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Transcriptome Analysis and Sensory Evaluation of Thai Native Chicken Raised in Conventional and Free-Range Conditions

P. Seritrakul* & P. Poommarin
Faculty of Animal Sciences and Agricultural Technology, Silpakorn University,
1 M.3 Samphraya, Cha-am, Phetchaburi, Thailand 76120
*Corresponding author: seritrakul_p@silpakorn.edu
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ABSTRACT

Ethically raised animal products are believed to be better for both the environment and consumer health. Despite advances in chicken genetics and production systems, we still know little about how free-range condition affects the birds at a molecular level, specifically their gene expression and the resulting meat characteristics. This study aims to compare the transcriptome of Thai Praduhangdum, a native meat chicken raised in conventional floor pen and free-range systems, and investigate how these conditions cause gene expression changes at the molecular level and phenotypic changes in terms of meat sensory evaluation score. A total of 100 Thai Praduhangdum chickens were raised under a free-range system and a conventional floor pen system. At 6 months, blood samples were collected for transcriptome analysis and verified by quantitative real-time PCR. Additionally, meat samples were collected and sensory panel evaluation was performed. A total of 278 unique genes showed significantly different expression levels in either up-regulated or down-regulated direction. Gene set enrichment analysis revealed that these genes are associated with multicellular organism processes, development, and cell differentiation. Meat sensory panel evaluation showed that consumers preferred the appearance of the breast meat from the free-range chicken over the conventional floor pen chicken. Overall, the free-range condition caused detectable differences in gene expression and meat quality of Thai native chicken. The genes and pathways identified in this study provide a starting point for further functional tests and investigations into the relationship between chicken welfare and the physiological response at a molecular level.

Keywords: conventional and free-range; gene expression; meat sensory evaluation; native chicken; RNA-seq; transcriptome

INTRODUCTION

Chicken meat is one of the most consumed animal products world-wide. A large proportion of chicken meat comes from commercial breeds that are raised using conventional farming practices, where birds are kept in high-density, confined spaces with minimal regard to animal welfare (Scott *et al*., 2017). These conditions limit the chicken's ability to display their natural behaviors, such as foraging, perching, and dust-bathing. This raises concerns regarding both animal welfare and consumer health perspectives. It has been shown that intensively farmed commercial chicken meat is inferior in quality when compared to meat from free-range birds (Stadig *et al*., 2016; da Silva *et al*., 2017). Industrially-raised chicken meat is often described as dry, bland, and tasteless (Cheng *et al*., 2008). Additionally, consumers are willing to pay a higher price for ethically raised food, which is perceived as being safer, more nutritious, and better quality (Bray & Ankeny, 2017).

The free-range poultry production system allows birds access to outdoor environments to forage for naturally occurring feed and display their natural behaviors. Thus, the resulting free-range chickens are often described as "happy" chickens due to their apparent happiness (Marino, 2017). The free-range standard generally requires lower stocking density than an industrial system, freedom for birds to move in and out of their housing during the day, access to the five freedoms (freedom from thirst, hunger, discomfort, pain, and fear) (Mellor, 2016), access to an outdoor environment, and minimal use of synthetic chemicals and antibiotics (Scott *et al*., 2017).

Thailand's native chicken breeds originated from the ancestral Red Jungle Fowl (RJF) and have been domesticated as livestock for thousands of years (Mekchay *et al*., 2014; Siriwadee *et al*., 2023). Their unique traits include strength, agility, scavenging ability, strong mothering ability, ease of care, and resistance to various tropical diseases and environmental conditions. Genetic evidence indicates that there are at least four recognized breeds of Thai native chicken, namely Dang, Chee, Luenghangkhao, and Praduhangdum, all of which still possess a high degree of genetic diversity, characteristic of RJF and are distinct from modern breeds (Mekchay *et al*., 2014). Praduhangdum is one of the best-recognized

and well-studied breed of Thai native chicken raised for meat due to their availability, resilience, and economic value (Swaeng-ngam *et al*., 2023; Yaemkong *et al*., 2024). In Thailand, native chicken meat is desirable both by the local population and high-end niche markets, where they can be sold at a significantly higher price than conventional broiler meat due to their superior taste, nutritional value, and potential as a functional food (Charoensin *et al*., 2021; Lengkidworraphiphat *et al*., 2021).

