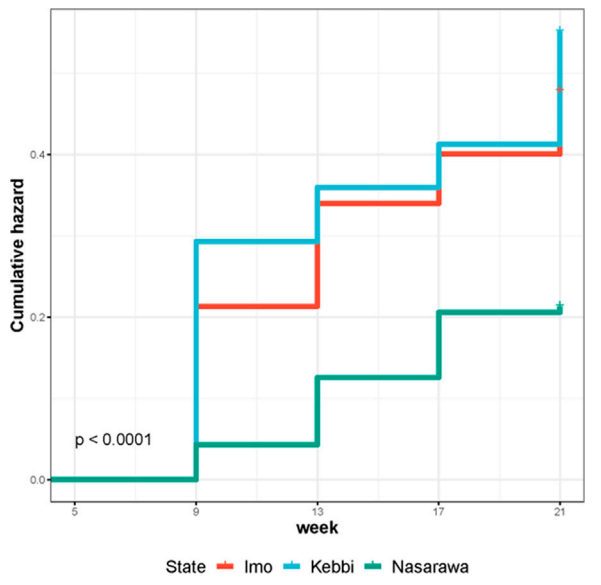

FIGURE 3
Effect of agro-ecological zone on the cumulative hazard of birds (5–21 weeks).

reported that genetic selection geared towards improvement in production traits could have an effect on the growth performance of the birds (Nematbakhsh et al., 2021). Both FUNAAB Alpha and Noiler have been genetically selected for improved dual-purpose (meat and eggs) performance in flocks owned by rural and peri-urban households. However, their body weight and weight gain performance appeared similar in the current study all through the growth phase (5–21 weeks). This is contrary to earlier findings (pre-COVID-19) on the same chicken genetics under similar environmental conditions, where the 18-week body weight (1,461.28 vs. 1,202.63 g) of Noiler birds was significantly higher than those of FUNAAB Alpha. The optimal performance of the birds could have been restricted as a result of certain nutritional limitations occasioned by the outbreak of COVID-19. Nutrition is a veritable component for the development of smallholder poultry, as it interacts with the genetics of the birds (Bamidele et al., 2020; Birhanu et al., 2022).

The feeds of birds under semi-intensive system of production are normally supplemented by the farmers (Tolasa, 2021). However, household food status and consumption patterns are some of the factors influencing the scavenging feed resource base available to chickens (Gondwe and Wollny, 2007). An earlier study revealed that COVID-19 had a negative effect on the average monthly income of farmers in the study area (Nigeria), where it was reduced from NGN 22,565 (USD 62.70) to NGN15,617 (USD 38.10) (Bamidele and Amole, 2021). This could have also reduced the ability of the farmers to supplement feeds quantitatively and qualitatively, with concomitant effect on the body size of the birds. Similar reports on the negative effects of the COVID-19 pandemic on avian species and the poultry industry generally have been documented (Esiegwu and Ejike, 2021; Seress et al., 2021). It is possible that Noiler birds will exhibit optimality under improved feeding conditions. However, the mature body weights (1,927.02 and 1,792.38 g) obtained in this study for Noiler and FUNAAB Alpha birds were higher than the values of 813.75 g, 1,400–1,660 g, 1,451–1716 g reported for mature indigenous chickens in Nigeria (Ajayi et al., 2020), Kenya (Mujyambere et al., 2022), and Algeria (Dahloum et al., 2016), respectively. Mortality rate did not differ between the two genotypes.

TABLE 6 Effect of antibiotics usage on survival performance of birds (5–21 weeks).

Drug	Week	IN	FN	NM	Surv. prob.± S.E	Cum. hazard ± S.E	Log-rank (*p*-value)
No	9	330	255	75	.773 ± .03	.258 ± .03	.0024
Yes	9	1,170	1,001	169	.856 ± .012	.156 ± .012	
No	13	255	221	34	.67 ± .039	.401 ± .039	
Yes	13	1,001	925	76	.791 ± .015	.235 ± .015	
No	17	221	212	9	.642 ± .041	.443 ± .041	
Yes	17	925	861	64	.736 ± .018	.307 ± .018	
No	21	212	200	12	.606 ± .044	.501 ± .044	
Yes	21	861	802	59	.685 ± .02	.378 ± .02	

IN and FN, initial and final number of birds; NM, number of mortality; Surv. prob., survival probability; Cum. hazard, Cumulative hazard; S.E, standard error; Log-rank, Test of homogeneity for differences in survival.

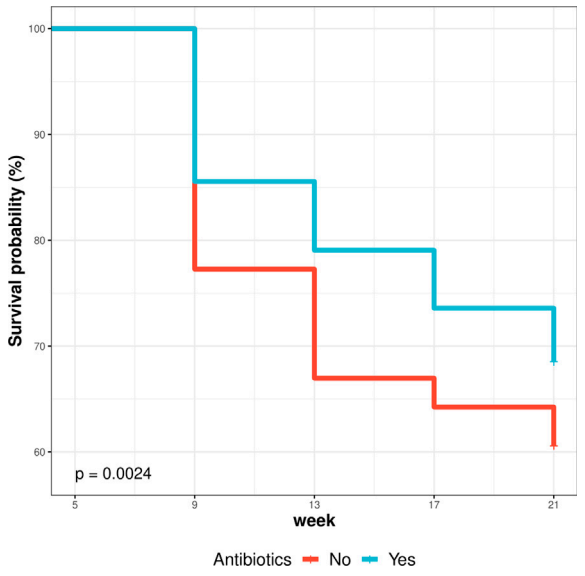

FIGURE 4
Effect of antibiotics usage on survival performance of birds (5–21 weeks).

This further confirms the potential of both tropically improved indigenous chicken genotypes to thrive in the smallholder production systems under the prevailing circumstances in Nigeria. It is congruous with the reports on the better performance of tropical breeds of chicken (Abegaz et al., 2019; Bamidele et al., 2020; Itafa et al., 2021; Kassa et al., 2021), which were primary developed for the improvement of genetics of growth among others (Zhang et al., 2020; Chomchuen et al., 2022).

The agro-ecology of the birds affected their body weight and weight gain performance. This could be attributed to varying environmental conditions, available scavenging resources, feed supplementation and managerial ability of the farmers in the different zones. Imo State is located in the wetter tropical rain forest zone of southern Nigeria while Nasarawa and Kebbi states are located in the hotter southern Guinea and Sahel savanna zones of northern Nigeria, respectively. Naturally, one would have expected birds in the rain forest zone to exhibit better performance. However, the reverse was the case in the current study, which was carried out from June–November 2021. This, probably, could be as a result of the fact that the stage of active growth of the birds coincided with the period of peak rainfall in the southern Guinea and Sahel savanna zones (between August and September) compared to the rain forest zone (July) (Ogungbenro and Morakinyo, 2014). A cool atmosphere could improve the comfort, feed efficiency, and overall productivity of birds. In a related study, Sztandarski et al. (2021) found associations between weather conditions and performance of birds. Alemu et al. (2021) also reported that climate may lead to body weight differences in different strains of improved tropically adapted chickens.

Male birds were heavier than their female counterparts, which could be attributed to sexual size dimorphism (SSD). According to Székely et al. (2007), SSD in most avian species favors male birds. Such dimorphism could have resulted from differential sexual- and natural-selection pressures experienced by both sexes (Yakubu et al., 2022), or from adaptive selection pressures which is a reflection of the evolution of males and females towards fitness optima divergence (Sztepanacz and Houle, 2021). At the level of smallholder poultry, it is possible that SSD may be influenced by differential ecological and socio-biological traits exhibited by male and female birds (Alarcón et al., 2017). This is more noticeable during the competition of male animals for mates in the context of polygyny (Cassini, 2022). Also, the sex-specific gene-regulation of body weight QTL (Johnsson et al., 2018) could be responsible for the differential expression of this trait in males and females. This study's findings are similar to those previously reported in poultry (Dahloum et al., 2016; Toalombo Vargas et al., 2019; Yakubu et al., 2022).

TABLE 7 Effect of genetics on profitability (LSM ± SE) of the improved smallholder chicken intervention.

Trait	Genetics	Average no. of birds	CV (%)	LSM ± SE	CV (%)
Average cost of feed and drugs per HH	FUNAAB Alpha	10		7,091.77 ± 326.34	33.79
	Noiler	10		7,311.62 ± 287.66	31.64
Cost of feed and drugs per bird	FUNAAB Alpha	1		709.18 ± 32.63	33.79
	Noiler	1		731.16 ± 28.77	31.64
Expected sale per bird	FUNAAB Alpha	1		3,241.75 ± 125.62	28.92
	Noiler	1		3,119.54 ± 110.73	30.02
Expected profit per bird	FUNAAB Alpha	1		2,532.58 ± 110.43	32.58
	Noiler	1		2,388.37 ± 97.34	35.99
Expected total profit (males only at 21 weeks)	FUNAAB Alpha	2.84 ± .18	36.24	7,278.84 ± 618.37	50.69
	Noiler	2.43 ± .16	56.67	6,206.81 ± 545.07	76.36

LSM ± SE, least square means ± standard error; CV, coefficient of variation; HH, household.

TABLE 8 Effect of agro-ecological zone on profitability (LSM ± SE) of the improved smallholder chicken intervention.

Trait	State	Average no. of birds	CV (%)	LSM ± SE	CV (%)
Average cost of feed and drugs per HH	Imo	10		7,860.74 ± 360.86^{b}	34.86
	Kebbi	10		6,550.69 ± 345.38^{a}	40.08
	Nasarawa	10		7,193.65 ± 384.57^{ab}	17.41
Cost of feed and drugs per bird	Imo	1		786.07 ± 36.09^{b}	34.86
	Kebbi	1		655.07 ± 34.54^{a}	40.08
	Nasarawa	1		719.37 ± 38.46^{ab}	17.41
Expected sale per bird	Imo	1		3,109.02 ± 138.9^{ab}	33.39
	Kebbi	1		2,915.88 ± 132.94^{a}	27.66
	Nasarawa	1		3,517.03 ± 148.03^{b}	25.1
Expected profit per bird	Imo	1		2,322.95 ± 122.11^{b}	39.32
	Kebbi	1		2,260.82 ± 116.87^{b}	32.35
	Nasarawa	1		2,797.66 ± 130.13^{a}	29.58
Expected total profit (males only at 21 weeks)	Imo	2.41 ± .2	56.69	5,875.89 ± 683.78	74.94
	Kebbi	2.63 ± .19	59.04	6,544.72 ± 654.45	86.28
	Nasarawa	2.86 ± .21	28.33	7,807.87 ± 728.71	34.25

^{ab}means within column sharing no common superscript were significantly different ($p < .05$);
LSM ± SE, least square means ± standard error; CV, coefficient of variation; HH, household.

The location, genetics and sex interaction effect on body weight where Noiler and FUNAAB Alpha birds seemed to have performed better in Nasarawa and Kebbi could be a reflection of the prevailing environmental conditions, available resources, management and socio-cultural practices of the smallholder farmers. We tried to compare our present results with earlier findings, and observed a similar trend as regards FUNAAB Alpha only. In an 18-week on-farm trial, the average body weights of FUNAAB Alpha in Imo, Nasarawa and Kebbi were reported as 1,072.33, 1,145.30, and 1,502.35 g (males) and 934.57, 1,001.91, and 1,294.52 g (females) (Ajayi et al., 2020). Thus, it appears that FUNAAB Alpha birds are more suitable to the environmental settings in Kebbi and Nasarawa compared to Imo. The present findings are consistent with the submission of Kassa et al. (2021) that breed-environment interaction could influence the phenotypic expression of traits. Similar genotype by location interaction effect on growth traits has

TABLE 9 Interaction effect of gender and location on profitability (LSM ± SE) of the improved smallholder chicken intervention.

Gender	Location	TCFD per HH	CFD per bird	EP per bird	ES per bird	ETP (males)
M	Imo	8,339.91 ± 417.17	833.99 ± 41.72	2,690.08 ± 113.97[a]	3,486.94 ± 216.22[a]	8,046.3 ± 1,071.09
F		8,132.38 ± 441.07	813.24 ± 44.11	2,687.35 ± 191.75[ab]	3,468.92 ± 128.52[ab]	8,020.64 ± 636.64
M	Nasarawa	7,995.88 ± 578.72	799.59 ± 57.87	2,254.09 ± 146.14[b]	3,085.34 ± 155.87[b]	6,601.7 ± 943
F		7,788.35 ± 343.98	778.83 ± 34.4	2,251.35 ± 138.22[b]	3,067.32 ± 164.8[b]	6,576.04 ± 671.44
M	Kebbi	6,993.72 ± 509.51	699.37 ± 50.95	2,205.06 ± 120.2[c]	2,901.69 ± 190.37[c]	5,999.26 ± 772.1
F		6,786.19 ± 362.78	678.62 ± 36.28	2,202.32 ± 168.81[c]	2,883.68 ± 135.55[c]	5,973.6 ± 816.33
Coefficient of variation		32.44	32.44	34.59	29.53	65.75
Source of variation (***$p < .001$,**$p < .01$, *$p < .05$)						
Gender		NS	NS	NS	NS	NS
Location		*	*	**	**	NS
Interaction		NS	NS	*	*	NS

[abc]means within column sharing no common superscript were significantly different ($p < .05$);
LSM±SE, least square means ± standard error; M, male; F, female; TCFD, total cost of feed and drugs; CFD, cost of feed and drugs; EP, expected profit; ES, expected sale; ETP, expected total profit; HH, household; NS, not significant.

been documented in improved indigenous and tropically adapted chickens (Ajayi et al., 2020; Guni et al., 2021b). The lower mortality of birds in Nasarawa State might be attributed to better management practices, which is consistent with the findings of Alemu et al. (2021).

Although antibiotics have been reported to act as growth promoters (Plata et al., 2022; a practice which is now banned in many countries due to antimicrobial resistance concerns), there was no clear-cut pattern of the effect of antibiotics usage on the growth traits of birds in the current study. While body weight was higher in flocks where antibiotics were used at 9 weeks of rearing, body weight gain of flocks without the use of antibiotics was higher. The same could be said of percentage mortality as there was no distinct difference between the two treatment groups. It is possible that other feeding and health management strategies including environmental conditions could have shaped the pattern of expression of both body weight and body weight gain as well as the mortality rate of the birds (Silveira et al., 2014; Nakkazi et al., 2015; El-Sabrout et al., 2022). Also, within the smallholder poultry production system in Nigeria, farmers' use of antibiotics is primarily for therapeutic purposes, and not growth promotion (Bamidele et al., 2022a). This study did not assess if farmers administering antibiotics observed the required withdrawal period prior to sale (Turcotte et al., 2020).

4.3 Survivability of birds

The fitness of an animal in the environment where it is being kept is a measure of survival. The probability to survive appears higher in FUNAAB Alpha than in Noiler birds. This can be attributed primarily to genetics and management practices. It has been established that there is a relationship between the genetic constitution of birds, and their ability to cope in a particular environment (Cheng and Muir, 2005; Peeters et al., 2012). Also, better nutrition and health management practices can make birds to survive within their rearing environments (Kalia et al., 2017; Bughio et al., 2021; Chebo et al., 2022; Mujyambere et al., 2022). The observed genetic effect on survivability is consistent with the findings of Ajayi et al. (2020). Similar observations have also been made on chickens in Nigeria (Ademola et al., 2020), Ethiopia (Kassa et al., 2021), and Tanzania (Guni et al., 2021b).

The ability to adapt to varying environmental conditions is one of the attributes of improved indigenous and tropically adapted chickens. This ability includes a strong and efficient immune response to pathogenic infection, which assists in their survival (Wondmeneh et al., 2015), and adaptability (Sankhyan and Thakur, 2018) in the smallholder production systems. When other factors such as productive abilities, choice of breeds and traits of preference (Yakubu et al., 2020) are put into consideration, the present information may guide improved chicken breeds distribution to farmers in different agro-ecologies in Nigeria. Such breed by agro-ecology interaction effect on survivability has also been advocated by Guni et al. (2021b) as a means of distributing improved chicken breeds for optimal performance.

Health-related problems cause chicken losses on farm, hence the inclusion of antibiotics either in the feed or water of birds to ameliorate health-related conditions. The use of antibiotics could have conferred some health advantages on birds in the current study. Such birds, according to Berghof et al. (2019), are more resilient and less susceptible to environmental perturbations

(diseases inclusive). Disease prevention in poultry is imperative for survival of the birds (Sargeant et al., 2019; Aboah and Enahoro, 2022), while intensifying the small-scale systems (Wilson et al., 2022). However, the abuse of antibiotics is of public health concerns, as it can lead to the multiplication of bacteria that are antibiotic resistant (Hafez, and Attia, 2020; Bamidele and Amole., 2021; 2022b; Zalewska et al., 2021). There was differential response of the sexes of the chickens in the different agro-ecologies to the use of antibiotics. However, as an alternative to antibiotics, probiotics and prebiotics are now being recommended as beneficial additives (Al-Khalaifah, 2018). It was difficult to compare our results with others due to dearth of information in literature. However, Nkansa et al. (2020) and Chah et al. (2022) reported the use of antibiotics for prophylaxis or treatment by backyard poultry farmers in Ghana and Nigeria, respectively.

4.4 Profitability of smallholder dual-purpose chicken enterprise

Considering the prevailing economic situation in Nigeria, post COVID-19, the profit level of both FUNAAB Alpha and Noiler birds, is an indication that investment in smallholder backyard poultry business is lucrative, provided all required inputs (housing, vaccination, supplementary feeding) are supplied. This, apart from contributing to household income, can improve food security and livelihoods of the farmers, as a way of attaining the Sustainable Development Goal of ending hunger and malnutrition by the year 2030 (Fang et al., 2021). Importantly, it will make available nutritious diets in form of quality meat and eggs ((Nuriliani et al., 2022). In this study, the average selling price (USD 7.7) for both chicken genetics was higher than the value (USD 1.6–2.4) reported for indigenous birds in Kenya (Otiang et al., 2020). The variation in price may however be attributed to the breed of chicken, size of bird, production cost, purchasing power, or the demand for local and improved chickens. On the other hand, the average selling price in Nigerian Naira (NGN 3,181) was similar to that previously reported (NGN 3,350) by Alabi et al. (2020) for both FUNAAB Alpha and Noiler birds.

The average cost of production, per bird per farmer from 5–21 weeks of production was NGN 720.2 (USD 1.8), at approximately NGN 45 (USD 0.1) per week. Transferring this cost to buyers at an average market price of NGN 3,181 (USD 7.8) per live-bird, yields an average profit of NGN 2,461 (USD 6.0) per cock sold. The average number of cocks and hens per farmer at the end of the study was three and six, respectively. When all the cocks are sold, this provides an average household income of NGN 7,383 (USD 18.0) to each farmer. In practice, cocks are sold more often than hens to meet urgent financial needs of smallholder poultry households while hens are kept for eggs (sale and consumption) as well as for breeding purposes (Alabi et al., 2020). Therefore, in additional to the sale of cocks, the hens (6 birds per household) are a potential source of extra household income (average of NGN 40–70/USD .10–.17 per egg) through sale of eggs (average of three eggs per week over a 6-month laying period) when properly kept under on-farm management conditions (Ajayi et al., 2020; Alabi et al., 2020). Also, chickens (cocks and hens) contribute to the nutritional and dietary requirements for animal-sourced proteins within the households. This is significant as it reduces household expenditure on eggs and meat, consequently increasing household savings, and improving the purchasing power for other basic necessities (Udo et al., 2011; Alabi et al., 2020). Our findings show that irrespective of gender, chickens can support household livelihoods as well as improve food security for resource-poor smallholder farmers. This is consistent with the findings of Agwu et al. (2020) where there was no gender differential in the profit made by male and female chicken producers.

The expected total profit realized from the sales of birds in Nasarawa, Kebbi, and Imo implies that smallholder poultry farming is a veritable venture in the three agro-ecological zones. This is in consonance with earlier submission (Alabi et al., 2020). It has also been reported in Vietnam that small-scale chicken production, which is dependent on the size of the flock is profitable (Truong et al., 2021). To boost production and increase profit under smallholder settings in Sub-Saharan Africa and Asia, some models such as microfranchising, microfinancing, cooperative farming, enterprise development, and out-grower model (Beesabathuni et al., 2018) have been proposed.

Use of ethnoveterinary medicines by farmers in the current study led to a reduction in cost of production, although it did not reflect in the overall profit. The continuous use of antibiotics on-farm by the smallholder farmers might have been encouraged by the patron-client relationship between farmers and dealers on pharmaceutical products (Masud et al., 2020). However, where resources are highly limited, the use of proven and effective ethnoveterinary medicines (Alders et al., 2018; Jambwa et al., 2022) is highly encouraged, as it has the potential to cut down production cost with possible increase in profit. The interaction between location, genetics and antibiotics did not significantly influence profitability. This is a further confirmation of the fact that both FUNAAB Alpha and Noiler chickens can be successfully reared in the three agro-ecologies of Nigeria with or without the use of antibiotics. The economic importance of these two improved indigenous chicken genetics, together with other tropically adapted breeds, has been highlighted in a scaling readiness study as part of a genetic solution strategy in Sub-Saharan Africa for smallholder poultry production systems (Sartas et al., 2021).

5 Conclusion and policy implications

Overall, this study demonstrates that provision of livestock, at 50% of the value of the cash transfer initiated by the Federal

Government of Nigeria, to vulnerable smallholder farming households during the recovery phase of the COVID-19 pandemic holds great potential for economic growth and resilience of rural and peri-urban communities in Nigeria. In addition to cash-based interventions, provision of improved, locally-adapted chicken genetics to the poorest of the poor will also serve as a quick means of mitigating the impact of the pandemic, prevent negative coping strategies, and offer immediate as well as sustainable long-term nutritional relief and livelihoods support. Since smallholder poultry is practiced by most rural and peri-urban households in Nigeria, where it accounts for about 50% of total household income, rural economic recovery strategies in a post COVID-19 era, should include a value-chain approach that maximizes the economic potentials and improves the overall efficiency within the production system. The FGN's poverty reduction policies aimed at lifting one hundred million Nigerians out of poverty within a decade should consider adopting women-friendly, agricultural technologies, such as the low-input, high-output, dual-purpose chicken genetics, for socioeconomic development of peri-urban and rural communities.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Institutional Review Board of International Livestock Research Institute (ILRI) (ILRI COVID-19 Project 03/2021). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

OB and TA obtained the grant, designed and supervised the project; OB, OA, AY, and TA drafted, revised and edited the article; OB, AY, WH, UO, and TA conducted the intervention and collected the data; OB, OA, and AY cleaned, analyzed and interpreted the data. All authors read and approved the final version of the article.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.1033654/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Effect of genetics on the cumulative hazard of birds (5–21 weeks).

SUPPLEMENTARY FIGURE S2
Effect of agro-ecological zone on survival performance of birds (5–21 weeks).

SUPPLEMENTARY FIGURE S3
Effect of antibiotics usage on the cumulative hazard of birds (5–21 weeks).

SUPPLEMENTARY TABLE S1
Effect of sex on body weight, weight gain, and mortality.

SUPPLEMENTARY TABLE S2
Effects of location, genetics and sex on body weight gain of the chickens.

SUPPLEMENTARY TABLE S3
Effects of location, genetics and sex on mortality of the chickens.

SUPPLEMENTARY TABLE S4
Effect of antibiotics use on body weight, weight gain, and mortality of the chickens.

SUPPLEMENTARY TABLE S5
Effect of antibiotics usage on profitability of the improved smallholder chicken intervention.

SUPPLEMENTARY TABLE S6
Interaction of location, genetics and antibiotics usage on profitability of the improved smallholder chicken intervention.

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Genetic and phenotypic correlations among feed efficiency, immune and production traits in indigenous chicken of Kenya

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This study aimed at estimating genetic and phenotypic relationships among feed efficiency, immune and production traits measured pre- (9–20 weeks of age) and post- (12 weeks from on-set of lay) maturity. Production traits were average daily gain (ADG) and average daily feed-intake ($ADFI_1$) in the pre-maturity period and age at first egg (AFE), average daily feed-intake ($ADFI_2$) and average daily egg mass (EM) in the post-maturity period. Feed efficiency comprised of residual feed intake (RFI) estimated in both periods. Natural antibodies binding to keyhole limpet hemocyanin (KLH-IgM) and specific antibodies binding to Newcastle disease virus (NDV-IgG) measured at 16 and 28 weeks of age represented immune traits pre- and post-maturity, respectively. In the growing period, 1,820 records on ADG, KLH-IgM and NDV-IgG, and 1,559 records on $ADFI_1$ and RFI were available for analyses. In the laying period, 1,340 records on AFE, EM, KLH-IgM and NDV-IgG, and 1,288 records on $ADFI_2$ and RFI were used in the analyses. Bi-variate animal mixed model was fitted to estimate (co)variance components, heritability and correlations among the traits. The model constituted sex, population, generation, line and genotype as fixed effects, and animal and residual effects as random variables. During the growing period, moderate to high heritability (0.36–0.68) was estimated for the production traits and RFI while the antibody traits had low (0.10–0.22) heritability estimates. Post-maturity, the production traits and RFI were moderately (0.30–0.37) heritable while moderate to high (0.25–0.41) heritability was estimated for the antibody traits. Genetic correlations between feed efficiency and production traits in both periods showed that RFI had negative genetic correlations with ADG (–0.47) and EM (–0.56) but was positively correlated with $ADFI_1$ (0.60), $ADFI_2$ (0.74) and AFE (0.35). Among immune and production traits, KLH-IgM and NDV-IgG had negative genetic correlations with ADG (–0.22; –0.56), AFE (–0.39; –0.42) and EM (–0.35; –0.16) but were positively correlated with $ADFI_1$ (0.41; 0.34) and $ADFI_2$ (0.47; 0.52). Genetic correlations between RFI with KLH-IgM (0.62; 0.33) and NDV-IgG (0.58; 0.50) were positive in both production periods. Feed intake, RFI and antibody traits measured in both production periods were positively correlated with estimates ranging from 0.48 to 0.82. Results from this study indicate selection possibilities to improve production, feed efficiency and immune-competence in indigenous chicken. The genetic correlations suggest that improved feed efficiency would be associated with high growth rates, early maturing chicken, high egg mass and reduced feed intake. In contrast, improved general (KLH-IgM) and specific (NDV-IgG) immunity would result in lower growth rates and egg mass but associated with early sexual maturation and

high feed intake. Unfavorable genetic correlations between feed efficiency and immune traits imply that chicken of higher productivity and antibody levels will consume more feed to support both functions. These associations indicate that selective breeding for feed efficiency and immune-competence may have genetic consequences on production traits and should therefore be accounted for in indigenous chicken improvement programs

KEYWORDS

indigenous chicken, feed efficiency, immunity, production, genetic parameters

Introduction

Indigenous chicken (*Gallus gallus domesticus*) play significant roles in nutrition, food and income security in many rural households in most countries in the tropical regions (Alders and Pym, 2009). In Kenya, indigenous chicken (IC) account for about 80% of the total chicken population and are kept by over 75% of the rural households (KNBS, 2019). Their popularity, particularly among rural households, is attributed to their ability to produce under low input systems and adapt to local environmental conditions (Olwande et al., 2010). Despite their adaptive ability, IC are predominantly raised under challenging environments which limit their optimal utilization. Seasonal variation in availability and quality of feed resources is a major challenge that limit productivity (Miyumo et al., 2018). Given that feed represents about 60%–70% of the total production costs, increased feed costs directly affect profitability of the enterprise (Besbes, 2009). Under low input systems, IC are exposed to a myriad of pathogens that cause various diseases which result in massive production and economic losses (Okeno et al., 2011). Of significance is the Newcastle disease (NCD) which is endemic among chicken in the tropics and is reported to be a major factor limiting productivity due to high prevalence and mortality rates (Lwelamira, 2012). These environmental conditions are expected to negatively influence the availability and quality of feed resources and pathogen epidemiology due to climate change impacts.

Genetic improvement of feed efficiency provides an avenue to select individuals that are able to efficiently convert available feed resources into products and support their maintenance requirements. Besides, selection for feed efficient animals contributes to reduced feed costs and nitrogenous wastes, and minimizes the environmental footprint (Moore et al., 2009; Zhang and Aggrey, 2003). Measures such as residual feed intake (RFI) and feed conversion ratio (FCR) have extensively been used to improve feed efficiency in chicken (Prakash et al., 2020), swine (Patience et al., 2015) and cattle (Berry and Crowley, 2012) due to their moderate to high heritability. Selective breeding for antibody traits related to general and specific immune-competence provide an opportunity to enhance disease resistance in animals (Cheng et al., 2013). Natural antibodies binding to keyhole limpet hemocyanin (KLH-IgM) antigen in chicken have previously been used to improve general disease resistance (Berghof et al., 2019) and are also associated with higher survival rates (Sun et al., 2013). On the other hand, specific antibodies binding to the NCD virus (NDV-IgG) have been found heritable and able to confer specific disease resistance against NCD (Lwelamira, 2012).

The Kenyan IC is a highly diverse population with a large plasticity in genomic regions that confer adaptability to challenging production conditions, poor nutrition and tolerance to pathogens (Ngeno et al., 2015). Such genetic advantage could be exploited to improve feed efficiency and immune-competence in this population. However, in cases of resource scarcity, trade-offs between different functions in the body may be expected when a negative dependency between resource acquisition and resource allocation exists (Zerjal et al., 2021). Even in environments where *ad libitum* feed resources are available, Rauw (2012) indicates that there is a limited amount of feed an individual can ingest, assimilate, use, and share among various functions in the body with preferential partitioning of resources to functions of interest. For instance, divergent selection for chicken lines of high growth rates (van der Most et al., 2011) or early maturing chicken lines with high egg numbers (Pinard-van der Laan et al., 1998) resulted in lower antibody responses upon disease challenge. Conversely, in a high egg laying line, van der Klein et al. (2015) estimated a positive genetic correlation between feed efficiency and natural antibodies indicating that immune-competent chicken required more feed to support both high maintenance and production requirements. These studies demonstrate competitive nutrient and energy partitioning between production and immune functions, and also suggest involvement of pleiotropic gene action among feed use efficiency, production and immune functions.

Understanding the nature of trade-offs between production and immune traits based on available feed resources is crucial when breeding for a robust chicken that is capable of high productivity while maintaining its adaptive potential to stressors in its production environment. Considering the ability of IC to produce and survive in poor conditions, studies on the impact of improving their resource use efficiency and immune-competence on growth and egg production are sparse. The current study aimed at estimating genetic and phenotypic correlations among feed efficiency, antibody and production traits measured pre- and post-maturity in indigenous chicken. Findings from this study would provide information on the genetic background of feed efficiency, antibody and production traits and the pleiotropic nature among the traits that could be applied in chicken breeding, especially when utilizing highly diverse populations as genetic resources.

Materials and methods

Experimental population

The study was conducted at the Non-Ruminant Research Institute of the Kenya Agriculture and Livestock Research Organization (NRI-KALRO) in Naivasha-Kenya. Two populations of chicken exist at the research station; an indigenous chicken (IC) population and a synthetic breed population known as KALRO chicken (KC). The IC population comprises of ecotypes from various agro-ecological zones grouped into three phylogenetic clusters based on major

histocompatibility complex (MHC) linked microsatellite markers (Ngeno et al., 2015). Cluster one constitutes ecotypes from the Western and South-Rift regions that exhibit warm and humid weather; cluster two constitutes ecotypes from the North-Rift and North-Eastern regions that are considered arid and semi-arid; and cluster three constitute ecotypes from the Coastal region that is hot and humid. Within the clusters seven genetic groups exist namely normal feathered, naked neck, frizzled feathered, crested head, feathered shanks, dwarf and game-gaited (*Kuchi*) structure (Magothe et al., 2012). The synthetic (KC) population, on the other hand, originated from a dual-purpose hybrid that was subjected to a systematic and continuous *inter-se* mating resulting to highly segregated individuals in subsequent generations (Ilatsia et al., 2017). Based on plumage dominance, two distinct groups were isolated; black and white barred plumage (KC1) and black plumage (KC2). The groups were subsequently subjected to within line mating to stabilize the respective plumage colour.

The two populations are under continuous selection to develop meat (ML) and egg (EL) lines (Ilatsia et al., 2017). The ML birds are selected for body weight at 12 weeks of age (BW_{12}) (Ngeno et al., 2013). The EL are selected based on age at first egg (AFE) (Dana et al., 2011). The chicken breeding program at NRI-KALRO is in its initial stages with a small population size and limited number of pedigree and performance records, therefore, selection for ML and EL is based on phenotypic information (Ilatsia et al., 2017). The selection criterion involves retaining males and females whose phenotypic BW_{12} is at least one standard deviation above average as meat lines. Chicken whose phenotypic BW_{12} is below average are considered for AFE evaluation. This involves assessing females using own phenotypic AFE records while males are assessed based on average phenotypic information on AFE from their respective daughters and dams. Individuals with below average AFE are retained. In this study, three generations (generations four to six) of these chicken comprising of both ML and EL were considered. A total of 1820 chicken were included in the study.

Management of the experimental population followed standard operating procedures of the breeding program at NRI-KALRO (Ilatsia et al., 2017). Birds were fed a starter ration (20% CP and 2800 Kcal ME/Kg) from day one to week 8 of age, growers ration (18% CP and 2750 Kcal ME/Kg) from week 8 to week 19 of age and layers ration (16% CP and 2600 Kcal ME/Kg) from week 20 of age to the rest of the test period. Routine health management involved vaccination against endemic diseases namely Marek's disease (MD), Newcastle disease (NCD), and infectious bursal disease (IBD). In addition, experimental chickens were vaccinated against fowl pox (week 6) and fowl typhoid (week 18), and dewormed and disinfected routinely.

Data collection

Ethical approval to conduct the study was provided by the Institutional Animal Care and Use Committee (IACUC) of KALRO - Veterinary Science Research Institute (VSRI) (KALROVSRI/IACUC019/30082019). Measurement of antibody traits during the growing period and laying period involved blood sampling of the experimental chicken *via* the wing vein at the ages of 16 and 28 weeks, respectively. These age points were considered based on the average age at point of maximum growth rate (16 weeks) and sexual maturity (28 weeks; age at first egg for the females and extended to their respective male relatives) of the third generation. Blood sampling procedure was carried out without anesthesia and no chicken was killed for sample collection. Post sampling, birds were given multi-vitamins and observed for any post-trauma effects. Plasma was extracted from the blood samples for further use in antibody measurement.

Natural antibodies binding to keyhole limpet hemocyanin (KLH-IgM) and specific antibodies binding to Newcastle disease (NCD) virus (NDV-IgG) were used to measure general disease resistance and specific resistance against NCD, respectively. The KLH antigen was considered due to its suitability to measure natural antibodies while NDV antigen was considered due to the negative impact of NCD on IC population (Lwelamira, 2012; Sun et al., 2013). Titers of KLH-IgM isotype was determined using an indirect two-step ELISA (Enzyme-linked Immunosorbent assay) as described by Berghof et al. (2015). Similarly, titers of NDV-IgG isotype were measured using indirect ELISA as described by Bell and Lelenta (2002). Absorbance levels were measured at 450 nm (reference wavelength at 620 nm) using a spectrophotometer ELISA reader (mrc Scientific Instrument-UT-6100, Israel). Pre-defined serial standard dilutions of the antibody traits (Bell and Lelenta, 2002; Berghof et al., 2015) and their respective absorbance reads were used to obtain standard curves by fitting a four-parameter logistic model (Herman et al., 2008) using GraphPad Prism 9.1 (GraphPad Software). Subsequently, concentration levels of the antibody traits in plasma samples were calculated from the standard curves using their respective absorbance reads. The concentration levels of the antibody traits were thereafter adjusted to their respective sample dilution factors (1:10 for KLH-IgM and 1:40 for NDV-IgG) and expressed as $\log_2$ values. This was done separately for each plate to partly correct for plate differences and allow values to be comparable across plates.

Residual feed intake (RFI) and feed conversion ratio (FCR) were used to assess feed efficiency during the growing and early laying phases. To estimate the efficiency measures, average daily feed intake (ADFI), average daily gain (ADG) and metabolic body weight (MBW) were considered during the growers' phase (from 8–20 weeks of age). At week eight of age, birds were weighed (initial weight), transferred to individual feeding pens and allowed a 1-week acclimatization period. Thereafter, daily feed intake recording commenced from week 9 and ended at week 20, upon which birds were weighed (final weight) again. Average daily gain was obtained as the difference between final and initial body weight divided by the test period. Cumulative feed intake from week 9 to week 20 was divided by the duration of test to obtain ADFI. Metabolic body weight was calculated based on the average between initial and final body weights raised to the power of 0.75 ($BW^{0.75}$). During the laying period, ADFI, average daily egg mass (EM), MBW and ADG were considered. At the onset of lay, initial body weight was measured, daily feed intake, egg number and weight recording commenced and continued until week 12, at which final body weight was measured. Estimation of ADG, ADFI and MBW were made as described in the growing period. Egg number was summed and egg weights averaged during the 12 weeks of test period. Thereafter, EM was calculated as the product of total egg number and average egg weight divided by the test period (12 weeks) (Yuan et al., 2015). Feed conversion ratio was estimated as a ratio of ADFI and ADG in the growing period and as a ratio of ADFI and EM in the laying period. Residual feed intake was computed as the difference between observed ADFI and expected ADFI. Observed ADFI is actual

measurement taken on feed intake while expected ADFI is feed intake predicted on basis of MBW and production traits.

Random effect models were fitted to compute RFI using Eq. 1 (growing period) and Eq. 2 (laying period) using the *lme4* package of R Software (R Core Team, 2021). The model allows parameters to vary between- and within-individuals hence, improves the accuracy of predicting feed intake and also aids in selection when confronted with birds with similar RFI values (Aggrey and Rekaya, 2013).

$$RFI_i = Y_i - (b_0 + b_1 ADG_i + b_2 MBW_i + \alpha_{1i} ADG_i + \alpha_{2i} MBW_i) \quad (1)$$

$$RFI_i = Y_i - (b_0 + b_1 EM_i + b_2 ADG_i + b_3 MBW_i + \alpha_{1i} EM_i + \alpha_{2i} ADG_i + \alpha_{3i} MBW_i) \quad (2)$$

Where: RFI_i is the estimated residual feed intake for ith bird; Y_i is the average daily feed intake record of ith bird; ADG_i, MBW_i and EM_j are the observed average daily gain, metabolic body weight and average daily egg mass of ith bird, respectively; b_k are the fixed regression coefficients (k = 0, 1, 2, 3) related to the population; α_{ki} is the random regression coefficient (k = 1, 2, 3) specific to i^{th} bird for the observed traits and assumed to have ~ N (0, $\sigma^2\alpha$) distribution.

Production traits included data on the traits under selection in the study population; BW_{12} and AFE. In addition, during the growing period, ADG, MBW, and ADFI were considered. In the laying period, body weight at first egg (BW_{AFE}), egg weight of first egg (EW_{AFE}), ADG, MBW, ADFI, EM, cumulative egg number (EN_{12}) and average daily egg weight (EW_{12}) 12 weeks post on-set of lay were considered.

Statistical analysis

A series of bi-variate animal models (Eq. 3) were fitted to estimate genetic and phenotypic parameters for antibody, feed efficiency and production traits by restricted maximum likelihood through the average information (AI-REML) algorithm of the WOMBAT software (Meyer, 2007).

$$\begin{bmatrix} \mathbf{y}_i \\ \mathbf{y}_j \end{bmatrix} = \begin{bmatrix} \mathbf{X}_i & 0 \\ 0 & \mathbf{X}_j \end{bmatrix} \begin{bmatrix} \mathbf{b}_i \\ \mathbf{b}_j \end{bmatrix} + \begin{bmatrix} \mathbf{Z}_{ii} & \mathbf{Z}_{ij} \\ \mathbf{Z}_{ji} & \mathbf{Z}_{jj} \end{bmatrix} \begin{bmatrix} \mathbf{a}_i \\ \mathbf{a}_j \end{bmatrix} + \begin{bmatrix} \mathbf{e}_i \\ \mathbf{e}_j \end{bmatrix} \quad (3)$$

where: $\mathbf{y}_i$ is the vector of observations for the antibody, feed efficiency and production traits (considering two at a time); $\mathbf{b}_i$ the vector of fixed effects; $\mathbf{a}_i$ the vector of random animal additive genetic effects assumed to be $\mathbf{a} \sim N(0, \mathbf{A}\sigma^2 a)$ in which $\mathbf{A}$ is the numerator relationship matrix and $\sigma^2 a$ is the additive genetic variance; $\mathbf{e}_i$ the vector of random residual effect assumed to be $\mathbf{e} \sim N(0, \mathbf{I}\sigma^2 e)$ in which $\mathbf{I}$ is an identity matrix and $\sigma^2 e$ is the residual variance; $\mathbf{X}_i$, and $\mathbf{Z}_i$ are incidence matrices relating records to fixed and random animal effects, respectively. The pedigree used to construct the numerator relationship matrix consisted of 2,013 individuals (inclusive of those with and without records) from three generations. Assumed covariance structures of the random model terms among the traits are presented below.

$$\mathrm{Var}\begin{bmatrix} \mathbf{a}_i \\ \mathbf{a}_j \\ \mathbf{e}_i \\ \mathbf{e}_j \end{bmatrix} = \begin{bmatrix} \sigma^2_\alpha \mathrm{ii}\mathbf{A} & \sigma^2_\alpha \mathrm{ij}\mathbf{A} & 0 & 0 \\ \sigma^2_\alpha \mathrm{ji}\mathbf{A} & \sigma^2_\alpha \mathrm{jj}\mathbf{A} & 0 & 0 \\ 0 & 0 & \sigma^2_e \mathrm{ii}\mathbf{I} & \sigma^2_e \mathrm{ij}\mathbf{I} \\ 0 & 0 & \sigma^2_e \mathrm{ji}\mathbf{I} & \sigma^2_e \mathrm{jj}\mathbf{I} \end{bmatrix} \quad (4)$$

where $\mathbf{a}_i$, $\mathbf{e}_i$, $\mathbf{A}$ and $\mathbf{I}$ were described in Eq. 3; $\sigma^2 a_{ii}$ is the additive genetic variance for trait i; $\sigma^2 a_{ij}$ is the additive genetic covariance between trait i and trait j; $\sigma^2 e_{ii}$ is the residual variance for trait i; $\sigma^2 e_{ij}$ is the residual covariance between trait i and trait j; zero covariance between additive genetic effect and residual effect was assumed.

From preliminary tests of fixed factors significantly ($p < 0.05$) affecting the traits, sex, population, generation, line and genotype were included in the bivariate analyses as fixed effects. Likelihood ratio test was used to determine whether genetic correlations among the traits were significantly different from zero, by comparing Eq. 3 to a bivariate model with additive genetic covariance fixed at zero. On the other hand, Fisher's *r* to *z*-transformation was used to test whether phenotypic correlations were significantly different from zero using the following test statistic:

$$z = 0.5 \ln \frac{(1 + r)}{(1 - r)}$$

where r is the estimated phenotypic correlation, and z follows a normal distribution with standard deviation $1/\sqrt{(n-3)}$, where n is the sample size. Various combinations of traits in different bi-variate models fitted resulted in several estimates of variance components and variance ratios for each of the traits. Therefore, to obtain a global estimate for the genetic parameters on each of the traits, pooling was carried out by weighting estimates by the inverse of their respective sampling variance.