Previous studies have shown that native chicken breeds possess characteristics that allow them to thrive better under free-range conditions when compared to commercial breeds (Michalczuk *et al*., 2016; Sadr *et al*., 2023; Stefanetti *et al*., 2023). For example, Iranian indigenous chicken showed several genes that are significantly and differentially expressed, such as those encoding heat-shock proteins (HSPs) (Sadr *et al*., 2023). A study comparing broiler and several native chicken breeds of Italy showed that these breeds are better suited for free-range systems than the fast-growing broiler chicken, resulting in better intestinal morphology and immune gene expression (Stefanetti *et al*., 2023). This indicates that there is a significant interaction between chicken genotype and their rearing systems.

The free-range poultry production system offers several benefits in terms of animal welfare and production quality. A study using Thai native chicken reported that free-range chicken showed lower feather damage resulting from aggressive behaviors, and the resulting carcass had significantly higher levels of collagen content, omega-3, and improved skin pigmentation, all of which are indicators of a healthier bird overall (Molee *et al*., 2022). A similar study using a Polish commercial crossbred chicken found that meat from birds with access to outdoor environments had significantly higher vitamin E, polyunsaturated fat, and lower saturated fat (Michalczuk *et al*., 2016). Free-range Beijing You native chicken with free dietary choice also showed higher value of blood platelets, richer microbial composition in their digestive system, better feather profile, and better meat quality, thus indicating an overall better health condition (Chen *et al*., 2018).

Despite advances in chicken genetics and production systems, we still know little about how these factors affect the birds at a molecular level, specifically the gene expression and resulting meat characteristics. In China, black-bone chicken raised in free-range and caged conditions show differences in meat quality parameters and expression of genes associated with meat flavor, including those related to muscle development, nucleotide, and amino acid metabolic pathways (Zhang *et al*., 2018; Zhang *et al*., 2022). A previous study compared chickens raised in caged and free-range conditions and found that those raised in free-range conditions had lower production performance and caused changes in several genes related to calcium and GnRH signaling, development, and immune response (Xiang *et al*., 2018). In laying hens, it has been shown that the stress caused by the cage-rearing system leads to changes in the expression of genes related to the development of the magnum tissue, which contributes to the albumen deposition in the eggs and the resulting nutritional values (Rodríguez-Hernández *et al*., 2021). Additionally, in Beijing You chicken, caged and free-range systems caused major changes in the behavior, gut microbiome, as well as the expression of genes and pathways associated with cellular differentiation, development, and response to a stimulus (Chen *et al*., 2019).

Transcriptome is a molecular technique that profiles the full range of mRNA transcripts expressed by an organism, which has been utilized in various animals, including chicken, for example, to identify differences between chicken breeds and to study physiological responses to different environmental conditions (Wu *et al*., 2018; Xiang *et al*., 2018; Park *et al*., 2019). However, no previous studies have been conducted on Thai native chicken to compare free-range and conventionally raised chicken at the genome-wide gene expression level and the resulting meat characteristics. Therefore, this study aims to compare the transcriptome and meat sensory scores between Thai Praduhangdum native chicken raised in conventional floor pens and free-range systems.

MATERIALS AND METHODS

Ethical Statement

Animal protocol in this study was reviewed and approved by the Silpakorn University Animal Care and Use Committee (protocol no. 13/2564). The sensory evaluation protocol was approved for exemption by the Silpakorn University Ethical Review Board (certificate no. COE 65.0304-042) in accordance with the Declaration of Helsinki.

Animal Care and Experimental Design

A total of 100 Praduhangdum native chicks were obtained from a local breeder. The chicks were 1 day old, unsexed, and weighed between 35-40 grams. They were divided into two groups of 50 each, with one group raised under the free-range "happy" system and the other under conventional housing (floor pen). Conventionally raised chickens were housed in a naturally ventilated shed with 12/12 lighting/dark cycle, with rice hulls as litter material, at a stocking density of 4 birds per square meter. Free-range chickens were housed in the same manner but had access to an outdoor environment (5 square meters per bird) where they could forage and exhibit natural behaviors during daylight hours except in severe weather conditions (Scott *et al*., 2017).

The free-range area consisted of primarily dirt ground with naturally occurring grass and small herbaceous plants (*Brachiaria ramosa, Cyperus rotundus, Chloris barbata, Chromolaena odorata, Cymbopogon citratus, Mimosa pudica, Ocimum tenuiflorum, Tridax procumbens*), enclosed by a wired fence and nylon net (Thai Agricultural Standard, TAS 6914-2017). Both groups of chickens had *ad libitum* access to water and complete native chicken feed (14% crude protein, 2% crude fat, 7% crude fiber, 13% moisture) and received vaccination and

regular health inspection by a veterinarian. The animal protocol for this study was reviewed and approved by the Silpakorn University Animal Care and Use Committee (protocol no. 13/2564).

Sample Collection

For molecular analysis, blood samples were collected from 6-month-old chickens from each group. The age of 6 months was selected because it is when native chickens commonly reach adulthood. A total of 1 ml of blood was drawn by a needle from the wing vein according to a previously published protocol (Pértille *et al*., 2017). Collected blood samples were preserved in RNAlater (Invitrogen, USA) solution for RNA stabilization before extraction. Three male birds were randomly sampled from each group (3 biological replicates). To eliminate the potential effects of sexual differences on gene expression, only male chickens were sampled. For meat quality analysis, 3 animals from each group were euthanized by cervical dislocation at 8 months, and carcasses were immediately processed for analysis. The age of 8 months was selected because it is when native chicken is commonly harvested for meat.

Sensory Evaluation of Chicken Meat

To evaluate consumers' perceptions of the resulting meat samples from chicken raised under conventional and free-range conditions, meat samples were collected from 3 male chickens from each group at 8 months of age. Leg and breast meat samples were roasted unseasoned at 150 °C until the internal temperature reached 76 °C and cut into 1-2 cm pieces. Sensory panel evaluation was done using blind tasting by 50 volunteer panelists aged 18-62. Panelists randomly sampled the meat without prior knowledge of the sample's identity. The attributes for evaluation were aroma, flavor, firmness, tenderness, juiciness, and overall acceptability. Each attribute was scored using a hedonic scale from 1-5 (extremely dislike, dislike, neutral, like, extremely like) (Cheng *et al*., 2008). Sensory scores were averaged and tested for statistically significant differences between each pair of samples (conventional floor pen vs. free-range) using 2-tailed t-test (SPSS v.20). Sensory evaluation protocol was reviewed and approved for exemption by Silpakorn University Ethical Review Board (certificate no. COE 65.0304-042) in accordance with the Declaration of Helsinki.

Transcriptome Analysis

Total RNA from chicken blood was extracted using an RNeasy mini kit (Qiagen, Germany) according to the manufacturer's protocol. We used blood for gene expression analysis because it is an easily accessible sample source that provides a comprehensive overview of chicken health and wellness. It has been investigated previously regarding chicken stress and rearing conditions (Pértille *et al*., 2017). Extracted RNA samples were first run on an agarose gel to visualize the overall samples, and then RNA quality was assessed by a Nanodrop spectrophotometer. TapeStation 2200 (Agilent Technologies, USA) was used to determine RNA concentration and integrity. Samples with an RNA integrity number (RIN) of 8 or above were processed for sequencing. RNA-seq libraries were prepared using TruSeq Stranded mRNA library prep kit (Illumina, USA) according to the manufacturer's protocol. Libraries were sequenced on a Next-Generation Sequencing (NGS) machine, Illumina NovaSeq 6000, with 100bp paired-end reads. Sequence mapping was done using HISAT2 (Kim *et al*., 2019) with the chicken reference genome (*Gallus gallus*; GRCg7), and transcript assembly was done using Stringtie (Pertea *et al*., 2016). For each sample, the expression value was quantified in TPM (transcripts per kilobase million). Differentially Expressed Genes (DEGs) analysis was done using DESeq2 to identify the genes that are expressed differently between chickens raised under conventional and free-range systems. Using the list of DEGs, Gene Ontology and KEGG pathway enrichment analysis were performed using g:Profiler (Reimand *et al*., 2007) and ShinyGO tool (Ge *et al*., 2020), respectively. All transcriptome sequences in this study are deposited to NCBI sequence read archive (SRA) under BioProject number PRJNA1046029 and BioSample numbers SAMN38469689, SAMN38469690, SAMN38469691, SAMN38469692, SAMN38469693, SAMN38469694.

Quantitative Real-time PCR Analysis

Extracted RNA was converted to cDNA by reverse transcription with oligo-dT primers using a Maxime RT Premix kit (Intron Biotechnology, Korea) according to the manufacturer's protocol and stored at –20 °C. Quantitative real-time PCR was performed using 10 μL of Luna universal qPCR master mix (New England Biolabs, USA), 0.5 μL each of forward and reverse primer (10 μM), 1 μL of template cDNA, and 8 μL of nuclease-free water for a total reaction volume of 20 μL. The RT-PCR experiment was done according to the MIQE guideline (Bustin *et al*., 2009). The reaction condition was as follows: 60 seconds at 95 °C for initial denaturation, followed by 15 seconds at 95 °C for denaturation, 30 seconds at 60 °C for extension, and repeated for 40 cycles. Primers for five up-regulated genes and 5 down-regulated genes were designed based on the transcript ID identified from transcriptome sequence by DEG analysis using NCBI Primer-BLAST (ncbi.nlm.nih.gov/tools/primer-blast). Real-time PCR cycling and signal detection were performed using qTower3 thermal cycler (Analytik-Jena, Germany). The Ct values from the real-time PCR reactions were calculated into gene expression fold change relative to the 28S reference housekeeping gene using the delta-delta Ct method (Livak & Schmittgen, 2001).