Results

Descriptive statistics on production, feed efficiency and immune traits measured during the growing and laying periods are presented in Table 1. Mean estimates of production traits under selection in the study population showed that BW_{12} was 1205.81 g and AFE was 23 weeks. During the growing period, chicken had a higher growth rate of 13.82 g/d compared to the laying period (3.21 g/d). In contrast, mean estimates of ADFI, MBW and ADFI were higher in the laying period than in the growing period. At the on-set of lay, mean estimates of BW_{AFE} and EW_{AFE} were 1616.81 g and 42.30 g, respectively. Twelve weeks post on-set of lay showed that the study population laid on average 54 eggs with an average daily egg weight of 47.86 g resulting to an average daily egg mass of 28.87 g/d. In feed efficiency measures (RFI and FCR), chicken had higher mean estimates during the laying period (RFI = 0.05 g/d; FCR = 7.47 g:g) compared to the growing period (RFI = 0.01 g/d; FCR = 4.69 g:g). In immune traits, mean estimates of KLH-IgM in both production periods were higher than the NDV-IgG estimates. Between the two production periods, the laying period had higher mean estimates for the antibody traits than the growing period. The coefficient of variation of traits measured in both production periods indicated wide dispersion of estimates from their respective means.

Variation due to additive genetic effects and residual effects, and heritability estimates are presented in Table 2. During the growing period, additive genetic effect contributed more to phenotypic variation of BW_{12}, ADG, RFI and FCR than residual effects. In contrast, residual effects were higher on MBW, ADFI, KLH-IgM and NDV-IgG than additive genetic effects. Across the traits measured during the laying period, residual effect was more eminent on AFE, ADG, MBW, EN_{12}, EM_{12}, KLH-IgM, and NDV-IgG than additive genetic effect. On the other hand, phenotypic variation due to additive genetic effect was highest in BW_{AFE}, EW_{AFE} and EW_{12} than additive genetic effects. Moderate to high

TABLE 1 Number of observations (N), mean, standard deviation (SD) and coefficient of variation (CV) of production, feed efficiency and immune traits measured during the growing and laying periods.

Period[a]	Trait[b]	N	Mean	SD	CV (%)
Growing	BW_{12} (g)	1,820	1205.81	84.55	7.01
	ADG (g/d)	1,820	13.82	3.80	27.50
	MBW (g)	1,820	188.43	18.42	9.78
	ADFI (g/d)	1,559	95.16	4.17	4.38
	RFI (g/d)	1,559	0.01	4.14	41400.00
	FCR (g:g)	1,559	4.69	2.27	48.40
	KLH-IgM (ng/mL)	1,820	7.08	1.24	17.51
	NDV-IgG (ng/mL)	1,820	5.48	0.94	17.15
Laying	AFE (weeks)	1,340	23.00	2.88	12.52
	BW_{AFE} (g)	1,340	1616.81	175.47	10.85
	EW_{AFE} (g)	1,340	42.30	9.11	21.53
	ADG (g/d)	1,340	3.21	1.44	44.90
	MBW (g)	1,340	271.67	20.98	7.72
	EN_{12} (number)	1,340	54.00	15.6	28.89
	EW_{12} (g)	1,340	47.86	6.97	14.56
	EM_{12} (g/d)	1,340	28.87	9.95	34.46
	ADFI (g/d)	1,288	115.21	16.65	14.45
	RFI (g/d)	1,288	0.05	13.79	27580.00
	FCR (g:g)	1,288	7.47	2.9	38.82
	KLH-IgM (ng/mL)	1,340	12.33	0.91	7.38
	NDV-IgG (ng/mL)	1,340	11.22	0.67	5.97

[a]Growing period was from 8 to 20 weeks of age; Laying period was 12 weeks from onset of lay.

[b]BW_{12} is body weight at week 12; ADG, is average daily gain; MBW, is metabolic weight; ADFI, is average daily feed intake; RFI, is residual feed intake; FCR, is feed conversion ratio; KLH-IgM, is natural antibodies of IgM isotype binding to KLH, antigen; NDV-IgG, is specific antibodies of IgG binding to NDV, antigen; AFE, is age at first egg; BW_{AFE}, is body weight at age at first egg; EW_{AFE}, is egg weight at age at first egg; EN_{12} is cumulative number of eggs 12 weeks from onset of lay; EW_{12} is average egg weight 12 weeks from onset of lay; EM_{12} is average daily egg mass 12 weeks from onset of lay.

heritabilities (0.32–0.68) were estimated for production traits (BW_{12}, ADG, MBW, and ADFI) measured during the growing period. Among the feed efficiency measures, heritability estimates for RFI and FCR were 0.43 and 0.45, respectively. Low to moderate heritability was estimated in KLH-IgM (h^2 = 0.22) and NDV-IgG (h^2 = 0.10). At the onset of lay, heritability estimates for AFE, BW_{AFE} and EW_{AFE} were 0.31, 0.37 and 0.59, respectively. For egg production traits measured 12 weeks post onset of lay, heritability estimates for EN_{12}, EW_{12} and EM_{12} were 0.23, 0.64, and 0.33, respectively. Contrary to the growing period, ADG (h^2 = 0.02), MBW (h^2 = 0.26) and ADFI (h^2 = 0.37) measured during the laying period had lower heritability estimates. Feed efficiency measures in the laying period had lower heritability (RFI = 0.30; FCR = 0.34) estimates than in the growing period. Heritability estimates for KLH-IgM and NDV-IgG in the laying period were 0.41 and 0.25, respectively. These estimates were higher compared to those obtained for traits in the growing period.

Genetic (r_g) and phenotypic (r_p) correlations among production, feed efficiency and immune traits measured during the growing period are presented in Table 3. Genetic correlations among the production traits (BW_{12}, ADG, MBW and ADFI) were highly positive (0.43–0.86; $p < 0.01$). Between feed efficiency and production traits, RFI was negatively correlated with BW_{12} (r_g = −0.66), ADG (r_g = −0.47) and MBW (r_g = −0.52) but positively correlated with ADFI (r_g = 0.60). On the other hand, FCR had positive (r_g = 0.44–0.49) correlations with BW_{12}, MBW and ADFI but was highly negatively correlated with ADG (r_g = −0.79). Among antibody traits and production traits, negative correlations were estimated between KLH-IgM with BW_{12} (r_g = −0.47; $p < 0.01$) and between NDV-IgG with ADG (r_g = −0.56; $p < 0.01$). Between feed efficiency and immune measures, ADFI and RFI was positively correlated with KLH-IgM (r_g = 0.41–0.62) and NDV-IgG (r_g = 0.34–0.58). Positive genetic correlation was estimated between RFI and FCR (r_g = 0.51). Although non-significant, KLH-IgM and NDV-IgG had a negative genetic correlation. Phenotypic correlations among the traits followed a similar pattern as genetic correlations, apart from correlations between FCR with BW_{12}, MBW, and RFI, and between RFI and BW_{12}. Significant positive phenotypic correlations (0.44–0.82; $p < 0.01$) were estimated among the production traits. Residual feed intake had positive phenotypic correlation with BW_{12} (0.36; $p <$

TABLE 2 Estimates of additive genetic variance (σ_a^2)[1], residual variance (σ_e^2)[1] and heritability (h^2)[1] of production, feed efficiency and immune traits measured in growing and laying periods.

Period[b]	Trait[c]	σ^2_a	σ^2_e	h^2
Growing	BW_{12} (g)	34.77 ± 0.03	10.98 ± 0.03	0.44 ± 0.07
	ADG (g/d)	5.15 ± 0.94	2.38 ± 0.68	0.68 ± 0.10
	MBW (g)	85.61 ± 9.27	184.56 ± 5.62	0.32 ± 0.10
	ADFI (g/d)	17.45 ± 1.40	21.03 ± 1.18	0.36 ± 0.05
	RFI (g/d)	23.15 ± 0.04	10.45 ± 0.26	0.43 ± 0.07
	FCR (g:g)	2.54 ± 0.25	1.86 ± 0.32	0.45 ± 0.04
	KLH-IgM (ng/mL)	0.20 ± 0.08	0.34 ± 0.16	0.22 ± 0.03
	NDV-IgG (ng/mL)	0.24 ± 0.03	0.41 ± 0.34	0.10 ± 0.02
Laying	AFE (weeks)	0.37 ± 0.14	3.35 ± 0.36	0.31 ± 0.09
	BW_{AFE} (g)	979.78 ± 0.04	957 ± 0.04	0.37 ± 0.12
	EW_{AFE} (g)	70.12 ± 13.79	7.72 ± 9.94	0.59 ± 0.13
	ADG (g/d)	0.04 ± 0.14	1.75 ± 0.17	0.02 ± 0.08
	MBW (g)	107.39 ± 47.90	300.66 ± 44.88	0.26 ± 0.11
	EN_{12} (number)	41.55 ± 19.91	137.28 ± 19.14	0.23 ± 0.11
	EW_{12} (g)	25.11 ± 5.70	8.64 ± 4.33	0.64 ± 0.14
	EM_{12} (g/d)	18.13 ± 6.75	36.85 ± 6.08	0.33 ± 0.12
	ADFI (g/d)	77.30 ± 28.31	133.66 ± 24.87	0.37 ± 0.13
	RFI (g/d)	61.73 ± 23.93	129.56 ± 21.86	0.30 ± 0.05
	FCR (g:g)	2.69 ± 0.82	5.51 ± 0.76	0.34 ± 0.04
	KLH-IgM (ng/mL)	0.62 ± 0.20	0.80 ± 0.04	0.41 ± 0.08
	NDV-IgG (ng/mL)	0.46 ± 0.02	0.50 ± 0.02	0.25 ± 0.06

[a]± SE, standard errors.

[b]Growing period was from 8 to 20 weeks of age; Laying period was 12 weeks from onset of lay.

[c]BW_{12} is body weight at week 12; ADG, is average daily gain; MBW, is metabolic weight; ADFI, is average daily feed intake; RFI, is residual feed intake; FCR, is feed conversion ratio; KLH-IgM, is natural antibodies of IgM isotype binding to KLH, antigen; NDV-IgG, is specific antibodies of IgG binding to NDV, antigen; AFE, is age at first egg; BW_{AFE}, is body weight at age at first egg; EW_{AFE}, is egg weight at age at first egg; EN_{12} is cumulative number of eggs 12 weeks from onset of lay; EW_{12} is average egg weight 12 weeks from onset of lay; EM_{12} is average daily egg mass 12 weeks from onset of lay.

TABLE 3 Estimates of genetic[a] (lower diagonal) and phenotypic[a] (upper diagonal) correlations among production, feed efficiency and immune traits measured during the growing period (9–20 weeks of age).

Traits[b]	BW_{12}	ADG	MBW	ADFI	RFI	FCR	KLH-IgM	NDV-IgG
BW_{12}		0.47 ± 0.04***	0.82 ± 0.01***	0.44 ± 0.04***	0.36 ± 0.09***	−0.42 ± 0.09**	0.04 ± 0.03	0.05 ± 0.03
ADG	0.43 ± 0.09***[c]		0.69 ± 0.02***	0.72 ± 0.04***	−0.03 ± 0.12	−0.94 ± 0.01**	0.18 ± 0.05***	−0.18 ± 0.05
MBW	0.75 ± 0.02***	0.86 ± 0.05***		0.61 ± 0.20**	−0.01 ± 0.14	−0.53 ± 0.03***	0.27 ± 0.06***	0.12 ± 0.06
ADFI	0.78 ± 0.05***[c]	0.58 ± 0.18**	0.44 ± 0.16**		0.05 ± 0.06	0.88 ± 0.06***	0.56 ± 0.22**	0.20 ± 0.18
RFI	−0.66 ± 0.18*	−0.47 ± 0.08**	−0.52 ± 0.12**	0.60 ± 0.15**		0.22 ± 0.04***	0.02 ± 0.06	0.04 ± 0.05
FCR	0.44 ± 0.18*	−0.79 ± 0.06	0.47 ± 0.14***	0.49 ± 0.16***	0.51 ± 0.11**		−0.18 ± 0.15	0.10 ± 0.08
KLH-IgM	−0.47 ± 0.15**	−0.22 ± 0.19	0.46 ± 0.17**	0.41 ± 0.13**	0.62 ± 0.17***	0.47 ± 0.34		0.01 ± 0.03
NDV-IgG	0.06 ± 0.66	−0.56 ± 0.11**	0.49 ± 0.15***	0.34 ± 0.09**	0.58 ± 0.13***	0.03 ± 0.57	−0.55 ± 0.38	

[a]± SE, standard errors.

[b]BW_{12} is body weight at week 12 of age; ADG, is average daily gain; MBW, is metabolic weight; ADFI, is average daily feed intake; RFI, is residual feed intake; FCR, is feed conversion ratio; KLH-IgM, is natural antibody of IgM isotype binding to KLH, antigen measured at 16 weeks; NDV-IgG, is specific antibody IgG isotype binding to NDV, antigen measured at 16 weeks of age.

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

TABLE 4 Estimates of genetic[a] (lower diagonal) and phenotypic[a] (upper diagonal) correlations among production, feed efficiency and immune traits during the laying period (12 weeks from on-set of lay).

Traits[b]	AFE	BW_{AFE}	EW_{AFE}	ADG	MBW	EN_{12}	EW_{12}	EM_{12}	ADFI
AFE		0.35 ± 0.04***	0.31 ± 0.24	−0.21 ± 0.19	0.28 ± 0.04***	−0.20 ± 0.05*	0.28 ± 0.04***	0.10 ± 0.05	0.04 ± 0.05
BW_{AFE}	0.46 ± 0.12*		0.13 ± 0.05*	−0.13 ± 0.04	0.94 ± 0.01***	0.20 ± 0.04***	0.18 ± 0.05***	0.10 ± 0.04*	0.32 ± 0.04***
EW_{AFE}	0.67 ± 0.20**	0.44 ± 0.11***		−0.15 ± 0.05	0.09 ± 0.05	−0.18 ± 0.05	0.52 ± 0.03***	0.10 ± 0.05	0.11 ± 0.06
ADG	−0.47 ± 0.42	−0.38 ± 0.09*	−0.49 ± 0.42		0.23 ± 0.04***	−0.16 ± 0.05	-0.06 ± 0.05	−0.18 ± 0.05	0.05 ± 0.05
MBW	0.25 ± 0.35	0.72 ± 0.01***	0.21 ± 0.20	0.39 ± 0.14***		-0.04 ± 0.04	0.17 ± 0.05**	0.04 ± 0.04	0.34 ± 0.04***
EN_{12}	-0.54 ± 0.10**	-0.59 ± 0.13**	-0.27 ± 0.21	0.28 ± 0.38	0.72 ± 0.18***		-0.05 ± 0.05	0.81 ± 0.01***	0.11 ± 0.06
EW_{12}	0.74 ± 0.29*	0.46 ± 0.20*	0.76 ± 0.08***	0.10 ± 0.22	0.24 ± 0.20	-0.52 ± 0.07***		0.41 ± 0.04***	0.13 ± 0.07
EM_{12}	0.41 ± 0.20*	0.62 ± 0.20**	0.27 ± 0.22	0.11 ± 0.31	0.75 ± 0.25**	0.93 ± 0.03***	0.66 ± 0.13***		0.23 ± 0.05***
ADFI	0.27 ± 0.34	0.53 ± 0.20**	0.34 ± 0.19	-0.28 ± 0.29	0.51 ± 0.20*	0.40 ± 0.12***	0.61 ± 0.16***	0.55 ± 0.17***	
RFI	0.35 ± 0.47	0.06 ± 0.28	0.46 ± 0.21	-0.69 ± 0.30	-0.61 ± 0.19**	-0.44 ± 0.10*	0.45 ± 0.21*	-0.56 ± 0.18**	0.74 ± 0.02***
FCR	-0.44 ± 0.42	-0.48 ± 0.26	-0.41 ± 0.21	-0.18 ± 0.33	0.54 ± 0.19**	-0.74 ± 0.09***	0.82 ± 0.11***	-0.82 ± 0.03***	0.51 ± 0.16**
KLH-IgM	-0.39 ± 0.10*	-0.41 ± 0.10*	0.29 ± 0.23	0.31 ± 0.22	0.51 ± 0.18**	-0.37 ± 0.09*	-0.43 ± 0.11*	-0.35 ± 0.09*	0.47 ± 0.13**
NDV-IgG	-0.42 ± 0.12*	-0.35 ± 0.29*	-0.27 ± 0.13	0.29 ± 0.24	0.44 ± 0.16*	-0.49 ± 0.12*	-0.37 ± 0.09*	-0.43 ± 0.11*	0.52 ± 0.15***

[a]±SE = standard errors

[b]AFE is age at first egg; BW_{AFE} is body weight at age at first egg; EW_{AFE} is egg weight at age at first egg; ADG is average daily gain; MBW is metabolic weight; EN_{12} is cumulative number of eggs 12 weeks from onset of lay; EW_{12} is average egg weight 12 weeks from onset of lay; EM_{12} is average daily egg mass 12 weeks from onset of lay; ADFI is average daily feed intake; RFI is residual feed intake; FCR is feed conversion ratio; KLH-IgM is natural antibody of IgM isotype binding to KLH antigen measured at 16 weeks; NDV-IgG is specific antibody IgG isotype binding to NDV antigen measured at 16 weeks of age.

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

TABLE 5 Estimates of genetic[a] (lower diagonal) and phenotypic[a] (upper diagonal) correlations among production, efficiency and immunity traits during the laying period (12 weeks from on-set of lay).

Traits[b]	RFI	FCR	KLH-IgM	NDV-IgG
AFE	-0.06 ± 0.04	-0.08 ± 0.04	-0.01 ± 0.05	-0.02 ± 0.06
BW_{AFE}	-0.05 ± 0.04	-0.01 ± 0.04	-0.08 ± 0.05	0.10 ± 0.06
EW_{AFE}	0.08 ± 0.05	-0.09 ± 0.05	0.12 ± 0.06	-0.13 ± 0.05
ADG	-0.11 ± 0.05	-0.05 ± 0.04	0.10 ± 0.05	-0.06 ± 0.06
MBW	-0.04 ± 0.04	0.06 ± 0.04	0.09 ± 0.05	0.08 ± 0.06
EN_{12}	-0.12 ± 0.09	-0.39 ± 0.02**	-0.07 ± 0.06	-0.02 ± 0.06
EW_{12}	0.10 ± 0.05	0.41 ± 0.04**	-0.10 ± 0.05	-0.10 ± 0.05
EM_{12}	-0.17 ± 0.05	-0.49 ± 0.02**[c]	-0.02 ± 0.05	-0.01 ± 0.06
ADFI	0.89 ± 0.01***	0.30 ± 0.05**	0.04 ± 0.05	0.03 ± 0.05
RFI		0.25 ± 0.07**	0.01 ± 0.05	0.15 ± 0.09
FCR	0.41 ± 0.10**		0.01 ± 0.06	0.07 ± 0.06
KLH-IgM	0.33 ± 0.15*	0.29 ± 0.24		-0.16 ± 0.13
NDV-IgG	0.50 ± 0.18*	0.34 ± 0.21	-0.49 ± 0.35	

[a]± SE, standard errors.

[b]AFE, is age at first egg; BW_{AFE}, is body weight at age at first egg; EW_{AFE}, is egg weight at age at first egg; ADG, is average daily gain; MBW, is metabolic weight; EN_{12} is cumulative number of eggs 12 weeks from onset of lay; EW_{12} is average egg weight 12 weeks from onset of lay; EM_{12} is average daily egg mass 12 weeks from onset of lay; ADFI, is average daily feed intake; RFI, is residual feed intake; FCR, is feed conversion ratio; KLH-IgM, is natural antibody of IgM isotype binding to KLH, antigen measured at 16 weeks; NDV-IgG, is specific antibody IgG isotype binding to NDV, antigen measured at 16 weeks of age.

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

0.001) while FCR was negatively (r_p = −0.42 to −0.94; $p < 0.01$) correlated to BW_{12}, ADG and MBW but was positively correlated to ADFI ($r_p = 0.88$; $p < 0.001$). There was a positive ($r_p = 0.22$; $p < 0.001$) correlation between the two measures of feed efficiency. Positive phenotypic correlations were estimated between KLH-IgM with ADG, MBW, and ADFI.

TABLE 6 Estimates of genetic[a] (r_g) and phenotypic[a] (r_p) correlations for production, feed efficiency and immune traits between the growing and laying periods.

Growing period[b]	Laying period[b]	r_g	r_p
BW_{12}	AFE	-0.88 ± 0.18**	-0.08 ± 0.05
ADG_1	ADG_2	-0.91 ± 0.12***	0.01 ± 0.05
MBW_1	MBW_2	0.20 ± 0.09*	0.59 ± 0.03***
$ADFI_1$	$ADFI_2$	0.80 ± 0.12***	0.01 ± 0.04
RFI_1	RFI_2	0.48 ± 0.32	0.55 ± 0.04***
FCR_1	FCR_2	0.19 ± 0.18	0.08 ± 0.04
KLH-IgM_1	KLH-IgM_2	0.62 ± 0.21**	0.05 ± 0.03
NDV-IgG_1	NDV-IgG_2	0.82 ± 0.17***	0.09 ± 0.06

[a]± SE, standard errors.

[b]BW_{12}, AFE, RFI, FCR, KLH-IgM, and NDV-IgG, are as described in Table 1: BW_{12}, ADG_1, MBW_1, $ADFI_1$, RFI_1, FCR_1, KLH-IgM_1, and NDV-IgG_1, are traits measured in the growing period; AFE, ADG_2, MBW_2, $ADFI_2$, RFI_2, FCR_2, KLH-IgM_2, and NDV-IgG_2, are traits measured in the laying period.

***$p < 0.001$· **$p < 0.01$.

Genetic (r_g) and phenotypic (r_p) correlations among production, feed efficiency and immune traits measured during the laying period are presented in Table 4; Table 5. Positive (0.44–0.67; $p < 0.01$) genetic correlations were estimated among egg-related traits (AFE, BW_{AFE} and EW_{AFE}) measured at the onset of lay. Age at first egg had negative correlation EN_{12} ($r_g = -0.54$) but was positively correlated with EW_{12} ($r_g = 0.74$) and EM_{12} ($r_g = 0.41$). Body weight at sexual maturity was positively ($r_g = 0.46$–0.72) correlated with MBW, EW_{12}, $EM_{12,}$ and ADFI but was negatively correlated with ADG ($r_g = -0.38$) and EN_{12} ($r_g = -0.59$). Egg weight of first egg had positive correlation with EW_{12} ($r_g = 0.76$; $p < 0.001$). Positive (0.39–0.75) genetic correlations were estimated between MBW with ADG, EN_{12}, EM_{12} and ADFI. Cumulative number of eggs 12 weeks' post-onset of lay was negatively ($r_g = -0.52$) correlated with EW_{12} but was positively correlated with EM_{12} ($r_g = 0.93$) and ADFI ($r_g = 0.40$). Average daily egg weight had positive correlations with EM_{12} ($r_g = 0.66$) and ADFI ($r_g = 0.61$). Between feed efficiency and production traits, RFI was negatively ($r_g = 0.44$ to -0.61; $p < 0.01$) correlated with MBW, EN_{12} and EM_{12} but positively ($r_g = 0.45$ to 0.74; $p < 0.001$) correlated with EW_{12} and ADFI. Feed conversion ratio had positive ($r_g = 0.51$–0.82) correlations with MBW, EW_{12} and ADFI but had negative ($r_g = -0.74$ to -0.82) correlations with EN_{12} and EM_{12}. Genetic correlations between immune and production traits showed that KLH-IgM was negatively ($r_g = -0.35$ to -0.43) correlated with AFE, BW_{AFE}, EN_{12}, EW_{12} and EM_{12} but was positively ($r_g = 0.47$–0.51) correlated with MBW and ADFI. Similarly, NDV-IgG was negatively ($r_g = -0.35$ to -0.49) correlated with AFE, BW_{AFE}, EN_{12}, EW_{12} and EM_{12} but was positively ($r_g = 0.44$–0.52) correlated to MBW and ADFI. Positive (0.33–0.50) genetic correlations were estimated between RFI and the antibody traits. Between RFI and FCR, positive ($r_g = 0.41$) genetic correlations were estimated. Similar to the growing period, although non-significant, KLH-IgM and NDV-IgG were negatively ($r_g = -0.49$) correlated. Although lower estimates, phenotypic correlations among production, feed efficiency and antibody traits followed a similar a pattern as the genetic correlations.

Genetic and phenotypic correlations of traits between the growing period and laying period are presented in Table 6. Highly negative genetic correlations were estimated between BW_{12} and AFE ($r_g = -0.88$; $p < 0.01$), and between ADG measured in both production periods ($r_g = -0.91$; $p < 0.001$). On the other hand, positive (0.62–0.82; $p < 0.01$) genetic correlations between two production periods were estimated for ADFI, KLH-IgM and NDV-IgG. Positive phenotypic correlation was estimated between MBW ($r_p = 0.59$; $p < 0.001$) and RFI ($r_p = 0.55$; $p < 0.001$) measured in both production periods.

Discussions

The high heritability estimates for growth-related (BW_{12} and ADG) and feed efficiency (RFI and FCR) traits during the growing period indicate that these traits could be selected for in indigenous chicken to develop meat lines of high growth rates and that are feed efficient. The magnitude of heritability estimates for these traits suggest relatively higher prediction accuracies would be expected and therefore, mass selection could be utilized to improve growth and feed efficiency (Falconer and Mackay, 1996). Besides, higher additive genetic variance than residual variance for the respective traits, imply that relatively high genetic progress on growth and feed efficiency can be achieved through selective breeding. Post-maturity, the moderate to high heritability estimates for the production traits (AFE, EN_{12}, $EW_{12,}$ and EM_{12}) and feed efficiency (RFI and FCR) suggest selection possibilities to breed for indigenous chicken egg lines that are early maturing, with high egg productivity and are feed efficient. Apart from average daily egg weight, higher residual variance than additive genetic variance for egg production and feed efficiency measures indicate that family selection would be a more reliable strategy to negate the residual environmental effects and improve accuracies of breeding values for these traits (Falconer and Mackay, 1996). Heritability estimates for production and feed efficiency traits in both production periods were within the range of values previously reported in local chicken in Tanzania (Lwelamira et al., 2009) but higher than those reported in commercial meat (Aggrey et al., 2010) and laying (van der Klein et al., 2015) chicken. Lower heritability estimates in the commercial population than the local chicken may be attributed to population differences. Studies by Aggrey et al. (2010) and van der Klein et al. (2015) used populations that have been subjected to intensive selection for production and feed efficiency and hence, are in general less diverse than the local chicken population.

Differences in heritability estimates for growth, metabolic weight, feed intake and feed efficiency traits between the growing period and the laying period may implicate physiological age as a source of variation. This could be in relation to gonadal hormones which are activated at point of sexual maturity and hence, influence body composition pre- and post-maturity (Loyd et al., 2011). Besides, Deeb and Lamont (2002) reported that sexual maturity coincides with the inflection of the growth curve, which corresponds to a shift in body composition away from protein accretion and towards fat deposition. Considering protein accretion is an energy expensive process than its maintenance while fat deposition requires less energy than its maintenance (Arthur et al., 2001), differences in body composition between pre- and post-mature age periods is likely to influence growth rate, maintenance requirement, feed intake and efficiency of feed use. Diet differences, in terms of energy density and protein content fed between the two production periods, may also be a contributing factor to variation in heritability estimates for these traits. In addition, considering that indigenous chicken are less active feeders (due to brooding behavior) during the laying period than during growing period (Dana et al., 2011), feeding behavior may also explain the differences in heritability estimates between the two production periods.

In immune traits, the moderate to high heritability estimates for KLH-IgM at 16 and 28−weeks of age indicate selection possibilities for natural antibodies to improve general immunity pre- and post-maturity. The advantage of using natural antibodies that bind to exo-antigens, such as KLH, to evaluate general immunity is that the study population did not and probably will not encounter KLH and therefore, reflects the capacity of innate immune function to respond to non-specific antigens not previously encountered (Star et al., 2007). Besides, KLH-IgM is significantly associated with survival in laying chicken and could be used as an accurate predictor of survivability in chicken (Sun et al., 2011). The low to moderate heritability estimates for NDV-IgG at 16 and 28 weeks of age indicate that genetic improvement of immune responses to vaccination against NCD pre- and post-maturity is feasible. Considering the study population encountered the NDV antigen *via* vaccination, presence of heritable variation for NDV-IgG could reflect an active status of memory immune cells specific to NDV antigen in both production periods (Walugembe et al., 2019).

Lower heritability and additive genetic variance estimates for the antibody traits (KLH-IgM and NDV-IgG) at 16 weeks than at 28 weeks of age suggests that relatively low prediction accuracies would be expected during the growing period compared to the laying period. In a commercial layer line divergently selected for natural antibodies at age 16 weeks for seven generations, Bovenhuis et al. (2022) estimated lower heritability and selection responses than expected in the high line compared to the low line. The study by Bovenhuis et al. (2022) attributed these observations to the effect of allele frequency changes in the *TLR$_1$A* gene polymorphism on genetic variance. The *TLR$_1$A* gene was previously identified on chromosome 4 and polymorphism of the gene was found as the most likely causal variant affecting the level of natural antibodies in chicken (Berghof et al., 2018). In this study, however, the experimental population was not subjected to artificial selection for antibody traits but rather production traits. Therefore, it is possible that either directional natural selection on immune-related genes at 16 weeks of age may be present in this population or that selection for body weight at week 12 of age may be having counter effects on antibody traits at 16 weeks of age. To this regard, there is need to carry out a genome wide association study in this study population to determine the genes that influence the antibody traits at 16 weeks of age and how allele frequencies of these genes are influenced by natural selection or by artificial selection for production traits. Conversely, higher heritability estimates for antibody traits measured post-maturity than pre-maturity may indicate effect of physiological age on immune function. Differences in physiological age has previously been linked to the development process of the immune function. For instance, Bernasconi et al. (2003) and Berghof et al. (2010) found that as animals advance in age, they encounter a variety of exogenous stimuli from either pathogens or environmental stressors which interact with genetic components to shape and enhance the formation of antibodies. Generally, in both production periods, higher residual variances than additive genetic variances for the antibody traits indicate lower repeatability for the immune measures and hence, response to selection would likely take a longer time, especially under mass selection. In this case, family selection coupled with improved management conditions (such as bio-security measures and vaccination) could optimize on accuracies of breeding value estimations while minimizing disease incidences (Farias et al., 2017).

Considering the study population is under selection for BW_{12} to develop meat lines, the positive correlation between BW_{12} and ADG indicate that improved body weight at 12 weeks will be associated with higher growth rates. Besides, BW_{12} is previously reported to have favorable genetic associations with hatch weight and market weight (24–26 weeks of age) in indigenous chicken (Ngeno et al., 2013). However, chicken of higher BW_{12} would have higher maintenance requirements during the growing period. Among the traits measured at the onset of lay, the genetic correlations indicated that early maturing birds had low mature body weight and low weight of first egg. The positive genetic correlation between BW_{AFE} and MBW suggest that chicken of low mature body weight are likely to have low metabolic body weights. In laying chicken, low mature body weight is mostly preferred as it is associated with low maintenance requirements and allows for more feed resources to be diverted towards egg production (Luiting, 1990). Knap and Rauw (2008), however, reports that focus to reduce maintenance requirement for the benefit of productivity compromises an individual's adaptive capacity and physiological balance to cope with stressors within its production environment. Genetic associations between AFE and egg production traits suggest that age at first egg could be used as an indicator trait for cumulative egg numbers and average daily egg mass during the early laying period. Among the egg-related traits during the early laying period, chicken of high daily egg mass would be associated with high cumulative egg numbers but produce eggs of low weights (Dana et al., 2011). In both production periods, the highly positive genetic correlation of ADFI with production traits and metabolic body weight imply that a considerable proportion of the genetic variation in daily feed intake is associated with genetic differences in growth-related traits, egg-related traits and maintenance requirements (Yuan et al., 2015).

Feed conversion ratio was strongly correlated to ADG (growth period) and egg production traits than with ADFI, implying that variability in FCR was majorly influenced by production traits than by feed intake. On the other hand, the genetic and phenotypic correlations indicated that FCR efficient chicken consumed less feed but had higher growth rates during the juvenile period and

higher egg production. Although FCR does not account for metabolic weight, the significant correlation with MBW suggest that efficient chicken had low maintenance requirement. These favorable associations imply that FCR is a trait that can be considered in breeding objectives intended to improve the efficiency of producing indigenous chicken during the growing and laying periods. Furthermore, FCR has extensively been used to measure feed efficiency due to its ease of computation and its direct association of costs and profits to quantities of feed (Aggrey et al., 2010). However, considering the strong genetic correlations between FCR and the production traits than with feed intake, Crews (2005) suggests that selection to reduce FCR may not necessarily be correlated specifically to improvements in efficiency, but may only reflect selection for increased productivity. Furthermore, FCR being a ratio trait, Aggrey et al. (2010) indicates that selection for such a trait may not translate into equivalent improvements in efficiency mainly because selection pressure may be disproportionately applied to the numerator or to the denominator (mostly in favor of the component trait with the most genetic variance). Besides, the confounding effects resulting from the relation between FCR and its component traits and the relation between its component traits, as observed in this study, prevent FCR from being an ideal measure of efficiency (Willems et al., 2013).

Due to the distributing properties of the regression procedure used to obtain RFI in this study, the efficiency measure was phenotypically independent from the production traits used in its estimation. Netter et al. (2004) reports that this phenotypic independence allows for comparison of individuals differing in the level of production traits. However, at a genetic level, Kennedy et al. (1993) found that genetic variability of RFI may not necessarily be independent of the production traits included in the model. In this study, genetic correlations between RFI and production traits indicated that feed efficient chicken during the growing period would be associated with higher growth rates while post-maturity, RFI efficient chicken would lay more eggs of low egg weights but have a higher egg mass. The magnitude of genetic correlation estimates was, however, higher between RFI with MBW than with production traits, an indication that RFI reflected more the variability in maintenance requirement than differences in production traits. Therefore, it is possible that the study population was diverting more feed resources to maintenance requirement rather than production requirement. Similar observations were previously reported by Aggrey and Rekaya (2013) in growing chicken and by Luiting (1990) in laying chicken. Besides, the negative genetic correlations between RFI and MBW in both production periods suggest that feed efficient chicken had a higher maintenance requirement. Aggrey et al. (2010) indicates that during the growing period protein accretion is at a higher rate than fat deposition due to muscle development and given that protein turnover is an energy expensive process, could perhaps explain the high maintenance requirement in feed efficient chicken during this production period. This, however, implies that feed efficient growers will likely produce lean carcasses at market point. Similarly, the high maintenance requirement in laying chicken that were feed efficient may suggest that post-mature metabolic weight was majorly composed of protein tissues and hence, the use of more feed resources to maintain these tissues.

The positive genetic correlation between RFI and ADFI in both production periods indicate that improved efficiency would be accompanied by lower feed intake levels. Selection for feed efficiency measures is mostly aimed at reducing feed intake levels with a consequential effect on reduced feed costs, nitrogenous wastes and environmental footprint (Zhang and Aggrey, 2003). To this regard, higher genetic associations of ADFI with RFI than with FCR suggest that selection for RFI would be more beneficial in reducing feed intake than FCR-based selection. Besides, the positive correlation between RFI and FCR imply that improved RFI would also result in FCR efficient chicken. Considering that BW12 and AFE were not included in the estimation of RFI and FCR, presence of significant genetic and phenotypic correlations indicates that selection for body weight at week 12 and early sexual maturity would be accompanied with feed efficient meat and egg lines, respectively.

Among immune and production traits, the negative correlations between KLH-IgM and BW_{12}, and between NDV-IgG and ADG implied immune deficiencies in chicken of high body weight at week 12 of age and growth rate during the juvenile period. At sexual maturity, the negative correlations between AFE and the antibody traits suggested that early maturing chicken had higher KLH-IgM and NDV-IgG levels. Similar observations by Zheng et al. (1998) indicated a physiological relationship between sexual maturation and development of the immune system exists, such that, earlier presence of circulating gonadal hormones positively influence the immune function. However, chicken of high mature body weights would be associated with low antibody levels. Similarly, genetic correlations between the antibody traits and egg-production traits indicated that chicken of high egg numbers, egg weight and egg mass had low KLH-IgM and NDV-IgG. Zerjal et al. (2021) reports that the immune system is heavily dependent on metabolic resources for proper functioning and therefore, it is expected to be in competition with other nutrient- and energy-demanding process, such as production, in an individual's resource allocation strategy. This could explain the unfavorable associations between the antibody traits and production traits in both production periods and may indicate presence of biological trade-offs among these energy demanding functions. On the other hand, genetic correlations between the antibody traits with MBW, ADFI, and RFI in both production periods suggested that immune-competent chicken had higher maintenance requirements, consumed more feed and were feed inefficient. Considering that the development and maintenance of a fully competent immune system, as well as, the mobilization of immune responses to external stimuli have a metabolic cost (Rauw, 2012), it is possible that the study population was consuming more feed to support these immune functions rather than production and hence, the inefficiencies. Based on resource allocation theory, these observations indicate that an indirect relationship between levels (KLH-IgM) and activation (NDV-IgG) of humoral immunity and feed efficiency may be present.

Between the immune traits, the negative correlation indicated an antagonistic association between KLH-IgM and NDV-IgG. However, the lack of significance of the estimate suggests the association may not be significantly different from zero and the traits are probably genetically different. Antibodies binding to KLH and NDV represent different functional B cell activities and mechanisms underlying their formation vary with respect to the nature of the antigen (Parmentier et al., 2004; Star et al., 2007). For instance, KLH, a glycoprotein, is recognized by TLR-1 which is associated with Th-2 type of immune responses while NDV is a double stranded RNA recognized by TLR-3 which induces Th-1 type of immune responses (Cheng et al., 2014; Berghof et al., 2018). Mangino et al. (2017)

indicates that the different TLRs and regulatory pathways are influenced by different genes and this could perhaps explain the genetic independence between KLH-IgM and NDV-IgG. Therefore, it is possible to simultaneously select for KLH-IgM and NDV-IgG to improve general immunity and specific immunity to NCD, respectively, with minimum to no adverse effects on either of the antibody traits.

For production traits measured pre- and post-maturity, the negative genetic correlation between BW_{12} and AFE implies that improved body weight at week 12 would lead to delayed sexual maturity. This may be undesirable for the egg lines given the negative associations between AFE and egg numbers (van der Klein et al., 2015). In ADG, the negative genetic correlation between the two production periods indicate that high growth rates during the juvenile period would result in low growth rates post-maturity. Growth curves of chicken follow a sigmoidal pattern in which point of growth inflection and attainment of asymptotic body weight coincides with point at sexual maturity (Ridho et al., 2021). Subsequently, post-maturity, growth rate tends to decelerate with increase in age and this could explain the observed genetic correlation between ADG measured pre- and post-maturity. Genetic associations between ADFI measured in both production periods suggests that feed intake in young and mature birds share a similar genetic background and therefore, chicken with high feed intake are likely to maintain this level of intake post-maturity.

Being a linear combination of production traits and metabolic weight, RFI aims to capture the variations among animals in energy utilization for production and maintenance requirement (Arthur et al., 2001). This ensures that the animals resulting from this form of selection would potentially be efficient both as producing individuals and breeding individuals. For instance, in developing feed efficient meat lines, it is expected that selection for RFI during the juvenile period will result in a breeding flock that will also be feed efficient when growth has virtually ceased and, maintenance and reproduction functions are a priority. Similarly, for egg lines, selected RFI efficient layers as the breeding flock are expected to produce progenies that can efficiently utilize feed resources for growth and development during the juvenile age to attain the required mature age and body weight. In this study, the positive genetic correlation between RFI measured pre- and post-maturity indicates that feed efficient chicken in the growing period would maintain their efficiency during the laying period. However, the non-significance level of the correlation estimate suggests that RFI determined prior to sexual maturity may not be an accurate predictor of post-maturity RFI. In this case, low RFI growers may or may not rank as efficient breeders and low RFI layers in the breeding flock may or may not produce progenies that efficiently utilize feed resources to attain expected sexual mature age and weight.

As animals grow, composition of their gain shifts from protein accretion to fat deposition with a substantial shift occurring around the time of sexual maturity and hence, variability in body composition between young and mature animals (Arthur et al., 2001). This could be supported by the low genetic correlation in MBW measured between the two production periods indicating that genetic influence of maintenance requirements at juvenile ages may not necessarily be same requirements post-maturity. Since energy expenditure associated with deposition and maintenance of tissues varies between protein and fat tissues (Loyd et al., 2011) and that MBW is a major contributor to variation in RFI (Luiting, 1990), the efficiency of feed use is expected to vary depending on the body composition. Therefore, changes in body composition associated with advancing physiological maturity could partially explain the low genetic correlations between pre- and post-maturity RFI. Besides, Luiting (1990) also reports that genetic differences between RFI measured pre- and post-mature periods could be related to differences in feeding behavior, physical activity, nutrient digestibility, heat increment, and energy homeostasis and partitioning. On the other hand, during the juvenile period the focus of selection for meat lines is to maximize on growth and development processes occurring during this period while post-maturity the focus is to have early maturing chicken with high egg production. These production functions contribute differently to variation in feed consumed (Zerjal et al., 2021) and therefore, the efficiency of feed utilization in the respective production periods is expected to vary. Higher phenotypic correlation than genetic correlation for RFI and MBW could suggest the traits measured in the two period share common environmental effects (Falconer and Mackay, 1996). This indicates that if favorable environmental conditions are experienced in both production periods, feed efficient chicken and those of low maintenance requirement during the growing period are likely to maintain the same performance during the laying period (Willems et al., 2013). Therefore, high producing individuals selected to be breeders should perform in the same environment to maintain the similar efficiency and maintenance requirement.

The strong and positive genetic correlations between antibody traits measured at 16 and 28 weeks of age could indicate that KLH-IgM and NDV-IgG share a common genetic background at juvenile age and at maturity. Similar observations by Bovenhuis et al. (2022) in laying chicken found that genetic correlations between natural antibodies measured at 16 and 32 weeks of age were not significantly different from one. Despite the strong genetic correlations, differences in heritability estimates of antibody traits between the two age periods suggest that environment-related effects have more influence than additive genetic effects (Bovenhuis et al., 2022). Boa-Amponsem et al. (1997) indicates that the regression of the bursa of Fabricius (production site of B cells) leading into maturation and differentiation of the immune system mostly occurs when birds are approaching maturity (9–20 weeks) and immediately post-maturity (20–40 weeks) and that these processes are influenced by similar genes. Therefore, chicken with higher levels of KLH-IgM and NDV-IgG at juvenile age are likely to rank as immune-competent at maturity. This implies that early selection for KLH-IgM and NDV-IgG at 16 weeks of age is feasible and may be effective on immune function at 28 weeks of age. Besides, presence of a linear association between immune functions and age (Berghof et al., 2010) indicate that selected individuals at 12 weeks of age will likely have enhanced antibody responses even at advanced ages, that is, beyond 28 weeks of age. This is based on the idea that environmental sensitization from exogenous stimuli an individual may encounter over time shapes and enhances antibody responses (Bernasconi et al., 2003). For instance, in an egg line, Sun et al. (2013) estimated positive correlations between KLH-IgM measured at 20 and 40 weeks of age and between KLH-IgM measured at 40 and 65 weeks of age, indicating a peak in antibody levels between ages 20–40 weeks, followed by a stable period until 65 weeks of age.

Conclusion

The low to high heritability estimates for growth-related, egg-related, feed efficiency and antibody traits measured during the growing and

laying periods indicate presence of heritable variation that could be exploited to improve production, feed efficiency and immunity in indigenous chicken. Genetic correlations among the traits suggest that improved feed efficiency would be associated with high growth rates, early maturing chicken, high egg mass and reduced feed intake. In contrast, improved general (KLH-IgM) and specific (NDV-IgG) immunity would result in lower growth rates and egg mass but associated with early sexual maturation and high feed intake. Unfavorable genetic correlations between feed efficiency and immune traits imply that chicken of higher productivity and antibody levels will consume more feed to support both functions. These associations indicate that selective breeding for feed efficiency and immune-competence may have genetic consequences on production traits and should therefore be accounted for in indigenous chicken improvement programs.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of KALRO-Veterinary Science Research Institute (VSRI) (KALROVSRI/IACUC019/30082019).

Author contributions

SM: Conceptualization (lead); Methodology (lead); Investigation (lead); Formal analysis (lead); Writing—original draft (lead); Visualization (lead); Writing—review and editing (equal) CW: Methodology (supporting); Writing–original draft (supporting); Writing—review and editing (equal). EI: Conceptualization (supporting); Funding acquisition (lead); Writing—review and editing (equal). JB: writing—review and editing (equal); Supervision (lead). MC: Writing–review and editing (equal); Supervision (lead).

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Uganda chicken genetic resources: I. phenotypic and production characteristics

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The extent of diversity in the indigenous chicken breeds of Uganda was assessed for their potential utilisation in breeding programmes. A total of 293 indigenous-chicken-keeping households in villages across 35 districts forming 12 sub-regions of the four regions were randomly sampled for 586 mature chickens of both sexes. While only 20.8% of households were headed by women, 40.6% of indigenous chicken keepers were women. The production objectives mainly focused on chickens as sources of income from sales and household food. The chickens were predominantly managed in a scavenging (94.2%) feeding system in a mainly (96.9%) mixed crop-livestock system. The average flock size was 19.80 ± 1.21 chickens with 3.83 ± 0.29 laying hens, each producing an average of 13.41 ± 0.20 eggs/clutch and 40.3 ± 0.6 eggs/annum of 5.20 ± 0.03-month egg production age. Normal-feather strains predominated (>90%), with scattered incidences of naked neck, frizzles, polydactyl, and ptilopody traits in both sexes. Dark (49.0% hen; 43.8% cock) and white (38.3% hen; 42.4% cock) skin colours were most common among the chickens compared to yellow skin. However, yellow-coloured shanks were proportionally the most observed (41% cock; 29% hen). Orange and brown iris (eye) colours were the most common in both sexes. The hens commonly had small round earlobes with varying colours, while cocks had large oval-shaped, mainly red (70%) earlobes. The single-comb type was dominant in both sexes, with wattles almost universally present. Frizzle and polydactyl allele frequencies were significantly lower ($p < 0.05$) than the expected Mendelian proportions, indicating a possible state of endangerment. Meanwhile, the estimated allele frequencies of ptilopody, tufted-crest, and rose comb alleles in the population were similar ($p > 0.05$) to the expected Mendelian frequencies. However, these strains did not show any significant ($p > 0.05$) influence on the body weight or the linear morphometric estimates except for being marginally higher than the normal strains. The phenotypic correlations of body weight and morphometric traits ranged from 0.457 to 0.668 and 0.292 to 0.454 in cocks and hens, respectively. These findings provide hints about the prospects for improved performance with modifications in the production environment. The wide phenotypic diversity would support management efforts for their sustainable utilisation and preservation.

KEYWORDS

indigenous chickens, qualitative traits, Gallus gallu, descriptive phenotypic traits, scavenging management system, phenotypic effect on morphological traits, genetic diversity, chicken strains

Introduction

Domestic free-range/scavenging indigenous chickens sustain the livelihoods of millions of people in smallholder subsistence economies (FAO, 2018a). They are amongst the most important livestock species, constituting the most popular domesticated animal in Uganda to provide a regular source of meat (65,000 tonnes per year) and eggs to a large proportion of the population (FAO, 2019a). Over 85% of the national chicken flock population of Uganda are mainly indigenous breeds (FAO, 2019a; NEMA, 2019), traditionally kept by smallholder backyard poultry farmers under a free-range system (MAAIF & UBOS, 2009; Vernooij et al., 2018; FAO, 2019b; MAAIF, 2019). Together with beef, chicken has been targeted in the Uganda Agriculture Sector Strategic Plan 2015/16–2019/20, as a priority commodity for development (FAO, 2018b). Tropical production environments are challenging, and most farmers raise their domestic indigenous chickens under no to minimal input conditions. Resilience to selective pressures is, therefore, what has allowed chickens to remain predominant in many villages, with wide phenotypic variability (Dessie et al., 2011; FAO, 2015; Mpenda et al., 2018; FAO, 2010). This indicates a huge genetic diversity requiring comprehensive characterisation, inventorying, and monitoring across different agroecological zones for their sustainable utilisation and conservation under the prevailing production system. Moreover, in the wake of the impact of climate change already dawning on Africa more intense and frequent climate stressors are expected to increase, particularly in East Africa, by 2050 (Waithaka et al., 2013; Girvetz et al., 2019). The rapidity with which mitigating adaptive measures are instituted will ensure that we can cope with climate change in the region, especially with the ongoing introgression of exotic breeds into the indigenous population to improve their performance. Examples of these introgressions include the Serere Agriculture and Animal Production Research Institute (SAARI) exotic chicken crossbreeding project (Ssewannyana et al., 2019), the National Animal Genetic Resources Centre and Data Bank (NAGRC & DB) Kuroiler crossbreeding projects (USAID, 2017), the *Rakai* (district) local chicken improvement with Bovans White sires (Roothaert et al., 2011) as well as the *Namasagali* (the local hen in Namasagali town of Kamuli district, Eastern Uganda) and Kuroiler sires crossbreeding project by National Livestock Resources Research Institute (NaLIRRI)-Gulu-Makerere University (Kayitesi, 2015; RUFORUM, 2016).

Therefore, defining the genetic attributes among the indigenous chicken genetic resources and determining the state of their available diversity are useful for an effective national breeding programme. This would involve a systematic identification, inventory, monitoring, and description of the production environment to set the entry point to the sustainable utilisation and conservation of these animal genetic resources (AnGR) (FAO, 2012; AU-IBAR, 2019). Our study, therefore, aimed to assess the production characteristics, phenotypic diversity, occurrence, and performance of major chicken genotypes/strains in the indigenous chicken population of Uganda under traditional husbandry conditions. Our findings will provide preliminary geographic scope information regarding the phenotypic characteristics of the indigenous chickens in Uganda and a description of their production system, thus setting a basis for wider genetic diversity studies to identify valuable chicken genetic resources for the selection and improvement of breeding programmes to mitigate the unavoidable climate change.

Materials and methods

Study area and study period

This study, covering 293 indigenous-chicken-keeping homesteads across 35 districts forming 12 sub-regions of the four regional clusters and spanning the diverse agricultural production/ecological zones (AEZs) of Uganda (Figure 1 and Supplementary Table S1), was conducted from January to March 2020. The Global Positioning System (GPS) map of the study location in Uganda is available as a Google map (here). Uganda is landlocked, bordered by the Congo Democratic Republic (DR), Kenya, Rwanda, South Sudan, and Tanzania. Much of its border is lakeshore and located astride the Equator (between latitudes 4° North and 1° South and longitudes 30° East and 35° East). The altitudes of the survey areas ranged from 614 to 2,261 m, averaging 1,184 m above mean sea level. Uganda has a total area of 241,550.7 km^2 of which about 197,065.91 km^2 is land area and 7,620.76 km^2 is swamp and inland water masses. Although generally equatorial, the climate is not uniform since the altitude modifies the climatic conditions and vegetation type. Hence, 10 AEZ exist, defined by similar ecological conditions and socioeconomic characteristics, farming systems, and practices (MAAIF, 2010). A unique AEZ has common crops and livestock types while zones cut across districts (Kraybill and Kidoido, 2009). The descriptions of the AEZs are shown in Supplementary Table S2. The Northeastern drylands AEZ was excluded from this study because the area is a semi-arid zone inhabited by nomadic pastoralists who derive their livelihoods from cattle keeping and so hardly keep chickens. Additionally, the subregion (Karamoja) is prone to insecurity due to cattle rustling.

Study design and data collection

Chickens were sampled mainly from rural households based on grid cells of 50 km^2 across the Ugandan landscape map (Figure 1). The wide landscape sampling ensured varied agro-climates, which have implications for genetic diversity through adaptive divergence. As such, sampling genetically diverse populations was maximised by randomly selecting at least three villages separated by at least 5 km in the grid-cell-identified districts. Then, with the help of the District Veterinary Officers (DVOs), reconnaissance surveys were conducted to sample one indigenous chicken-keeping household from each village for the study. Data on a total of 293 farmer-households and their flock profiles with production history were obtained through interviews (in the language they understood) using the African Union-InterAfrican Bureau for Animal Resources (AU-IBAR) harmonised standard questionnaire, the AnGR-CIM Tool ENFR v2.1 (deployed on the ODK Collect v1.29.5 application for Android devices). Following the AnGR guidelines for phenotypic characterisation (FAO, 2012), two mature unrelated chickens (a hen and a cock) were used for the phenotypic study in each of the farmer-households, totalling 586 (299 hens: 287 cocks) birds across sites. Pictorial field guidebooks (AU-IBAR, 2015a) aided in the description of the qualitative phenotypic characteristics regarding body condition score, body colours (skin, shank, eyes, ear lobe, and beak); plumage and feathering features (feather structure, feather distribution, and body plumage pattern); head features (earlobe size, earlobe shape, comb type, wattles, and crests and beak shapes); and skeletal variance (body shape/conformation, frame, spur size, and tail length). Body weights

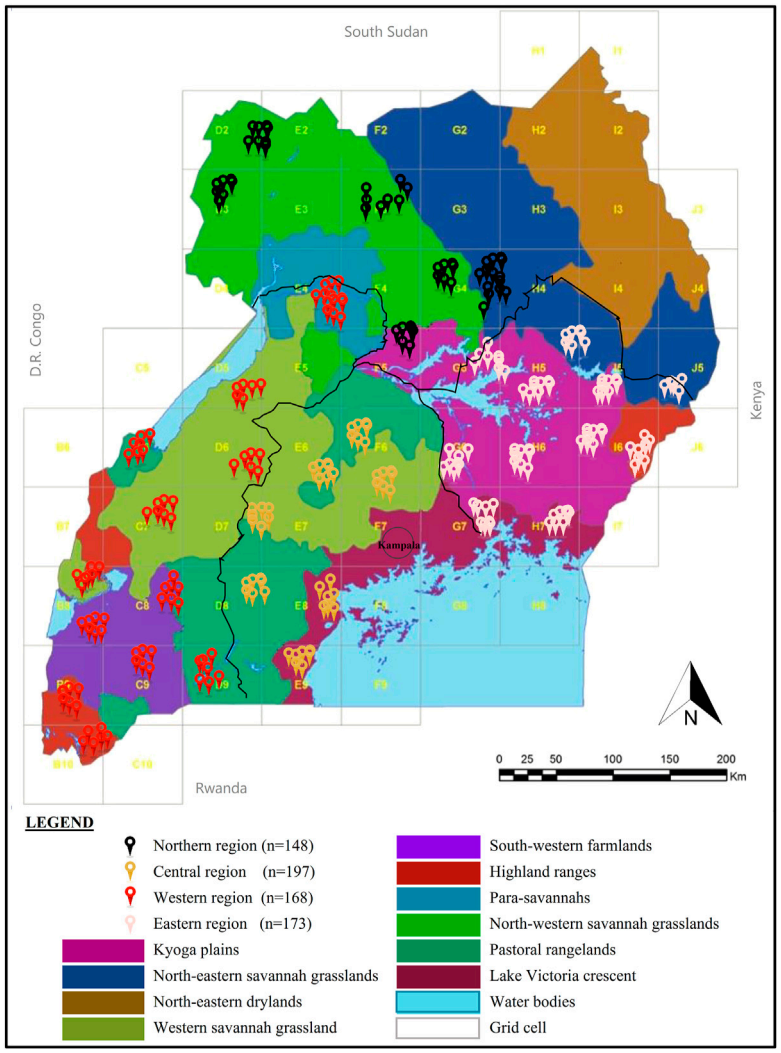

FIGURE 1
Map of the study locations of the indigenous chickens in Uganda. The sample distribution of the 293 randomly selected households from villages (>5 km apart) was obtained from grid cells of approx. 50 km^2 across the AEZs of Uganda to ensure the collection of landscape data.

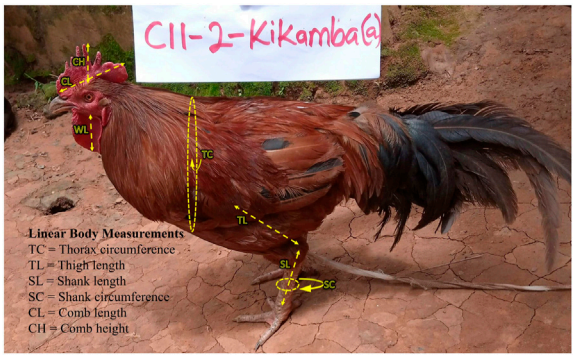

FIGURE 2
Linear measurements (in cm) of the indigenous chickens in Uganda, as described by AU-IBAR (2015b).

(in grams) were measured using a standard electronic weighing scale (WH-A08; 0–10 kg) while the linear body measurements of the live chickens (Figure 2) were taken using a tailors tape measure. The GPS location of each sample homestead and digital photographs of the studied chickens were captured with the integrated AnGR-Photo Tool (ENFR v2.1). The ages of the chickens were obtained from farmers' records or estimated (where records were unavailable) as recalled by the owner or judged by visual appraisal as in the AnGR guidelines for phenotypic characterisation (FAO, 2012) and described by Birteeb et al. (2016). The body conditions of most of the matured cocks and hens were scored as emaciated, thin, good, or fat, on a scale of 1, 2, 3, and 4, respectively.

Estimation of the effective population size (Ne) for the randomly mated indigenous chicken populations across the household flocks in each region and their corresponding rate of inbreeding (ΔF) were executed as described by Falconer and Mackay (1989) using the equations:

TABLE 1 Demographic analysis of indigenous chicken farmers in Uganda.

Variable (n = 293)	Farmer-households [n (%)]					χ^2, region	χ^2, category	*p*-value[1]
	Northern n = 74	Central n = 49	Western n = 84	Eastern n = 86	Total n = 293			
***Gender of respondents* (*farmers*)**						7.29^{ns}	10.32^{**}	
Male	48 (64.9)	22 (44.9)	47 (56.0)	57 (66.3)	174 (59.4)			
Female	26 (35.1)	27 (55.1)	37 (44.0)	29 (33.7)	119 (40.6)			
Position of the respondent within the household						4.85^{ns}	105.55^{**}	
Household head	48 (64.9)	28 (57.1)	46 (54.8)	54 (62.8)	176 (60.1)			
Spouse	20 (27.0)	12 (24.5)	26 (31.0)	24 (27.9)	82 (28.0)			
Other-household member	6 (8.1)	9 (18.4)	12 (14.3)	8 (9.3)	35 (11.9)			
Gender of household head						15.38^{**}	99.80^{***}	
Male	67 (91)	31 (63)	70 (83)	64 (74)	232 (79.2)			
Female	79)	18 (37)	14 (17)	22 (26)	61 (20.8)			
***Age of respondents* (*farmers*)**						3.4^{ns}	225.10^{***}	
22–30 years	10 (13.5)	3 (6.1)	5 (6.0)	8 (9.3)	26 (8.9)			
31–60 years	52 (70.3)	38 (77.6)	64 (76.2)	64 (74.4)	218 (74.4)			
Over 60 years	12 (16)	8 (16.3)	15 (17.9)	14 (16.3)	49 (16.7)			
Age (*Mean ± SE*)	46.6 ± 1.6	48.0 ± 1.6	48.7 ± 1.4	46.2 ± 1.4	47.3 ± 0.8			0.577
Household size (*Mean ± SE*)	7.91 ± 0.56	8.24 ± 0.67	7.68 ± 0.37	7.22 ± 0.46	7.64 ± 0.27			0.628
***Average distance from farmers' homestead* (*Mean±SE*)**								
Road[1] (km)	1.16 ± 0.29	4.57 ± 1.02	2.50 ± 0.35	5.31 ± 4.65	33.30 ± 1.38			0.691
Market (km)	3.16 ± 0.26	6.58 ± 0.93	4.28 ± 0.41	8.45 ± 4.64	5.60 ± 1.38			0.497
***The most important household income of source* (*n = 213*)**						7.74^{ns}	217.55^{***}	
Crop products sales	53 (71.6)	13 (26.5)	20 (23.8)	57 (66.3)	143 (48.8)			
Sale of L&LP*	11 (14.9)	8 (16.3)	10 (11.9)	14 (16.3)	43 (14.7)			
Trade in L&LP	1 (1.4)	-	-	1 (1.2)	2 (0.7)			
Off-farm	9 (12.2)	2 (4.1)	3 (3.6)	11 (12.8)	25 (8.5)			
Undisclosed	-	26 (53.1)	51 (60.7)	3 (3.5)	80 (27.3)			

Numbers in brackets are the percentages of total respondents in each location; n = chickens sampled (-) = not reported; * livestock and livestock-products; χ^2 = chi-square tests for among region and within variable categories; $^{ns}P>0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; [1]*p*-value of one-way ANOVA; SE, standard error. Means with no superscripts within rows did not differ significantly ($p > 0.05$). [1]Distance from household to an all-weather road.

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Effective population; $Ne = \frac{4(N_m \times N_f)}{N_m + N_f}$

Rate of inbreeding; $\Delta f \frac{1}{2N_e}$

where N_e is the net effective population size, N_m is the number of breeding cocks in the flock, N_f is the number of breeding hens in the flock, and ΔF is the rate of inbreeding per generation.

Records of indigenous chicken strains showing major genes that characterise genotypes with known adaptive values like the allele for frizzling [F/f], ptilopody [Pti/pti], tufted crests [Cr/cr], rose comb [R/r], and Polydactyl [P/p] were used to estimate their allele frequencies. The proportions of phenotypic counts were computed as:

$$Phenotypic\ frequency = \frac{Number\ of\ individuals\ carrying\ trait}{Total\ number\ of\ individuals\ sampled} \times 100$$

Chi-squared goodness of fit analyses of the proportions of the observed chicken strains against the expected Mendelian proportions (25% and 75%, respectively for incidence and absence of the genotype) were conducted using the `chisq.test` method in R software. The estimates of allele frequencies using the Hardy-Weinberg principle (Falconer and Mackay, 1989) were based on the models below:

$$q = \sqrt{\frac{m}{t}} \text{ and } p = 1 - q$$

where q is the frequency of recessive allele, m is the observed number of indigenous chickens expressing the recessive phenotypes under consideration, t is the total number of chickens sampled, and p is the frequency of dominant allele expressed in the chickens not showing the major trait considered.

Statistical analysis

With sample locations (geographical regions) and chicken sex categories fitted as fixed independent variables, the dependent variables were subjected to descriptive-analytical procedures in IBM SPSS Statistics for Windows, version 20.0.0.0. Kendall's concordance coefficient *W* tests were applied to test and rank agreement among farmers regarding their rated household chicken production objectives at a 5% significance level. Analysis of variance (ANOVA) was conducted using the general linear models in IBM SPSS Statistics for Windows, version 20.0.0.0. The least-square means of significant differences were compared using Scheffe's adjustment to account for unequal sample sizes per category. The model for the morphometric traits (body weights and linear body measurements), which excluded the interaction effects because they were non-significant, took the form: $\mathcal{Y}_{ijk} = \mu + l_i + s_j + e_{ijk}$; where $\mathcal{Y}_{ijk}$ is the observation of the morphometric trait in chicken location *i*, for chicken sex *j*, μ is the overall mean, l_i is the effect of location (*i* = Northern, Central, Western, or Eastern region); s_j is the effect of sex (*j* = hen or cock), and e_{ijk} is the random effect. The overall correlation among these traits was tested using Pearson correlations.

Results

Demographic profile of indigenous chicken-rearing households in Uganda

The demographic analysis of the indigenous chicken-keeping households in Uganda, presented in Table 1, showed relatively similar proportions of male and female respondent farmers ($p > 0.05$) across the regions. While male respondents were mostly higher ($p < 0.001$) in Northern (64.9%), Western (56.0%), and Eastern (66.3%) Uganda, the opposite was true for Central Uganda, where female respondents (55.1%) were observed. The responses were largely ($p < 001$) obtained from the household heads (60.1%), followed by the spouses of the household heads (28.0%), with only a few coming from other household members in similar ($p > 0.05$) proportions across the regions. Most households ($p < 0.001$) were male-headed (79.2%), with significantly ($p < 0.01$) different proportions across the regions. However, the aggregate proportion of male respondents, was only slightly higher (59.4%) across the regions, in a society where most households are headed by men ($p < 0.001$). The age composition of the respondents was characteristically similar to the population pyramid in most developing countries, in which the active working group (30–60-year-olds) accounted for most (74.4%) of the respondents against those aged below 30 down to 22 (8.9%) or above 60 years (16.7%) in this study. The mean age across regions was similar ($p > 0.05$), with an overall mean age of 47.3 ± 0.8 years (range: 22–88 years). The average household sizes were also comparable ($p > 0.05$) across regions, with an overall mean size of 7.6 members. The mean distance from the farmers' homestead to the market was 5.6 km and to an all-weather road was 33.3 km, both of which were similar ($p > 0.05$) across regions. The proportions of the most important household income source were similar ($p > 0.05$) across regions. In most households, the sale of crop (48.8%) and livestock and livestock-products (14.7%) respectively formed the most significant economic activity, which provided incomes for most families ($p < 0.001$). Only a few (12%) indigenous chicken-keeping families derived some income from off-farm activities or the trading of livestock and livestock-products. Some (27.3%) of households did not disclose information regarding their income source, likely due to a belief that one loses wealth upon disclosure, especially among farmers in western and central Uganda.

Flock descriptions, ownership, and production objectives of indigenous chickens in Uganda

The number and proportion of the different chicken age and sex categories in a flock describe the structure of the flock. The results presented in Table 2 and Supplementary Figure S1A–D show that all households kept indigenous chickens of different flock compositions and ownership characteristics. The composition of flocks varied ($p < 0.01$) with the regions and the lowest flock numbers per category were in Central and Western Uganda whilst the highest were observed in Northern and Eastern Uganda. An overall average of 19.80 ± 1.21 chickens was held in indigenous-chicken-keeping households. Similarly, the flock size per chicken category differed significantly ($p < 0.001$) with the proportions of households owning them. Most households held between one and five chickens for the different flock compositions, except for chicks that were also held substantially in all sizes by relatively larger proportions of households. Cock numbers were mainly low, between one to five in most (92.8%) households and across the regions, with an average of 2.6 ± 0.2. Up to 10 hens and growers (pullets/cockerels) were kept in most households, with averages of 6.9 ± 0.4 hens, 4.8 ± 0.5 cockerels, and 5.5 ± 0.4 pullets, respectively. In most households, adult men owned the highest (37.4%) number of chickens in the flock compared

TABLE 2 Composition of household indigenous chicken flocks in Uganda.

Flock description	Farmer-households					*p*-value
	Northern	Central	Western	Eastern	Overall	
Flock composition (Mean ± SE)						
Breeding Cock	3.1 ± 0.4[a, b]	2.1 ± 0.2[a, b]	1.8 ± 0.2[a]	3.2 ± 0.4[b]	2.6 ± 0.2	0.002
Breeding hens	7.0 ± 0.6[a, b]	7.1 ± 1.5[a, b]	4.8 ± 0.5[a]	8.9 ± 0.9[b]	6.9 ± 0.4	0.004
Cockerels	7.6 ± 0.8[a]	1.9 ± 0.7[b]	1.5 ± 0.2[b]	7.5 ± 1.3[a]	4.8 ± 0.5	<0.001
Pullets	9.0 ± 1.2[a]	3.0 ± 0.8[b]	2.1 ± 0.3[b]	7.0 ± 0.8[a]	5.5 ± 0.4	<0.001
Chicks	17.7 ± 2.4[a]	8.7 ± 1.3[b, c]	6.5 ± 0.8[c]	15.4 ± 1.7[a, b]	12.3 ± 0.9	<0.001
Total flock size	26.8 ± 2.6[a]	14.0 ± 2.3[b]	10.1 ± 0.8[b]	26.6 ± 2.7[a]	19.8 ± 1.2	<0.001
***Flock category size, n = 293*(%)**	**1–5 chickens**	**6–10 chickens**	**11–15 chickens**	**16–20 chickens**	**20+ chickens**	χ^2
Cock	272 (92.8)	15 (5.1)	3 (1.0)	2 (0.7)	1 (0.3)	973.60**
Hens	169 (57.7)	87 (29.7)	17 (5.8)	7 (2.4)	13 (4.4)	332.20**
Cockerels	216 (73.7)	42 (14.3)	12 (4.1)	14 (4.8)	9 (3.1)	504.46**
Pullets	191 (65.2)	64 (21.8)	18 (6.1)	9 (3.1)	11 (3.8)	408.42**
Chicks	99 (33.8)	72 (24.6)	48 (16.4)	28 (9.6)	46 (15.5)	51.52**

[a]Means within rows with different superscripts indicate significant differences ($p < 0.05$).
[b]SE, standard error of means.
[c]Percentages (in brackets) are based on the total number of respondents (household) per each category in rows. χ^2 = chi-square test.**$p < 0.01$; **$p < 0.001$.
They are the flock size range (as heading) per category of chickens kept.

TABLE 3 Reasons/purposes for keeping chickens.

Ranking variables (n = 293)	Household ranking[a]					Total (%)	Mean ranks
	1st	2nd	3rd	4th	5th		
Purpose of keeping chickens							
Income (cash from sales)	91	115	11	-	-	217 (45.5)	5.40
Food (meat and egg source)	130	72	4	-	-	206 (43.2)	5.02
By-products (dropping)[b]	1	12	8	2	1	24 (5.0)	2.81
Socio-economic/prestige and Culture	1	7	9	4	-	21 (4.4)	2.77
Gift/barter[c]	1	2	2	1	-	6 (1.3)	2.53
Leisure	1	-	1	1	-	3 (0.6)	2.47
Kendall's W[d]							0.701
Chi-square test (χ^2)							782.12**

[a]The purposes for keeping chickens in households were ranked from 1 (most) to 5 (least) in corresponding order of importance.
[b]By-product (droppings) for use as organic fertiliser.
[c]Gifts/slaughtered for visitors or exchanged for other livestock species.
[d]Kendall's coefficient of concordance W. % = relative proportion, (-) = not reported. **$p < 0.001$.

to adult women (24.6%) Joint ownership by adult men and women household members was also common (28.6%), whilst children under 18 years owned just a few chickens in a flock. In rare instances, a few chickens were kept elsewhere from the household flock or were not owned but kept on behalf of others (Supplementary Figure S1A). The overall effective population size (*Ne*) and rate of inbreeding (ΔF) estimated for the indigenous chicken flock kept across the farmer-households were, therefore, 2,200 and 0.023%, respectively (Supplementary Figure S1B). Regionally, the net effective population size was higher in the Eastern region (802), followed by the Northern (636), Western (441), and Central regions (313), respectively; with a corresponding trend in the rate of inbreeding, which was lower in the Eastern (0.062%) and Northern regions (0.079%) but higher in the Central (0.160%) and Western regions (0.113%). The selling price of chickens differed ($p < 001$) with age category (Supplementary Figure S1C) with the highest average price observed for mature cocks (UGX 24,110.8/ = ~ US$ 6.42). The average price of a mature hen (UGX 16,576.27/ = ~ US$ 4.48) was similar to that of a cockerel (UGX 16,421.05/ = ~ US$ 4.44) and was lowest for a pullet (UGX 9,555.56/= (US$ 2.58). The purpose of selling chickens among the farmers varied significantly ($p < 0.01$) with only 47.6% degree of concordance. However, selling chickens to meet planned household expense was ranked first by the majority (69.2%) of households (Supplementary Figure S1D). The body condition scores of most of the breeding cocks and hens were similar ($p > 0.05$) across households (Supplementary Figure S2). Few thin (7.2% cocks; 8.9% hens), fat (2.4% cocks; 1.7% hens), or emaciated (0.3% cocks; 1.7% hens) chickens were observed, with most of the cocks (90.1%) and hens (87.7%) showing good body condition. Flock numbers showed an increasing trend among farmers with smaller flock sizes when analysed retrospectively across 12 calendar months (Supplementary Figure S3).

Chicken exit from the flock showed different forms among indigenous chicken-producing households in Uganda. While most households allowed cocks to stay in the flock for up to 1 year (41%) or longer

TABLE 4 Indigenous chicken production and management practices in Uganda.

Production and management practices	Farmer-households, n (%)				Total	χ^2
	Northern	Central	Western	Eastern		
***Production system* (*dry or wet season*)**						11.833[ns]
Agro-pastoral	2 (2.7)	2 (7.7)	-	-	4 (1.8)	
Mixed crop-livestock	70 (94.6)	24 (92.3)	36 (97.3)	86 (100)	216 (96.9)	
Scavenging-poultry[a]	2 (2.7)	-	1 (2.7)	-	3 (1.3)	
Total	74	25	37	86	223	
Feeding management practised						13.394[ns]
Scavenging (free-range)	71 (95.9)	25 (96.2)	31 (83.8)	86 (96.5)	214 (94.2)	
Restrictive scavenging feeding[b]	2 (2.7)	-	1 (2.7)	1 (1.2)	3 (1.3)	
Scavenging-supplementation[c]	-	1 (3.8)	4 (10.8)	1 (1.2)	5 (2.2)	
Nutritionally complete feeding[d]	1 (1.4)	-	1 (2.7)	1 (1.2)	1 (0.4)	
Total	74	26	37	86	223	
***Feed available to chickens*[e]**						71.138***
Concentrate (*grains*)	31 (53.4)	-	-	28 (46.6)	59 (11.7)	
Pasturage (*forage, seeds, worms*)	46 (29.3)	25 (16.6)	36 (22.9)	50 (31.2)	157 (31.6)	
Agro-by-product (*spent grains/bran*)	18 (12.9)	23 (17.3)	25 (20.1)	68 (49.6)	133 (28.0)	
Kitchen residue (*leftovers*)	43 (31.2)	24 (16.7)	30 (22.5)	40 (29.7)	137 (27.8)	
Nutritionally complete feed[d]	1 (20.0)	-	1 (20.0)	3 (60.0)	5 (1.0)	
Water provision to chicken flocks						71.141***
Water is fetched/provided	69 (93.2)	25 (51.0)	36 (42.9)	76 (88.4)	206 (70.3)	
Chicken searches for water	5 (6.8)	24 (49.0)	48 (57.1)	10 (11.6)	87 (29.7)	
Total	74	49	84	86	293	
Quality of water available to chickens						100.622***
Muddy	2 (2.7)	24 (49.0)	46 (54.8)	1 (1.2)	73 (24.9)	
Good/clear	72 (97.3)	25 (51.0)	38 (45.2)	85 (98.8)	220 (75.1)	
Total	74	49	84	86	293	

[a]Production of only poultry species under scavenging management.
[b]Chickens are only fed leftovers, grains, brans, etc. within a tethered radius.
[c]Scavenging with some supplementation.
[d]Chickens fed manufactured or formulated feed balanced for all nutrients.
[e]Feed available row percentages are based on the sum of responses for each category. The row total percentages are based on the overall responses (i.e., households feed more than one type). Wet and dry = rainy and dry seasons. (-) = not reported. [ns]$P>0.05$; *$p < 0.05$;**$p < 0.001$.

(44%), only about 15% of households kept their cocks for just a few months (Supplementary Figure S4). One-third (29%) of farmers exchanged their chickens to acquire other livestock species (Supplementary Figure S5), usually (25.0%) goats but also occasionally pigs (1.4%), chickens with unique traits (1.45%), cattle (1.0%), and sheep (0.3%).

The production objectives of indigenous chickens among households across Uganda were significantly ($p < 0.001$) similar. The farmers were 70.1% concordant with one another regarding the ranked purposes for keeping chickens (Table 3). The most significant production objectives of indigenous chickens, ranked first and second across the regions, were income generation from the sale of the chickens and consumption as food in the form of meat and eggs, respectively. The lowest ranked production objectives included the benefits obtained from by-products in the form of chicken droppings for use as organic fertilisers in their crop gardens, socio-economic/prestige and cultural reasons, and keeping chickens for gifts to visitors and/or barter trading to obtain other livestock species and for leisure purposes, respectively.

Indigenous chicken production/management systems and their performance in Uganda

Feed resources, feeding and watering management practices

The production system and feeding management practised were similar across the regions ($p > 0.05$). The mixed crop-livestock system, where farmers kept some livestock alongside crop farming, was the

TABLE 5 Egg production and sale of indigenous chickens in Uganda.

Production variable	Regional households [n (%)]				Overall n = 253	χ^2	*p*-value
	Northern n = 71	Central n = 42	Western n = 59	Eastern n = 80			
Laying hen in the flock?						19.528	<0.001
No	3 (4.1)	6 (12.2)	20 (25.3)	6 (7.0)	35 (12.2)		
Yes	71 (95.9)	43 (87.8)	59 (74.7)	80 (93.0)	253 (87.8)		
Laying hens (Mean ± SEM)	5.23 ± 0.52[a]	3.56 ± 0.67[a, b]	2.56 ± 0.58[b]	3.96 ± 0.49[a, b]	3.83 ± 0.29		0.008
Egg numbers per laying hen							
Age at laying (month)	5.21 ± 0.05[a]	5.02 ± 0.06[a]	5.07 ± 0.05[a]	5.50 ± 0.04[b]	5.20 ± 0.03		<0.001
Average eggs per clutch	13.06 ± 0.37	12.95 ± 0.47	13.98 ± 0.40	13.64 ± 0.35	13.41 ± 0.20		0.234
Average, 3 clutches (a year)	39.17 ± 1.10	38.86 ± 1.42	41.95 ± 1.21	40.91 ± 1.04	40.22 ± 0.60		0.234
Eggs sold per laying cycle						36.122	0.002
0	63 (88.7)	34 (79.1)	41 (69.5)	65 (81.3)	203 (80.2)		
1–10	-	1 (2.3)	-	8 (10.0)	9 (3.6)		
11–20	6 (8.5)	3 (7.0)	6 (10.2)	5 (6.3)	20 (7.9)		
21–30	-	3 (7.0)	7 (11.9)	2 (2.5)	12 (4.7)		
31–60	2 (2.8)	1 (2.3)	4 (6.8)	-	7 (2.8)		
61–90	-	1 (2.3)	1 (1.7)	-	2 (0.8)		
Egg sales points						28.924	0.004
Not sold, consumed/incubated	64 (90.1)	30 (69.8)	40 (67.8)	67 (83.8)	201 (79.4)		
Local market	6 (8.5)	4 (9.3)	8 (13.6)	7 (8.8)	25 (9.9)		
Within neighbourhood	1 (1.4)	3 (7.0)	6 (10.6)	1 (1.3)	11 (4.3)		
Retail shops	-	2 (4.7)	3 (5.1)	5 (6.3)	10 (4.0)		
Small-scale hatcheries	-	4 (9.3)	2 (3.4)	-	6 (2.4)		

[a]Means within rows with different superscripts differ significantly ($p < 0.05$).

[b]Means with no superscripts within rows do not differ significantly ($p > 0.05$). χ^2 = chi-square test (-) = not reported, SEM, standard error of the mean.

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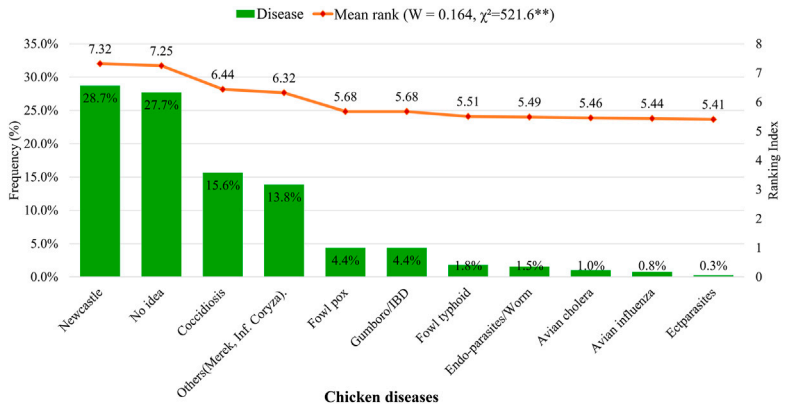

FIGURE 3
Disease prevalence in indigenous chickens of Uganda as described by households. W = Kendall's coefficient of concordance. x^2 = chi-squared.**p < 0.001.

predominant (96.9%) agricultural production system in most farmer-households (Table 4). However, the agro-pastoral system (1.8%) which combines crop and pastoral livestock production and the farming of only poultry species under scavenging management (1.3%) formed isolated cases of production systems in households in the Northern, Central, and Western regions. Across the regions, scavenging (free-range) feeding management was mainly (94.2%) practised in most indigenous-chicken-keeping households, irrespective of the season, followed by restrictive scavenge-feeding (1.3%), where the flock only has access to leftovers, brans or grains within a confined area; or scavenging-supplementation (2.2%), where the flock is provided supplemental feed as they scavenge for feed near and around the homestead (Supplementary Figure S6). Hardly any formulated feed (0.4%) balanced for all the required nutrients was provided to the chickens in most households except in one case each from the Northern, Western, and Eastern regions.

The three major feed resources available for the chicken flock were pasturage (31.6%) near or around the homesteads for the chickens to forage, followed by agricultural by-products like spent grains from breweries, residues and bran from grain mills (28.0%), and kitchen refuse/leftovers (27.8%). Only a small proportion of households (11.7%) provided grain concentrates to supplement their flock's scavengeable feed resources, with rare cases of households (1.0%) providing manufactured or formulated nutritionally complete feed to their chickens. In most households in the Western region (57.1%) and a good number in the Central region (49.0%), chickens were not provided water for drinking; rather, the chickens searched or walked to sources of water within their scavenging range. However, most households in the Northern (93.2%) and Eastern (88.4%) regions provided drinking water to their flocks. The water sources within the scavenging radius of the flock were good in most (75.1%) of the households except for one-third of those in Central and Western Uganda, in which the water sources were muddy.

Housing systems for indigenous chickens in Uganda

Most households provided chickens with some form of housing to shelter both young and old birds ($p > 0.05$). Predominant among the housing units across the households were shelters constructed on the side of the main house (43.9% young; 46.9% adult) and sheds (43.4% young; 37.2% adult) in the homestead. A few other households sheltered their chickens in human dwellings (10.2%, young and adults)), off-ground perches/kitchens/trees (2.0% young; 4.6% adult), and stalls (0.5% young; 1% adult) (Supplementary Figures S7A, S8). Regional differences were observed in the housing structures of chickens ($p < 0.001$). Sheds were more popular in the Northern and Eastern regions whilst shelters constructed on the side of the main house dominated the Central and Western regions (Supplementary Figure S7B, S8). However, the proportions of households keeping chickens in human-dwelling units were similar across regions.

Chicken health services and disease control

Disease incidence and treatment dynamics in the chicken flock differed significantly ($p < 0.001$) across the households (Supplementary Table S3). Holistically, disease episodes in most household indigenous chicken flocks were reported to occur once in a while (35.5%) and seasonally (32.4%), as almost a quarter of the households (24.2%) reported seldom experiencing disease challenges. Most of the households reporting a low disease incidence were in the Central and Western regions, whereas households in Northern and Eastern regions mostly reported occasional disease episodes. Most farmer-households across the regions treated diseased chickens only when they were very sick. Farmers, especially those in Northern (45.9%) and Eastern (33.7%) regions, had to treat their diseased chickens, or they died of their ailment during the disease episodes. Many of the farmers reported that their chickens recovered on their own without treatment during disease episodes or died, while farmers in the Northern and Eastern regions reported losing their birds before any treatment could be administered. A small proportion of farmers across the regions did not encounter disease episodes in their flock, which they attributed to their use of phyto-prophylaxis.

Poultry health services available to chickens differed ($p < 0.001$) across regions, with curative treatment the most common (49.5%), followed by vaccination (45.3%) which was higher in the Northern (36.2%) and Eastern (48.5%) regions (Supplementary Table S3 and Supplementary Figure S9A). Antihelminthic deworming and ectoparasite control were minimal, at 6.3% and 1.0% respectively. Most farmers (87%) relied on ethnoveterinary concoctions to treat

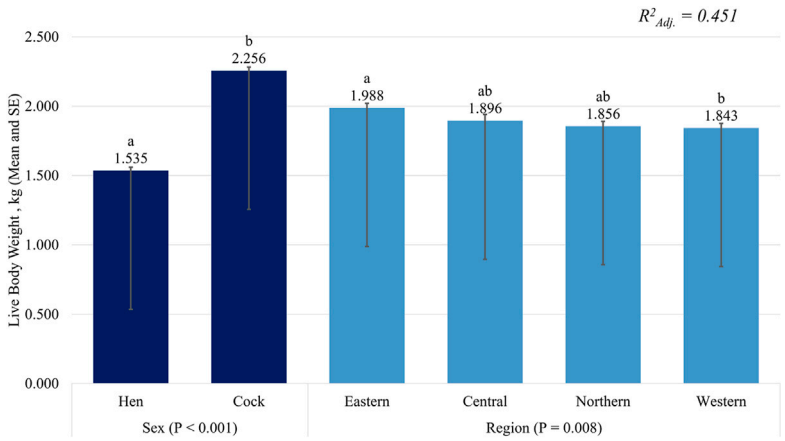

FIGURE 4
Mature body weight (mean and SE) of indigenous chickens in Uganda. [a,b]Significant differences in within-factor mean bars ($p < 0.05$). x^2 = chi-squared.