RESULTS

Chicken Housing and Behavior

During the entire length of the experiment, the Praduhangdum native chickens in both the free-range

and conventional floor pen housing systems were found to be healthy overall. There was no apparent illness or symptoms of diseases and no adverse effect from the environmental conditions (such as wind, rain, storm, etc). At 6 months of age, the chicken housed in the conventional system had a feed conversion ratio (FCR) of 5.70, while those housed in the free-range condition had a slightly higher FCR of 6.39. The average weight was 1.7 kg and 1.5 kg for conventional and free-range chicken. Chicken in both groups exhibit some natural behaviors such as scratching, perching, and crowing. However, the free-range chicken also displayed additional foraging behavior in the native vegetation and dust-bathing outdoors (Figure 1).

Differences in Gene Expression Profiles

Blood samples were collected from three male chickens from each group. Total RNA samples were extracted, sequencing libraries were prepared (TruSeq standard mRNA library prep), and transcriptome sequencing (Illumina PE 100) was performed. In total, each sample generated over 15 Gb of paired-end sequencing reads. The sequencing reads were quality-trimmed for a Phred score over 30 (Q30). The trimmed sequencing reads were aligned to the domestic chicken genome (*Gallus gallus*; GRCg7) using HISAT2, with overall read mapping over 93% for all samples (Figure 1). Transcripts were quantified, and DEG analysis was performed to compare gene expression and identify top candidate genes that were differentially expressed between chicken-raised under floor pens and free-range systems. Overall, 278 unique genes showed significantly different expression values at the level of over 2-fold change in either direction (up-regulated or down-regulated; $|FC| >= 2$, $p < 0.05$). Of these, 174 genes were up-regulated, where the free-range groups showed significantly higher expression than the conventional group (Table 1), and 104 genes were down-regulated, where the free-range groups showed significantly lower expression than the conventional group (Table 2). The top 30 genes from the up-regulated and down-regulated groups are listed in Tables 1-2, with the expression difference measured in fold-change.

To visually represent the similarity of gene expression patterns between chickens raised under conventional and free-range systems, a hierarchical clustering analysis was performed. The resulting heatmap showed the clustering of the 278 DEGs into two distinct groups (control = conventional, treatment = free-range) (Figure 2). To further investigate the biological functions of these candidate DEGs, gene set enrichment analysis was done for the gene ontology (GO) of biological process (BP) using gProfiler (https://biit.cs.ut.ee/gprofiler/orth). The result showed 14 enriched GO-BP terms, with the top terms with the largest intersecting size being multicellular organismal process, anatomical structure development, multicellular organism development, negative regulation of the cellular process, and cell differentiation (Figure 3A). KEGG pathway analysis was also performed using the up-regulated and down-regulated gene lists. The results showed several pathways that are enriched, such as fatty acid biosynthesis, amino acid and nucleotide metabolism, cell adhesion, and cell signaling pathways (Figure 3B).

Gene Expression Detection by Real-Time PCR

From the list of DEGs identified from transcriptome sequence, primers for five up-regulated genes and 5 down-regulated genes were designed for quantitative

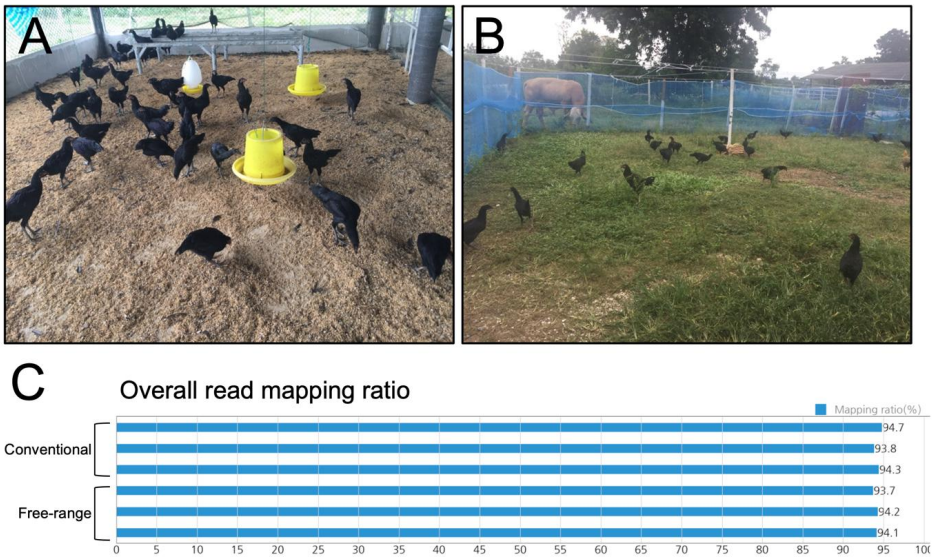

Figure 1. Praduhangdum Thai native chicken raised in conventional floor pen (A) and free-range conditions (B). Conventionally raised chickens were housed in a naturally ventilated shed, while free-range chickens had access to an outdoor environment. The free-range area was primarily dirt with naturally occurring grass and small herbaceous plants, enclosed by a wired fence and net. All animals had access to water, complete feed, and regular health inspections. Transcriptome analysis was performed using the RNA extracted from the blood of 6-month-old chickens in both groups. The overall RNA sequencing read mapping ratio is shown (C).

Table 1. The list of top 30 up-regulated Thai Praduhangdum chickens genes raised under a free-range system and a conventional floor pen system identified by transcriptome analysis

Transcript ID	Gene Symbol	Description	Gene biotype	Free-range/conventional fold-change
NM_001301787, XM_040655089, XM_040655090	OTX5	Orthodenticle-related homeobox 5	Protein coding	21.256.899
XR_005840870	LOC121107182	Uncharacterized LOC121107182	lncRNA	16.966.648
XM_040648898	LOC121106975	C-type lectin domain family 2 member B-like	Protein coding	10.232.319
NM_204955, XM_015291978, XM_015291979	ACAN	Aggrecan	Protein coding	7.283.499
XM_040653985	LOC107049489	Leukocyte immunoglobulin-like receptor subfamily A member 2	Protein coding	7.258.002
XM_015278992, XM_015278993, XM_015278995	FAM189A1	Family with sequence similarity 189 member A1, transcript variant X8	Protein coding	7.236.052
XM_040653991	LOC107049796	Platelet glycoprotein VI-like	Protein coding	6.779.064
XM_004944808, XM_025154911	ATP10B	Atpase phospholipid transporting 10B (putative), transcript variant X2	Protein coding	6.206.068
XR_005858584	LOC121110282	Uncharacterized LOC121110282	lncRNA	5.957.987
XM_004947560, XM_425760	STC1	Stanniocalcin 1, transcript variant X1	Protein coding	5.182.148
XR_003071476	LOC112530123	Uncharacterized LOC112530123, transcript variant X1	lncRNA	5.103.768
XM_015280699	CR1	Complement c3b/C4b receptor 1 (Knops blood group)	Protein coding	5.078.917
XM_025143258	CDON	Cell adhesion associated, oncogene regulated, transcript variant X1	Protein coding	4.822.762
NM_001001754, XM_015282913, XM_015282914	LOC414835	Chz-cadherin	Protein coding	4.811.930
XM_015298676	CFAP45	Cilia and flagella associated protein 45	Protein coding	4.735.108
XR_005855761	LOC121109763	Uncharacterized LOC121109763, transcript variant X1	lncRNA	4.733.088
XM_004940761, XM_004940762, XM_025150362	CETN1L	Centrin 1-like, transcript variant X5	Protein coding	4.664.924
XM_426171	SLC35D3	Solute carrier family 35 member D3	Protein coding	4.547.081
XR_005840222, XR_005840223	LOC121106965	Uncharacterized LOC121106965, transcript variant X1	lncRNA	4.527.153
XM_015298463, XM_040652517, XM_040652518	LOC101750908	T-lymphocyte surface antigen Ly-9-like, transcript variant X2	Protein coding	4.451.296
NM_001389721, XM_040649099	C17H9ORF172	Chromosome 17 open reading frame, human c9orf172	Protein coding	4.389.634
XM_015297825, XM_040651777, XM_040651778	CSMD2	CUB and Sushi multiple domains 2, transcript variant X2	Protein coding	4.358.111
XM_001234025	PODXL2	Podocalyxin like 2	Protein coding	4.335.057
XM_040656141, XM_416268	C22orf23	C22orf23 homolog, transcript variant X2	Protein coding	4.261.318
XM_004948336, XM_025143601, XM_025143602	LOC425662	Methanethiol oxidase-like, transcript variant X1	Protein coding	4.142.942
XR_001465799	LOC101750502	Uncharacterized LOC101750502	lncRNA	4.106.257
NM_205209	SLC2A1	Solute carrier family 2 member 1	Protein coding	4.100.535
XM_040701938	LOC121113280	Scavenger receptor cysteine-rich type 1 protein M130-like	Protein coding	4.074.822
XM_040705963	C3orf33	Chromosome 3 open reading frame 33	Protein coding	4.033.723
XR_005858901, XR_005858902, XR_005858903	LOC121110450	Uncharacterized LOC121110450, transcript variant X1	lncRNA	3.942.512