FIGURE 5
Feather distribution and structure in the indigenous chicken population in Uganda. **(Al, A2)** Normal feather distribution. **(Bl, B2)** Naked-neck. **(Cl, C2)** Ptilopody (feathered shanks/tarsus). **(Dl, D2)** Frizzle feather structure. **(E)** Smooth/neat plane feather. **(F)** Silky and **(G)** superficially silky feather structures.

their morbid chickens compared to the few (13%) who used conventional medication ($p < 0.001$). Predominant among the ethno-materials were plant leaves (68%) while stems, seeds, fruits, barks, and wood ash were also sometimes used (Supplementary Figure S9B).

The farmers showed significant ($p < 0.001$) agreement in their ranking of the diseases that challenged their chicken flocks, based on experience (not pathological diagnosis). However, a significant proportion (27.7%) of the farmers had no idea about the diseases that challenged their production. Newcastle disease was ranked first (28.7%) as the most prevalent disease, followed by coccidiosis (15.6%). Other diseases of importance were Marek's disease and infectious coryza (13.8%), Gumboro/infectious bursal disease (IBD) (4.4%), and a host of other minor health challenges including fowl typhoid, helminths, avian cholera/influenza, and some ectoparasites (Figure 3).

Most (38.4%) of the farmers administered healthcare services to their flock themselves or through a neighbour without any professional advice, while 22.0% sought professional advice (Supplementary Figure S10). Farmers reaching out to animal health service providers and governmental veterinarians constituted 17.3% and 17.0% respectively, as 4.3% accessed treatment from veterinary supply shops in their neighbourhoods. Community vaccinations were accessed by only a few (0.9%) indigenous chicken farmers.

Chicken identification and pedigree information

Regarding chicken identification and pedigree information, 95% of indigenous-chicken-keeping farmers hardly kept any form of

FIGURE 6
Shank (tarsus) colours. **(A)** White. **(B)** Grey/blue-grey. **(C)** Black/dark grey. **(D)** Yellow. **(E)** Orange. **(F)** Brown. **(G)** Green. **(H)** Pink.

written records on their production (Supplementary Figure S11). Nonetheless, 90% of them claimed they could recognise their chickens individually, though a planned outline of naming or identification of individual members of their chicken flock was non-existent in most (95%) of the households.

Productive performance and sales from indigenous chicken flocks in Uganda

Table 5 shows that most households (87.8%) generally had an average of 3.83 ± 0.29 laying hens in their flock; however, this number varied significantly across the regional households ($p < 0.05$). The Northern region had a higher ($p < 0.01$) average number of laying hens compared to the Western region but not the Central and Eastern regions. The average age at first lay was 5.20 ± 0.03 months (21 weeks). The average of 13.41 ± 0.20 eggs per hen per clutch was similar ($p > 0.05$) across regions. Consequently, an average of 40.22 ± 0.60 eggs were produced per year.

A high proportion (80.2%) of farmers across the regions ($p < 0.01$) had not sold any eggs in the 12 months before the study. About 11.5% of them sold 20 eggs at most while a small proportion (8.3%) sold at least an egg crate (30 eggs) from their flock. Most (79.4%) of the eggs produced were used as food in the homestead or were incubated to hatch chicks, leaving a few for sale in local markets (9.9%) within the neighbourhood (4.3%), at retail shops (4.0%), and to small-scale hatcheries, mainly in the Central and Western regions (2.4%).

The mature chicken body weights (Figure 4) for market differed between sexes, with the cocks heavier ($p < 0.001$) than the hens. Regional differences ($p < 0.05$) were also observed, with chickens in the Western region lighter ($p < 0.05$) than those in the Eastern region but as heavy ($p > 0.05$) as those from the other regions.

Phenotypic characteristics of indigenous chickens in Uganda

The total of 586 indigenous breeding chickens sampled for the phenotypic characterisation study were of similar ($p > 0.05$) sex categories, with overall comparable ($p > 0.05$) regional proportions (Supplementary Table S1 and Figure 1). Most of the breeding chickens in the household flocks from which samples were taken were in generally good body condition with moderately developed concave breast muscle and less prominent keel ($p > 0.05$) across households.

Feather morphology, distribution, and plumage patterns

The feather structure and distribution of the chickens varied significantly ($p < 0.05$) across the study locations. Most of the chickens across the regions (97.7% of hens and 95.5% of cocks) presented smooth/neat plane feather structures irrespective of sex ($p > 0.05$). However, a few isolated birds with frizzle, silky, and superficially silky feathers were also observed (Supplementary Table S4 and Figure 5). Normal feather structure was generally prevalent across the regions (90.3% of hens and 89.2% of cocks), with the sparse occurrence of naked neck trait and ptilopody (feathered shanks) in both sexes ($p > 0.05$) (Supplementary Table S4 and Figure 5).

The chickens displayed diverse plumage patterns (Supplementary Table S4 and Supplementary Figure S12) in both sexes across the regions ($p < 0.001$), most commonly the partridge (37.8%) and birchen (19.8%) patterns in cocks overall. In contrast, the hens generally presented mixed plumage patterns (25.2%), which were a blend of the eight- plumage patterns observed. The hens also presented high proportions of uniform (23.2%) and pencilled (21.5%) plumage patterns. The cocks showed the highest proportions of birchen (39.7%) plumage pattern in the Northern region but the lowest proportion (10.7%) in the Western region. Notably, the birchen pattern only existed (8.0%) in hens ($p < 0.001$) in the Northern region. Partridge was the predominant pattern in cocks in the Central, Western and Eastern regions, observed in 44.4%, 44.0%, and 34.9% of the birds, respectively. In hens, pencilled plumage pattern was dominant in the Central and Western regions, while uniform plumage occurred more in the Northern region. The Eastern region instead had a higher proportion of mixed plumage patterns in this study. Mottled and spotted patterns were the least frequent plumage across the regions.

TABLE 6 Variations in the descriptive head features of indigenous chickens in Uganda.

Qualitative traits [n (%)]	Northern		Central		Western		Eastern		Uganda		χ^2	
	Hen n = 75	*Cock* n = 73	*Hen* n = 58	*Cock* n = 54	Hen n = 78	*Cock* n = 75	*Hen* n = 87	*Cock* n = 86	*Hen* n = *298*	*Cock* n = *288*	*Sex*	*Region*
Earlobe size											304.3***	16.4**
Small[a]	61 (81.3)	13 (17.8)	57 (98.3)	18 (33.3)	77 (98.7)	27 (36.0)	83 (95.4)	7 (8.1)	279 (93.3)	65 (22.6)		
Large[b]	14 (18.7)	60 (82.2)	1 (1.7)	36 (66.7)	1 (1.3)	48 (64.0)	4 (4[b]6)	79 (91.9)	20 (6.7)	223 (77.4)		
Earlobe shape											161.8***	60.2***
Oval	33 (44.6)	66 (90.4)	4 (6.9)	32 (59.3)	5 (6.3)	43 (57.3)	27 (31.0)	78 (90.7)	69 (23.2)	219 (76.0)		
Round	41 (55.4)	7 (9.6)	54 (93.1)	22 (40.7)	74 (93.7)	32 (42.7)	60 (69.0)	8 (9.3)	229 (76.8)	69 (24.0)		
Comb type											13.5**	22.5*
Absent	2 (2.7)	2 (2.7)	NR	NR	4 (5.1)	NR	2 (2.3)	2 (2.3)	8 (2.7)	4 (1.4)		
Single	70 (93.3)	69 (94.5)	58 (100.0)	52 (96.3)	73 (93.6)	68 (90.7)	82 (94.3)	80 (93.0)	283 (95.0)	269 (93.4)		
Double	3 (4.0)	NR	NR	NR	NR	NR	NR	NR	3 (1.0)	NR		
Rose	NR	2 (2.7)	NR	1 (1.9)	1 (1.3)	6 (8.0)	3 (3.4)	4 (4.7)	4 (1.3)	13 (4.5)		
Pea	NR	NR	NR	1 (1.9)	NR	1 (1.3)	NR	NR	NR	2 (0.7)		
Presence of wattles											8.2**	6.0[ns]
Absent	2 (2.7)	NR	3 (5.2)	NR	1 (1.3)	NR	NR	NR	6 (2.0)	NR		
Present	73 (97.3)	73 (100.0)	55 (94.8)	54 (100.0)	77 (98.7)	75 (100.0)	87 (100.0)	86 (100.0)	292 (98.0)	288 (100)		
Presence of crest											9.8**	2.1[ns]
Absent	67 (89.3)	70 (95.9)	48 (82.8)	52 (96.3)	70 (89.7)	73 (97.3)	77 (88.5)	79 (91.9)	262 (87.9)	274 (95.1)		
Present	8 (10.7)	3 (4.1)	10 (17.2)	2 (3.7)	8 (10.3)	2 (2.7)	10 (11.5)	7 (8.1)	36 (12.1)	14 (4.9)		
Beak shape											4.6*	327.7***
Hooked	56 (74.7)	59 (80.8)	9 (15.5)	16 (29.6)	4 (5.1)	16 (21.3)	87 (100)	85 (98.8)	156 (52.3)	176 (61.1)		
Straight	19 (25.3)	14 (19.2)	49 (84.5)	38 (70.4)	74 (94.9)	59 (78.7)	NR	1 (1.2)	142 (47.7)	112 (38.9)		

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns = non-significant; χ^2 = chi-square test of fixed variables; n = chickens sampled; NR, not reported.

[a]Rudimentary-form earlobes for each chicken category.

[b]Prominent earlobes.

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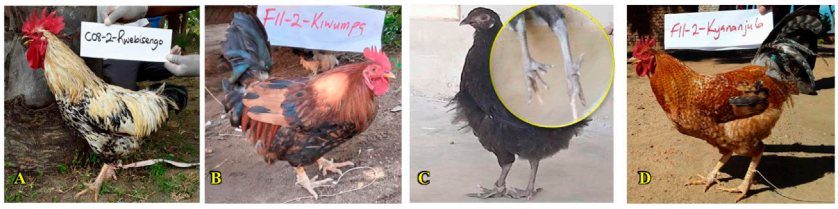

FIGURE 7
Skeletal variance. **(A)** Rumpless. **(B)** Dwarf. **(C)** Polydactyl. **(D)** Normal skeletal structure.

Body colours of indigenous chickens in Uganda

The skin colours of the chickens differed ($p < 0.001$) with location irrespective of sex ($p > 0.05$) (Supplementary Table S5). The proportion of purplish-brown/dark-skinned chickens was slightly higher (49.0% of hens and 43.8% of cocks) than those with white (38.3% of hens and 42.4% of cocks) and yellow (12.8% of hens and 13.9% of cocks) skins. The dominant tarsus (shank) colour varied widely across regions and between sexes ($p < 0.01$). However, yellow tarsi were more frequent, followed by grey/blue-grey, black/dark-grey, white, and other less occurring tarsi colour variants including pink, green, brown, and orange, in varying proportions (Figure 6 and Supplementary Table S5). The eye (iris) colours also differed across regions ($p < 0.01$) irrespective of sex ($p > 0.05$). The most common eye colours were orange, brown, and yellow in both sexes, with comparable proportions ($p > 0.05$). Very low proportions of chickens across locations exhibited dark-green, pink, red, and cyan/blue eye colours. Black eye colour was rare, with only one occurrence in a hen in Western Uganda (Supplementary Table S5).

The location and sex of the chickens influenced the incidence of the earlobe and beak colours in the chicken populations ($p < 0.01$) (Supplementary Figure S13 and Supplementary Table S5). Large variations were observed in the earlobe colour diversity of the chickens, with instances of red and pink earlobes blemished with white, cyan, or yellow. This made it challenging to characterise them into distinct phenotypes. Consequently, the dominant earlobe colours were grouped into broader phenotypes from a pool of related ones. The hens presented pink and dark-red earlobes particularly in similar proportions (21%), followed by light red (16.8%), yellow (15.1%), cyan-blue (10.7%), and white (9.7%) earlobes, compared to the cocks, which mostly showed light (70.1%) or dark (13.2%) red earlobes, with other colours not common. Regionally, yellow (20.3% of hens and 20.3% of cocks) and dark red (18.9% of hens and 28.8% of cocks) earlobes were more pronounced in hens from Northern and Central respectively. Hens in Western Uganda showed quite a wide variability in earlobe colours, including white (29.1%), cyan/blue (19.0%), yellow (17.7%), and light red (16.5%) variants, as well as other low-occurring earlobe colours. Similarly, 28.7%, 27.6%, and 21.8% of hens from Eastern Uganda had dark red, light red, and pink earlobe variants, respectively. Green and grey earlobes variants were subtle across regions and in both sexes. The combs and wattles of indigenous chickens in Uganda were generally red (data not shown), except for a few variants with white and black wattles. The beak colours (Supplementary Figure S14 and Supplementary Table S5) in most cocks were grey, brown, and yellow (31.6%, 18.8%, and 18.4%, respectively), whereas the hens presented wide variations in beak colour, with 21.5% yellow, 19.5% brown to grey, and 18.1% brown-purple.

Head features of indigenous chickens in Uganda

All the descriptive head features (Table 6 and Supplementary Figure S15) were significantly ($p < 0.05$) associated with the sex of the chickens. However, the locations were not significantly associated with the presence of wattles and crests ($p > 0.05$). Hens presented mostly small earlobes compared to cocks, which had large, prominent earlobes. Oval-shaped, large earlobes were more predominant amongst cocks than in hens, which mostly mostly had the round-shaped variant across Uganda. The single-comb type was almost universal (>93%) in both sexes, with rose combs occurring in low proportions scattered across the regions. The double-comb variant occurred exclusively in the Northern region, while the pea comb occurred mainly in cocks from the Central and Western regions. Only a few birds had rudimentary or lacked combs. The presence of wattles was universal, especially in cocks. Crests, on the other hand, mainly existed at low frequencies with just a few occurring across locations in both hens (12.1%) and cocks (4.9%). Bearded chickens (Supplementary Figures S16E1, E2) also occurred (data not shown) but were rare. Hooked beaks generally occurred in both hens and cocks. However, straight beaks were observed more often in the chicken population from the Central and Western regions.

Skeletal variance among indigenous chickens of Uganda

The skeletal variance among the chicken population (Supplementary Table S6) was significantly associated with region ($p < 0.05$) but not the sex of the birds. Generally, the chicken populations were skeletally normal across Uganda. Traces of polydactylous chickens (more than the normal four-digit toes) as well as dwarf and rumpless chickens were also observed in mainly the Northern, Central, and Western regions (Figure 7). The body frame of most of the chickens was medium (79.2% of hens and 60.1% of cocks), with significant ($p < 0.05$) differences across the regions in both sexes. Hens showed a higher proportion of medium body frames across the regions except in Western Uganda, where most hens had rudimentary frames. Cocks were more likely to have long body frames across the regions, while hens were more likely to have rudimentary frames (Supplementary Figure S17 and Supplementary Table S6). Higher proportions of hens with medium body conformation were observed in the Northern and Eastern regions than those in the Central and Western regions, which showed more blocky-compact body conformations. Hence, medium and blocky-compact conformation was more likely in hens, whereas tall-angular body shape was more

TABLE 7 Occurrence and allele frequencies of rare major genotypes in the indigenous chicken population.

Strains [allele]	N	Observed frequency %	χ^2	Expected frequency %	Calculated allele frequency	Expected allele frequency	χ^2
Frizzle [F]	10	1.71[a]	*169.6****	25	0.13[a]	0.25	*7.680***
Other [f+]	576	98.29[b]		75	0.87[b]	0.75	
Ptilopody [Pti]	24	4.10[a]	*136.6****	25	0.20[a]	0.25	*1.333*[ns]
Other [pti+]	562	95.90[b]		75	0.80[a]	0.75	
Crest [Cr]	50	8.53[a]	*84.8****	25	0.29[a]	0.25	*0.853*[ns]
Other [cr+]	536	91.47[b]		75	0.71[a]	0.75	
Polydactyl[c] [P]	11	1.88[a]	*167.1****	25	0.14[a]	0.25	*6.453**
Other [p+]	575	98.12[b]		75	0.86[b]	0.75	
Rose comb [R]	17	2.90[a]	*152.6****	25	0.17[a]	0.25	*3.413*[ns]
Other [r+]	569	97.10[b]		75	0.83[a]	0.75	

[a]Values followed by different superscripts in the same column differ significantly ($p < 0.05$).

[b]Other = Individuals not displaying the trait.

[c]Feathered shank trait; n = counts. χ^2 = chi-squared estimate. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, [ns] not significant.

likely in cocks across the regions (Supplementary Figure S18). Rudimentary to small spurs were mostly observed in hens across regions whilst cocks largely had well-projected spurs across the regions (Supplementary Figure S19). Similarly, short to medium tail lengths were observed in hens whereas the cocks developed medium to long tails (Supplementary Figure S17).

Allele and phenotypic frequencies of indigenous chicken strains in Uganda

The distributions and allele frequencies of the indigenous chicken strains were 0.13, 0.20, 0.29, 0.14, and 0.17 for frizzle, ptilopody, tufted crest, polydactyl, and rose comb chickens (Table 7). Generally, the occurrence of chickens exhibiting the genotype that characterised major traits were relatively low. However, the tufted crest, which was the most common (50; 8.53%) genotype, had an allele frequency of 0.29, which was statistically ($p > 0.05$) similar to the expected Mendelian proportion of 0.25. Likewise, the allele frequencies for ptilopody and rose comb had similar ($p > 0.05$) estimates to the Mendelian proportion of 0.25. However, the frizzling and polydactyl allele frequencies, 0.13 and 0.14 respectively; were significantly lower ($p < 0.05$) than the expected Mendelian frequency of 0.25.

Morphometric traits of the major strains (genotypes) among indigenous chickens in Ugandan

The body weight and linear measurements of the circumferences of the thorax and shanks; lengths of the thigh, shank, comb, and wattle; and the comb height were not significantly ($p > 0.05$) associated with genotypes (strains having major alleles) in either sex (Table 8). However, naked-necked cocks had significantly ($p < 0.05$) taller combs compared to rose comb cocks (5.05 ± 0.37 cm vs 2.46 ± 0.51 cm). Wattle length in both naked-neck hens and cocks was longest compared to the normal, frizzle, polydactyl, rose comb, and tufted crest strains. The shank (tarsus) length in both the hen and cock of rose comb chickens was larger than those in the other chicken strains. Numerically, shank length and comb height and length in hens were higher in the polydactylous and frizzle chicken strains respectively. Whereas in cocks, marginally higher shank length and comb height and length, were observed in the naked-neck and polydactylous strains compared to the other strains.

Correlations between morphometric traits of indigenous chicken strains in Uganda

The Pearson correlations between the morphometric traits of indigenous chicken strains in Uganda for both cocks and hens are shown in Supplementary Table S7. The correlation coefficients generally differed from zero ($p < 0.05$), except for comb height (CH) and shank circumference (SC), CH and shank length (SL), comb length (CL) and SL, and WL and TL in hens. The correlation coefficients for all pairs of morphometrics measured were much stronger in cocks than in hens, with only LW and TC and CH and TC being comparable.

Discussion

This study sought to perform extensive and comprehensive across-country investigations to characterise the indigenous

TABLE 8 Least-square means (LSM ±S.E) of morphometric measurements among the strains of the indigenous chicken population in Uganda.

Morphometric variables	Strains (major phenotypes)						
	Normal feather	Naked neck	Frizzle	Polydactyl	Ptilopody	Rose comb	Tufted crest
Hens							
Live weight (LW), kg	1.54 ± 0.02	1.55 ± 0.08	1.66 ± 0.16	1.27 ± 0.23	1.78 ± 0.10	1.58 ± 0.16	1.47 ± 0.06
Thorax circumference (TC), cm	33.61 ± 0.32	35.13 ± 1.14	35.13 ± 2.41	32.5 ± 3.41	36.80 ± 1.53	32.98 ± 2.41	34.11 ± 0.85
Thigh length (TL), cm	13.16 ± 0.09	13.59 ± 0.34	13.88 ± 0.71	12.50 ± 1.01	13.60 ± 0.45	13.75 ± 0.71	13.03 ± 0.25
Shank circumference (SC), cm	3.93 ± 0.04	3.90 ± 0.13	3.93 ± 0.27	3.75 ± 0.38	3.89 ± 0.17	4.15 ± 0.27	3.85 ± 0.10
Shank length (SL), cm	7.82 ± 0.07	8.03 ± 0.24	7.75 ± 0.52	8.50 ± 0.73	7.81 ± 0.33	8.15 ± 0.52	7.29 ± 0.18
Comb height (CH), cm	1.29 ± 0.05	1.28 ± 0.17	1.60 ± 0.35	0.40 ± 0.50	1.59 ± 0.22	0.75 ± 0.35	1.25 ± 0.12
Comb length (CL), cm	3.05 ± 0.06	3.17 ± 0.22	3.53 ± 0.46	2.50 ± 0.65	3.53 ± 0.29	2.43 ± 0.46	2.52 ± 0.16
Wattle length (WL), cm	1.21 ± 0.06	1.59 ± 0.19	1.15 ± 0.41	0.50 ± 0.58	1.17 ± 0.26	1.18 ± 0.41	1.17 ± 0.15
Cocks							
Live weight (LW), kg	2.22 ± 0.03	2.58 ± 0.13	2.33 ± 0.21	2.18 ± 0.21	2.35 ± 0.16	2.27 ± 0.18	2.32 ± 0.16
Thorax circumference (TC), cm	38.1 ± 0.41	39.56 ± 1.59	40.17 ± 2.52	37.58 ± 2.52	37.55 ± 1.86	37.25 ± 2.18	40.91 ± 1.86
Thigh length (TL), cm	16.24 ± 0.11	16.53 ± 0.41	15.00 ± 0.65	15.92 ± 0.65	16.77 ± 0.48	15.38 ± 0.56	16.59 ± 0.48
Shank circumference (SC), cm	4.76 ± 0.04	4.98 ± 0.17	4.82 ± 0.26	4.85 ± 0.26	4.85 ± 0.19	5.04 ± 0.23	4.68 ± 0.19
Shank length (SL), cm	9.77 ± 0.08	10.63 ± 0.31	9.08 ± 0.49	10.42 ± 0.49	9.67 ± 0.36	10.00 ± 0.42	9.86 ± 0.36
Comb height (CH), cm	$3.84^{a,\ b}$ ± 0.10	5.05^{a} ± 0.37	$3.48^{a,\ b}$ ± 0.59	$4.25^{a,\ b}$ ± 0.59	$3.85^{a,\ b}$ ± 0.44	2.46^{b} ± 0.51	$4.21^{a,\ b}$ ± 0.44
Comb length (CL), cm	7.40 ± 0.14	8.45 ± 0.55	6.70 ± 0.87	8.67 ± 0.87	7.56 ± 0.64	5.83 ± 0.75	7.52 ± 0.64
Wattle length (WL), cm	3.74 ± 0.08	4.61 ± 0.33	3.77 ± 0.51	3.77 ± 0.51	3.59 ± 0.38	3.61 ± 0.45	3.83 ± 0.38

[a]Means with different superscripts within rows differ significantly ($p < 0.05$).
[b]Means with no superscripts within rows do not differ significantly ($p > 0.05$).

chicken production dynamics and the phenotypic and morphologic differentiation of chickens across the Ugandan landscape. Such large-scale characterisation reports are scarce, thus making this study foremost within the country. These results indicated that the flock description, production dynamics, performance, and phenotypic characteristics of indigenous chickens were similar to those reported elsewhere within the continent (Njenga, 2005; Adomako, 2009; Dana, 2011; Dessie et al., 2011; Marwa et al., 2016; Mahoro et al., 2017; Hirwa et al., 2019; Otecko et al., 2019).

Demographic profile of indigenous chicken-rearing households in Uganda

The description of the demographic profile of the indigenous chicken-keeping households is key to determining the socio-economic features of the household, which is necessary for the implementation of sustainable improvement programmes. Across the studied households in Uganda, higher proportions of male chicken farmers were observed, except for the Central region, which showed higher numbers of female chicken keepers. For such a society more likely to be headed by a man, having women contend favourably in chicken rearing supports the claim that household chicken keeping is sometimes the only livestock species under the control of women (Kyarisiima et al., 2004; Dana, 2011; Mahoro et al., 2017). Gender roles imposed by the traditional setting in Northern Uganda and the capacity to exert control over income from household chicken production, especially by women who headed households, influence women's participation in the chicken value chain (Akite et al., 2018). The responses obtained in this study were mainly from adults (≥22 years old) who were household heads, spouses, or other household members. Therefore, they were deemed reliable to infer the production characteristics of indigenous chickens in Uganda. Besides, the age range between 31 and 60 years of most of the farmers was consistent with earlier findings in Uganda (Kugonza et al., 2008; Kayitesi, 2015) and neighbouring Rwandan local chicken keepers (Mahoro et al., 2017; Hirwa et al., 2019). The similar mean distances from a farmer's homestead to an all-weather road (33.3 km) and the market (5.6 km) across the regions indicated the use of representative samples, including those from the hinterlands. This further ensured that the sampling of related chickens was avoided. The medium average agrarian household size of 7.6 observed across the regional households was above the reported average of five in rural residents (UBOS, 2021). The households derived their livelihoods mostly from the sale of crop products and livestock and livestock products. A few households traded livestock and livestock products and participated in off-farm activities. About 3.3 million households in Uganda live in a subsistence economy, which is defined to include 62% subsistence-farming households engaged in agriculture mainly for household consumption and sale/barter according to the UBOS (2021). This emphasises the importance of household livestock and the role of indigenous chicken farming under the low input management in peoples' livelihoods in Uganda.

Ownership and flock description of household chickens in Uganda

The flock size averaging 19.80 ± 1.21 chickens across the regions in this study is consistent with earlier findings in Eastern Uganda (Kugonza et al., 2008), along with the increasing trend of small flock holdings. The low numbers of cocks in the flock but higher numbers for hens, growers, and chicks are also consistent with earlier reports (Kyarisiima et al., 2004; Ssewannyana et al., 2008; Nakkazi

et al., 2014). The flock sizes held by women were small (1–10 chickens) compared to those of men. The opposite was true, as 53.6% of men kept 16 to over 20 chickens. The African Livestock Futures (Herrero et al., 2014) describes women's small chicken flock size as a special policy problem emanating from their typically lower mobility due to domestic work. Thus, the women can only manage small flocks along with their chores. As such, women are more likely to lose on policies seeking to cushion large-scale enterprises like public subsidies, which could boost their markets and their corresponding income. Those that could benefit them as smallholders like animal health services, when underserved also result in a disproportionate loss. Therefore, women are usually not able to grow their flock size. Mature cocks, followed by mature hens or the other chicken categories were usually the first to exit the flock. Most farmers usually kept cocks for up to or over 1 year before they were sold. The exchange of chickens to acquire other livestock species was consistent with reports of the acquisition of cattle and goats through barter trading or the use of cash proceeds from chicken production (Iisa, 2003; Kugonza et al., 2008).

Indigenous chicken farmers in Uganda shared similar production objectives. The generation of income from sales and the consumption of chicken meat and eggs were considered the most important, consistent with previous reports in Uganda (Kugonza et al., 2008; Natukunda et al., 2011). The minor production objectives pertained to the benefits derived from by-products including chicken droppings as organic fertilizer, socio-economic/prestige and cultural heritage, gifts to visitors, and/or barter trading to obtain other livestock species and leisure purposes.

Indigenous chicken production, management system, and performance in Uganda

The mixed crop-livestock systems fall under one of the broad classifications of livestock production systems in Africa, mainly in places with high rainfall and crop production potential (AU-IBAR, 2019). This could explain the predominance of mixed crop-livestock farming across Uganda, as reported previously (Kugonza, 2008). The scavenging feeding management across indigenous chicken-keeping households in Uganda, with only a few households providing supplementation, is consistent with earlier reports in Uganda (Kugonza et al., 2008; Natukunda et al., 2011; MAAIF, 2019) and findings elsewhere (Moreda et al., 2013; Mahoro et al., 2017; Hirwa et al., 2019). Scavenging chicken production is popular among resource-challenged rural communities in most parts of the developing world to derive their livelihoods (Melesse, 2014).

The feed resources available to the indigenous chickens were mainly pasturage, from which they foraged on grasses, seeds, lush leaves, and other plant resources near and around the homestead. Other scavenged feed resources were kitchen refuse or agro-by-products, with little grain concentrates. Ssewannyana et al. (2008) found that indigenous chickens are valued for their ability to scavenge in Uganda. Uncontrolled mating characterises a scavenging feeding system, which limits the production of indigenous chickens due to inbreeding. Intensively fed indigenous chickens performed better than those kept under semi-scavenging conditions (Magala et al., 2012; Nakkazi et al., 2015). Interestingly, water was not consciously provided to the chickens. The chickens must, therefore, search for water as they scavenge near and around their homestead, as most households had good clear water resources within the scavenging radius of their flock.

Housing for both adult and young chickens varied from predominantly bespoke enclosures constructed on the side of the main house, sheds, and human dwelling units to less common shelters like above-ground perches/kitchens and stalls (Supplementary Figure S7–S9). A similar situation occurs in Rwanda, where most farmers house their flocks in enclosures (Hirwa et al., 2019). The occasional observation of chicken flocks housed in human-dwelling units and stalls in some households is consistent with reports of chickens not being provided with specific housing in Uganda (Kyarisiima et al., 2004; Natukunda et al., 2011). Like in many African countries, housing is either specifically fabricated for indigenous chickens or the chickens seek shelter in the natural surroundings under the prevailing scavenging management (FAO, 2008).

The results of the present study generally revealed a mix of different episodes of disease challenges in indigenous chicken flocks across the regions. Most were once-a-while disease challenges occurring at random and yearly/seasonal episodes. Flocks in which disease episodes were rare, especially in Western Uganda, might be due to the adaptive survival of the chickens to agro-ecologic conditions, which impact disease incidence and severity. Host genetics also impact resistance in diverse ways (Khobondo et al., 2015; Richardson, 2016). Berghof et al. (2019) posited differences in immunity and disease resistance because of growth differences in individuals. As such, the body weight deviations in the chickens from the Western region might have conferred resilience in the flock to some extent. Most indigenous farmers across households in Uganda treat their sick chickens, while only a quarter leave their sick chickens to recover on their own or die without any treatment during disease episodes. Meanwhile, a small proportion of farmers across Uganda seldom have disease situations in their flock, which they associated with their use of phyto-prophylactic remedies. Newcastle disease, as the major disease challenging indigenous chicken farming in Uganda in this study, corroborates earlier reports in Uganda (Kugonza et al., 2008; Natukunda et al., 2011), with significant minor diseases including coccidiosis, and a host of others. Contrary to the case in Uganda, coccidiosis is as most significant disease challenge in Rwandan indigenous chicken production, while Newcastle disease constituted a third major disease (Hirwa et al., 2019). Healthcare services for indigenous chickens are mainly curative treatments during the disease incidence, while vaccinations are also administered. A total of 13.2% of chicken keepers did not provide any health service to their flock. Deworming and ectoparasite control are less commonly practiced. Likewise, providers of healthcare services for indigenous chicken production are limited, as most farmers resort to self-services with or without professional advice. The use of local or herbal remedies as treatments against chicken diseases has been the main practice by most indigenous chicken farmers in Uganda (Kyarisiima et al., 2004; Natukunda et al., 2011).

Record-keeping, which is relevant for farm evaluation for key decision-making among most indigenous chicken farmers, was non-existent. Recognition of chickens is, therefore, mostly based on visual cues such as plumage colours or patterns and chicken types to differentiate chicken flocks.

Regarding the production performance of the hens, most farmers across Uganda had an average of 3.83 ± 0.29 laying hens, which was higher than the estimate in the Western region. The average age at first egg production of 5.20 ± 0.03 months ranged between 5.1 and 7.0 months, within the reported range of 20–21weeks for hens managed under an extensive system (FAO, 2003), as reported in

Eastern Uganda (Kugonza et al., 2008). An average of 13.41 ± 0.20 eggs/hen/clutch was produced, resulting in 40.22 ± 0.60 eggs/hen/year. These findings matched those reported previously (Mogesse, 2007; Mahoro et al., 2017; Hirwa et al., 2019). However, the estimates were below the reported range of 50–60 eggs per annum of 10–12 eggs per clutch in the Domestic Animal Diversity information system (DAD-IS, 2022). The egg production potential of indigenous chickens, as reviewed by Padhi (2016) is low compared to dual-purpose breeds or commercial laying chickens, which produce about six times that of indigenous chickens (Farooq et al., 2002). Low egg production in indigenous chickens is associated with broodiness, which involves incubating their eggs to hatch embryos, as well as caring for their hatchlings. This period is accompanied by frequent nesting, reduced feed and water intake, increased body temperature, and, ultimately, cessation of laying during the broody periods. However, management interventions to preclude broodiness increase the laying ability of broody hens (Kugonza et al., 2008; Hossen, 2010; Jiang et al., 2010). Additionally, the average mature live body weights of chickens to be sold were 1.535 ± 0.025 kg and 2.256 ± 0.026 kg for hens and cocks, respectively, within the ranges of 1.03–2.05 kg and 1.25–2.86 kg reported for hens and cocks in Uganda (Kyarisiima et al., 2004).

Phenotypic characteristics of the indigenous chickens in Uganda

The description of livestock breed characteristics is necessary to guide decision-making and is valuable for strategic development and breeding programmes (FAO, 2007). The general observation of the good body condition of most of the breeding chickens across households affirmed the ability of indigenous chickens to thrive well under the prevailing low-input conditions (Dessie et al., 2011).

Feather morphology of the indigenous chickens in Uganda

Feather morphology is often used to group chicken populations into sub-groups due to its importance in evolutionary biology and the socioeconomics of farmers. This trait influences preferences for a particular type of chicken, which determined its importance across the surveyed households. Most farmers in Uganda kept chickens with smooth-neat plane feather structures whilst frizzles, silkies, and chickens with superficial feathering were less common. Consequently, most of the indigenous chickens of both sexes had normal feather distributions. Special phenotypes (strains), including naked necks, feathered-shanks (ptilopody), and frizzle chickens, were present at low proportions similarly in both sexes, corroborating reports of the predominance of normal-feathered chickens in the chicken genetic resources in Rwanda (Hirwa et al., 2019), Ethiopia (Fistum, 2016), Ghana (Mensah, 2016; Brown et al., 2017), Nigeria (Yakubu, 2010), and Algeria (Dahloum et al., 2016). The low proportion of naked neck, feathered-shank (ptilopody), and frizzle traits, despite their favourable effects on production and tropical adaptation (Adomako, 2009; Mensah, 2016), is linked to socio-cultural/religious reasons (Njenga, 2005; Desta, 2020). Naked neck and frizzle-feathered phenotypes are negatively selected since they do not fetch a premium market price on grounds of undesirable aesthetic value (Yakubu, 2010; Desta et al., 2013). Moreover, anecdotal reports and personal interactions with the farmers in this study suggested that such chicken strains are ordinarily for home consumption and ritual sacrifices, as reported elsewhere (Njenga, 2005; Desta et al., 2013); hence, considered fetish. Moreover, the frizzle gene is reportedly detrimental in unfavourable environmental conditions in its homozygous state and causes internal organ abnormalities (Fathi et al., 2013), suggesting their very low frequencies. We observed a high variation in plumage patterns across the regions. Most of the hens in Eastern Uganda did not have a defined plumage pattern. However, the hens with patterns that could be described were of similar frequencies as the pencilled (in Western and Central regions), and uniform (in the Northern region) patterns of white, and black plumage colours. Cocks more often showed a partridge pattern, followed by the birchen pattern, with a host of other undefined patterns. Similar observations were made by Ssewannyana et al. (2008) who described the feathering in most indigenous chickens in Uganda as having no definite patterns. The low frequencies of some of the plumage patterns, and colours might be due to the preference of such chickens exclusively for sociocultural/religious ritual practices (Desta, 2020) and the lack of particular consumer demand for certain plumage patterns, consequently leading to a decline in population genetic structures. Whereas the largely non-descript hen plumage is perhaps due to farmers' understanding of multiple plumage colours as camouflage against aerial predators (Besbes et al., 2007) and as part of the hen's mothering ability (Kyarisiima et al., 2004).

Body colours of indigenous chickens in Uganda

The proportions of dark-skinned (purplish-brown/dark) and white-skinned chickens were similar, while that of yellow skin colouration was lowest among the observed skin phenotypes. This is contrary to earlier reports of subtle (1%) dark skin colour phenotype in Ugandan indigenous chickens (Ssewannyana et al., 2008) and the dominance (69%) of yellow-skinned chickens in the Rwandan indigenous breeds (Hirwa et al., 2019) and the Ethiopian chicken population (Desta et al., 2013; Negassa et al., 2014). Skin colour variations are influenced by a combination of genes and modifiers that influence melanin pigmentation and carotenoid deposition in the skin, in addition to environmental factors such as diet and physiological state (Jin et al., 2016). Dark skin colour correlates with melanin pigmentation, caused by the absence of sex-linked dermal melanin-inhibiting mutation (*Id*) and some plumage colour-influencing genes like the extension (*E*) locus alleles. Additionally, the fibromelanosis (*Fm*) locus gene together with dermal melanin id^+ causes melanised pigments in the skin and connective tissues (Jacob, 2022). White skin colour, however, is associated with the autosomal dominant white (*W*) locus allele and the non-extended black (*e*) allele or the combination of the *Fm* gene and *Id* mutation, which results in no visible skin colouration (Jin et al., 2016).

The white-skin gene reportedly originated from red jungle fowl (believed to be the ancient ancestor of domestic chickens), while the yellow-skin gene is from the grey jungle fowl (Eriksson et al., 2008), suggesting a dual origin of the trait. The recessive yellow skin (*w*) allele is related to carotenoid pigmentation (Jin et al., 2016).

Regarding shank/tarsi colours, the yellow dominant (primary) phenotypes were the most common in both sexes across the locations, indicating carotenoid deposition in the skin of the shanks (Gunnarsson, 2009). Grey/blue-grey shank was the second most frequent phenotype; however, hens were more likely to have grey-to-black shanks compared to cocks. The white shank phenotype

ranked third in occurrence in both sexes. These findings are consistent with those of Ssewannyana et al. (2008) regarding the order of dominant shank colours among indigenous chickens in Uganda. The dominance of yellow tarsi in chickens has been reported elsewhere (Desta et al., 2013; Negassa et al., 2014; Hirwa et al., 2019). Contrarily, white tarsi are predominant in the Algerian chicken population (Dahloum et al., 2016). The pink and green-shank phenotypes in the present study were more frequently observed in cocks than in hens.

The variability of skin colours of the chicken body and shank is influenced by sex, genotype, and physiological state and is particularly prominent in laying hens (Eriksson et al., 2008). Dark-pigmented or dull (dark) skins are observed in poor layers; moreover, during the laying period, yellow pigments from the body of laying hens are diverted to the egg yolks (Singh, 2022). Hence, the combined effect of these factors and the subjective colour determination used in the present study may explain the higher frequency of yellow shanks in the hens, which otherwise commonly exhibited dull skins.

The predominant eye colour was orange, followed by yellow and brown. Dark-green, pink, dark-red, and cyan/blue variants of eye colour were rarely observed. These findings confirm an earlier report of the dominance of orange-eyed chickens in Uganda (Ssewannyana et al., 2008). A similar finding was also reported in Algerian indigenous chickens (Dahloum et al., 2016).

Regarding earlobe colour, our findings were contrary to those of Ssewannyana et al. (2008), who reported no particular colour occurring in the earlobes of hens in Uganda. The hens in the present study showed wide variations in prominent earlobe colours. Our observations of red or pink earlobe blemished with white, yellow, or cyan/blue are consistent with observations in Ethiopia (Desta et al., 2013). The red (light and dark variants) dominant earlobe colour variant in this study is consistent with reports in Ethiopian village chicken populations (Dana et al., 2010; Desta et al., 2013). The observed proportions of white earlobes were much lower than in previous reports in Uganda (Ssewannyana et al., 2008), Algeria (Dahloum et al., 2016), and Rwanda (Hirwa et al., 2019). Earlobe colour traits are reportedly sex-linked and polygenic (Wragg et al., 2012). Incidentally, the pink, green, cyan, and grey earlobe colours observed have not been previously characterised in Uganda. Thus, this study highlights the large variability of earlobe colours among indigenous Ugandan chickens. The hens were more likely than the cocks to have earlobe colours other than light red.

Similarly, the beak colours of the chickens varied widely. The hens mostly showed yellow (21.5%), brown (21.5%), grey (19.5%), and purple/brown-purple beaks, whereas cocks mainly showed grey (31.6%), yellow (18.4%), and brown (18.8%) beaks. Generally, the grey beak occurred more frequently in aggregate, followed by brown and yellow in equal proportions (25.4%, 20.1%, and 20.0% respectively). The proportion of green beak colour was higher than that in Rwandan chickens (Hirwa et al., 2019) whilst it was not reported in the Ethiopian population. The yellow to brownish beaks could be due to carotenoid pigmentation from xanthophylls in feed whilst the grey to dark beaks occur due to high melanin concentrations.

Head features of indigenous chickens in Uganda

The association of all descriptive head features of the Ugandan indigenous chickens (Supplementary Figure S15 and Table 6) with the sex category may be corroborative evidence of the sex-linked nature of earlobe shape and size, comb type as well as the presence of wattles and crests. The hens in general had typically small round, earlobes, and a single-type comb with small wattles. Cocks on the other hand typically presented large oval-shaped earlobes with single-type combs as well as large floppy wattles.

Despite the low incidence, hens across the regions were more likely than cocks to have tufted crests. This is consistent with the report by Desta et al. (2013) but contrary to the report of more tufted crest cocks than hens by Wang et al. (2012). Tuft crests in chickens are caused by a non-sex-linked dominant gene and it is considerably more noticeable in hens than in cocks. Therefore, having more tufted crest hens than cocks could be due to the farmer breeding practices of culling cocks earlier from their flock across the regions.

Comb type is regulated by two dominant genes, *R* and *P* for rose and pea combs, respectively. The absence of these dominant genes results in a single-comb type (Jacob, 2022). Therefore, the rose comb trait occurring at low proportions indicates a low frequency of the gene, consistent with reports elsewhere (Desta et al., 2013; Negassa et al., 2014; Dahloum et al., 2016; Brown et al., 2017).

The occurrence of hooked beaks supports the general observance of the curved beak in Rwandan indigenous chickens (Hirwa et al., 2019). Beak shapes play a vital role in scavenging for feed resources around the homestead. In the wild, avian beak conformation is influenced by adaption to behaviours such as preening, probing for food, feeding, killing small prey/pests, manipulating objects, and feeding offspring (Jacob, 2022). However, no studies have detailed the evolution and morphometrics of the beak of domestic chickens (Iqbal and Moss, 2021).

The low proportion of pea comb in the study may be related to its irrelevance in tropical climates as it is an adaptive trait for colder climates (Lee, 2009). However, comb and wattles have a significant function in reducing body temperature. This is consistent with the nearly universal occurrence of wattles (98.0% of hens and 100% of cocks, $p < 0.001$) and single-type comb (95.0% of hens and 93.4% of cocks), which was similar ($p > 0.05$) across regions. The majority of indigenous chickens bearing combs with only a few combless hens agrees with an earlier report in Uganda (Ssewannyana et al., 2008), although, rose, double, and pea combs were not featured in previous reports. The low proportions of the rose contrasted with the report of its dominance in Ethiopian indigenous cocks (Negassa et al., 2014). The dominance of the single comb type in both sexes, followed by rose combs, was also reported within the Kaffa Zone of Ethiopia (Tadele et al., 2018).

Skeletal variance in the Ugandan indigenous chicken population

A significant ($p < 0.001$) association was observed in the skeletal variance of chickens across the regions (Supplementary Table S6 and Figure 7; S17-19), which could be attributed to the disproportionately low frequency of polydactyl, dwarf, and rumpless traits. Polydactyl and rumpless phenotypes were restricted to Northern and Western Uganda, suggesting their localised acceptance. The skeletal variance of most Ugandan chickens, which was mainly normal in both sexes and with medium body frame and conformation, supports earlier reports of 62% medium body size and 25% small body size among Ugandan indigenous chickens (Ssewannyana et al., 2008). The hens mainly had medium to small or rudimentary body frames whilst cocks showed medium to long body frames across the regions. Chickens with larger body frames grow faster and yield more meat. Regarding spur size, spurs were not noticeable or were rudimentary (39.9%) on some hens

with most (59%) having small spurs. The incidence of medium and large spurs was higher ($p < 0.001$) in cocks across the region. This contrasted with the report of a nearly universally absent spur in the Ethiopian indigenous chicken population (Fistum, 2016). Reviews of earlier studies on the inheritance of spur described its incidence as a secondary sex characteristic inhibited by the ovarian hormones in most hens. As such, hens with removed ovaries all grew spurs (Hutt, 1949). The sex-influenced nature of spur incidence is supported in other studies (Fairfull and Gowe, 1986; Oguntunji and Ayorinde, 2009; Egena et al., 2011). The tail length, which is made up of feathers for balance while walking and a steering mechanism while in flight, was mainly medium across the regions. The cocks were more likely to possess a long tail than the hens across the regions, suggesting the sex-linked nature of the trait.

Allele and phenotypic frequencies of major traits in the Ugandan indigenous chicken population

Despite the considerably low distribution of the major chicken strains in the Ugandan indigenous chicken population, the allele frequencies of the tufted crests (0.29), ptilopody (0.20), and rose comb (0.17) strains were consistent with the expected Mendelian proportions of 0.25; indicating their preference for selection by the farmers who kept them. This finding is contrary to the lower-than-expected allele frequencies of the tufted crest, ptilopody, and rose comb in the Algerian (Dahloum et al., 2016), Ghanaian (Mensah, 2016; Brown et al., 2017), and Nigerian indigenous chicken populations (Ikeobi et al., 2001). Meanwhile, the low frequencies of frizzle and polydactyl alleles, despite their association with heat stress adaptation and higher body weight, respectively, were suggestive of their endangerment, partly due to selection against their population and their neglect in breeding programmes in Uganda.

Morphometric traits of indigenous chicken strains in Uganda

The chicken strains did not significantly differ in body weight and linear measurements within the sex categories, except for taller comb height in the naked-neck cocks compared to the rose comb cocks. The marginally longest wattle length in naked-neck hens and cocks compared to the normal, frizzle, polydactyl, rose comb, and tufted crest strains could be the effect of the naked-neck gene on wattle length as a heat dissipation trait. The naked-neck gene interacts with the environment, thereby impacting the productive performance of the strain at high temperatures (Chen et al., 2008). Additionally, the shank circumferences were largest among the rose comb hens and cocks compared to the rest of the strains, consistent with the report by Adekoya et al. (2013). This suggests local physiologic adaptation to the prevailing production system in Uganda like in other African countries; however, the mechanism is unclear since the rose comb gene is considered to be of European origin (Ikeobi et al., 2001). Amongst hens, the feathered-shank (ptilopody) strain was marginally heavier, with a larger thorax circumference and longer comb length compared to the normal, naked-neck, frizzle, polydactyl, and rose comb strains. However, thigh length was on the upper limit in the frizzle chickens compared to the rest of the strains. Meanwhile, amongst the cocks, the naked-neck strain showed marginally heavier body weight compared to the rest of the strains, while the thorax circumference and thigh length were on the upper limit for the tufted crest and feathered shank strains. The estimates of the effect of the chicken strains on the body weight and linear measurements recorded in this study are comparable to those in previous reports (Mensah, 2016; Brown et al., 2017; Machete et al., 2017). In contrast, higher body weight has been reported in naked-neck chickens compared to the other chicken strains (Njenga, 2005; Birteeb et al., 2016). The favourable marginal estimates for the chicken strains under the prevailing low input condition could be explored further for their potential under improved keeping conditions.

The general strong phenotypic correlation coefficients for all pairs of morphometric variables (Supplementary Table S7) for the chicken strains in this study, except for CH and SC, CH and SL, CL and SL, and WL and TL of the hens corroborate the reports in most studies (Egena et al., 2011; Dahloum et al., 2016; Hirwa et al., 2019; Otecko et al., 2019). In addition, the correlation coefficients for all pairs of morphometric traits were much stronger in cocks than in hens, with only LW and TC and CH and TC pairs comparable, which follows among other various selective forces and the evolution of sexual dimorphism in chickens (Karubian and Swaddle, 2001). This provides a good proxy for the estimation of the live body weight of chickens based on other morphometric values, especially those that indicate intrinsic body size.

Conclusion

The results of this study revealed a great pool of production and phenotypic diversity in the genetic resources of Ugandan indigenous chicken typically reared in extensive scavenging, mixed crop-livestock production system to support household livelihoods; even in smallholdings of a little over 20 chickens. The vital roles of indigenous chickens in Uganda were further emphasised in a society of only 20.8% female-headed households in which 40.6% of women were responsible for keeping indigenous chickens. This forms part of a balanced system mainly focused on income generation from sales and household food sources. The production performance in terms of egg production of the hens was about 40.3 ± 0.6 eggs/year, while the weights of mature chickens at sale averaged 1.535 kg and 2.256 kg, respectively, for hens and cocks. The eggs produced in most indigenous-chicken-keeping undertakings were usually consumed within the household or incubated to hatch the replacement stock. Despite their low production performance, partly from the challenges imposed by the husbandry practices, indigenous chickens in Uganda were hardy and in good body condition to offer multipurpose functions, including serving as capital for the acquisition of other livestock flocks, mainly goats. As such, improvements in management practices and healthcare and supplementary feeding, in addition to selection within the indigenous chickens for increased productivity and conservation under the prevailing environment, could help increase their productivity.

Wide phenotypic variation was observed in all the traits studied as well as a generally strong phenotypic correlation between all pairs of morphometric variables measured, especially for those of the cocks. However, only marginally higher estimates in the morphometric traits of the chicken strains were detected, which did not clearly infer higher performance, despite the notion of better production performance over the normal counterparts. The allele frequencies were higher among those characterising the major chicken strains including the tufted crest, rose comb, and ptilopody. This demonstrated their local acceptance and prospects for sustainable utilisation and conservation worthiness in Uganda. In contrast, the low gene frequencies of frizzle

and polydactyl genes despite being associated with adaptability to a low-input management system and tropical environment, suggest their low preference and risk of being lost from the chicken genetic resources in Uganda. This situation calls for a scientific drive to ensure that such traits of adaptive essence are maintained to ensure their sustainable development, utilisation, management, and conservation. The use of molecular genetics techniques will be useful in confirming the phenotypic diversity.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Uganda National Council for Science and Technology (UNCST) (clearance no. A154ES) and Gulu University Research Ethics Committee (permission GUREC-2020-18). Written informed consent for participation was not obtained from the owners because verbal consent was mandatory for obtaining household data in the electronic consent form on the AnGR-CIM tool used for the characterisation study and the participants gave their verbal consent to participate after written consent from the District Veterinary Offices in our community entry protocol.

Author contributions

IY, DK, MO, PA, RC, and FD conceptualised and designed the work; IY and DK collected the data; IY and DK analysed and interpreted the data; IY and DK drafted the manuscript; DK, PA, and RC critically revised the manuscript; DK, CM, and FD supervised the study; FD, DK, MO, PA, and RC secured the funding; and FD, DK, and MO provided project administration; All authors read approved the final version of the manuscript to be published.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.1033031/full#supplementary-material

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Production performance of four lines of Japanese quail reared under tropical climatic conditions of Tamil Nadu, India

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This research investigated the growth and other production traits of four distinct lines (L1, L2, L3, and L4) of Japanese quail (Cortunix japanoica) kept in the tropical climate of Tamil Nadu, India. The traits related to body weight at different weeks and weight gain were measured in 180 birds (90 males and 90 females) per line up to the fifth week of age, and then 90 birds (females only) from the sixth to the 16th week of age, with egg production and feed efficiency parameters measured in 10 observations per line. The traits were analysed using the General Linear Model procedure, and Tukey's HSD was used to test for statistical differences ($p < 0.05$) between the means for subclasses under a specific effect. The results revealed a highly significant difference ($p < 0.01$) in body weight from hatch to fifth week of age. At 5th week of age, the L3 and L2 did not differ ($p > 0.05$) based on Tukey test; therefore, both presented the highest values and were statistically significant ($p < 0.01$) with L4 with a lowest value of 203.62 g. The cumulative fifth week feed conversion ratio showed non-significant difference ($p > 0.05$) at first and second week, and highly significant difference ($p < 0.01$) from third to fifth week of age. The age at 50 per cent egg production was 60.2 (L4), 61.4 (L2), 65.1 (L3) and 66.0 (L1) days and the L1 egg production differed significantly ($p < 0.01$) from L4 line. The highest bodyweight (g) during the laying period (at 15 weeks of age) was observed in L2 (327.08) and L3 (326.54) and differed significantly ($p < 0.01$) from L1 (309.24) as well as L4 (288.69) lines. The mean egg weight (g) of different lines showed non-significant difference ($p > 0.05$) at all weeks, except at 11th week of age ($p < 0.01$). The mean feed consumption (g)/bird/day differed significantly ($p < 0.01$) from 6th to 16th week of age, except at sixth and eighth week of age, where it was non-significant ($p > 0.05$). The overall feed efficiency/dozen of eggs (from 6th to 16th weeks) ranged from 1.33 (L1) to 1.98 (L3). The livability from 6 to 16 weeks of age was 100 per cent in all the lines. In order to boost Japanese quail production in the tropics, L3 and L4 may be selected for body weight and egg production, respectively.

KEYWORDS

Japanese quails, breeding lines, production traits, livability, tropics

1 Introduction

Poultry production is one of agriculture's fastest growing subsectors, producing a variety of commodities for the world's population. Broiler chickens, commercial layers, turkeys, ducks, and quails are common meat and egg producers. These high-yielding birds have been genetically improved to have higher growth and egg-laying rates. The goal of developing such strains was to meet the global population's dietary needs, particularly for proteins (Hussain et al., 2018; Ghayas et al., 2020). The Japanese quails (*Coturnix japonica*) are the smallest possible avian species raised for meat and egg production. Several factors contribute to their utility and cost-effective alternative to chicken's production (Kayang et al., 2004; Sezai et al., 2010), such as: shorter production cycle, early maturity and lower initial investment required for the commercially rearing of quails. In addition, they are associated with healthier meat and eggs (Ghayas et al., 2017; Ahmad et al., 2018), present a meat with a distinct flavor and high nutritional value (Kayang et al., 2004), and the resource poor people all over the world are interested in commercially rearing quails due to the lower initial investment. Many meat consumers prefer quail meat because of its low fat content, primarily saturated fatty acid and cholesterol level, when compared to similar cuts of red meat such as beef and mutton (Boni et al., 2010) and white meat such as broiler chicken and duck (Ionita et al., 2008). According to Genchev et al. (2008), a daily intake of two quails provides the human body with 27–28 g protein, 11 g of essential amino acids, and covers 40 per cent of the human protein requirement.

In general, the production performance of the Japanese quail can be improved by increasing their genetic potential and providing favourable management conditions. One of the most crucial economic variables in any genetic improvement programme is body weight, along with egg production, for a number of reasons, including its relationship to other meat production traits and their relative simplicity of measuring. The true economic and commercial value of this amazing bird is found in its egg production, as domesticated lines of Japanese quail can lay up to 290 to 300 eggs in their first year of lay (Jatoi et al., 2015). At the age of 4 weeks, it has the potential to gain 160–170 g of body weight (Akram et al., 2014). The fundamental tool for maximising the productive potential of birds is by selection. Around the world, a variety of selection techniques have been in use, ranging from mass selection to complete pedigree selection (Krishna et al., 2016; Narinç et al., 2016; Durmuş et al., 2017; Pandian et al., 2017) to improve the Japanese quails' performance.

Quail farming is a growing industry in India, because of the relatively higher profit margins (Pandian et al., 2017; Prabakaran and Ezhil Valavan, 2020). As there is a need to promote the growth of these birds through selection programmes, different production performances were utilized for the genetic selection in the four different lines (L1, L2, L3, and L4) of Japanese quail maintained at the Poultry Farm Complex of Department of Poultry Science, Veterinary College and Research Institute, Namakkal, Tamil Nadu, India for the production of parent breeders and the characteristics that are taken into account are body weight, growth rate and egg production. Selection experiments provide background knowledge of complex trait inheritance and allow for the evaluation of hypothetical predictions by comparing observations to expectations. Long-term response to selection is more focused on fixing the probabilities of alleles responsible for the trait(s) under consideration and short-term selection response, on the other hand, could be attributed to segregating alleles in the population (Fuller et al., 2005). Therefore, the present study was conducted to evaluate the production performance of four different lines of Japanese quail maintained under the tropical climatic conditions of India, and to select the best lines for different purposes including commercial production for economic rearing for the betterment of smallholder farming communities.

2 Materials and methods

2.1 Study area and genetic information of the lines

The experiment was conducted at the Poultry Farm Complex, Department of Poultry Science, Veterinary College and Research Institute, Namakkal, This complex is located at an elevation of 192 m above mean sea level and at 11°2′N latitude and 78°2′ E longitudes. The average minimum and maximum temperatures as well as the relative humidity were 20.39°C and 33.64°C and 46.94 and 87.27 per cent, respectively. For this study, four Japanese quail lines, L1, L2, L3, and L4 were used and lines were genetically unrelated and purchased from different sources to maximise heterosis in the year 2005. The lines L1, L2, and L4 were obtained from three different quail breeding companies located in different parts of Southern India, while the L3 line was obtained from a quail breeder cum farmer in Tamil Nadu. The lines were maintained for several generations and subjected to genetic selection for improving the meat and egg production with different traits, which would include L1 for egg weight, L2 for age at 50% egg production and medium body weight, L3 for higher body weight at 6 weeks of age, and L4 for egg number.

2.2 Experimental design and birds' management

Hatching eggs from four different lines of Japanese quail, namely, L1, L2, L3, and L4, were incubated separately, and the chicks that hatched were used in the study. The experiment was divided into two parts: growth performance of four lines of Japanese quail from 0 to 5 weeks of age, and growth and egg production performance of four lines of Japanese quail from 6 to 16 weeks of age. The birds were used as parent stock for the production of day-old chicks for the experiment when they were 16 weeks old. A total of 20 males and 80 females were randomly selected from each line (L1, L2, L3, and L4), and half-sib mating was used within each line, with one male and four females of selected birds housed together in each breeding cage. Eggs were collected separately and stored in a cold room before being placed in an incubator to hatch. After hatching, the 180 day-old straight-run Japanese quail chicks from each line were weighed individually and immediately transferred to the brooding cages. A total of 18 chicks per cage were reared up to 2 weeks of age in the brooding cages. From third week of age, birds were transferred to grower cages and kept up to 5 weeks of age. The

TABLE 1 Means (±S.E.) of body weight (g) of different lines of Japanese quail from hatch to fifth week of age.

Effect	Number of observations	Body weight (g)						Body weight gain (g)/week					
		Hatch weight	First week	Second week	Third week	Fourth week	Fifth week	First week	Second week	Third week	Fourth week	Fifth week	Overall (hatch to fifth week)
Overall	**720**	9.15 ± 0.02	27.35 ± 0.07	67.15 ± 0.14	114.32 ± 0.52	177.27 ± 0.66	222.25 ± 0.73	18.20 ± 0.06	39.80 ± 0.20	47.17 ± 0.52	62.95 ± 0.36	44.98 ± 0.44	213.10 ± 0.72
LINE		**	**	**	**	**	**	**	**	**	**	**	**
Line 1	**180**	8.99^{a} ± 0.05	28.32^{c} ± 0.14	67.52^{b} ± 0.28	113.85^{b} ± 1.04	172.36^{a} ± 1.33	217.05^{b} ± 1.45	19.33^{c} ± 0.11	39.80^{ab} ± 0.41	46.33^{b} ± 1.04	58.51^{a} ± 0.72	44.69^{b} ± 0.88	208.06^{b} ± 1.44
Line 2	**180**	9.02^{a} ± 0.05	27.34^{b} ± 0.14	67.42^{b} ± 0.28	119.16^{c} ± 1.04	182.63^{b} ± 1.33	232.99^{c} ± 1.46	18.32^{a} ± 0.11	40.08^{b} ± 0.41	51.74^{c} ± 1.04	63.47^{b} ± 0.72	50.36^{c} ± 0.88	223.97^{c} ± 1.44
Line 3	**180**	9.24^{b} ± 0.05	27.34^{b} ± 0.14	67.60^{b} ± 0.28	117.17^{bc} ± 1.05	186.48^{b} ± 1.33	235.31^{c} ± 1.46	18.10^{a} ± 0.11	40.26^{b} ± 0.41	49.57^{bc}± 1.04	69.31^{c} ± 0.72	48.83^{c} ± 0.88	226.07^{c} ± 1.44
Line 4	**180**	9.33^{b} ± 0.05	26.41^{a} ± 0.14	66.05^{a} ± 0.28	107.10^{a} ± 1.04	167.60^{a} ± 1.33	203.62^{a} ± 1.46	17.08^{a} ± 0.11	39.64^{a} ± 0.41	41.05^{a} ± 1.04	60.50^{a} ± 0.72	36.02^{a} ± 0.88	194.29^{a} ± 1.44
F value		11.87	33.19	6.96	25.95	43.66	103.29	70.66	9.02	17.68	44.41	40.99	96.86
***p*-value**		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Means with at least one common superscript within age group do not differ significantly ($p \geq 0.05$).

[a]Highly Significant ($p < 0.01$).

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TABLE 2 Means (±S.E.) of cumulative feed conversion ratio of different lines of Japanese quail from first to fifth week of age.

Effect	Number of observations	Age in weeks				
		1	2	3	4	5
Overall	**40**	2.04 ± 0.03	2.37 ± 0.01	2.78 ± 0.02	2.83 ± 0.01	3.24 ± 0.02
LINE		**NS**	**NS**	**	**	**
Line 1	10	1.93 ± 0.07	2.36 ± 0.03	2.73^{a} ± 0.04	2.87^{bc} ± 0.02	3.22^{a} ± 0.03
Line 2	10	2.02 ± 0.07	2.36 ± 0.03	2.69^{a} ± 0.04	2.79^{ab} ± 0.02	3.14^{a} ± 0.03
Line 3	10	2.12 ± 0.07	2.37 ± 0.03	2.76^{a} ± 0.04	2.74^{a} ± 0.02	3.16^{a} ± 0.03
Line 4	10	2.11 ± 0.07	2.40 ± 0.03	2.94^{b} ± 0.04	2.92^{c} ± 0.02	3.43^{b} ± 0.03
F value		1.72	0.56	8.41	11.44	16.88
***p*-value**		0.18	0.64	0.00	0.00	0.00

Means with at least one common superscript within age group do not differ significantly ($p \geq 0.05$).
NS Not, significant **Highly Significant ($p < 0.01$).

birds were wing banded at third week for identification and proper recording of production data. After the completion of the growth study, ninety females from each line were taken and reared in layer cages by keeping nine females per cage up to 16 weeks of age to study the growth and egg production performances. The traits such as body weight, body weight gain, cumulative feed conversion ratio were recorded up to fifth week of age in both males and females and then to study the laying performances the traits *viz.*, age (days) at first egg, age at 50 per cent egg production, age at 90 per cent egg production, hen-housed egg production, hen-day egg production, egg weight, feed consumption (g)/bird/week and weekly feed efficiency/dozen eggs were recorded. At each experimental stage, the quails were given weighed amounts of feed to eat *ad libitum*, along with unrestricted access to clean water. The overall amount of light exposure per day was 16 hours. Other routine management practices were carried out.

2.3 Data collection

During the growth period, body weight at weekly interval, feed consumption and egg production on daily basis and mortality, if any, were recorded during the laying period. The weight of the eggs was measured once a week; in addition, age at sexual maturity, hen-day egg production, hen-housed egg production, livability, and feed efficiency in terms of feed per dozen eggs were calculated. To assess production performance, the individual weights of all chicks from four lines (L1, L2, L3, and L4) were recorded at the 0^{th} day (hatch weight), first, second, third, fourth and fifth week of age, and subsequently measured at fortnightly intervals from the 7th to 15th week of age. Throughout the study, the daily feed consumption (g) per bird was recorded and the average feed consumption per bird was calculated at the end of each week by subtracting the leftover feed from the total amount of feed given. The feed conversion ratio was calculated by dividing the amount of feed consumed by the bird by the amount of weight gain. The individual egg weight (g) was recorded with an electronic balance with an accuracy of 0.001 g. The Hen-day egg production was calculated by using the following formula.

$$\text{Hen} - \text{day egg production for the period} = \frac{\text{Number of eggs laid during the period}}{\text{Sum of number of hens survived per day for the period}} \times 100$$

The Hen-housed egg production (eggs per bird) from 6th to 16th weeks of age was calculated by

$$\text{Hen housed egg production for the period} = \frac{\text{Average number of eggs produced during the period}}{\text{Total number of hens housed in the beginning}} \times 100$$

The feed efficiency was expressed in kilograms of feed consumed to produce one dozen eggs. The total feed consumed (kg) during 6–16 weeks of age was calculated in all the four lines. The total number of eggs produced during the respective periods was arrived by adding the number of eggs produced during the period for each experimental line. The feed efficiency was calculated as follows:

$$\text{Feed efficiency (per dozen of eggs)} = \frac{\text{Total kilograms of feed consumed during the period}}{\text{Total number of eggs produced during the period}} \times 12$$

Mortality was recorded as at occurrence during the study period and breeder house livability (%) was calculated by using the following formula.

$$\text{Livability} = \frac{\text{Total number of live birds during the period}}{\text{Total number of birds present in the breeder house at the beginning of study}} \times 100$$

2.4 Statistical analysis

In order to ascertain the normality of the data and the residuals, they were subjected to Shapiro-Wilk's test ($p > 0.05$), while homogeneity of variances ($p > 0.05$) was confirmed using Levene's test as described by Yakubu et al. (2022). The traits such as body weight, body weight gain, cumulative feed conversion ratio, age (days) at first egg, age at 50 per cent egg production, age at 90 per cent egg production, hen-housed egg

TABLE 3 Means (±S.E.) of body weight (g) of different lines of Japanese quail layers from 7th to 15th week of age.

Effect	Number of observations	Age in weeks				
		7	9	11	13	15
Overall	**360**	266.87 ± 1.51	308.90 ± 1.59	319.11 ± 1.72	319.23 ± 1.65	312.89 ± 1.67
LINE		**	**	**	**	**
Line 1	90	252.99^{a} ± 3.01	297.04^{a} ± 3.19	310.89^{b} ± 3.43	316.85^{b} ± 3.30	309.24^{b} ± 3.33
Line 2	90	270.01^{b} ± 3.01	319.13^{b} ± 3.19	326.82^{c} ± 3.43	333.52^{c} ± 3.30	327.08^{c} ± 3.33
Line 3	90	286.25^{c} ± 3.01	329.36^{b} ± 3.19	341.44^{d} ± 3.43	333.60^{c} ± 3.30	326.54^{c} ± 3.33
Line 4	90	258.23^{a} ± 3.01	290.06^{a} ± 3.19	297.27^{a} ± 3.43	292.94^{a} ± 3.30	288.69^{a} ± 3.33
F value		24.01	33.44	31.23	33.94	29.66
***p*-value**		0.00	0.00	0.00	0.00	0.00

Means with at least one common superscript within age group do not differ significantly ($p \geq 0.05$).
[a]Highly Significant ($p < 0.01$).

production, hen-day egg production, egg weight, feed consumption (g)/bird/week and weekly feed efficiency/dozen eggs were analyzed using General Linear Model procedure of Statistical Package for Social Sciences (SPSS) 25th version (IBM Corp, 2019) and the statistical model used for the analysis was $Y_{ij} = \mu + L_i + e_{ij}$ (where Y_{ij} is the observed production and reproduction parameters analysed individually; μ = Overall mean; L_i = effect of *i*th line (i = 1–4) and e_{ij}=random errors). Statistical differences ($p < 0.05$) between the means for subclasses under a particular effect were tested by Tukey's HSD test.

3 Results

3.1 Body weight and body weight gain from hatch to fifth week

Table 1 shows the means (±S.E.) of body weight (g) and body weight gain (g/week) of various lines of Japanese quail from hatch to fifth week of age. The body weight of the four lines of Japanese quail differed significantly ($p < 0.01$) from hatch to the fifth week of age. However, the difference between lines was not uniform and varied with different ages. Highest fifth week body weight was recorded in L3 (235.31 g) and L2 (232.99 g) and did not differ significantly ($p < 0.01$) with them but both differed significantly ($p < 0.01$) from L1 (217.05 g) and L4 (203.62 g). From hatch to the fifth week of age, the average body weight gain of L2 (223.97 g) and L3 (226.07 g) was substantially identical and non-significant ($p > 0.05$), but they differed significantly ($p < 0.01$) from L1 (208.06 g) and L4 (194.29 g).

3.2 Feed consumption and feed conversion ratio from 0 to fifth week of age

Table 2 depicts the mean (±S.E.) of the cumulative feed conversion ratio for different lines of Japanese quail from the first to fifth week of age. The feed conversion ratio observed between the lines were non-significant ($p > 0.05$) in the first and second week, but differed significantly ($p < 0.01$) in subsequent weeks with varied response. At three and 5 weeks of age, the L1, L2, and L3 generally had feed conversion efficiencies that were nearly similar and did not differ significantly ($p > 0.05$) from one another. These three lines also had higher feed conversion efficiencies than L4 and differed significantly ($p < 0.01$). Among the four lines evaluated, the L4 feed conversion efficiency was low.

3.3 Body weight of females of different lines at the laying period

The mean body weight of breeder female quail lines increased progressively from the 7th to 11th week of age (Table 3). Later, the increase was meagre up to 13th week as well as reduction of body weight was noticed at 15th week of age. At the 13th week of age, body weight obtained in L2 and L3 were not different ($p > 0.05$) from each other, but they were higher than that observed for L1 and L4 lines. A similar pattern was observed at the 15th week of age. In all the lines, the body weight at the 15th week was slightly lower than the values observed at the 13th week of age.

3.4 Age at sexual maturity and egg production parameters

For the four lines of Japanese quail, the mean (±S.E.) of age (days) at first egg, age at 50 and 90 per cent of egg production are shown in Table 4. For the age of sexual maturity as determined by the first egg deposited, there was no statistically significant ($p > 0.05$) difference between the four lines. However, the age at which 50 per cent of eggs produced, revealed a significant variability ($p < 0.01$) with the earliest production occurring in L4 (60.2 days) and differed significantly only with L1 (66.0 days) line. There was no statistically

TABLE 4 Means (±S.E.) of age (days) at first egg, age at 50 per cent and 90 per cent egg production of different lines of Japanese quail.

Effect	Number of observations	Age at first egg	Age at 50% egg production	Age at 90% egg production
Overall	**40**	50.78 ± 0.88	63.18 ± 0.74	75.88 ± 1.29
LINE		**NS**	*	**NS**
Line 1	**10**	51.70 ± 1.76	66.00^{b} ± 1.48	78.90 ± 2.58
Line 2	**10**	52.50 ± 1.76	61.40^{ab} ± 1.48	78.40 ± 2.58
Line 3	**10**	51.00 ± 1.76	65.10^{ab} ± 1.48	74.20 ± 2.58
Line 4	**10**	47.90 ± 1.76	60.20^{a} ± 1.48	72.00 ± 2.58
F value		1.30	3.63	1.68
***p*-value**		0.29	0.02	0.19

Means with at least one common superscript within the economic traits do not differ significantly ($p \geq 0.05$).
NS Not, significant *Significant ($p < 0.05$).

TABLE 5 Means (±S.E.) of hen-housed egg production (eggs per bird) of different lines of Japanese quail from 6th to 16th week of age.

Effect	N	Age in weeks											
		6	7	8	9	10	11	12	13	14	15	16	Overall (6–16)
Overall	**40**	0.01 ± 0.01	0.33 ± 0.07	0.92 ± 0.13	2.36 ± 0.16	4.07 ± 0.16	5.16 ± 0.13	5.43 ± 0.13	5.70 ± 0.14	6.04 ± 0.10	6.26 ± 0.09	6.42 ± 0.08	42.70 ± 0.69
LINE		**NS**	**NS**	*	**	**NS**	*	**	**	**	**NS**	**NS**	**
Line 1	10	0.00 ± 0.01	0.17 ± 0.14	0.67^{ab} ± 0.26	1.74^{a} ± 0.31	3.87 ± 0.32	4.57^{a} ± 0.25	4.82^{a} ± 0.25	4.90^{a} ± 0.28	5.22^{a} ± 0.20	5.97 ± 0.17	6.34 ± 0.15	38.27^{a} ± 1.38
Line 2	10	0.00 ± 0.01	0.30 ± 0.14	0.89^{ab} ± 0.26	2.33^{ab} ± 0.31	4.18 ± 0.32	4.95^{ab} ± 0.25	4.98^{a} ± 0.25	5.40^{ab} ± 0.28	6.24^{b} ± 0.20	6.33 ± 0.17	6.40 ± 0.15	42.00^{ab} ± 1.38
Line 3	10	0.02 ± 0.01	0.24 ± 0.14	0.56^{a} ± 0.26	1.91^{a} ± 0.31	3.66 ± 0.32	5.57^{b} ± 0.25	6.17^{b} ± 0.25	6.52^{c} ± 0.28	6.54^{b} ± 0.20	6.45 ± 0.17	6.60 ± 0.15	44.24^{b} ± 1.38
Line 4	10	0.01 ± 0.01	0.61 ± 0.14	1.58^{b} ± 0.26	3.44^{b} ± 0.31	4.59 ± 0.32	5.55^{b} ± 0.25	5.76^{ab} ± 0.25	5.76^{bc} ± 0.28	6.29^{b} ± 0.20	6.29 ± 0.17	6.32 ± 0.15	46.30^{b} ± 1.38
F value		0.73	1.85	3.19	6.08	1.63	3.68	6.54	6.30	8.09	1.51	0.69	6.18
***p*-value**		0.54	0.16	0.04	0.00	0.20	0.02	0.00	0.00	0.00	0.23	0.56	0.00

Means with at least one common superscript within age group do not differ significantly ($p \geq 0.05$).
NS Not, significant *Significant ($p < 0.05$) **-Highly Significant ($p < 0.01$).

significant ($p > 0.05$) difference in the age at which 90 per cent of egg production between the lines.

The overall hen-housed egg production pooled over 6th to 16th week of age (Table 5) was much higher in L4 (46.30) and L3 (44.24) and differed significantly ($p < 0.01$) from L1 (38.27), but there were no significant difference ($p > 0.05$) between L2, L3, and L4 as well as L1 and L2. The overall hen-day egg production (Table 6) observed for the L3 and L4 lines differed significantly ($p < 0.01$) from L1 line.

3.4 Egg weight, feed efficiency/dozen eggs and livability

The means (±S.E.) of egg weight (g) for various lines of Japanese quail from the 6th to 16th week of age are displayed in Table 7. The overall egg weight (g) from 6th to 16th week revealed that the L2 (12.77 g) differed significantly ($p < 0.01$) from L4 (12.36 g) only. The mean feed consumption/bird/week is presented in Table 8. The mean feed consumption (g)/bird/day differed significantly ($p < 0.01$) from 6th to 16th week of age, except at sixth and eighth week of age, where it was non-significant ($p > 0.05$). The means of weekly feed efficiency/dozen eggs (Table 9) indicated non-significant ($p > 0.05$) differences at weeks 7, 8, 10, and 15; significant differences ($p < 0.05$) at weeks 9, 13, and 16, and highly significant differences ($p < 0.01$) at weeks 11, 12, and 14. In general, after reaching 50 percent egg production, L4 (0.45) had the best feed efficiency and differed significantly ($p < 0.01$) from L3 (0.50), L2 (0.54), and L1 (0.57) lines. The livability observed from day-old to 16th week of age was 100 per cent.

In comparison between lines, the study found that the growth performance of L2 and L3 was higher in terms of maximum fifth

TABLE 6 Means (±S.E.) of hen-day egg production (%) of different lines of Japanese quail from 6th to 16th week of age.

Effect	N	Age in weeks											Overall (6–16)
		6	7	8	9	10	11	12	13	14	15	16	
Overall	**40**	0.12 ± 0.09	4.72 ± 1.03	13.17 ± 1.83	33.69 ± 2.22	58.17 ± 2.28	73.69 ± 1.81	77.58 ± 1.78	81.47 ± 2.00	86.31 ± 1.43	89.45 ± 1.21	91.67 ± 1.08	61.39 ± 1.88
LINE		**NS**	**NS**	*	**	**NS**	*	**	**	**	**NS**	**NS**	**
Line 1	10	0.00	2.38 ± 2.05	9.52^{ab} ± 3.67	24.92^{a} ± 4.43	55.24 ± 4.55	65.24^{a} ± 3.62	68.89^{a} ± 3.56	70.00^{a} ± 4.01	74.60^{a} ± 2.86	85.24 ± 2.42	90.64 ± 2.17	56.27^{a} ± 3.81
Line 2	10	0.00	4.29 ± 2.05	12.70^{ab} ± 3.67	33.33^{ab} ± 4.43	59.68 ± 4.55	70.79^{ab} ± 3.62	71.11^{a} ± 3.56	77.14^{ab} ± 4.01	89.05^{b} ± 2.86	90.48 ± 2.42	91.43 ± 2.17	61.76^{ab} ± 3.81
Line 3	10	0.32 ± 0.18	3.49 ± 2.05	7.94^{a} ± 3.67	27.30^{a} ± 4.43	52.22 ± 4.55	79.52^{b} ± 3.62	88.10^{b} ± 3.56	93.18^{c} ± 4.01	93.49^{b} ± 2.86	92.22 ± 2.42	94.29 ± 2.17	62.32^{b} ± 3.73
Line 4	10	0.16 ± 0.18	8.73 ± 2.05	22.54^{b} ± 3.67	49.21^{b} ± 4.43	65.56 ± 4.55	79.21^{b} ± 3.62	82.22^{ab} ± 3.56	85.56^{bc} ± 4.01	88.10^{b} ± 2.86	89.84 ± 2.42	90.32 ± 2.17	65.21^{b} ± 3.73
F value		0.73	1.84	3.19	6.08	1.62	3.67	6.55	6.32	8.11	1.51	0.70	0.98
***p*-value**		0.54	0.16	0.04	0.00	0.20	0.02	0.00	0.00	0.00	0.23	0.56	0.41

Means with at least one common superscript within age group do not differ significantly ($p \geq 0.05$).
NS Not, significant *Significant ($p < 0.05$) **-Highly Significant ($p < 0.01$).

TABLE 7 Means (±S.E.) of egg weight (g) of different lines of Japanese quail from 6th to 16th week of age.

Effect	N	Age in weeks											
		6	7	8	9	10	11	12	13	14	15	16	Overall (6–16)
Overall	**80**	9.62 ± 0.07	11.05 ± 0.11	11.84 ± 0.11	12.86 ± 0.25	12.36 ± 0.08	12.76 ± 0.05	12.82 ± 0.10	12.76 ± 0.73	12.73 ± 0.09	12.71 ± 0.08	12.94 ± 0.09	12.56 ± 0.04
LINE			**NS**	**NS**	**NS**	**NS**	**	**NS**	**NS**	**NS**	**NS**	**NS**	**
Line 1	20	-	10.83 ± 0.22	11.82 ± 0.22	12.35 ± 0.51	12.37 ± 0.17	12.83^{b} ± 0.10	12.88 ± 0.20	12.79 ± 0.15	12.42 ± 0.19	12.64 ± 0.16	12.80 ± 0.19	12.51^{ab} ± 0.07
Line 2	20	-	11.27 ± 0.22	12.02 ± 0.22	13.47 ± 0.51	12.47 ± 0.17	12.96^{b} ± 0.10	12.95 ± 0.20	12.85 ± 0.15	12.93 ± 0.19	12.84 ± 0.16	13.16 ± 0.19	12.77^{b} ± 0.07
Line 3	20	9.84 ± 0.48	11.06 ± 0.22	11.88 ± 0.22	12.98 ± 0.51	12.37 ± 0.17	12.82^{b} ± 0.10	12.84 ± 0.20	12.77 ± 0.15	12.88 ± 0.19	12.78 ± 0.16	12.94 ± 0.19	12.59^{ab} ± 0.07
Line 4	20	9.18 ± 0.00	11.05 ± 0.22	11.63 ± 0.22	12.63 ± 0.51	12.24 ± 0.17	12.43^{a} ± 0.10	12.62 ± 0.20	12.60 ± 0.15	12.66 ± 0.19	12.59 ± 0.16	12.84 ± 0.19	12.36^{a} ± 0.07
F value		-	0.68	0.55	0.91	0.34	4.95	0.53	0.56	1.60	0.58	0.76	5.36
***p*-value**		-	0.57	0.65	0.44	0.79	0.00	0.67	0.64	0.20	0.63	0.52	0.00

Means with at least one common superscript within age group do not differ significantly ($p \geq 0.05$).
NS Not, significant **Highly Significant ($p < 0.01$).

week body weight and better feed conversion ratio, and hence both may be used to improve growth features (Table 10). The line L4 had a younger age of sexual maturity with superior egg production performance, including hen-day and hen-housed egg production, feed consumption, and feed efficiency per dozens of eggs. As a result, L4 line is better for reproductive performance, but a detailed repeated measures analysis with more samples is needed to make a meaningful judgment for utilizing the L4 lines for the improvement of reproduction parameters. The outcomes of the present study is in consonance with the selection criteria of the different lines, which included L2 for age at 50% egg production and medium body weight, L3 for greater body weight at 6 weeks of age, and L4 for egg number.

4 Discussion

In the current study, four lines of quails were compared to determine which one produced the best egg and meat characteristics. As a result, several measurements were taken and

TABLE 8 Means (±S.E.) of feed consumption (g)/bird/week of different lines of Japanese quail from 6th to 16th week of age.

Effect	N	Age in weeks											
		6	7	8	9	10	11	12	13	14	15	16	Overall (6–16)
Overall	**40**	205.65 ± 5.02	203.93 ± 3.09	251.92 ± 2.30	238.62 ± 2.03	223.53 ± 1.65	231.83 ± 1.62	224.53 ± 1.81	245.27 ± 3.31	222.62 ± 1.88	221.88 ± 1.85	203.25 ± 1.34	224.82 ± 1.09
LINE		**NS**	**	**NS**	**	**	**	**	*	**	**	**	**
Line 1	10	195.92 ± 10.03	179.35^{a} ± 6.17	244.93 ± 4.61	220.83^{a} ± 4.06	221.91^{ab} ± 3.29	233.82^{b} ± 3.24	227.92^{b} ± 3.63	249.73^{ab} ± 6.63	218.06^{ab} ± 3.76	213.18^{a} ± 3.70	198.08^{a} ± 2.67	218.52^{a} ± 2.19
Line 2	10	212.86 ± 10.03	216.54^{b} ± 6.17	257.67 ± 4.61	247.32^{b} ± 4.06	228.66^{b} ± 3.29	239.23^{b} ± 3.24	232.67^{b} ± 3.63	251.73^{b} ± 6.63	226.90^{bc} ± 3.76	223.92^{ab} ± 3.70	210.76^{b} ± 2.67	231.66^{b} ± 2.19
Line 3	10	221.94 ± 10.03	231.11^{b} ± 6.17	252.68 ± 4.61	245.95^{b} ± 4.06	231.59^{b} ± 3.29	235.15^{b} ± 3.24	227.47^{b} ± 3.63	253.79^{b} ± 6.63	234.23^{c} ± 3.76	237.16^{b} ± 3.70	214.63^{b} ± 2.67	235.06^{b} ± 2.19
Line 4	10	191.90 ± 10.03	188.73^{a} ± 6.17	252.38 ± 4.61	240.37^{b} ± 4.06	211.94^{a} ± 3.29	219.11^{a} ± 3.24	210.05^{a} ± 3.63	225.84^{a} ± 6.63	211.28^{a} ± 3.76	213.28^{a} ± 3.70	189.53^{a} ± 2.67	214.04^{a} ± 2.19
F value		1.99	15.17	1.30	9.10	7.03	7.34	7.5	3.88	7.15	9.42	18.71	21.41
***p*-value**		0.13	0.00	0.29	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00

Means with at least one common superscript within age group do not differ significantly ($p \geq 0.05$).
NS Not, significant **Highly Significant ($p < 0.01$).

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TABLE 9 Means (±S.E.) of weekly feed efficiency/dozen eggs of different lines of Japanese quails from 6th to 16th week of age.