real-time PCR analysis to independently test the differences in gene expression levels compared to the reference 28S rRNA housekeeping gene (Bhanja *et al*., 2014) (Table 3). The expression of each gene generally followed the same trend as the transcriptome results. The down-regulated genes were expressed at lower levels in chickens raised in the free range compared to the floor pen, and the up-regulated genes were expressed at higher levels in chickens raised in the free range than those in the floor pen. However, the expression differences were not found to be statistically significant ($p>0.05$) (Figure 4).

Difference in Meat Sensory Evaluation Score

To determine whether the floor pen and free-range growth conditions affected the meat of the resulting chicken, sensory evaluation was done for roasted breast and leg meat in terms of aroma, flavor, firmness, tenderness, juiciness, and overall acceptability. The evaluation score was given using the hedonic scale (Cheng *et al*., 2008) from 1 (extremely dislike) to 5 (extremely like). The overall score was over 2 in all categories, with the lowest score being 2.42 for the juiciness of chicken breast meat from chickens raised under conventional floor pen conditions. The highest score was 3.98 for the overall satisfaction of

Table 2. The list of top 30 down-regulated Thai Praduhangdum chickens genes raised under a free-range system and a conventional floor pen system identified by transcriptome analysis

Transcript ID	Gene Symbol	Description	Gene biotype	Free-range/conventional fold-change
XM_415522, XR_001464480	PAPPA	Pappalysin 1, transcript variant X1	Protein coding	-17.059.235
XM_015277049, XM_040705895, XM_040705896	CLSTN2	Calsyntenin 2, transcript variant X1	Protein coding	-14.875.856
NM_205430, XM_015295857, XM_040669158	EPHA3	EPH receptor A3	Protein coding	-9.252.392
XM_040653183	LOC100859420	SID1 transmembrane family member 2-like	Protein coding	-8.303.889
XM_040702693	LOC121113330	Endogenous retrovirus group K member 8 Gag polyprotein-like	Protein coding	-7.665.751
XR_005857890	LOC121110094	Uncharacterized LOC121110094	lncRNA	-7.331.750
XM_040706196, XM_040706198, XM_040706201	LOC107052456	FH2 domain-containing protein 1-like, transcript variant X2	Protein coding	-6.752.535
XM_015280937	CNGA4	Cyclic nucleotide gated channel alpha 4	Protein coding	-6.074.211
XM_040699770	LOC770574	Uncharacterized LOC770574	Protein coding	-5.836.740
XM_025154651	LOC112533348	GRF-interacting factor 1-like	Protein coding	-5.433.123
NM_001030731, XM_015301204, XM_015301418	CD36	CD36 molecule	Protein coding	-5.283.103
NM_204447	FGFBP2	Fibroblast growth factor binding protein 2	Protein coding	-5.204.680
XM_040654400	LOC121107862	T-cell-interacting, activating receptor on myeloid cells protein 1-like, transcript variant X1	Protein coding	-5.187.150
NM_001012295, XM_025151643	PRLHR	Prolactin releasing hormone receptor	Protein coding	-4.831.984
XM_015294504	HS3ST2	Heparan sulfate-glucosamine 3-sulfotransferase 2	Protein coding	-4.481.527
XR_005860675, XR_005860676, XR_005860677	LOC121113299	Uncharacterized LOC121113299, transcript variant X1	lncRNA	-4.086.265
XM_015273518	NR4A1	Nuclear receptor subfamily 4 group A member 1	Protein coding	-4.082.082
XM_015282340, XM_015282341, XM_015282342	PTPN3	Protein tyrosine phosphatase, non-receptor type 3, transcript variant X7	Protein coding	-4.057.964
XM_015299781, XM_015299795, XM_015299796	EVA1C	Eva-1 homolog C, transcript variant X5	Protein coding	-4.014.690
XM_015282405, XM_015282406, XM_015282407	NR4A3	Nuclear receptor subfamily 4 group A member 3, transcript variant X1	Protein coding	-3.980.346
NM_001328490, XM_004942106, XM_004942108	ABCG2	ATP binding cassette subfamily G member 2 (Junior blood group)	Protein coding	-3.979.869
XM_015277814, XM_025146651, XM_040657368	IL1RL2	Interleukin 1 receptor-like 2, transcript variant X7	Protein coding	-3.957.947
XR_005840435, XR_005840436, XR_005840437	LOC121107059	Uncharacterized LOC121107059, transcript variant X1	lncRNA	-3.905.443
XR_005862123	LOC101751200	Uncharacterized LOC101751200	lncRNA	-3.889.497
XR_001467241	LOC101749597	Uncharacterized LOC101749597, transcript variant X1	lncRNA	-3.803.663
XM_040669166	LOC121110514	Uncharacterized LOC121110514	Protein coding	-3.586.025
XM_025148185	LOC112531906	Endogenous retrovirus group K member 10 Gag polyprotein-like	Protein coding	-3.578.625
NM_001318978, NM_001318979, NM_001318980	OPRL1	Opioid related nociceptin receptor 1	Protein coding	-3.516.973
NM_001277619, NM_001277620, NM_001277621	NQO1	NAD(P)H quinone dehydrogenase 1	Protein coding	-3.488.322
XM_040702723, XM_040702726, XM_040702733	CLC2DL5	C-type lectin domain family 2 member D-like 5, transcript variant X1	Protein coding	-3.454.981