Effect	N	Age in weeks												
		6	7	8	9	10	11	12	13	14	15	16	After 50% egg production (10–16)	Overall (6–16)
Overall	**40**	21.27	9.17 ± 2.20	4.86 ± 1.26	1.53 ± 0.15	0.71 ± 0.04	0.56 ± 0.02	0.52 ± 0.02	0.55 ± 0.03	1.57 ± 0.20	0.43 ± 0.01	0.38 ± 0.01	0.51 ± 0.10	1.57 ± 0.20
LINE			**NS**	**NS**	*	**NS**	**	**	*	**	**NS**	*	**	**NS**
Line 1	10		9.02 ± 5.37	4.25 ± 2.43	2.07^{b} ± 0.30	0.75 ± 0.07	0.63^{b} ± 0.03	0.59^{b} ± 0.03	0.67^{b} ± 0.06	1.33 ± 0.41	0.43 ± 0.01	0.38^{a} ± 0.01	0.57^{c} ± 0.20	1.33 ± 0.41
Line 2	10		7.03 ± 4.65	6.30 ± 2.60	1.26^{ab} ± 0.31	0.71 ± 0.07	0.60^{ab} ± 0.03	0.59^{b} ± 0.03	0.59^{ab} ± 0.06	1.35 ± 0.42	0.44 ± 0.01	0.40^{a} ± 0.01	0.54^{c} ± 0.20	1.35 ± 0.42
Line 3	10	17.58	15.32 ± 3.80	3.43 ± 2.81	1.88^{ab} ± 0.30	0.83 ± 0.07	0.51^{ab} ± 0.03	0.44^{a} ± 0.03	0.47^{ab} ± 0.06	1.98 ± 0.41	0.44 ± 0.01	0.39^{a} ± 0.01	0.50^{b} ± 0.20	1.98 ± 0.41
Line 4	10	24.95	5.32 ± 3.52	5.46 ± 2.17	0.91^{a} ± 0.30	0.57 ± 0.07	0.49^{a} ± 0.03	0.44^{a} ± 0.03	0.46^{a} ± 0.06	1.60 ± 0.40	0.41 ± 0.01	0.36^{a} ± 0.01	0.45^{a} ± 0.20	1.60 ± 0.40
F value			1.35	0.23	3.36	2.32	4.54	6.02	3.51	4.51	0.54	3.11	6.72	0.54
***p*-value**			0.29	0.87	0.03	0.09	0.01	0.002	0.025	0.009	0.65	0.04	0.00	0.65

Means with at least one common superscript within age group do not differ significantly ($p \geq 0.05$).
NS Not, significant *Significant ($p < 0.05$) **Highly Significant ($p < 0.01$).

TABLE 10 Order of preference of the different lines for the different production and reproduction characters.

Parameters	Order of performance of lines			
Body weight (5th week) (g)	L3 (235.31^{c} ± 1.46)	L2 (232.99^{c} ± 1.46)	L1 (217.05^{b} ± 1.45)	L4 (203.62^{a} ± 1.46)
Body weight of layers (15th week) (g)	L2 (327.08^{c} ± 3.33)	L3 (326.54^{c} ± 3.33)	L1 (309.24^{b} ± 3.33)	L4 (288.69^{a} ± 3.33)
Cumulative feed conversion ratio (5th week)	L2 (3.14^{a} ± 0.03)	L3 (3.16^{a} ± 0.03)	L1 (3.22^{a} ± 0.03)	L4 (3.43^{b} ± 0.03)
Feed efficiency after 50% egg production	L4 (0.45^{a} ± 0.20)	L3 (0.50^{b} ± 0.20)	L2 (0.54^{c} ± 0.20)	L1 (0.57^{c} ± 0.20)
Age at 50% egg production (days)	L4 (60.20^{a} ± 1.48)	L2 (61.40^{ab} ± 1.48)	L3 (65.10^{ab} ± 1.48)	L1 (66.00^{b} ± 1.48)
Egg weight (g)	L2 (12.77^{b} ± 0.07 g)	L3 (12.59^{ab} ± 0.07 g)	L1 (12.51^{ab} ± 0.07 g)	L4 (12.36^{a} ± 0.07 g)
Hen day egg production (%)	L4 (65.21^{b} ± 3.73)	L3 (62.32^{b} ± 3.73)	L2 (61.76^{ab} ± 3.81)	L1 (56.27^{a} ± 3.81)
Hen housed egg production (number)	L4 (46.30^{b} ± 1.38)	L3 (44.24^{b} ± 1.38)	L2 (42.00^{ab} ± 1.38)	L1 (38.27^{a} ± 1.38)

NB: Please remove vertical lines from all the tables above.

these cumulative measurements may provide a solid foundation for selecting the best egg/meat productive line to meet the needs of the desired breeders.

4.1 Body size and weight gain from hatch to fifth weeks of age

The body weight of the different lines of Japanese quails observed a progressive increase from hatch to fifth week of age and the significant differences between lines at different ages were in agreement with the findings of the earlier researchers in Japanese quails in India (Vali et al., 2005; Akram et al., 2014). The differences at all ages of L1, L2, L3, and L4 could be due to genetic variation among them. Body weights of the progeny varied, possibly due to differences in selection strategies for the different lines. The L1, L2, and L3 lines were chosen for heavier body weight, while L4 was chosen for medium body weight. In many other studies, improved body weight in Japanese quail was also observed in birds selected for higher body weight (Akram et al., 2014; Ahmad et al., 2019).

Petek et al. (2003) discovered that hatch weight of chicks produced from smaller eggs was higher than hatch weight of chicks produced from larger eggs, which is similar to the results of this study in the case of line 4. The observed body weights from hatch to fifth week were larger than those of Dauda et al. (2014), who reported body weights of 5.74 ± 1.10 g, 10.88 ± 1.10 g, 23.70 ± 1.18 g, 34.73 ± 1.18 g, 54.54 ± 1.19 g and 76.08 ± 1.20 g, respectively at hatch (day 0), first, second, third, fourth and fifth weeks of age under the humid environment conditions of Central Nigeria. The differences could be due to climatic conditions, and

the lines differ from our research. Similarly, Feroz Mohammed et al. (2006) and Taskin Atilla et al. (2017) recorded lower body weight under tropical climatic conditions of India and Turkey, respectively. Krishna and Sahitya Rani (2017), and Sangilimadan and Richard Churchil (2018) also found significantly higher body weight and body weight gain than the results obtained in the current study for the Japanese quails maintained under wet and dry semi-arid climate and hot and humid tropical climatic conditions of India respectively. The variation in body weight between the studies may primarily be due to genetic variation, management procedures, and environmental factors, all of which play a significant role in the growth performance of Japanese quails.

4.2 Feed intake and its conversion ratio from hatch to fifth week of age

In the present study, the selection for high and medium body weights resulted in corresponding improvement in feed conversion ratio, and is consistent with the reports of earlier researchers (Khalid and Ali, 2017; Taskin Atilla et al., 2017; Sangilimadan and Richard Churchil, 2018). As a result of their larger bodies, certain birds may consume more feed on a daily and cumulative basis (Khaldari et al., 2010; Akram et al., 2014). The best FCR to a specific body weight may be explained in part by lower maintenance costs and less fat build-up in birds with faster growth rates. The feed conversion ratio observed in different lines were higher in the first week, but Krishna and Sahitya Rani (2017) showed a lower first week feed conversion ratio of 1.89 ± 0.02 to 4.73 ± 0.07 at the sixth week of age in a Japanese quail lines maintained under tropical climatic condition of Telangana, India. Due to differences in the genetic background of the breeds, significant differences in feed intake were observed in different breeds of Japanese quails and varied climatic conditions also played a role in variation between the different studies.

4.3 Body weight of females at laying period

The highly significant difference in body weight between the selected lines is in accordance with the earlier reports (Vieira Filho et al., 2016; Dzuriatmono et al., 2019). The mean body weight at seventh week observed in the current study (266.87 g) was higher than the value of 244.10 g by Lofti et al. (2012). Furthermore, Daikwo et al. (2014) and Dauda et al. (2014) both showed significantly lower body weights of 145 ± 0.74 g and 138.91 ± 0.64 g, respectively than those observed in the present study. However, greater than the present weights of 332.33 ± 2.45 and 346.3 ± 2.71 g, respectively, as determined by Arumugam et al. (2011) and Lukanov et al. (2018). The variation in body weight between studies may be primarily due to genetic variation and environmental factors between studies conducted in different locations. The reduction in body weight observed in the 15th week due to increased egg production were in line with those of Ojo et al. (2011), who claimed that body weight appeared to drop as egg production rises.

4.4 Age at sexual maturity and egg production characteristics

The age at first egg reported in different lines is very close to the value of 47–53 days reported by other researchers (Ashok and Mahipal Reddy, 2010; Lofti et al., 2012; Daikwo et al., 2014; Dzuriatmono et al., 2019). However, higher values of 54.49 ± 0.20, 55.79 and 64.17 ± 1.17 were reported by Dauda et al. (2014), Krishna and Sahitya Rani (2017) and Arumugam et al. (2011). Lower than the presented estimated value of 44.71 ± 0.62 days was also reported by Hussain et al. (2016) in pure line grand parent stock.

The hen-housed and hen-day egg production performance of Japanese quails in the present study were found to be in agreement with the findings of earlier researchers (Arumugam et al., 2011; Akram et al., 2014; Hussain et al., 2016; Taskin Atilla et al., 2017; Dzuriatmono et al., 2019). Subramaniam (2004) observed much higher mean per cent hen-day egg production of 76.05 ± 2.56 per cent to 85.92 ± 1.51 per cent and hen-housed egg production of 74.19 ± 3.74 to 95.15 ± 0.80 per cent in different lines of Japanese quails maintained under the tropical climatic conditions. Similar results were observed by Subhashini et al. (2018) with mean overall hen day egg production of 79.40 ± 6.00 per cent and Prabakaran and Ezhil Valavan (2020) reported overall hen day egg production and hen-housed egg production (6–46 weeks) of 79.00 ± 1.13 and 76.41 ± 1.31 per cent, respectively. When compared to the current four lines of Japanese quails, Lofti et al. (2012), Dauda et al. (2014), and Krishna and Sahitya Rani (2017) found reduced egg production in Japanese quails maintained at varied climatic conditions of India.

4.5 Egg weight from 6th to 16th week of age and feed efficiency

Non-significant to significant differences in egg weight between the lines were also reported by other researchers in Japanese quails in tropical climatic conditions. Ashok and Mahipal Reddy (2010) observed that brown strain exhibited significantly higher egg weight (11.04 ± 0.07 g) than white breasted (10.63 ± 0.06 g) and dark cinnamon brown (10.43 ± 0.02 g) strains, respectively. Genchev (2012) also indicated that egg weight of Pharaoh breed (13.71 ± 0.022 g) was significantly higher than that of the Manchurian golden breed (13.25 ± 0.029 g). However, Sakunthala Devi et al. (2010) observed no significant difference among the black and brown strains for egg weight (14.13 ± 0.10 and 14.32 ± 0.09 g) at 16th weeks of age. The egg weight observed at different ages in the present study are in accordance with Hrnčár et al. (2014), who reported that the average egg weight ranged between 11.48 ± 1.72 g and 13.06 ± 2.05 g, respectively. Similarly, Inci Hakan et al. (2015) reported average egg weight of 11.65 ± 0.14 g, 12.04 ± 0.15 g and 11.96 ± 0.15 g in white, dark brown and golden strains of Japanese quails. Prabakaran and Ezhil Valavan (2020) reported an average egg weight of about 13.12 ± 0.08 g and a value of 13.63 ± 0.34 g was also observed by Subhashini et al. (2018).

The heavy class of birds laid heavier eggs than the other classes combined. Similarly, Jatoi et al. (2013) observed that heavier birds lay heavier eggs at the beginning and end of the productive period. It could be explained by the positive relationship between egg weight and selection for increased body weight and egg number. As a result of this change in allele frequency controlling egg weight, ova size and

albumen secretions increase (Altinel et al., 1996; Ahmad et al., 2019). The current study supports the findings of Alkan et al. (2010), who discovered a significant difference in egg weight for both lines chosen for higher body weight and egg production in Japanese quails. El-Deen et al. (2015) also reported increased egg weight after two generations of selection for higher egg production compared to the control line.

Feed consumption during the laying period is influenced not only by body weight, but also by genetics and the time of year (Jatoi et al., 2013; Guimarães et al., 2014). There was a significant difference in feed efficiency per dozen egg in the present study, which is similar to the findings of Arumugam et al. (2011) in pure line Japanese quail breeder birds. Rehman et al. (2022) also reported significant difference in feed per dozen eggs of genetic lines and generations as well as their interaction. Better and increased feed efficiency in selected birds could explain the improved feed efficiency per dozen egg. The current study supports the findings of Kosba et al. (2003), who reported improved FCR in selected lines of Japanese quail compared to the control group. Similarly, another study found that birds selected for higher 4-week body weight in Japanese quail had higher feed efficiency (Khaldari et al., 2010). In another experiment, however, there was no difference in feed efficiency per dozen egg between groups of local and imported Japanese quail (Rehman, 2006). The obtained results in the present study showed lower feed consumption and higher feed efficiency than those reported by Subramaniam (2004) and Akram et al. (2014) in Japanese quails maintained under various tropical conditions in India. Taskin Atilla et al. (2017) found that heavier birds consumed more feed than lighter birds. Similar findings were obtained by Arumugam et al. (2011), Hussain et al. (2016), and Lukanov et al. (2018).

The livability from day-old to 16th week of age was 100 per cent. This is because of selection of quality chicks at the day-old stage itself and standard management practices. Livability standards are also attributed to other environmental factors responsible for the health of the birds along with the purpose of selection. This 100 per cent livability is supported by Almeida et al. (2002), who observed no mortality during the experimental period, and Subramaniam (2004), who found no mortality up to fourth week in L1, L2, L3, and L4. However, Prabakaran and Ezhil Valavan (2020) observed an average livability rate from 0–6 weeks of age of about 94.54 percent and Subhashini et al. (2018) reported overall livability of Japanese quail of 96.00 ± 0.88 percent. For further improving meat production, the traits such as body weight, body weight gain, and feed efficiency may be taken into consideration. For egg production, age (days) at first egg, age at 50%, age at 90% of egg production, and feed efficiency/dozen eggs may be taken into consideration.

5 Conclusion

The findings of the current study demonstrated that the growth performance and egg production features of Japanese quails were significantly influenced by pedigree-based selection. There were significant differences between the offspring of the various lines in terms of growth performance or egg production. It can be determined from the present findings that the performance L2 and L3 was better with regards to higher body weight; and this can inform decisions on subsequent genetic improvement programs and also recommend additional research for utilization of L4 for egg production. Based on the favourable overall growth performance, Japanese quails' production can be widely promoted as a new venture to provide nutritional security and employment to rural youth as an alternative to poultry farming in north-western agroclimatic conditions of Tamil Nadu, India.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The ethical review and approval was not required for the routine animal study in our country. Because the current study was conducted under normal management conditions, ethical review and approval were not necessary.

Author contributions

KA collected samples and recorded data. KA and AT were in charge of data analysis and interpretation. AY, DK, and RA critically reviewed the submission manuscript. AY, DK, and RA provided critical feedback on the proposal manuscript, which was written by AT. The final manuscript was read and approved by all authors.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Improving hydrocarbon toxicity tolerance in poultry: role of genes and antioxidants

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Sustenance of smallholder poultry production as an alternative source of food security and income is imperative in communities exposed to hydrocarbon pollution. Exposure to hydrocarbon pollutants causes disruption of homeostasis, thereby compromising the genetic potential of the birds. Oxidative stress-mediated dysfunction of the cellular membrane is a contributing factor in the mechanism of hydrocarbon toxicity. Epidemiological studies show that tolerance to hydrocarbon exposure may be caused by the activation of genes that control disease defense pathways like aryl hydrocarbon receptor (AhR) and nuclear factor erythroid 2p45-related factor 2 (Nrf2). Disparity in the mechanism and level of tolerance to hydrocarbon fragments among species may exist and may result in variations in gene expression within individuals of the same species upon exposure. Genomic variability is critical for adaptation and serves as a survival mechanism in response to environmental pollutants. Understanding the interplay of diverse genetic mechanisms in relation to environmental influences is important for exploiting the differences in various genetic variants. Protection against pollutant-induced physiological responses using dietary antioxidants can mitigate homeostasis disruptions. Such intervention may initiate epigenetic modulation relevant to gene expression of hydrocarbon tolerance, enhancing productivity, and possibly future development of hydrocarbon-tolerant breeds.

KEYWORDS

antioxidants, epigenetic modulation, gene expression, hydrocarbon pollutants, oxidative stress, toxicity

Introduction

Hydrocarbon pollution from oil spillage, natural gas flaring and organic pollutants is ubiquitous and of some fundamental health welfare concern in communities exposed to that pollution such as the Niger Delta region of Nigeria (Sam and Zabbey, 2018; Srivastava et al., 2019). The degradation of the environment by these pollutants destabilizes the ecosystem

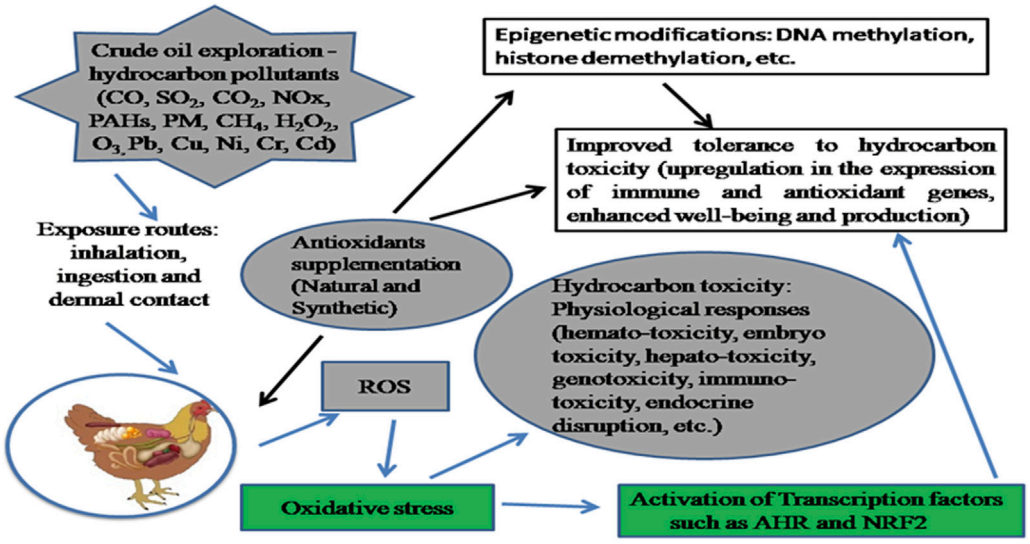

FIGURE 1
Consequence of hydrocarbon exposure and mitigation effect of antioxidant modulation. Crude oil exploration results in the release of hydrocarbon pollutants to the environment. Exposure to these pollutants via inhalation, dermal contact and/or ingestion results in interactions between different classes of pollutants, generation of reactive oxygen species and onset of oxidative stress conditions which are partly modulated by redox-sensitive signalling proteins and transcription factors. The mechanism of tolerance to hydrocarbon toxicity involves the activation of the aryl hydrocarbon receptor (AHR) and nuclear factor erythroid 2p45-related factor 2 (Nrf2) signalling pathways which regulates the expression of xenobiotic metabolism genes such as the cytochrome P450. To mitigate the impact of exposure on metabolic processes and enhance productivity, dietary interventions in regulating environmental perturbances could be beneficial.

(Anejionu et al., 2015; Kponee et al., 2015; Croitoru et al., 2020), leading to the loss of biodiversity of organisms and low productivity of aquatic organisms, which serve as the major source of livelihood and animal protein supply to rural households (Izah, 2015; Osuagwu and Olaifa, 2018; Ansah et al., 2022). Diversification of income sources was recommended as a crucial strategy for achieving poverty reduction and economic stability in order to increase resilience and reduce the vulnerability of the rural poor to the effects of environmental deterioration (Yakubu, 2017). Poultry may be a good substitute due to its intrinsic qualities of having a short generational gap, high prolificacy, rapid turnover, and acceptance across all socio-cultural, economic, and religious strata (Oleforuh-Okoleh, 2010).

Due to their toxic effects, environmental stresses such as those caused by hydrocarbon pollutants may prevent the full expression of the genetic potential of these birds. The severity of hydrocarbon toxicity is dependent on the exposure route, type of chemical compound, dosage, and duration of exposure. Hydrocarbon tolerance is regulated by biological (metabolic, genetic, and signal transduction) pathways whose expression upon exposure may create species-specific tolerance variances (Miller et al., 2018). Interactions between different classes of pollutants, the generation of reactive oxygen species (ROS), and the onset of oxidative stress conditions are partly modulated by changes in the levels and functions of redox-sensitive signaling proteins and transcription factors (Regoli and Giuliani, 2014). Modulation of the biological pathways using enzymatic and non-enzymatic molecules that can prevent metabolic dysfunctions caused by oxidative stress becomes expedient (Figure 1). There is evidence of effective dietary interventions using exogenous antioxidants to regulate the impact of environmental perturbances on organisms and hence gene expression with regards to tolerance (Hennig et al., 2007; Hoffman et al., 2017; Andreescu et al., 2018; Ideraabdullah and Zeisel, 2018).

Epidemiological studies indicate that oxidative stress modulates the organism's epigenome and thus regulates gene expression (Malireddy et al., 2012; Hedman et al., 2016). Karchner et al. (2006) and Bianchini and Morrissey (2020) highlighted the fact that there are species-specific differences in the mechanism and level of tolerance to hydrocarbon fragments. This disparity could be observed between chickens raised in severely hydrocarbon-polluted environments and those raised in unaffected areas. The extent of vulnerability could be due to evolutionary capability being dependent on factors such as the genetic structure and strength of the selection pressure (Dutilleul et al., 2015). Modifications of the genetic structures, such as those associated with fecundity, survival, and morphology, are experiential (Nacci et al., 2002; Charmantier and Garant, 2005). Whitehead et al. (2017) asserted that the genetic architecture core to adaptive traits may interrelate to form the possibility of evolutionary rescue from pollution. Thus, genomic variability is critical for adaptation and serves as a survival mechanism in response to environmental pollutants (Oziolor and Matson, 2015).

Crucial to the successful establishment of smallholder poultry production in regions affected by hydrocarbon pollution is, therefore, an understanding of the impact of hydrocarbon pollutants on poultry with respect to gene-environment interaction as well as the mitigation effect of such an impact using exogenous antioxidants. This review highlights some physiological dysfunctions arising from exposure to hydrocarbon

TABLE 1 Hydrocarbon pollutants and their effect on physiological responses of different organisms.

Hydrocarbon pollutant	Sample	Population	Physiological response	References
Particulate matter $(PM)_{2.5}$	Lungs	Human	PAH-Coated with PM induced gene expression of CYPIAL, NQO1, GST-P, 1 and GST mu-3	Abbas et al. (2009)
Biomass fuel (BMF) smoke	Blood and Lungs	Human	Downregulation of Nuclear factor Keap1 experimentation in BMF users, activation of Nrf2 and upregulation of NQO1	Mondal et al. (2018)
Ozone	Lungs	Rats, mice, guinea pig	Elevated lung enzyme activities, alveolar duct fibrosis in rat and guinea pig	Dormans et al. (1999)
Polycyclic Aromatic Hydrocarbons (PAHs)	Liver	Double crested cormorants	Mutations in DNA microsatellites of birds closest to pollutionsites	King et al. (2021)
PAHs			Epigenetic modifications (changes in DNA methylation, histone modification and miRNA regulation) resulting in chronic diseases	Das and Ravi (2022)
Ozone	Lungs	Quail	Loss of cilia in bronchi and trachea, necrosis of air capillary epithelium, inflammatory response	Rombout et al. (1991)
SO_2, NOx, PM $_{2.5}$	Blood	Passerine birds	Decrease in red blood cell count, beta globulins and body weight, increase in ESR size and liver transaminase	Llacuna et al. (1996)
Air pollutants	Lungs, feathers	Sparrow	Retention of particulate matters in the lungs and accumulation of toxic metals in the feather as well as lower T-AOC, SOD, immunoglobulin concentrations	Li et al. (2021)
PM	Genes		Epigenetic alterations (DNA methylation)	Ferrari et al. (2019)
Benzo{a}pyrene (BaP)	Blood	Broilers	Evidence of hemato- and hepatoxicity due to BaP oxidative stress	Latif et al. (2010)

pollutants while noting some mechanisms of hydrocarbon toxicity and connecting dietary modulation to possible epigenetic changes towards improving tolerance to enhance productivity.

Hydrocarbon pollution and physiological responses of poultry

Pollutants (both primary and secondary) from crude oil exploration activities like gaseous emissions from incomplete fossil fuel combustion and petrochemical industries, particulate matter (PM), polycyclic aromatic hydrocarbons (PAH), volatile organic compounds (VOC), and heavy metals (Pb, Cu, Ni, Cr, and Cd) pose adverse health challenges not only to humans but to other organisms within the ecosystem (Table 1). The interference of free radicals and reactive oxygen species (ROS) on the function of the cellular membrane as well as the enzymatic systems could possibly explain the mechanism of hydrocarbon toxicity. Though free radicals in the body system are involved in normal cellular functions and are essential in reduction-oxidation reactions and other physiological responses, maintenance of the balance between oxidation and anti-oxidation is critical (Bouayed and Bohn, 2010). Exposure of populations to hydrocarbon pollutants results in an imbalance between free radicals and the cellular antioxidant defense system, which culminates in various mutagen-induced deleterious effects. Overproduction of ROS causes them to behave as molecular sharks whose function is to damage molecules of the cell membrane, mitochondria, and nucleic acids, leading to oxidative stress (Aher et al., 2011). In other words, active oxygen species serve as mediators of the pollutants induced physiological responses by acting as precursors to various types of diseases, thereby affecting production (Ekweozor et al., 2002), fitness, and survivability (Cohen et al., 2017).

The accumulation of petroleum hydrocarbons in different tissues of exposed organisms has been reported (Bursian et al., 2017; Alzahrani and Rajendran, 2019). Increased stress levels, elevated detoxification efforts, and impairment of reproductive fitness were reported in birds following inhalation of pollutants (Sanderfoot and Holloway, 2017). A varied degree of biochemical and cellular responses linked to protein catabolism, bile acid metabolism, glucose homeostasis, and lipid peroxidation was observed in birds found in oil spill regions (King et al., 2014; Bianchini and Morrissey, 2018). Certain diseases such as lipid pneumonia, pulmonary dysfunction, decreases in hemocrit values, and immune-toxic effects were diagnosed in exposed birds (Szaro, 1991; Leighton, 1993; Trust et al., 1994). Al-Badri et al. (2019) found early and progressive stages of apoptosis in the hepatocytes of ducks exposed to various sources of air pollution. Pollutants substantially impacted osmoregulatory mechanisms, resulting in a decline in kidney function in birds exposed to pollutants (Amakiri et al., 2009; Dean et al., 2017). In the human population, there is a correlation between maternal exposure to air pollution and an increased risk of impaired lung development in the progeny (Saha et al., 2018). Pre- and post-hatching examinations of fertile eggs of *Larus marinus* (Lewis and Malecki, 1984) exposed to petroleum revealed developmental abnormalities of the embryo, morbidity, and high mortality in various avian species (Albers, 1978; Albers, 2006; Dubansky et al., 2018). Ekweozor et al. (2002) noted

that hens exposed to crude oil had diminished growth, egg production, egg quality, and hatchability. Kubale et al. (2018) and Jiang et al. (2019) reported incidences of cardiovascular diseases such as atherosclerosis, right ventricular failure, and developmental cardiotoxicity in poultry.

Genes' function in hydrocarbon toxicity

Variations in single-nucleotide polymorphisms from xenobiotic response elements (like hydrocarbon pollutants) influence gene expression (Liu et al., 2018). Lodovici and Bigagli (2011) reported on the mechanisms and pathways by which this occurs. There is evidence that these pathways are stimulated by oxidants in a number of cell types and may be involved in the activation of transcription factors (Samet et al., 2002; Øvrevik et al., 2015). The resultant physiological dysfunctions (Låg et al., 2020) are evidenced by abnormal cell differentiation and transcriptional activation of xenobiotic-activated receptors such as the aryl hydrocarbon receptor (AHR), nuclear factor erythroid-derived 2 (Nrf2), and nuclear factor (NF)-kappa B-related genes (Shukla et al., 2000; Watzky et al., 2022). A redox balance in oxidant-antioxidant synthesis is critical to cell signaling mechanisms, which are crucial for regulation of different gene expressions, adaptation to stress, and ultimately homeostasis maintenance in the body (Malireddy et al., 2012; Hedman et al., 2016; Surai et al., 2019).

AhR-mediated signaling is known for its role as a sensor for environmental stimuli (Karchner et al., 2006; Bessede et al., 2014; Zhou, 2016); it regulates the expression of many genes in response to xenobiotics. It has been proposed as a signal transducer of hydrocarbon-induced oxidative stress and inflammation (Dietrich, 2016; Jang et al., 2019), involved in the regulation of biological responses to polycyclic aromatic hydrocarbons (Ma and Baldwin, 2000; Zhou, 2016). It also regulates angiogenesis, hematopoiesis, drug and lipid metabolism, cell motility, and immune modulation (Puga et al., 2002). The cellular mechanisms of the gene showed that when a ligand binds to AhR, it moves into the nucleus, where it forms heterodimers and triggers transcription by binding to xenobiotic response enzymes like cytochrome P450 monooxygenases, aldehyde dehydrogenases 3, glutathione-S-transferases, and NADPH/quinone oxidoreductases (Gomez et al., 2018). Reitzel et al. (2014) indicated that different populations of Atlantic killfish showed strong genetic structure at the AhR-related loci studied.

Furthermore, genes that protect the cells against damage from oxidative stress (cytoprotective genes) are induced by antioxidant response elements at the transcription level mediated by Nrf2 (Jaiswal, 2004). Expression of Nrf2 occurs mostly in tissues easily affected by external stimuli like the gastrointestinal tract, lungs, and skin, as well as those that function in detoxification (Speciale et al., 2011). It plays a key role in the regulation of antioxidant biomarkers such as superoxide dismutase, glutathione peroxidases, glutathione S-transferases, and catalase (Zazueta et al., 2022). Evidence from chromatin immune-precipitation indicates that Nrf2 regulates the expression of AhR by binding to an antioxidant response element region in the AhR promoter (Shin et al., 2007). For instance, Shukla et al. (2000) reported an increase in transcriptional activation of NF-keppa B-dependent (NF-κB) gene expression, which was inhibited in the presence of catalase in murine exposed to $PM_{2.5}$ at non-cytotoxic concentrations. Inhalation and absorption of pollutants in different respiratory organ epithelial cells have been observed to induce oxidative stress (Liu et al., 2018), causing an increase in the translocation of some NF-κB to the nucleus and their increased binding to DNA, leading to the expression of genes associated with NF-κB such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) (Jiang et al., 2019). A study on genes associated with fat deposition in sanderling (*Calidris alba*) revealed that exposure to PAH downregulated the liver basic fatty acid binding protein 1 (Lbfabp) and hepatic lipase (Lipc) expression (Bianchini et al., 2021). There is evidence of inflammatory and immune diseases associated with respiratory disorders arising from the AhR-dependent disease pathway (Neavin et al., 2018).

Antioxidants and hydrocarbon toxicity

Antioxidants are molecules that interact with reactive oxygen species to cease their oxidative reaction. They function by neutralizing free radicals (nitrogen, oxygen, and lipidic radicals) and protecting the body's systems. Exposure to hydrocarbon pollutants induces oxidative stress, which stimulates injurious, inflammatory, adaptive, and reparative processes that overwhelm the antioxidant. The toxicity effect on the health and wellbeing of the organism arises from the level of genomic instabilities and alterations expressed as damage to biological molecules like proteins, lipids, and nucleic acids (Ren et al., 2017), resulting in oxidative stress, morbidity, mutation, and mortality. Oxidative stress is considered one of the critical pathways for the metabolism of hydrocarbon pollutant effects in the body (Tashakkor et al., 2011). The maintenance of the antioxidant status of exposed poultry is critical to halting cell damage and disrupting normal physiological processes. Antioxidants function in three ways to lessen the harm oxidative stress causes. These include enhancement of the expression of intracellular antioxidant enzymes, inhibition of the activity of ROS-generating enzymes, and direct reactions with the ROS (Lü et al., 2010; Poljšak and Fink, 2014).

The use of cellular antioxidants in prevention and protection against the metabolic dysfunctions can be achieved through enzymatic and non-enzymatic antioxidant defense systems (Bagyi et al., 2021) Consequently, dietary supplementation of exogenous antioxidants to compensate for the deficit in endogenous ones is imperative (Santos-Sánchez et al., 2019). Exogenous antioxidants rich in phenolic compounds have been proven to possess high antioxidant capacity owing to their redox properties, which enable them to adsorb and neutralize or minimize ROS, quench, and decompose peroxides (Osawa, 1994; Chen et al., 2020). Dietary antioxidants have been demonstrated to influence gene expression involving biochemical and pathological changes with respect to metabolic tissues, immune function, and disease risk factors induced by oxidative stress (Dincer and Yuksel, 2021; Dunisławska et al., 2022). The anti-oxidative effects of dietary antioxidants in a bid to compensate for the deficit in the endogenous system are achieved through various mechanisms and the interplay of signalling molecules, including the mediation of AhR, Nrf2, and CYP1A1 (Grishanova, 2022). Food polyphenolics

TABLE 2 Impact of antioxidant dietary modulation on different cell functions.

Dietary contents	Population	Type of intervention	Physiological response	References
Eucalyptus leaf polyphenol	Chickens	Dietary supplementation	Upregulation of antioxidant genes like HAO_2, GGT_1, GSTA4L, MGSTI.Inclusion of extract enhanced GSH–Px activity and reduced MDA in muscle tissues thereby improving antioxidant states of chickens	Li et al. (2020)
Tea extract granule	Chickens	Oral administration (drinking water)	Induction of oxidative stress via intramuscular injection of cyclophosphamide	Chi et al. (2020)
			Supplementation increased body weight and elevated the activity of SOD, CAT, GPx, with reduced MDA	
Vitamin C and Vitamin E	Chickens (Lveyang black-boned breeder rooster)	Dietary supplementation	Oxidative stress induced by subcutaneous injection of dexamethasone. Induced stress decreased SOD, IgM and mRNA expression of SOD and GSH-Px. Supplementation with Vitamin C and E had beneficial effect during early growth phase with increased body weight, improved antioxidant ability and immune performance in oxidative stressed roosters through upregulation of the expression of GSH–Px gene	Min et al. (2018)
Nettle (*Urtica dioica*)	Broiler chickens (Ross 380)	Dietary supplementation	Over expression of catalase, superoxide dismutase 1 in the lungs and liver of treated group. Attenuation of the right ventricular hypertrophy. Upregulation of hepatic and pulmonary antioxidant genes	Ahmadipour and Khajali (2019)
Vitamin C	Broiler chicken eggs (Arbor Acres)	Inovo injection	Improved growth performance traits, hatchability, total antioxidant capacity, immune status, and splenic expression of IL-4 and DNMT1, increased in expression of 1L-6, IFN-γ and TNF—α	Zhu et al. (2020)
Genistein	Breeder hens	Dietary supplementation	Maternal supplementation of genistein alters lipid metabolism in offspring through epigenetic modification resulting in improved antioxidant capacity	Lv et al. (2019)
			Upregulation in the expression of peroxisome proliferator-activated receptor (PPAR) genes, induced histone trimethylation and acetylation in chick liver	
Pterostilbene	broilers	Dietary supplementation	Attenuates diquat induced hepatic injury and oxidative stress of broilers via significant increase expression of Nrf2, heme oxygenase1, SOD and glutamate-cystein ligase catalytic subunit	Chen et al. (2020)
Folic acid	broiler	*In ovo* feeding at embryonic age 11 days	Increased hepatic folate content and expression of methlenetetrahydrofolate reductase and methionine synthase reductase. Increased plasma lysozyme activity and IgG and IgM concentray = tion, Histone methylation in IL-2 and IL-4 promoters. Immune function and epigenetic regulation of immune genes enhanced	Li et al. (2016)
Ginger extract	Rat	Oral administration	Exposure to lead via drinking water to induce oxidative-hepatic toxicity. Treatment with ginger extract resulted in upregulation of mRNA expression of antioxidant genes GST-α1, GPx1 and CAT in ginger extract supplemented group. GE had an antioxidant protective effects against lead acetate induced hepatotoxicity	Mohamed et al. (2016)
Ginger	Humans	Dietary supplementation	Increase in the expression of FoxP3 and PPAR-gamma genes in treated group and downregulation of the expression of T-bet and RORyt genes	Aryaeian et al. (2019)
Phytogenic premix (ginger, lemon balm, oregano and thyme)	Broiler chickens	Dietary supplementation	Supplementation of phytogenic premix upregulated the expression of cytoprotective genes (SOD1, GPx2, NQO1 AND HMOX1) and modulated the expression of Nrf2 and Keap1	Mountzouris et al. (2020)

showed AhR-based interactions at high concentrations (Amakura et al., 2008). Administration of a 6-shogaol-rich extract from ginger resulted in the induction of Nrf2 and Ho-1 regulated by mitogen-activated protein kinases (Bak et al., 2012). Numerous studies have shown potential benefits of antioxidant supplementation in the upregulation of transcription factor genes (Nrf2 antioxidant genes) and downregulation of inflammatory pathways triggered by oxidative stress (Table 2). Much as phytonutrients are capable

of down- or up-modulating AhR signalling, the mechanism of their action on AhR and the Nrf2 system is not clear.

Dietary modulation of genes encoding xenobiotic metabolizing enzymes to speed up the onset of disease tolerance, like those associated with hydrocarbon toxicity, would be beneficial even in poultry. Proteomic and transcriptomic analysis identified nine candidate genes and two candidate proteins that improved antioxidant status in chickens given eucalyptus leaf polyphenol extract (Li et al., 2020). Broiler chickens that were fed nettle (*Urtica dioica*) had a significantly upregulated expression of some antioxidant genes (SOD1 and CAT), which obviously prevented pulmonary hypertension (Ahmadipour and Khajali, 2019). The expression of CAT and SOD genes in the heart and lung of chickens reared in cold and high-altitude environments was increased when birds were fed *Securigera securidaca* (Ahmadipour, 2018). Curcumin and ginger were found to induce a protective effect by upregulating the activities of antioxidative enzymes, thereby modulating oxidative stress (Lin et al., 2019; Gao et al., 2022). A reduction in inflammation from oxidative stress was seen in mice administered a polyherbal mix of ginger, Chinese date, and wood ear mushroom (Nakyam et al., 2022).

Gene-antioxidant modulation and hydrocarbon tolerance

During the selection process for economically important traits, genetic and environmental factors play a crucial role. Observed phenotypic characteristics, such as hydrocarbon tolerance in poultry, will be dependent on various mechanisms and pathways involving gene expressions pertinent to maintaining the integrity of cells in the face of pollutant-induced oxidative stress. Homeostatic misbalance due to oxidative stress during exposure to hydrocarbon pollutants may induce modifications in specific epigenetic markers. To this extent, the accessibility of genes to the cellular proteins that modulate gene transcription–how, when and where a gene is silenced or activated is required (Burton and Lillycrop, 2019).

Epigenetic changes are reversible mitotically stable modifications that do not necessarily change the DNA sequence but affect the way the sequence is transcribed (Ibeagha-Awemu and Ying, 2021; Dunislawska et al., 2022). There are therefore heritable alterations in gene expression that do not necessitate any change in the DNA sequence (Jirtle and Skinner, 2007; Guerrero-Bosagna and Skinner, 2012). Epigenetic processes influence the ability of an organism to adjust to prevailing environmental perturbance and include DNA methylation, histone acetylation, and non-coding ribonucleic acids (Ju et al., 2020; Corbett et al., 2021). These modifications are classical epigenetic mechanisms involved in packaging the chromatin structure, regulating DNA damage, and repressing gene expression, among other transcriptional activities (Bannister and Kouzarides, 2011; Hunt et al., 2013). The alterations that occur through DNA methylation inhibit gene expression by silencing the gene (Dhar et al., 2021); that due to histone acetylation can be in the form of post-transcriptional and post-transitional modifications (Wang and Ibeagha-Awemu, 2021); while the non-coding ribonucleic acid is essential for regulating cellular differentiation and organism development. Of the three mechanisms, DNA methylation causes differentiated cells to develop a more stable and long-lasting methylation pattern that regulates tissue-specific gene expression by recruiting proteins involved in gene repression or inhibition of the binding of transcription factor(s) to DNA (Moore et al., 2013; Dhar et al., 2021).

The use of dietary antioxidants has been shown to be an effective strategy involving epigenetic DNA methylation and cessation of oxidative stress. Nayak et al. (2016) emphasized the role of epigenetic mechanisms in stress regulation. Improving production, immune-competence, general health and wellbeing of poultry, through dietary intervention to assure tolerance level maintenance could involve epigenetic mechanisms. In addition, diets rich in folates, vitamins C, E, A, and D, as well as polyphenol metabolites found in phytobiotics, have been shown to suppress certain diseases via modulation of DNA methylation, histone modifications, and subsequent epigenetic regulation of gene expression (Malireddy et al., 2012). Ideraabdullah and Zeisel (2018) provide a straightforward illustration of how diet regulates genomic responses and potential physiological outcomes by establishing, recognizing, and responding to epigenetic markers. Hong and Gurjit (2012) assert that exposure to particulate matter can induce glutathione depletion in the methylation cycle, thereby promoting epigenetic changes, whereas Madrigano et al. (2012) established that PAHs form adducts inducing DNA methylation (hypo and hyper) of specific genes linked to physiological dysfunctions and contribute more to the occurrence and development of diseases (Cao et al., 2020). Hernández-Cruz et al. (2022) also reviewed cadium-induced epigenetic alterations and the use of antioxidant compounds to counteract Cd-induced epigenetic alterations.

Future perspective

Evaluation of the genetic architecture and risk assessment of various chicken strains exposed to hydrocarbon contaminants is crucial for the development of intensive, commercial, and cost-effective smallholder poultry production in hydrocarbon-polluted communities. Studies on the regulation of target genes associated with environmental stress and immune-regulatory mechanisms related to hydrocarbon pollutants can provide a better understanding of how the phenotype plasticity of chickens exposed to hydrocarbon pollution can be utilized to increase productivity. Since the primary mechanism of hydrocarbon toxicity is oxidative stress, supplementing the diets of chickens exposed to pollutants with antioxidants is crucial, and the possibility of the supplement inducing epigenetic changes that promote hydrocarbon tolerance should be investigated. Based on the premise that expression patterns regulated by epigenetic processes, particularly DNA methylation, serve as a conduit for transmitting environmental information across generations via parental germ lines, such a change could be transgenerational (Weyrich et al., 2016). These studies may contribute to the identification of molecular markers for the creation of hydrocarbon-tolerant breeds.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Author contributions

Conceptualization of the study was done by VO-O. VO-O, ABS, AA, and OE were involved in literature search and writing the original draft. ABS, AJS, IK, and AA edited the manuscript. Substantial and intellectual contributions were made by VO-O, ABS, IK, BF, OO, UO, and OE. VO-O made major revisions to the manuscripts and prepared it for publication. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Decoding the fibromelanosis locus complex chromosomal rearrangement of black-bone chicken: genetic differentiation, selective sweeps and protein-coding changes in Kadaknath chicken

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Black-bone chicken (BBC) meat is popular for its distinctive taste and texture. A complex chromosomal rearrangement at the fibromelanosis (*Fm*) locus on the 20th chromosome results in increased endothelin-3 (*EDN3*) gene expression and is responsible for melanin hyperpigmentation in BBC. We use public long-read sequencing data of the Silkie breed to resolve high-confidence haplotypes at the *Fm* locus spanning both Dup1 and Dup2 regions and establish that the *Fm_2* scenario is correct of the three possible scenarios of the complex chromosomal rearrangement. The relationship between Chinese and Korean BBC breeds with Kadaknath native to India is underexplored. Our data from whole-genome re-sequencing establish that all BBC breeds, including Kadaknath, share the complex chromosomal rearrangement junctions at the fibromelanosis (*Fm*) locus. We also identify two *Fm* locus proximal regions (~70 Kb and ~300 Kb) with signatures of selection unique to Kadaknath. These regions harbor several genes with protein-coding changes, with the bactericidal/permeability-increasing-protein-like gene having two Kadaknath-specific changes within protein domains. Our results indicate that protein-coding changes in the bactericidal/permeability-increasing-protein-like gene hitchhiked with the *Fm* locus in Kadaknath due to close physical linkage. Identifying this *Fm* locus proximal selective sweep sheds light on the genetic distinctiveness of Kadaknath compared to other BBC.