leg meat from chicken raised under conventional floor pen conditions (Table 4). There were no statistically significant differences between any pairs of samples (conventional vs. free-range) for all attributes ($p>0.05$), except for the appearance of breast meat, where the free-range chicken had a significantly higher score than the conventional chicken ($p=0.011$).

DISCUSSION

Over the past decades, various methods of gene expression profiling have been widely utilized to examine differences in commercially farmed animals, including chickens raised under different conditions. Our study profiled the gene expression at the genome-wide level and identified gene expression differences due to growing conditions (conventional floor pen vs free-range) in Thai Praduhangdum native chickens. In this study, we identified a set of differentially expressed genes by transcriptome profiling and detected a significant difference in the consumer's sensory evaluation scores of the chicken raised under conventional and free-range conditions.

Although studies had been previously conducted on some breeds of chicken in other countries, such as China and Italy (Xiang *et al*., 2018; Stefanetti *et al*., 2023), to our knowledge, this is the first study focusing on Thai native chicken. This is important because, although

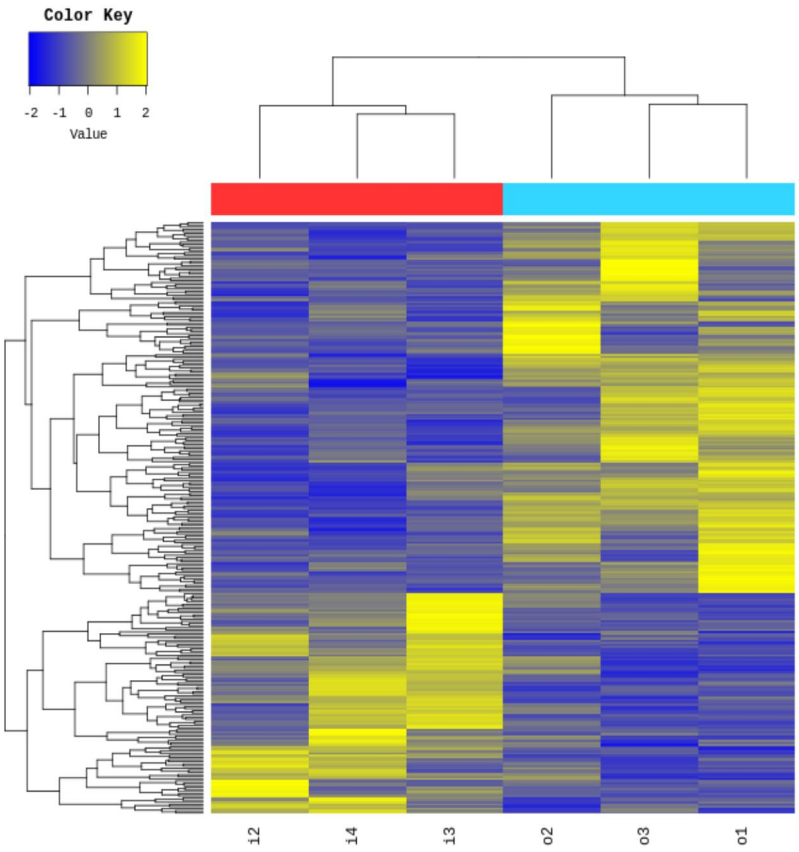

Figure 2. Heatmap from the one-way hierarchical clustering analysis of differentially expressed genes (DEGs) of Thai Praduhangdum chickens raised under a free-range system and a conventional floor pen system. The 278 DEGs were clustered into two distinct groups (control, ■= conventional, treatment, ■= free-range). For each group, the down-regulated genes are shown in blue (negative value) and the up-regulated genes are in yellow (positive value).