KEYWORDS

Kadaknath, black-bone chicken, genetic linkage, fibromelanosis, *Fm* locus

Introduction

Domestication of chicken during the Neolithic period involved a complex pattern of interbreeding with various jungle fowl species (Eriksson et al., 2008; Tixier-Boichard et al., 2011; Wang et al., 2020a; Lawal and Hanotte, 2021). After domestication, chickens have spread worldwide and occur as commercial, exotic, and indigenous village breeds. Humans use chickens as a research model due to their physiology and behavior (Cogburn et al., 2007), as game fowl and for religious reasons, or more commonly for egg or meat production

(Rubin et al., 2010). Chicken is the most preferred source of meat for humans due to its easy availability and affordability (Kralik et al., 2016). Hence, understanding the genetics of meat features is commercially relevant (Mir et al., 2017). Although understudied, indigenous village chicken breeds with unique properties provide an opportunity to understand the genetics of meat. For instance, the black-bone chicken (BBC) is a delicacy due to its texture, color, firmness, flavor, and use in traditional medicine (Jaturasitha et al., 2008; Dou et al., 2022). The black color of the BBC results from melanin deposition throughout the body, i.e., melanin hyperpigmentation or fibromelanosis caused by the *Fm* allele (Muroya et al., 2000). Bateson and Punnet were the pioneers in identifying the autosomal dominant *Fm* allele (Bateson et al., 1911). Modern studies found a chromosomal rearrangement on chromosome 20 is involved in the *Fm* locus (Dorshorst et al., 2011; Dharmayanthi et al., 2017; Sohn et al., 2018). The overexpression of the endothelin-3 (*EDN3*) gene located within the *Fm* locus is responsible for hyperpigmentation seen in the BBC (Dorshorst et al., 2011; Shinomiya et al., 2012; Zhang et al., 2022).

BBC breeds have anti-fatigue and anti-hypoxic abilities, with their meat having antioxidant properties (Tu et al., 2009; Dou et al., 2022), high carnosine (Tian et al., 2007; Sharma et al., 2022b), and lower fat and cholesterol content (Jaturasitha et al., 2008; Tian et al., 2011). Some BBC breeds also have local adaptations. For instance, the Korean Ogye has improved fetal viability and innate immunity against microbial and viral infections (Cho et al., 2022). BBC breeds occur globally and have distinct names, such as Ayam Cemani (Indonesia), Black H'Mong (Vietnam), Tuzo (Argentina), Svarthöna (Sweden) (Johansson and Nelson, 2015; Dharmayanthi et al., 2017), Yeonsan Ogye (Korea) (Sohn et al., 2018), and Thai BBC (Thailand) (Buranawit et al., 2016). China has a high diversity of BBC breeds, including Silkie, Jiangshan, Lueyang, Sichuan, Xingwen, Yugan, Dehua, Jinhu, Muchuan, Wumeng, Yanjin, Xichuan, Tuanfu, Wuliangshan, Emei, and Miyi fowl (Zhu et al., 2014; Huang et al., 2020b; Li et al., 2020; Kriangwanich et al., 2021; Dou et al., 2022). India has a single breed of BBC, commonly known as Kadaknath (Sharma et al., 2022b).

Kadaknath is considered native to the Jhabua, Alirajpur, and Dhar districts of Madhya Pradesh, but its farming has recently spread across India (Jadhao et al., 2022; Tripathi et al., 2022). The Kadaknath breed has long been documented as a distinctive indigenous Indian breed and is also called Kali Masi or Karaknath (Slater, 1945). At least three distinctive phenotypes (i.e., jetblack, pencil, and golden) occur within the Kadaknath breed (Haunshi and Prince, 2021). In jetblack, all the body parts like plumage, comb, internal organs, eyes, skin, beak, shank, and claw are entirely black, whereas pencil and golden have white and golden color patches on the plumage, respectively (Haunshi and Prince, 2021). Several unique characteristics, such as earlier egg-laying maturity, high-protein content, better disease resistance, and adaptation to the local environment, are attributed to the Kadaknath chicken (Rout et al., 1992; Haunshi et al., 2011; 2022; Thakur et al., 2015; Jena et al., 2018; Shanmathy et al., 2018; Sahu et al., 2019; Haunshi and Prince, 2021; Sehrawat et al., 2021; Sharma et al., 2022b; Jadhao et al., 2022). Understanding the genetics behind these traits will help establish the uniqueness of Kadaknath and guide de-extinction efforts and breeding programs.

Despite its immense popularity and commercial value, the genomics of the Kadaknath chicken breed has received limited attention. Therefore, further research and genomic analyses are required to understand adaptations in this breed and its genetic history. The aims of this study are as follows: (1) Performing whole-genome re-sequencing of Kadaknath and evaluating its relationship with other black-bone and non-black-bone chicken breeds. (2) Assessing whether Kadaknath and other BBC breeds share a common origin for the *Fm* locus by comparing the chromosomal rearrangement junction and resolving the correct arrangement of duplicated regions at the *Fm* locus. (3) Evaluating how the BBC breeds dispersed to various parts of Asia. (4) Using population genetic statistics to identify signatures of selection in Kadaknath compared to other BBC breeds.

Materials and methods

Population sampling

The study was approved by the Institutional Ethics Committee (IEC) of the Indian Institute of Science Education and Research, Bhopal, vide reference number IISERB/IEC/Certificate/2018-11/03 dated 8th June 2018. We purchased the meat of six individuals (two individuals each from jetblack, pencil, and golden morphs (Supplementary Figure S1)) of Kadaknath from an Food Safety and Standards Authority of India (FSSAI)-licensed shop in Bhopal, Madhya Pradesh, India. We procured two other individuals with a black-bone phenotype from the same FSSAI-licensed shop to examine hybrids. One had a golden–pencil external appearance, and another was completely white on the exterior. We also sampled two non-BBCs from the same shop to determine the genetic relationship of Kadaknath with the native-village chicken and broiler reared in the same poultry (Supplementary Figure S1). To reduce the chances of inter-breeding and have relatively pure Kadaknath samples, we obtained three (one individual each from jetblack, pencil, and golden morphs) additional Kadaknath breed chickens from Jhabua, Madhya Pradesh. Whole-genomic DNA with high purity and quality was extracted from the liver tissue samples using DNeasy Blood & Tissue Kits (QIAGEN). We generated >25x coverage whole-genome short-read paired-end data (using Illumina Novaseq) for all 13 individuals sampled (Supplementary Table S1).

To evaluate the relationship of Kadaknath chicken globally, we compared our dataset with publicly available chicken re-sequencing data. High-quality re-sequencing datasets of 88 chicken individuals from other BBCs, commercial chicken lines, and other distinctive chicken breeds with a coverage >20x were selected and obtained from European Nucleotide Archive (ENA) and Korean National Agricultural Biotechnology Information Center (KNABIC) (https://nabic.rda.go.kr/). Out of 88 individuals, 23 are BBCs, which we considered for further analysis. Hence, we analyze a dataset of 101 individuals (88 public +13 sequenced as part of this study) from different breeds (Supplementary Table S2 for more details) (Fan et al., 2013; Roux et al., 2014; Wang et al., 2015; Ulfah et al., 2016; Sohn et al., 2018; Qanbari et al., 2019; Li et al., 2020;

Mariadassou et al., 2021; Cho et al., 2022). Transcriptomic datasets from four individuals each for five native Indian breeds (Kadaknath, Ankleshwar, Aseel, Punjab brown, and Nicobari) and broiler chicken from India generated from the breast muscle are publicly available (Supplementary Table S2). We only include these 24 transcriptome samples with the 101 WGS samples to assess the population structure.

Read mapping, variant calling, and phylogeny

We mapped the paired-end raw reads of 101 individuals to the chicken genome assembly (genome assembly version is Gallus_gallus.GRCg6a) using the BWA (Li and Durbin, 2009) (Burrows–Wheeler aligner) v0.7.17-r1188 mem read mapper with default parameters. We added the read group information using Picard tools and removed duplicate reads in all 101 individual BAM files (https://github.com/broadinstitute/picard). We performed the variant calling using FreeBayes (Garrison and Marth, 2012) with different quality control flags such as -min-alternate-count -C 10, -min-mapping-quality -m 20, -min-base-quality -q 20, and -min-coverage 10. Similarly, we used the bcftools (Li and Barrett, 2011; Danecek et al., 2021) with mapping quality flags such as -mapping quality -C 50, -min base quality -Q 20, and -min mapping quality -q 20 for robust variant calling. We removed the indels from variant calls using VCFtools (Danecek et al., 2011) with the -remove-indels flag and extracted the common SNPs using both SNP callers for retaining reliable SNP calls. The single-nucleotide polymorphisms (SNPs) identified by both variant callers (bcftools v1.9 and FreeBayes v1.0.0) were used for subsequent analysis. To identify the effect of genetic variants, we used the snpEff v SnpEff 4.3t and GRCg6a.96″databases for annotation (Cingolani et al., 2012). For the prediction of the possible impact of non-synonymous fixed variants in BPIL on the protein's structure and function, we used the Polymorphism Phenotyping v2 (PolyPhen-2) tool (Adzhubei et al., 2010). Common SNPs from both vcf files were used to construct phylogeny for 34 and 101 individuals using SNPhylo (Lee et al., 2014) v20180901, based on a maximum likelihood tree with 1,000 bootstrap values. We excluded the scaffolds, Z, W, and MT chromosomes in phylogeny analysis. The local phylogeny for Dup1 and Dup2 regions was generated using the vk phylo command (using both NJ and UPGMA methods) implemented in the VCF-kit (Cook and Andersen, 2017). The Mt genome haplotype median-joining networks were constructed using PopART (Leigh and Bryant, 2015) and SplitsTree (Huson, 1998).

We mapped the paired-end transcriptomic raw read data of 24 individuals to chicken genome assembly (genome assembly version is Gallus_gallus.GRCg6a) using the STAR (Dobin et al., 2013) (v2.7.0d read mapper with default parameters. We perform the variant calling using bcftools with flags -mapping quality -C 50, -min base quality -Q 20, and -min mapping quality -q 20. Using VCFtools, we extracted 67,617 SNPs only considering autosomes where no data were missing in any individuals using the flag–max-missing 1. For assessing the population structure of all 125 individuals (101 with whole genomic data +24 transcriptomic data), we performed the variant calling only on 67,617 SNPs (extracted from transcriptomic data of 24 individuals) using bcftools.

Fm locus junction identification

The *Fm* locus consists of a complex chromosomal rearrangement composed of two different non-paralogous regions (Dup1 (~127 Kb) and Dup2 (~170 Kb)) separated by an intermediate (Int) region. Dup1 and Dup2 regions are both duplicated and are involved in a complex rearrangement consisting of two junctions: (**A**) Dup1 + (inverted Dup2) and (**B**) (inverted Dup1) + Dup2. To identify the base-pair level positions of Dup1, Dup2, and Int regions, we compared the short-read coverage of black and non-black chicken in 1 Kb windows along chromosome 20. We used the makewindows command of bedtools (Quinlan and Hall, 2010) (v2.26.0) to create 1 Kb non-overlapping windows along chromosome 20. The number of reads in each 1 Kb window was calculated using the bedtools coverage command. We shortlisted adjacent windows with drastically different read coverage in black individuals but not in non-black individuals. The base-pair level coordinates of Dup1 and Dup2 in the Gallus_gallus.GRCg6a genome were narrowed down further using coverage estimates in 1bp windows. Dup1 starts at 20:10766772 and ends at 20:10894151. Dup2 occurs further along the chromosome and starts at 20:11306686 and ends at 20:11477501. In addition to Dup1, Dup2, and Int regions, we defined ~500 Kb flanking regions as Flank1 (20:10263555-10766771) and Flank2 (20:11477502-11980000).

Black-bone-specific *Fm* locus junction

The junction between the rearranged regions in the BBC does not occur in the Gallus_gallus.GRCg6a genome (Supplementary Figure S2, 3). In searching for a completely assembled *Fm* locus, we check the previously published assembly of BBC breeds. We find that the two genome assemblies of Silkie (Silkie2 (GCA_024679325.10 and Silkie3 (GCA_024653025.1) generated by Li et al. (2022b) and one genome assembly of Yeonsan Ogye generated by Sohn et al. (2018) have been published. We generated the dot plot of chromosome 20 of chicken genome assembly (Gallus_gallus.GRCg6a) with chromosome 20 of Yeonsan Ogye (CM008847.1) and Silkie3 (CM045235.1) using the Gepard tool (Krumsiek et al., 2007). The rearranged *Fm* locus is partially assembled at Chr 20 in Yeonsan Ogye (Supplementary Figure S2, 3). In the Silkie3 genome, parts of the *Fm* locus occur on chr20 and unplaced scaffold (JAJMOI010001544.1) (Supplementary Figure S4, 5). However, in Silkie2 genome assembly, chr20 is not assembled, so Dup1 and Dup2 are independently present on four different unplaced scaffolds (JAJMOM010018106.1, JAJMOM010006651.1, JAJMOM010000156.1, and JAJMOM010000154.1). None of the existing assemblies provide the full-length assembly of the *Fm* locus in BBC breeds. While the junction sequences have been reported earlier (Dorshorst et al., 2010) for the Silkie breed, it is unclear whether all BBC breeds

share the same junction sequences. Hence, we searched for the *Fm* locus junction in the Korean BBC PacBio data from ENA (SRR6189090). Based on our search of PacBio reads that mapped to the Gallus_gallus.GRCg6a genome, we shortlisted reads that simultaneously aligned to two of the five (Flank1, Dup1, Int, Dup2, and Flank2) genomic regions we have defined. We found several reads spanning Flank1-Dup1, Dup1-Int, Int-Dup2, and Dup2-Flank2, which is unsurprising because these regions are adjacent.

We found five reads (SRR6189090.111279, SRR6189090.56386, SRR6189090.880702, and SRR6189090.387043, and SRR6189090.54824) that span across both Dup1 and Dup2. Dup1 and Dup2 are far apart in their genomic location in Gallus_gallus.GRCg6a assembly. Hence, these reads support a rearrangement that leads to junctions between these two regions. Three of these five reads (SRR6189090.111279, SRR6189090.880702, and SRR6189090.387043) support the junction Dup1 + (Inverted Dup2) (i.e., START-DUP1-END-END-DUP2-START), and the other two reads (SRR6189090.54824 and SRR6189090.56386) support the junction (inverted Dup1) + Dup2 (i.e., END-DUP1-START-START-DUP2-END). The read coverage at these junctions consistently differed between all black-bone and non-black-bone chickens. Based on the read coverage across the Fm locus junctions, we identified 34 out of 101 samples analyzed as BBC.

Long-read data mapping and haplotype-specific read identification

Recently, a new high-coverage multi-platform genomic public dataset for the Silkie BBC became available on the European Nucleotide Archive (ENA) as part of the Bioproject# PRJNA805080 (we thank China Agricultural University for publishing these data). With a ~65X (Nanopore) and >660X coverage (PacBio) of the same Silkie individual, this dataset is well suited to resolve the haplotypes at the *Fm* locus and identify the exact order of rearrangement. For mapping the PacBio and Nanopore long-read data of this Silkie individual, we used the Gallus_gallus.GRCg6a (galgal6a genome assembly) genome with the BWA (Burrows–Wheeler aligner) v0.7.17-r1188 bwasw read mapper with the flags -t 24, -a2, -b3, -q2, -r2, and -z1 to obtain high-quality read mapping.

We used the bam-readcount (Khanna et al., 2022) tool to obtain the number of reads supporting each of the four nucleotide bases at each position along the Dup1 (~127 Kb) and Dup2 (~170 Kb) regions. Potentially heterozygous sites with reliable read support were shortlisted as sites with at least 10 reads supporting each of the two alleles. Sites with at least 10 reads supporting three or more bases were excluded as potentially tri-allelic sites. Using these criteria, we found around 1% of the sites that are potentially heterozygous in Dup1 (1,214/127000) and Dup2 (1774/170000) regions. Subsequent steps were performed using the phasing.sh script (provided on our GitHub page). For each of these 2,988 sites, we labeled the reads based on the nucleotide base (i.e., A, T, G, and C) present in that read at each position using the biostar214299 program from jVarkit (Lindenbaum, 2015). We prepared the list of reads supporting each of the four bases for each site using the reads labeled in the previous step. Starting from the sites (i.e., 10766895 for Dup1 and 11476819 for Dup2) identified by visual inspection of the long-read alignments in IGV, we extended the haplotypes using the phasing.sh script. The rationale of the script is to find the next site which can distinguish the haplotypes with at least five reads supporting each haplotype. The number of reads supporting each of the four nucleotide bases at the two sites is calculated for every pair of sites the script considers. Hence, the number of reads supporting each of the 16 possible combinations of the four bases at the two sites is counted. We used this script sequentially to extend the haplotypes by identifying haplotype-defining sites by manually inspecting these counts. Of the 2,988 sites considered, 49 haplotype-defining sites were sufficient to span entire Dup1 and Dup2 regions (Supplementary Table S3).

Principal component analysis and admixture

Principal components analysis (PCA) was performed using PCAngsd (Meisner and Albrechtsen, 2018) based on genotype likelihood estimates from Analysis of Next-Generation Sequencing Data (ANGSD) v0.935 (Korneliussen et al., 2014). PCA was performed both genome-wide and *Fm* locus region-wise (Flank1, Dup1, Int, Dup2, and Flank2) for the 34 black-bone and all 101 chicken individuals. We used several flags in ANGSD for population structure analysis as follows: -GL 2, -doMaf 1, -minMapQ 30, -minQ 20, -doGlf 2, and -SNP_pval < 1e-6. Genotype likelihood values from ANGSD were used to identify principal components using PCAngsd and genome-wide admixture proportions using NGSadmix (Skotte et al., 2013). NGSadmix was run for different values of K from K1 to K10 using each K with 15 iterations with flags -minMaf 0.05 and -minInd as specified. Admixture analysis suggests Kadaknath has a sub-structure but remains distinct from the other black-bone breeds at best K = 7 using median values of Ln (Pr Data) or log probability of the data (referred to as ln Pr (X|K), i.e., k for which Pr(K = k) is highest, while according to ΔK (Evanno's best K method), the best K is 8. We used the Evanno (Evanno et al., 2005) method implemented in the Clumpak (Kopelman et al., 2015) web server to find the best K.

Using the variant calls of 125 individuals for 67,617 SNPs, we performed the admixture analysis through AdmixPipe v3 (Mussmann et al., 2020) for K1– K15, and we ran 10 iterations for each K. During the admixture analysis, two individuals of the Brown layer (BROL1 and BROL2, Supplementary Table S2) got excluded due to the poor quality of variant calls. In the admixture analysis of 123 individuals, we found that according to Evanno's best K method, the best K is 2. The observation of K = 2 in admixture analysis using ΔK (Evanno's best K method) is reported in more than 50% of recent studies (Janes et al., 2017). Hence, we followed the recommendations of Gilbert et al., (2012) and Janes et al. (2017) to assess the population structure. We re-run the admixture pipeline for 109, 100, and 88 individuals after removing the populations (Rhode Island Red, White Leghorn, and broiler), respectively, which separated at K = 2. As per the suggestion by Janes et al. (2017) to infer the optimal K value, we are providing the Ln Pr (X|K) and ΔK plots and bar plots for the multiple K values in supplementary. PCA was also performed using PCAngsd for *Fm* locus region-wise

(Flank1, Dup1, Int, Dup2, and Flank2) using 123 individuals (BROL1 and BROL2 are excluded).

Population genetic analysis

Chinese black-bone breeds have diverged to differing extents from Kadaknath and have limited sample sizes. Hence, we combined the individuals from closely related XBBC (Xichuan black-bone chicken), LCEM (Emei black fowl), and LCMY (Miyi black fowl) breeds into a single population representative of Chinese black-bone (CHIN, n = 9) chicken and JETB, PENC, and GOLD into another population representative of Kadaknath (KADK, n = 9) (Supplementary Table S4). We calculated population genetic statistics genome-wide to identify signatures of selection. However, to avoid false positives, we excluded genomic regions (50 Kb windows with <80 percent callable sites) with poor callability. We used the CallableLoci walker of GATK (McKenna et al., 2010) on the BAM files to quantify callability. For identifying callable regions from mapped BAM files for CHIN and KADK individuals, we used GATK with different flags such as -minMappingQuality 20, -minBaseQuality 20, -minDepth 10, -minDepthForLowMAPQ 20, and -maxFractionOfReadsWithLowMAPQ 20. Furthermore, we filtered 50 Kb windows with >0.1 repeat element fraction to rule out the possibility of false positives (Supplementary Figure S6–8). We used stringent coverage and quality criteria (-GL 2, -dosaf 1, -baq 1, -C 50, -setMinDepthInd 6, -minInd 3, 4 or 9, -minMapQ 30, -minQ 20, and -doCounts 1) in ANGSD to calculate all population genetic statistics. The list of individuals in each population and the population pairs compared is in Supplementary Table S4. We found that at least 20,000 of the 21,659 50 Kb windows had sufficient high-quality data in all populations.

We used the folded site frequency spectrum (SFS) approach implemented in ANGSD to calculate genome-wide population-specific estimates of π (the average pairwise differences), Watterson's θ (the average number of segregating sites), τ (Tajima's D), and Fu and Li's D. We estimated inter-population genomic differentiation (F_{ST}) and divergence (D_{xy}) using ANGSD and popgenWindows python script (https://github.com/simonhmartin/genomics_general) for each population pair. The F_{ST} estimates from the two methods were highly correlated (Pearson's ρ = 0.98, p-value <2.2 e−16) (Supplementary Figures S9,10). We defined FST outlier regions as 50 Kb windows in the top 1 percent of the genome-wide estimates and merged adjoining windows using bedtools. Similarly, the 50 Kb windows in the top 10 percent of the genome-wide estimates of Dxy were deemed to have high levels of divergence. We identified fixed sites as those sites with FST >0.9. We estimate haplotype-based statistics iHS and XP-EHH in the rehh (Gautier and Vitalis, 2012) R package with option polarized = FALSE using genotype data phased with SHAPEIT (Delaneau et al., 2011).

Results

Whole-genome re-sequencing

We generated >25X coverage whole-genome re-sequencing Illumina data for 13 chicken individuals from India. Our collection includes nine Kadaknath samples as representatives of all three extant morphs jetblack (JETB n = 3), golden (GOLD n = 3), and pencil (PENC n = 3). We also sampled one individual with a golden–pencil-like phenotype (GOPE n = 1), one with complete white plumage with black bone (CROS n = 1), one non-black-bone individual where plumage was black (NONB), and one individual of broiler breed (Supplementary Figure S1; Supplementary Table S1). We compared these Kadaknath chicken genomes with public re-sequencing datasets of chicken breeds. The sampling location (Figure 1A for black-bone chicken) of individuals analyzed in this study is spread across Asia (for more detail, see Supplementary Table S2, which includes the complete list of all chicken breeds examined). A detailed map of China depicts the locations of all the Chinese black-bone breeds (Supplementary Figure S11).

Kadaknath is a distinct breed of black-bone fowl

The major axes of genetic variation in the BBC (PC1:18.9% and PC2:13%) separated the Kadaknath, Yeonsan Ogye (YOSK n = 4), and Chinese BBC (n = 17) (see Figure 1B; Supplementary Figure S12). We observed that all Kadaknath individuals form a single cluster distinct from other black-bone breeds (from Indonesia, China, and South Korea) in the PCA (Figure 1B; Supplementary Figure S12). Similarly, in the genome-wide PCA analysis of 101 individuals consisting of both BBCs and non-BBCs, Kadaknath forms a separate cluster from other commercial, native, and BBC breeds in different PC comparisons (Supplementary Figure S13A–C).

Admixture analysis suggests that Kadaknath has sub-structure but remains distinct from the other black-bone breeds at best K = 7 (see Figure 1C and Supplementary Figure S14A–C; Supplementary Figure S15). In Chinese BBC, the admixture analysis identified three groups supporting the PCA clustering pattern, while YOSK forms a separate cluster (see Figure 1C; Supplementary Figure S12). We observed lower genetic diversity (π = 0.002, θ = 0.002) in YOSK (n = 4), while Kadaknath (n = 9) and CHIN (n = 9) BBC have comparable genetic diversity (π = 0.004 and 0.003, θ = 0.003 in both) (Supplementary Figure S16A, B). Genetic diversity can be affected by crossbreeding with native chicken breeds. Genome-wide admixture analysis of 101 individuals consisting of both BBCs and non-BBCs supports gene flow between Chinese BBC and native Chinese non-BBC breeds (Supplementary Figure S17A–C). Crossbreeding between Ogye and broiler is also supported by the genome-wide admixture analysis. Including native Indian non-BBC breeds in the admixture analysis suggests crossbreeding between Kadaknath and Ankleshwar breeds (Supplementary Figure S18A–C). However, Kadaknath is distinct from other native Indian breeds, and the Kadaknath samples from the transcriptome dataset cluster with the genome sequencing data generated in this study (Supplementary Figure S19–21A–C).

In contrast to the nuclear genome, the mitochondrial genome haplotype network did not separate Kadaknath from other BBC breeds (Supplementary Figure S22, 23). The *Fm* locus region on chromosome 20, which codes for the black-bone phenotype, is the defining feature of all BBCs. Even after excluding chromosome 20,

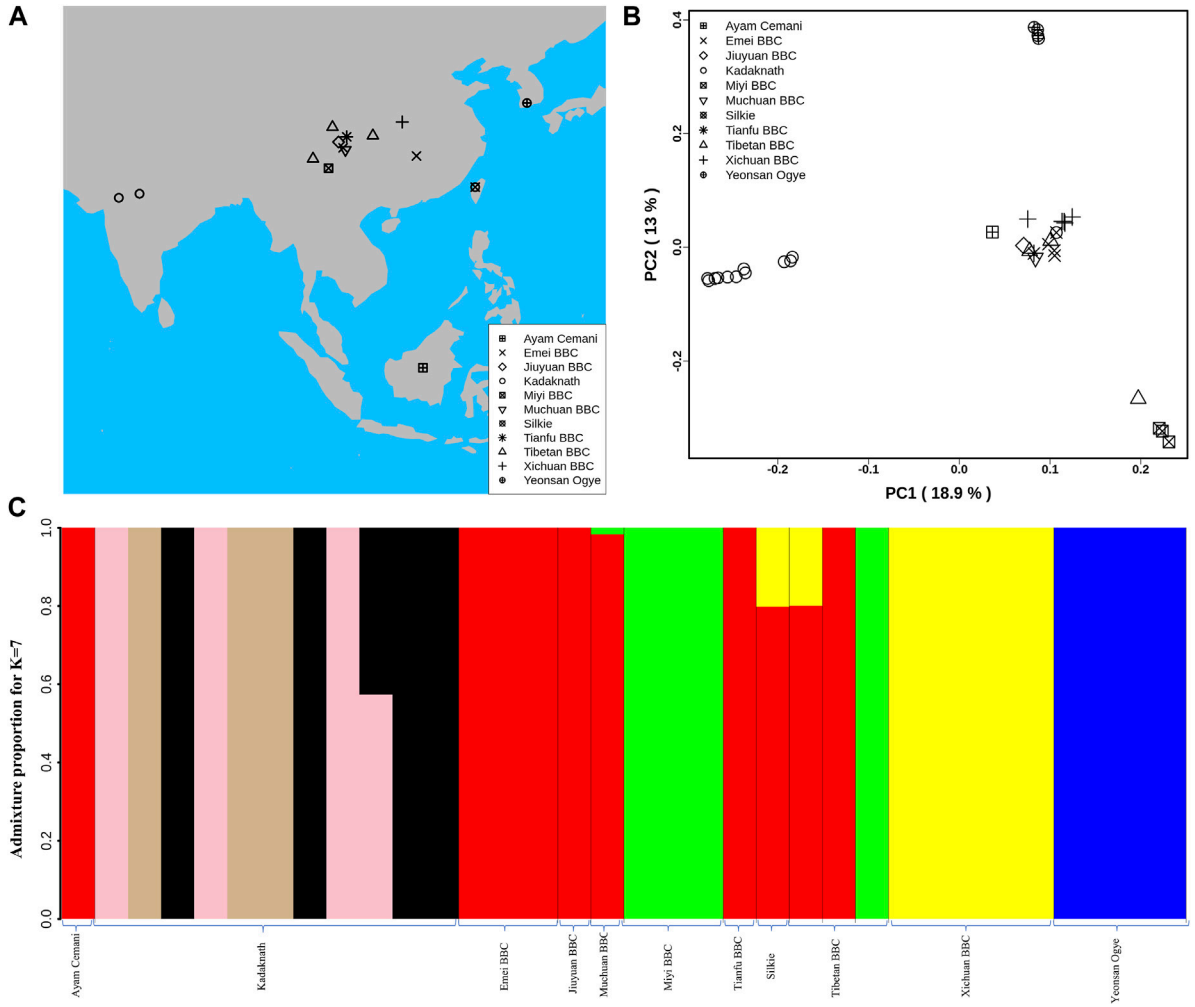

FIGURE 1
Population structure analysis: **(A)** Geographical locations of different BBC breeds used in this study are shown on the map using different shapes. The map was generated using rworldmap, map, and mapdata R packages. **(B)** Genome-wide principal component analysis reveals the genetic relationship of 34 BBC individuals. PC1 and PC2 explained 18.9% and 13% variance, respectively. **(C)** Population genetic structure and individual ancestry were estimated using NGSadmix for 34 BBCs from different breeds based on best K = 7.

the PCA of the remaining chromosomes finds that Kadaknath is genetically distinct from other BBC breeds (Supplementary Figure S24A–C). Hence, the genetic distinctiveness of Kadaknath is spread across the entire genome.

**Fm_2* is the correct arrangement of duplicated regions at the *Fm* locus

In the non-black chicken, Dup1 (~127 Kb), Int (~412 Kb), and Dup2 (~170 Kb) regions occur in a single copy, are arranged sequentially, and are flanked by Flank1 (~500 Kb) and Flank2 (~500 Kb) regions (Figure 2A). While the Dup1 region contains five protein-coding genes (*EDN3*, *ZNF831*, *SLMO2*, *ATP5E*, and *TUBB1*), the Dup2 region probably consists of only long-non-coding RNA genes. All non-BBCs have a single copy of this region, referred to as **N* locus (Figure 2A). The corresponding locus in the BBC is known as the *Fm* locus. The *Fm* locus consists of three different non-paralogous regions (Dup1, Int, and Dup2) that form a complex chromosomal rearrangement in which both Dup1 and Dup2 regions are duplicated, giving rise to two junctions: (**A**) Dup1 + (inverted Dup2) and (**B**) (inverted Dup1) + Dup2. Although the exact ordering of these regions in the rearrangement is not conclusively established, both Dup1 and Dup2 regions are known to be duplicated due to these regions having a sequencing coverage that is twice the genomic average (Dorshorst et al., 2011). The presence of Dup1 + (inverted Dup2) and (inverted Dup1) + Dup2 junctions has been verified in several BBC breeds (Dorshorst et al., 2011; Dharmayanthi et al., 2017; Sohn et al., 2018). Based on this information, three possible scenarios have been proposed by earlier studies (Figure 2B). The **Fm_2* scenario is supported based on crosses between black and non-black-bone chicken (Dorshorst et al., 2011). However, both **Fm_2* and **Fm_3* scenarios require two rearrangement events compared to a single rearrangement event needed for the **Fm_1* scenario (Sohn et al., 2018).

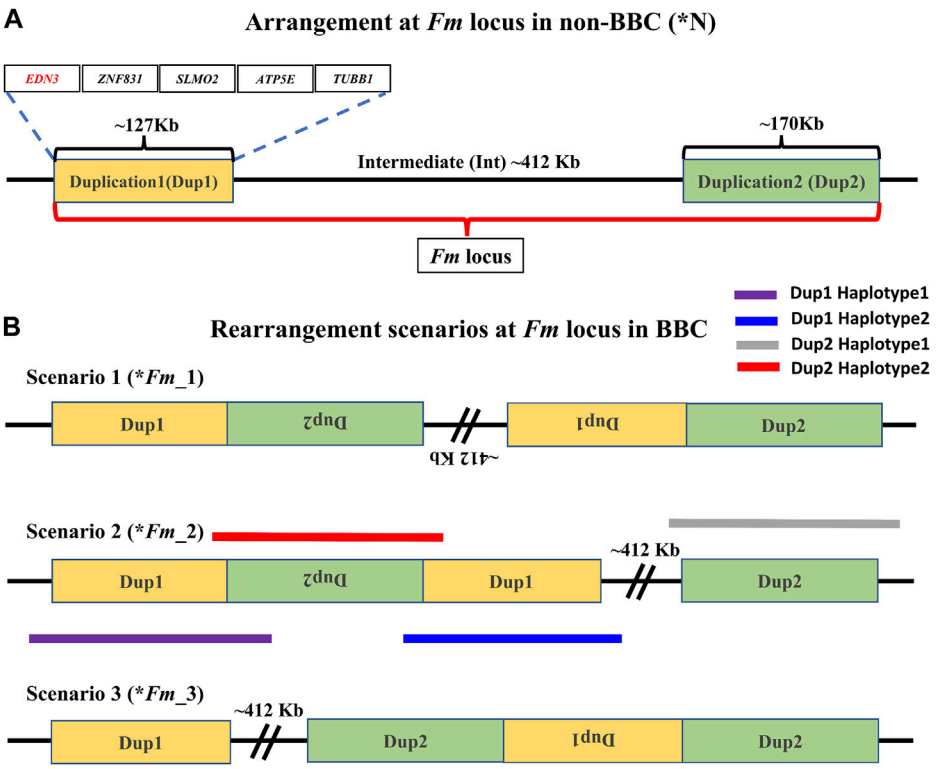

FIGURE 2
Arrangement of the *Fm* locus region in non-BBC and BBC breeds: two different non-paralogous regions on chromosome 20 are referred to as Duplication 1 (Dup1) and Duplication 2 (Dup2), shown in gold and light green colors, respectively. The length of the Dup1 region is ~127 Kb, the intermediate (Int) is ~412 Kb, and Dup2 is ~170 Kb in size. **(A)** These regions are neither duplicated nor rearranged in non-BBC (*N). Dup1, Int, and Dup2 regions are collectively referred to as the *Fm* locus. Dup1 contains five genes, *EDN3*, *ZNF831*, *SLMO2*, *ATP5E*, and *TUBB1*, whereas the Dup2 region does not have any protein-coding genes. **(B)** Three possible scenarios (**Fm_1*, **Fm_2*, and **Fm_3*) for *Fm* locus have been proposed in the BBC (earlier described in Dorshorst et al., 2011; Dharmayanthi et al., 2017; Sohn et al., 2018). The dark solid purple line represents haplotype-1, which spans across Dup1 from Flank1 to inverted Dup2 (i.e., Flank1 + Dup1 + (inverted Dup2)). The blue line represents haplotype-2, which spans Dup1 from inverted Dup2 to Int (i.e., (inverted Dup2) + Dup1 + Int).

Distinguishing between these three (**Fm_1*, **Fm_2*, and **Fm_3*) scenarios requires long-range connectivity information such as long-read sequencing data (PacBio, Nanopore, Synthetic long reads, *etc.*), Hi-C (high-resolution chromosome conformation capture) (Kronenberg et al., 2021; Wang et al., 2022), or optical mapping (Weissensteiner et al., 2020). Both **Fm_1* and **Fm_2* contain the same adjacent regions for Dup1 (defined as haplotype-1 and haplotype-2 in Figure 2B) inconsistent with **Fm_3*. Hence, long-range information that can span the entire ~127 Kb Dup1 region in a single read will be able to distinguish between (**Fm_1* and **Fm_2*) vs. **Fm_3*. However, to differentiate between **Fm_1* and **Fm_2* scenarios, we must span the entire ~170 Kb Dup2 region in a single read. Spanning Dup1 or Dup2 regions with PacBio (average read lengths of 10–25 Kb) or Nanopore (average read lengths of 10–30 Kb) technologies with a single read is challenging but not impossible as reads longer than 1 Mb are possible (Amarasinghe et al., 2020; Hon et al., 2020). Without such individual reads that can span the entire region, it is possible to perform read-based phasing to infer haplotypes that extend to distinct junctions (Patterson et al., 2015). Using a public long-read dataset, we have inferred distinct high-confidence Dup1 and Dup2 haplotypes (supporting the **Fm_2* scenario).

Upon visual inspection of the long-read alignments in IGV at the Flank1–Dup1 junction region, three heterozygous sites (10766895, 10766948, and 10767078 at chromosome 20) were recognized at the Dup1 start region potentially separating the two haplotypes of Dup1 (Supplementary Figure S25). We extended these haplotypes toward the end of Dup1 by relying upon overlapping long-reads at haplotype-defining sites (**Methods**). To ensure the robustness of our read-backed phasing approach, we required that more than 10 sequencing reads support haplotype-specific alleles at each pair of adjacent sites (Supplementary Table S3). Dup1 haplotypes are anchored by (Flank1 end)/(Dup2 start) at the Dup1 start and (Int start)/(Dup2 end) at the Dup1 end (Figure 3). The Flank1 + Dup1 junction containing haplotype is referred to as Dup1 haplotype-1 (D1H1) and contains the allele T at position 10766895. The (inverted Dup2) + Dup1 junction containing haplotype is referred to as Dup1 haplotype-2 (D2H2) and contains the allele A at position 10766895. Each haplotype can be distinguished at 24 sites (Supplementary Table S3) along the Dup1 region. Each haplotype-defining site shares more than 10 long reads with haplotype-specific alleles at adjacent positions. Dup1 haplotype-1 (Figure 3A; Supplementary Figure S26A, B) spans Dup1 from Flank1 to Dup2 haplotype-2 (D2H2) end, and haplotype-2

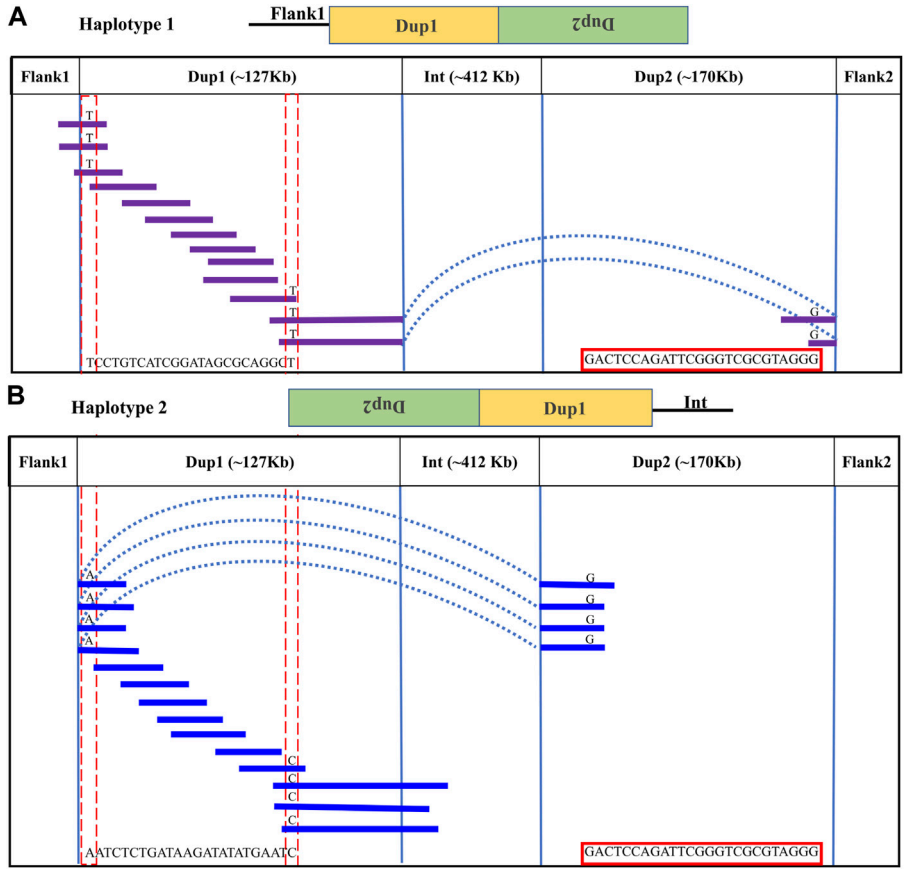

FIGURE 3
Long-read-based haplotypes resolve the sequence spanning the Dup1 region at *Fm* locus: the two haplotypes spanning the Dup1 region are distinguished by distinct alleles at 24 positions using long sequencing reads. Red dotted vertical boxes highlight the alleles that differ between haplotype-1 and haplotype-2 at the same positions. The alleles at 24 sites that separate these two haplotypes at various positions along Dup1 are presented sequentially between the red dotted boxes. **(A)** Haplotype-1 of Dup1 spans from Flank1 to inverted Dup2 (i.e., Flank1 + Dup1 + (inverted Dup2)). The purple lines represent overlapping reads of Nanopore containing Dup1 haplotype-1 alleles. The light blue dotted line represents the span of the same read from the end of haplotype-1 of Dup1 to the end of haplotype-2 of Dup2. **(B)** Haplotype-2 spans Dup1 from haplotype-2 of Dup2 to the Int region (i.e., (inverted Dup2) + Dup1 + Int). The blue lines represent the overlapping reads of Nanopore containing haplotype-2 alleles. The light blue dotted line represents the span of the same read from the start of haplotype-2 of Dup1 to the start of haplotype-2 of Dup2. Both haplotypes of Dup1 are connected to the single haplotype of Dup2 (i.e., haplotype-2 of Dup2) but at different ends.

(Figure 3B; Supplementary Figure S26A, B) spans Dup1 from Dup2 haplotype-2 (D2H2) start to Int start. Our inference of Dup1 haplotype-1 (i.e., Flank1 + Dup1 + (inverted Dup2)) and haplotype-2 (i.e (inverted Dup2) + Dup1 + Int) sequences rule out the **Fm_3* scenario, suggesting that either **Fm_1* and **Fm_2* scenario is possible.

In the Dup2 region, one haplotype was anchored to the Dup2+ Flank2 junction and another haplotype to the Dup2 + (inverted Dup1) junction. The Dup2 + Flank2 junction containing the haplotype is Dup2 haplotype-1 (D2H1) and contains the allele A at position 11476819. The Dup2 + (inverted Dup1) junction containing the haplotype is Dup2 haplotype-2 (D2H2) and contains the allele G at position 11476819 (Supplementary Figure S27). To verify the correct arrangement, we used the same read phasing approach on the Dup2 region, and we could distinguish the two haplotypes at the Dup2 region based on 25 sites (Supplementary Table S3). Haplotype-1 of the Dup2 region (Figure 4A; Supplementary Figure S28, 29) spans Dup2 from the end of Int to the start of Flank2, and haplotype-2 (Figure 4B; Supplementary Figure S28, 29) spans Dup2 from Dup1 haplotype-2 (D1H2) start to Dup1 haplotype-1 (D1H1) end. Our inference of haplotype-1 of Dup2 (i.e., Int+ Dup2 + Flank2) and haplotype-2 (i.e., start of Dup1 + (inverted Dup2) + Dup1 end) sequences rule out the **Fm_1* and **Fm_3* scenarios, suggesting that the **Fm_2* scenario is correct (Figure 5).

All black-bone chicken breeds share the same rearrangement junctions at the *Fm* locus

Conclusive inference regarding which scenario is present in the BBC has been extremely challenging due to the large (~1 Mb) size and complexity of the rearrangement. However, in the case of Kadaknath, it has not even been established whether Dup1 + (inverted Dup2) and (inverted Dup1) + Dup2 junctions identified

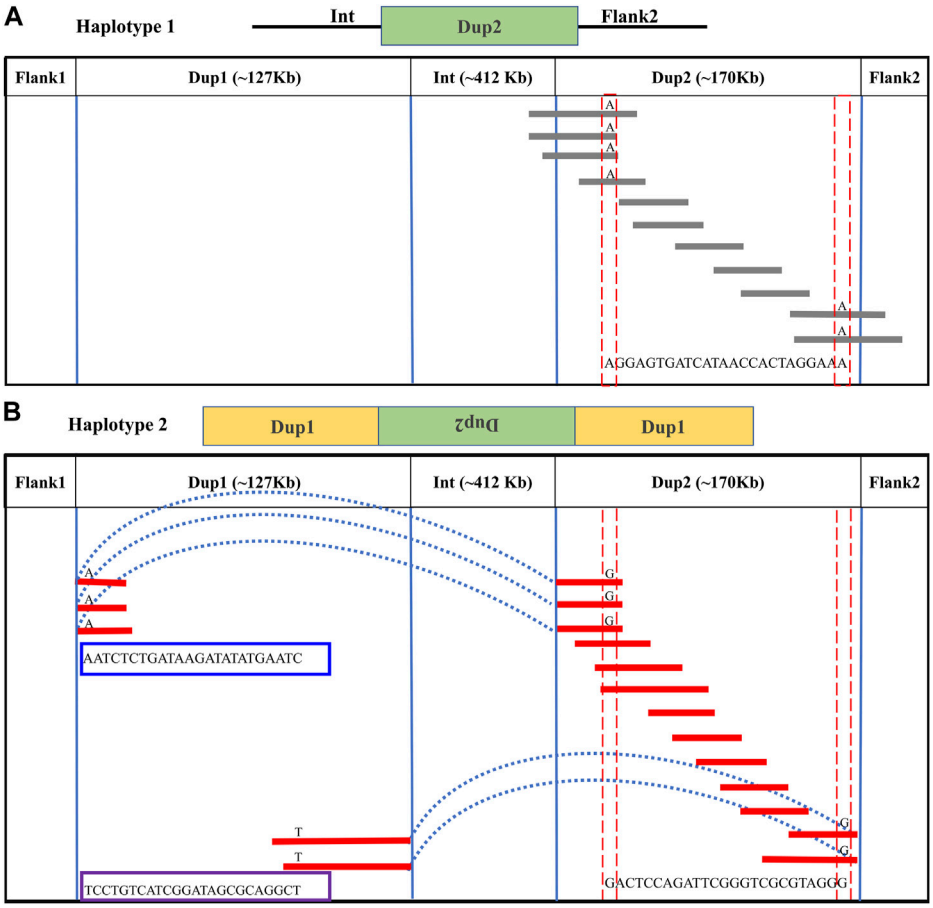

FIGURE 4
Long-read-based haplotypes resolve the sequence spanning the Dup2 region at *Fm* locus: The two haplotypes spanning the Dup2 region are distinguished by distinct alleles at 25 positions using long sequencing reads. Red dotted vertical boxes highlight the alleles that differ between haplotype-1 and haplotype-2 at the same positions. The alleles at 25 sites that separate these two haplotypes at various positions along Dup2 are presented sequentially between the red dotted boxes. **(A)** Haplotype-1 of Dup2 spans from Int to Flank2 (i.e., Int + Dup2 + Flank2). The gray lines represent overlapping reads of Nanopore containing Dup2 haplotype-1 alleles. **(B)** Haplotype-2 of Dup2 spans from the start of haplotype-2 of Dup1 to the end of haplotype-1 of the Dup1 region (i.e., Dup1 +(inverted Dup2)+ Dup1). The red lines represent the overlapping reads of Nanopore containing Dup2 haplotype-2 alleles. The light blue dotted line represents the span of the same reads from the start of haplotype-2 of Dup1 to the start of haplotype-2 of Dup2. Similarly, the same reads span the end of haplotype-1 of Dup1 to the end of haplotype-2 of Dup2.

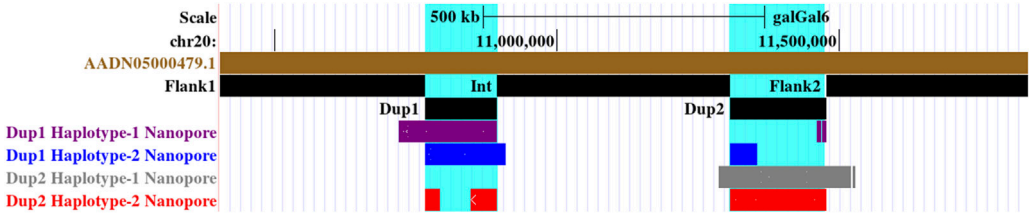

FIGURE 5
Screenshot of the UCSC genome browser of two haplotypes at Dup1 and Dup2 regions highlighted in cyan color identified using Nanopore long reads. Haplotype-1 of Dup1, shown in purple, spans from Flank1 to the end of inverted Dup2 (i.e., Flank1 + Dup1 + (inverted Dup2)). Haplotype-2 of Dup1, shown in blue, spans from the start of inverted Dup2 to the Int region (i.e, (inverted Dup2) + Dup1 + Int). Haplotype-1 of Dup2, shown in gray, spans from Int to Flank2 (i.e., Int + Dup2 + Flank2). Haplotype-2 of Dup2, shown in red, spans from the start of haplotype-2 of Dup1 to the end of haplotype-1 of the Dup1 region (i.e., Dup1 +(inverted Dup2)+ Dup1).

in other BBCs are present. We have compared the normalized short-read coverage at these junctions to evaluate whether all BBC breeds share the same rearrangement junctions at the *Fm* locus. The normalized short-read coverage in BBCs (Figures 6A–E) abruptly increases at Dup1 and Dup2 regions, while no such increase occurs in non-BBCs (Figure 6F). The drastic

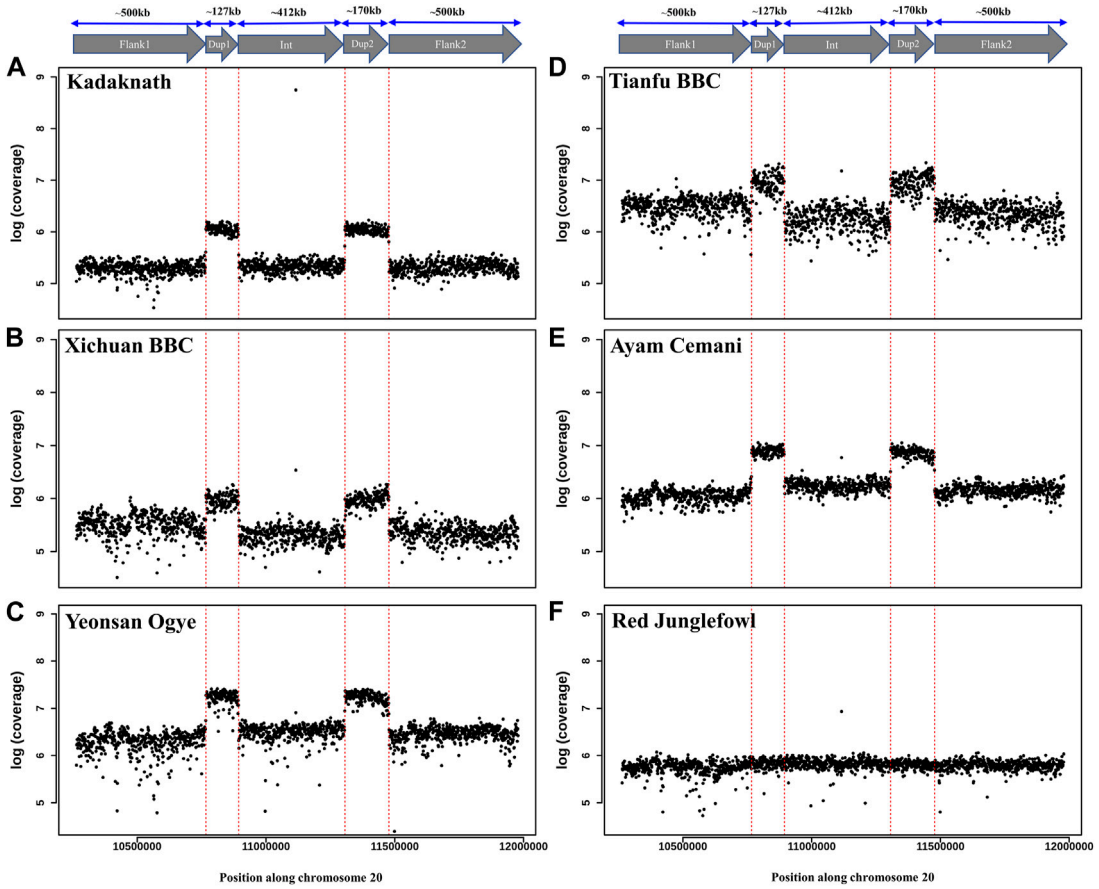

FIGURE 6
Comparison of *Fm* locus read coverage in black-bone vs. non-black-bone chicken breeds: read coverage along chromosome 20 at the *Fm* locus is shown in 1 Kb sliding windows. Two duplicated genomic loci, Dup1 and Dup2, are denoted by vertical dotted red lines and have a higher coverage in BBC breeds: **(A)** Kadaknath, **(B)** Xichuan, **(C)** Yeonsan Ogye, **(D)** Tianfu, **(E)** Ayam Cemani than non-BBC, and **(F)** red jungle fowl (RJF).

change in coverage at Dup1 and Dup2 boundaries occurs at the same base in all BBCs (Supplementary Figure S30–34). In the case of non-BBCs, no change in coverage occurs at this position (Supplementary Figure S35). We also identified the black-bone-specific *Fm* locus junctions Dup1 + (inverted Dup2) and (inverted Dup1) + Dup2 using published PacBio data (Figures 7A,B and Methods). Short-read coverage spanning these black-bone-specific *Fm* locus junctions is present in the BBC and missing in the non-BBC (Supplementary Figure S36–38). We can infer that BBC defining *Fm* locus originated through a single event based on coverage and rearranged junction sequence.

Crossbreeding with native and commercial breeds affects the genome-wide patterns of genetic variation in black-bone breeds (Supplementary Figure S17-21A–C; Supplementary Figure S39, 40). Hence, we focused on the *Fm* locus to investigate the history of the BBC breeds. The F_{ST} between the BBC and non-BBC along chromosome 20 is elevated at Dup1 and Dup2 regions (see Supplementary Figure S41). However, other population genetic parameters, such as genetic diversity (π and θ) and divergence (D_{xy}), lack any prominent signatures (Supplementary Figure S42–44). As evident from the elevated F_{ST}, the major axis of genetic variation (assessed using local PCA) in Dup1 and Dup2 regions separates the BBC from non-BBCs (Supplementary Figure S45, 46A–E). A phylogenetic tree of the SNPs from Dup1 and Dup2 regions also largely separates the BBC breeds from non-BBC breeds (Supplementary Figure S47A, B). Our evaluation of the genetic differentiation (F_{ST}) landscape between BBC breeds found reduced F_{ST} at Dup1 and Dup2 regions compared to the genomic background (Supplementary Figure S48). However, comparing individual BBC breeds with non-black chicken breeds showed the opposite pattern with elevated F_{ST} at Dup1 and Dup2 regions (Supplementary Figure S49, 50). Hence, the patterns of genetic differentiation (F_{ST}), local PCA, and phylogenetic tree also support a common origin of the *Fm* locus in all BBC breeds.

Isolation by distance pattern suggests dispersal between India and China

Our analysis discovered that the rearrangement junctions in all BBCs are identical to the one in Kadaknath and strongly support a common origin for the *Fm* locus. An independent origin for the *Fm* locus would mean separate rearrangement events have created identical junction sequences. Given the lack of repeat sequences at the junctions, such independent origins

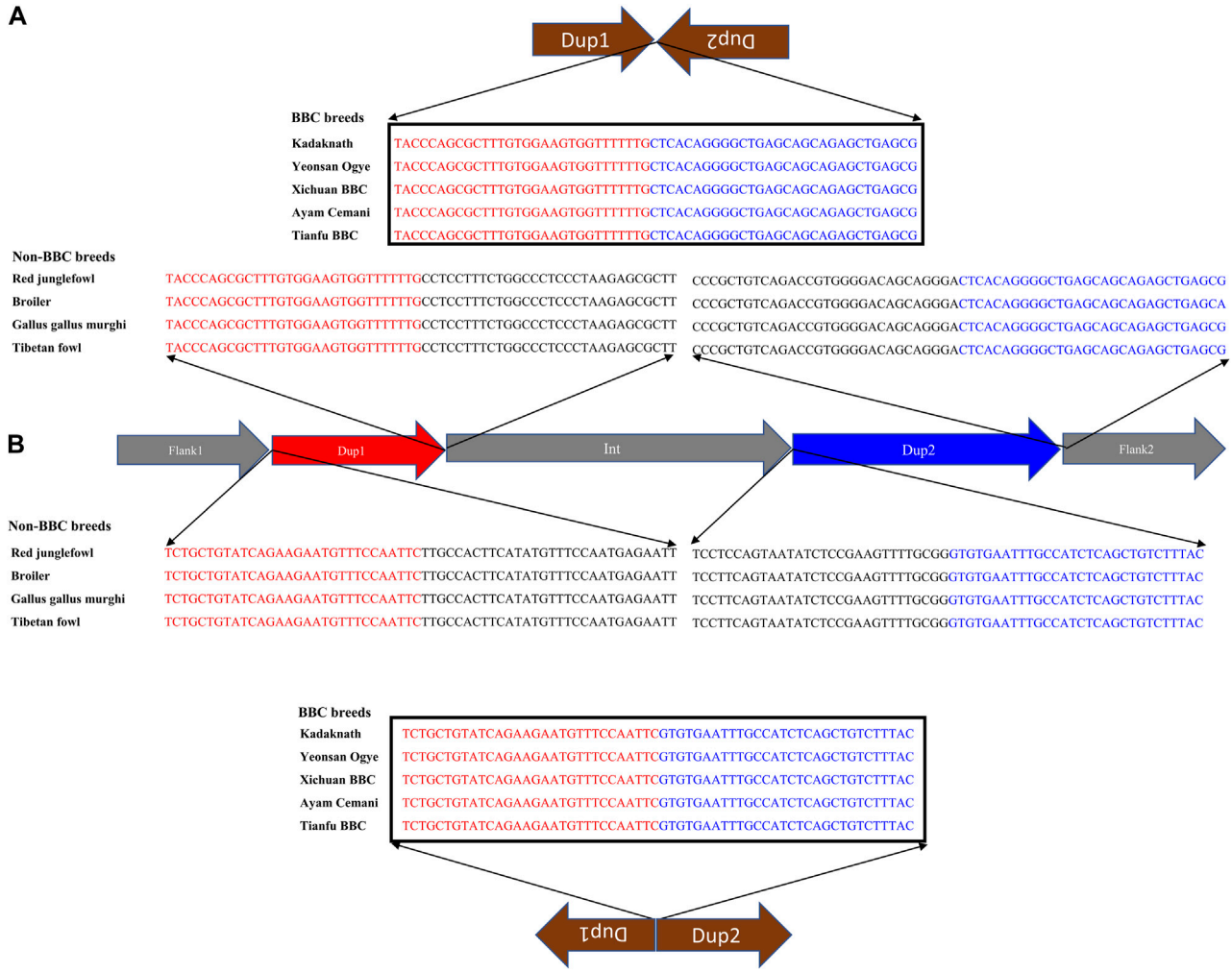

FIGURE 7
Nucleotide sequence of rearranged junctions at the *Fm* locus in black-bone vs. non-black-bone chicken: **(A)** Dup1 + (inverted Dup2) **(B)** (inverted Dup1) + Dup2. Brown arrows represent the direction of rearranged junction sequence, which is present as a continuous sequence (verified by their presence in a single read) in BBC breeds. In contrast, non-BBC breeds did not contain these junctions. Sequences highlighted in red and blue represent the Dup1 and Dup2 sequences, respectively. Sequences in black color represent the subsequent nucleotides.

seem highly unlikely and are not consistent with the genetic relatedness of BBC and non-BBC breeds at the *Fm* locus. Hence, the current distribution of BBC breeds across Asia needs an explanation. We hypothesized (see a schematic of the proposed scenario in Supplementary Figure S51) that the dispersal of BBC occurred after a common origin for the *Fm* locus, followed by recent crossbreeding with native and commercial breeds.

To test our hypothesis and delineate the dispersal route of different black-bone breeds, we first evaluated if a pattern of isolation by distance (IBD) is prevalent. The IBD plot shows a consistent increase in genetic distance with an increase in the geographic distance (Supplementary Figure S52, mantel's r = 0.64, *p*-value = 0.0002). The IBD pattern persisted even after we repeated the analysis using ANGSD/variant-call-based estimates of nucleotide differentiation (F_{ST}) after excluding various populations to avoid errors due to auto-correlation and biased estimates from isolated populations (Supplementary Figure S53–59). Among the black-bone breeds, Tibetan black-bone (TBTC) and Sichuan black-bone (LCTMJ) chickens are genetically closest to Kadaknath (mean F_{ST}~0.17). The other black-bone breeds that are geographically more distant from Jhabua (LCEM, mean F_{ST} = 0.21; XBBC, mean F_{ST} = 0.22; LCMY, mean F_{ST} = 0.3; and YOSK, mean F_{ST} = 0.36) occur at increasing genetic distances from Kadaknath (Supplementary Table S5). The pattern of IBD spanning India and China suggests potentially human-mediated dispersal. We lack conclusive evidence to identify the direction of dispersal. However, the analysis of our dataset, even after excluding alleles found in dbSNP (Sherry et al., 2001) (Supplementary Figure S60, 61), found that Kadaknath has almost twice the number of private alleles than Chinese and Korean BBC breeds and suggests an India-to-China dispersal. More extensive fine-scale sampling may provide a definitive answer regarding the direction and timing of the dispersal.

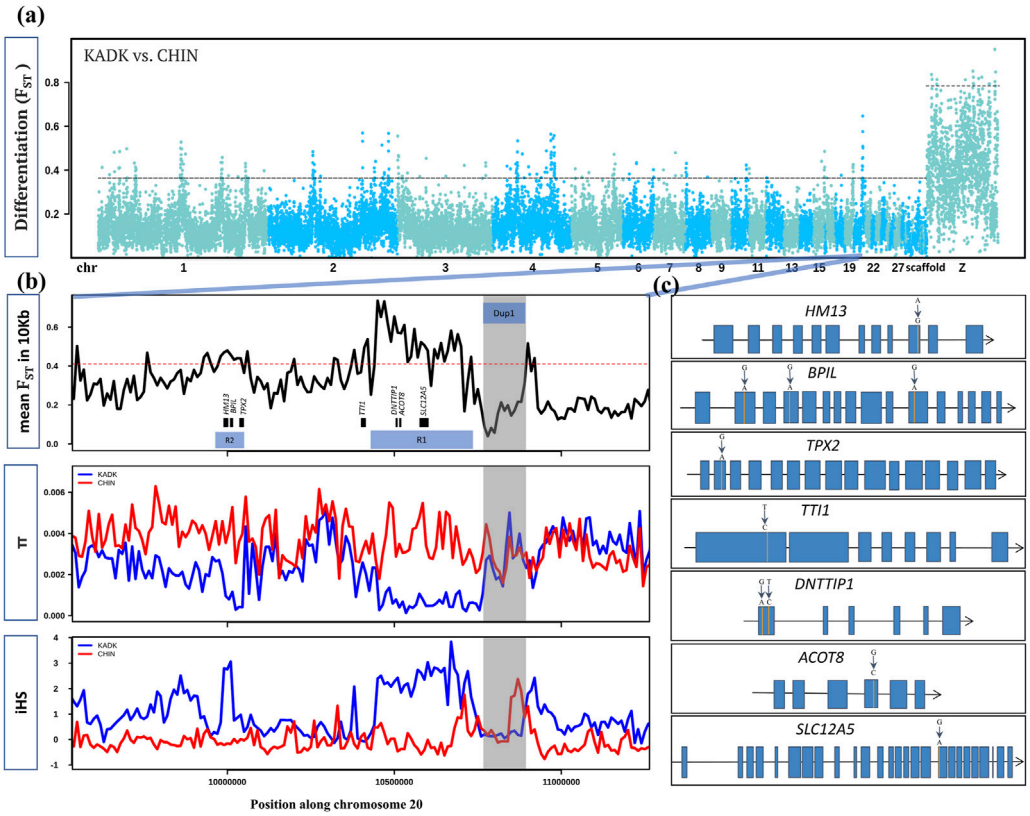

FIGURE 8
Within and between population comparison of KADK with CHIN: **(A)** genome-wide landscape of pairwise genetic differentiation (F_{ST}) between KADK and CHIN in 50 Kb non-overlapping windows. The dark slate gray and deep sky blue colors represent the alternative chromosomes. The dotted horizontal black line marks the 99th percentile outlier of estimated F_{ST} for autosome and the Z chromosome, respectively. **(B)** Highest F_{ST} region with a prominent pattern occurs on chromosome 20. The panels from top to bottom show estimates of F_{ST} in 10 Kb non-overlapping windows within population pairwise nucleotide diversity (π) and integrated haplotype score (iHS) with the major allele as ancestral and the minor allele as derived. The genes are represented as black boxes with names. The horizontal red dotted line marks the 99th percentile 10 Kb F_{ST} outlier. The transparent gray color highlights the Dup1 region in all panes in the B panel, while R1 and R2 represent the selective sweep regions. **(C)** Non-synonymous changes (shown above the exon) within genes in the regions R1 and R2 are denoted by vertical orange lines. Blue boxes are the exons connected by black lines representing introns.

Genome-wide signatures of selection in Kadaknath chicken

We screened the genome for selection signatures to identify all Kadaknath-specific regions by comparing Kadaknath with Chinese BBC. We also compared Kadaknath and a Chinese BBC population (XBBC, n = 4) with the Korean Yeonsan Ogye to identify population-specific signatures. Despite the sizeable geographic separation, the genome-wide mean F_{ST} between KADK and CHIN is 0.16, making it amenable to identifying selection signatures (Figure 8A; Supplementary Table S6). A pairwise comparison between KADK and CHIN revealed 137 genic regions in the top 1% F_{ST} windows (Supplementary Table S7; Supplementary Figure S62–94). We further shortlisted candidates using the additional criteria that genetic diversity (π and θ) and integrated haplotype score (iHS) should be strikingly different between KADK and CHIN. Two prominent signatures from these shortlisted regions indicative of selective sweeps in the KADK breed are evident on the 20th chromosome in the vicinity of the Dup1 region. The first region (R1) of ~300 Kb is ~160 Kb before Dup1, and the second region (R2) of ~70 Kb is ~0.7 Mb before Dup1. In both regions, the genetic diversity (π and θ) and Tajima's D (τ) are strongly reduced in KADK compared to CHIN (Figure 8B; Supplementary Figure S81). Compared to CHIN, the elevated iHS in KADK and the positive value of extended haplotype homozygosity (XP-EHH) support a selective sweep in KADK. This region also occurs in the top 10% genome-wide genetic divergence (D_{xy}) windows (Supplementary Figure S95.

In the first region (R1), deoxynucleotidyltransferase terminal interacting protein 1 (*DNTTIP1*), acyl-CoA thioesterase 8 (*ACOT8*), and solute carrier family 12 member 5 (*SLC12A5*) genes contain non-synonymous changes. The TELO2-interacting protein 1 homolog (*TTI1*), which occurs near the first sweep region (R1), also has a non-synonymous change (Figure 8C; Supplementary Table S8, 9). The second sweep region (R2) contains three genes with non-synonymous changes: histocompatibility minor 13 (*HM13*), bactericidal/permeability-increasing protein-like (*BPIL*), and targeting protein for Xklp2 (*TPX2*). The most striking differences were in the *BPIL* gene, which has three fixed sites with non-synonymous changes. Of these changes, two alleles are

unique to the KADK population (Supplementary Figure S96). The first non-synonymous change (C**G**G->C**A**G, R->Q) occurs at position 69 in exon 2. The second non-synonymous change (**G**AG->**A**AG, E–>K) is within the first BPI superfamily protein domain at position 159 in exon 4. The third and last non-synonymous change (C**G**C->C**A**C, R->H) is in the second domain at position 405 in exon 11. The impact of non-synonymous changes (R69Q, E159K, and R405H) in *BPIL* specific to Kadaknath is annotated as a moderate effect in the snpEff database. The PolyPhen-2 (Polymorphism Phenotyping v2) tool found that the non-synonymous changes R69Q and E159K are possibly damaging, while R405H is probably damaging (Supplementary Figure S97–99). We also found that *HM13*, *SLC12A5*, and *DNTTIP1* have novel Kadaknath-specific changes. The *HM13* gene is highly expressed in the muscle tissue, and we could assess the Kadaknath-specific change in all the native Indian breeds. Only the Kadaknath transcriptome contained the change identified in the genome sequencing data.

The Korean Yeonsan Ogye breed is closer to the Chinese XBBC (mean F_{ST} = 0.26) than to Kadaknath (mean F_{ST} = 0.3). However, the genome-wide mean F_{ST} is still low enough to distinguish selection signatures. A comparison of YOSK with KADK confirmed that R1 and R2 regions are specific to Kadaknath (Supplementary Figure S100; Supplementary Table S6). Surprisingly, our comparisons with YOSK identified a third region (R3) of ~570 kb, which is ~2.16 Mb before Dup1 with a sweep specific to YOSK (Supplementary Figure S101; Supplementary Table S9). Local PCA of the *Fm* locus, R1, and R2 regions demonstrates that Flank1, R1, and R2 regions have changed only in Kadaknath and not in other BBC (Supplementary Figure S102A–E; Supplementary Figure S103, 104). A careful examination of entire chromosome 20 failed to identify any other sweep regions (Supplementary Figure S105). Similar to the sweep in KADK, we found two genomic regions (on Chr 4 and Chr 9) with signatures of a sweep in the CHIN population (Supplementary Table S9). The sweep region on Chr 4 contains the *PCDH7* gene, and the region on Chr 9 contains the *COL4A3* and *MFF* genes (Supplementary Figure S65, 70). However, none of these genes have any non-synonymous fixed differences.

Discussion

All BBC breeds share a common origin of the *Fm* locus

As part of this study, we have generated the first whole-genome dataset of the Kadaknath breed spanning all three morphs. Hence, we can evaluate the relationship between all BBC breeds by comparing our data with public datasets. Our comparison of the *Fm* locus junction region in genome sequencing data from BBC breeds conclusively establishes a common origin for the complex chromosomal rearrangement (Dharmayanthi et al., 2017). The following four lines of evidence support this conclusion: (1) the short-read coverage along the *Fm* locus, (2) the high-sequence identity of the rearranged junctions across the BBC breeds, (3) the local and phylogenetic relationship between chicken breeds at the *Fm* locus, and (4) the patterns of pairwise genetic differentiation between BBC and non-BBC breeds. Independent structural variants can produce the same phenotypes, as seen in the case of blue eggshell (Wang et al., 2013). However, in the case of BBC breeds, the hyperpigmentation phenotype results from a single shared complex chromosomal rearrangement.

Spread of BBC across Asia

The earliest records of BBC have been found in the writings of Marco Polo, the Venetian explorer–writer who traveled through Asia (Dorshorst et al., 2011). The Compendium of Materia Medica or Bencao Gangmu, compiled and edited by Li Shizhen and published in the late 16th century, attributes various medicinal properties to BBCs (Wang et al., 2021). In Korea, the BBC is thought to have had a royal connection, and "Dongui Bogam," a traditional Korean medical encyclopedia compiled and edited by Heo Jun in 1,613, records the medicinal use of BBC (Sohn et al., 2018). While an earlier study notes that "In 1635 AD, the finding of chickens with black meat (typical of fibromelanosis, FM mutation) in Mozambique suggested direct introductions from India" (Tixier-Boichard et al., 2011), we could not find literature on how Kadaknath ended up in Jhabua, India. However, Indian chickens are thought to have entered Africa through Egypt (contributing to breeds such as Fayoumi) before dispersing into Europe (Eltanany and Hemeda, 2016).

Jhabua is close to the ancient port cities of Bharuch (also known as Bharukaccha, Barygaza, and Broach) (260 Km) and Lothal (300 Km) on the west coast of India. Given the proximity to port cities and the prevalence of BBC only in Western India (and the spread of BBC to Africa), we suspected that Kadaknath may have traveled through a marine route. The ancient sea trade between India, Korea, and other parts of Asia are well documented (Acri, 2018). Moreover, the movement of domesticated breeds during the period of colonialism was also facilitated by the common rule of large parts of Asia by various European powers (Sudrajad et al., 2020). We find evidence of crossbreeding between Kadaknath and Ankleshwar chicken breeds. The Ankleshwar breed of chicken is named after the city of Ankleshwar, located near modern-day Bharuch. Similar to Kadaknath, the Ankleshwar breed is reared by tribal communities of southern Gujarat. Evidence of gene flow between these native Indian breeds suggests that the rearing of Kadaknath and Ankleshwar breeds overlapped. None of the other native Indian breeds share ancestry with Kadaknath and support the movement of Kadaknath through the port city of Bharuch via a marine route.

Historical records are patchy, prone to error/obfuscation, and fail to provide conclusive information about the spread of BBC across Asia (Peters et al., 2022). Hence, we evaluated the relationship between BBC breeds and patterns of isolation by distance to infer the ancient dispersal route. Although each BBC breed has considerable genetic distinctiveness, population genetic analyses support the common heritage of all BBC breeds and reveal a trend of isolation by distance. While IBD patterns are well established in Chinese native chicken breeds (Nie et al., 2019), our dataset spans BBC breeds from India, China, Indonesia, and Korea. The sample size of some BBC breeds in our analysis is limited, and the sampling does not cover the entire geographic distribution of some breeds. However, our analysis achieves genome-wide pan-BBC breed sampling by including all the major breeds from Asia.

The BBC chicken from Tibet is genetically closest to the Kadaknath breed and suggests that the old Tibet–Nepal salt trade route or the maritime silk route may have facilitated the spread of BBC. Interestingly, the Tibetan BBC is genetically more similar to Kadaknath than some Chinese BBC breeds. Unfortunately, we lack BBC samples from Nepal. However, we believe BBC poultry in Nepal is Kadaknath chicken, recently imported from India. Hence, the ancestral stock of the BBC spread across Asia may not be currently available. Changes in trade routes and the introduction of commercial poultry breeds limit our ability to trace the historical prevalence of BBC breeds.

Did black-bone chicken originate in India?

The lack of data from India and the considerable interest in BBC breeds in Europe, China, and Korea have meant that studies have focused mainly on non-Indian BBCs. Hence, Southern China and Tibet are considered the source of all BBC breeds (Zhu et al., 2014; Zhang et al., 2018; Xue et al., 2023). However, despite a traditionally restricted geographic distribution, the Kadaknath breed has nucleotide diversity comparable to Chinese BBC breeds and much higher than the Korean BBC. The number of private alleles identified in Kadaknath is also greater than that in Chinese and Korean BBC breeds. Moreover, the mean genome-wide F_{ST} of 0.11 between Jhabua and Bhopal is comparable to the differentiation between some Chinese BBC breeds. The presence of three distinct morphs within Kadaknath chicken suggests phenotypic diversity derived either from commercial breeds, native Indian breeds such as Ankleshwar, or existing variations within Kadaknath. The extent of phenotypic diversity within BBC breeds from China and Korea is not documented to allow a fair comparison. The high genetic diversity in Kadaknath supports the potential origin of all BBC in Jhabua, India. The export of black chicken from India to Africa in ~1600 AD also supports that BBC was present in ancient India (Tixier-Boichard et al., 2011).

While we cannot conclude whether BBC had an Indian, Chinese, or more Southeast Asian origin, our data suggest that any of these sources are plausible. More widespread geographic sampling and analysis of allele-sharing patterns may provide a definitive answer regarding the origin of BBC. For instance, the human-aided dispersal of the ginkgo tree out of China could be traced back to samples from eastern China using extensive sampling (Zhao et al., 2019). Future studies using such large-scale sampling could provide a more definitive answer regarding the origin and dispersal of BBC. Unlike the initial domestication of the chicken, which may have occurred independently (Tixier-Boichard et al., 2011; Mariadassou et al., 2021) in several locations, the BBC has a single origin linked to the rearrangement at the *Fm* locus. Irrespective of the source of all BBCs, we identify several Kadaknath-specific genetic changes. Hence, the genetic distinctiveness of the Kadaknath breed has long diverged from other BBC breeds and is a result of its unique heritage sustained by the Bhil and Bhilala tribal communities of Madhya Pradesh. The beliefs and practices of tribal communities may have contributed to the domestication and conservation of Kadaknath (Dar et al., 2019).

Conclusive resolution of the complex chromosomal rearrangement

Earlier studies of the *Fm* locus have proposed three possible scenarios for the complex chromosomal rearrangement. Although the **Fm_2* scenario was favored based on crosses between BBC and non-BBC breeds, the correct scenario was not established by previous studies as the genome assembly of this region is challenging. We use a haplotype phasing approach relying on published long-read datasets to conclusively resolve the correct arrangement to be the **Fm_2* scenario at the *Fm* locus. In the **Fm_2* scenario, the distal region (Dup1+Int + Dup2+Flank2) resembles the **N* arrangement found in non-BBC breeds. However, the proximal region (Flank1+Dup1+(inverted Dup2)) has a very different arrangement than the non-BBC **N* arrangement. In contrast to the **Fm_2* scenario, the proximal region would be similar to the **N* arrangement in **Fm_3* scenario. In the **Fm_1* scenario, only the first occurrence of Dup1 and the last occurrence of Dup2 are similar to the **N* arrangement. Hence, in the **Fm_2* and **Fm_3* scenarios, recombination with **N* arrangement may be easier (i.e., recombination is suppressed only in the inverted ~127 Kb (Dup1) or 170 Kb (Dup2) region) compared to the ** Fm_1* scenario (i.e., recombination is suppressed in ~709 Kb (inverted (Dup2+Int + Dup1)) region). The ease of recombination with **N* arrangement could explain the prevalence of the **Fm_2* scenario, which requires two rearrangement events. Ongoing improvements to genome assembly methods such as haplotype-aware *de novo* assembly (Chin et al., 2016; Korlach et al., 2017; Garg et al., 2018; Sohn and Nam, 2018) and/or Strand-seq (Falconer et al., 2012; Sanders et al., 2017; Ghareghani et al., 2018) should allow easier resolution of such complex chromosomal rearrangements.

Selective sweep near the *Fm* locus may be a consequence of linkage

Our analyses identified two genomic regions (R1 and R2) in the vicinity of the *Fm* locus with prominent signatures of a selective sweep in the KADK population. The most promising candidate gene in this region that may have been the focus of selection is the *BPIL* gene which has accumulated three non-synonymous changes predicted to be functionally important. The *BPIL* gene is part of the innate immune defense system, which binds and neutralizes lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria (Holweg et al., 2011; Sun and Sun, 2016; Monson et al., 2019). *BPIL* is associated with upregulated expression after exposure to heat stress and LPS treatment in a comparative transcriptomic study between Fayoumi and broiler chicken breeds (Monson et al., 2019). The Fayoumi chicken breed is relatively resistant to Newcastle disease virus (NDV) compared to Leghorns (Deist et al., 2017) and potentially shares ancestry with Indian chicken breeds (Eltanany and Hemeda, 2016). Earlier studies have shown that the Kadaknath has a high level of disease tolerance against Newcastle disease, also known as Ranikhet disease, compared to other chicken breeds (Kokate et al., 2017; Gumasta et al., 2021; Malarmathi et al., 2023). Along with resistance against Newcastle disease, it has been reported that the Kadaknath breed is less sensitive to coccidial infection (Thakur et al., 2015). The non-synonymous variants in the *BPIL* gene might enhance the disease resistance in Kadaknath against various bacterial and viral infections and be associated with a better immune response. Further work to

functionally evaluate the effect of these changes will provide a clear answer.

Five *BPI*-like genes occur near *BPI* (Chiang et al., 2011), which help arrest bacterial growth, are prominent in neutrophil phagocytosis, and work as a bactericidal protein (Elsbach and Weiss, 1998; Weiss, 2003). In chickens, defense against Gram-negative bacteria may be especially important as they lack the complement *C9* gene required to effectively eliminate pathogens through the membrane attack complex (MAC) formation (Sharma et al., 2022a). Notably, another BBC breed from Korea, YOSK, has signatures of selection at the *TLR4* gene involved in detecting Gram-negative bacteria (Cho et al., 2022). Hence, various immune genes may have been selected in different chicken breeds to protect against Gram-negative bacteria.

Several other genes with non-synonymous changes occur in R1 and R2 genomic regions. Although we cannot identify the functional consequences of these changes, the strong signature of selection and KADK-specific protein-coding alterations suggest phenotype-altering ability. Predictions from snpEff and PolyPhen-2 also suggest a major effect of these protein-coding changes located within the domain regions. The genomic region of high differentiation is close to the *Fm* locus, while weak genome-wide differentiation (except for chr 4 and chr9 regions) between Kadaknath and Chinese BBCs is strongly suggestive of hitchhiking. Moreover, the sweep signature is in the Chinese BBC in chr 4 and chr 9 cases. Hence, the proximity of R1 and R2 regions to the *Fm* locus indicates that selective sweep may be due to close physical linkage with the *Fm* locus. Such co-selection of traits due to linkage and pleiotropy has occurred during animal domestication, including in chicken (Rubin et al., 2010; Feder et al., 2014; Webster et al., 2015). Hitchhiking of alleles can increase the frequency of both beneficial and mildly deleterious alleles (Kirkpatrick and Barton, 2006; Bierne, 2010; Huff et al., 2012; Flaxman et al., 2013; Franssen et al., 2015; Martin et al., 2020; Hale et al., 2021; Weist et al., 2022). Whether the changes in KADK are beneficial or deleterious need to be investigated in future studies.

The genomic co-occurrence of economically important traits in domesticated plants and animals is also known (Zhang et al., 2010; Kijas et al., 2012; Beissinger et al., 2014; Fang et al., 2017; Kong et al., 2018; Huang et al., 2020a; Wang et al., 2020b; Jayakodi et al., 2020; Guan et al., 2021; Li et al., 2022a). The alleles causing phenotypic changes can occur in closely linked genes and will undergo selection for any of the phenotypes being favored by the breeding process. Notably, R1 and R2 selective sweeps are found only in KADK, R3 in YOSK, and none in Chinese BBC breeds. Hence, the genetic variants and the selective sweep may represent recent events after the BBC breeds separated from each other. Comparing different BBC breeds provides snapshots of the selection process and the build-up of genetically linked co-selected allelic changes and may help understand the hitchhiking process during domestication.

Genome-wide data generated as part of this study and comparative analysis with other BBC breeds establish the genetic uniqueness of Kadaknath that extends beyond the *Fm* locus. We also identify specific genes with selection signatures that are likely responsible for the Kadaknath-specific phenotypes. The co-selection of genes that are in linkage, as shown in Kadaknath, is widespread in domestic species (Rubin et al., 2010; Zhang et al., 2010; Webster et al., 2015; Fang et al., 2017; Kong et al., 2018; Li et al., 2022a). Such clustering of linked alleles under selection is favored in low recombination regions and near chromosomal rearrangements. Our work exemplifies the interaction of artificial selection and chromosomal rearrangement-linked traits in domesticated species.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/ena, PRJEB51457. Scripts and data are available at: https://github.com/ceglabsagarshinde/Kadaknath_Project and https://doi.org/10.17632/8f9dn6h76h.1.

Ethics statement

The study was approved by the Institutional Ethics Committee (IEC) of the Indian Institute of Science Education and Research, Bhopal, vide reference number IISERB/IEC/Certificate/2018-11/03 dated 8th June 2018.

Author contributions

Conceptualization: SSS, AS, and NV. Methodology: SSS and AS. Investigation: SSS, AS, and NV. Visualization: SSS and AS. Supervision: NV. Writing—original draft: SSS and AS. Writing—review and editing: SSS, AS, and NV. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2023.1180658/full#supplementary-material

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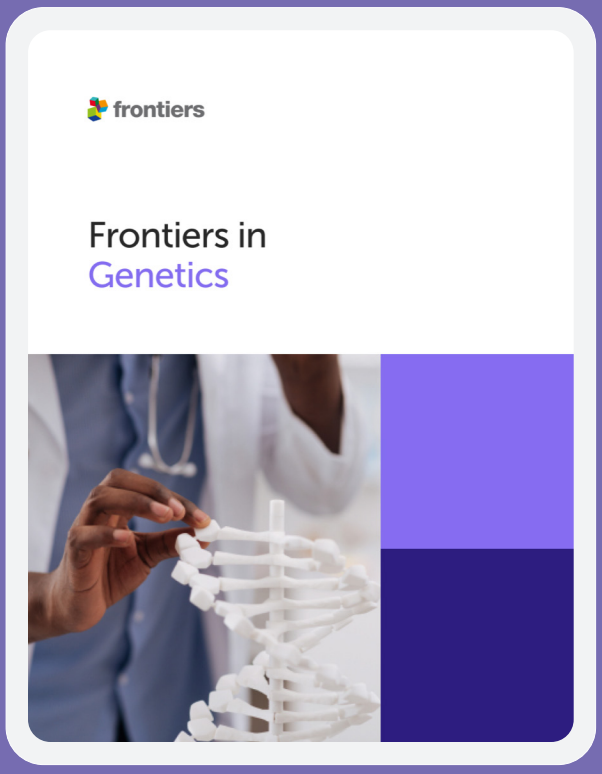

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