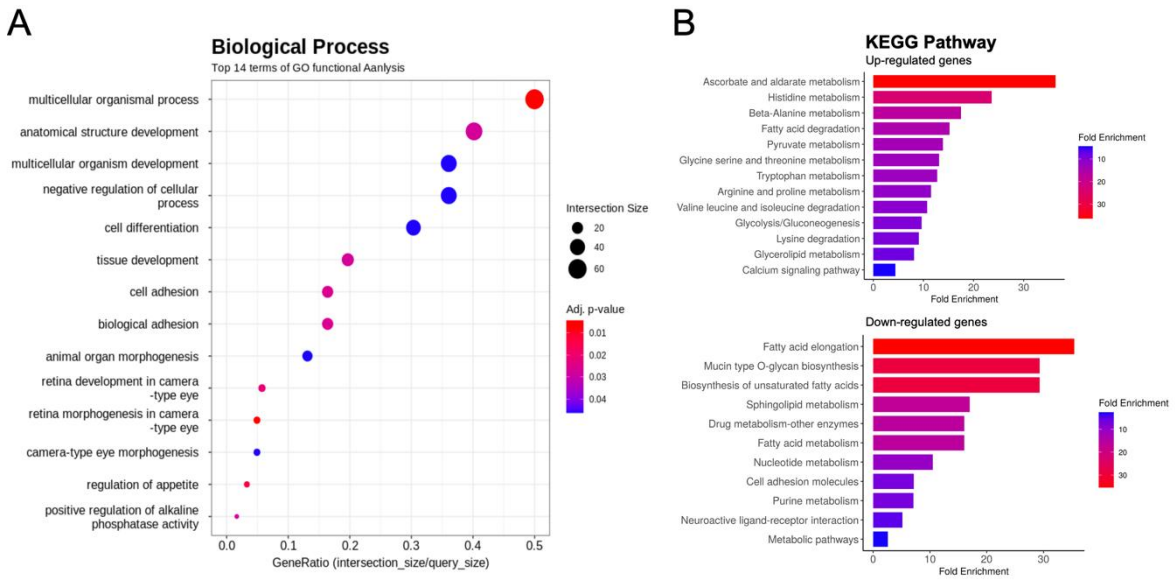

Figure 3. Bioinformatic analysis results from the transcriptome analysis of Thai Praduhangdum chickens raised under a free-range system and a conventional floor pen system. (A) Gene ontology (GO) functional analysis for terms related to biological processes (BP), performed using transcriptome-identified differentially expressed genes (DEGs). The top 14 enriched biological terms from the list of DEGs are shown. (B) KEGG pathway analysis performed using the up-regulated and down-regulated genes from the list of DEGs.

various native chicken breeds have been shown to thrive better in the free-range condition than in floor pen condition, each breed of native chicken from its region likely possesses unique genetics that facilitated it to adapt to the specific local environment of its region (Mekchay *et al*., 2014; Teinlek *et al*., 2018). Additionally, population genetics studies have shown that the wild RJF, the ancestor of the domestic chicken, originated in

Table 3. The list of primers used for quantitative RT-PCR of Thai Praduhangdum chickens genes raised under a free-range system and a conventional floor pen system

Gene	Transcript ID	Forward primer sequence	Reverse primer sequence	Ta (°C)	Product size (bp)
SIK1	NM_204682	5′ CGA AGA AGG GTT AGA GCT GGG 3′	5′ TTT TAT GGC AAC CTG CGT CT 3′	60	300
HPRT1	NM_204848	5′ CTC TGT CCG CTC GTC GC 3′	5′ TGCCAG TCT CTC TGT CCT GT 3′	60	205
CLC2DL4	XM_046909565	5′ CTC TTA CCG ACA CGG GCT 3′	5′ GCC CCA TTG GGA TTG TTG GT 3′	55	480
NR4A1	XM_015273518	5′ CCG GAA CGC GGC CAT 3′	5′ CAC CGT CGT AAT GGG GTG AA 3′	50	455
ABCG2	NM_001397253	5′ GCA CAC AGC CTC GGA GTA GA 3′	5′ TCC ACT GGA CTG CTT AAC GG 3′	60	253
GRIN3B	XM_015299988	5′ TTC CAC TTC CAC ATG GAC CG 3′	5′ GGT GTT GGA TAG GAA CCG GG 3′	59	597
DNAJC17	XM_040701136	5′ CGC GGG CCC AAC AGG 3′	5′ TTG TCA TAT GCC GCC CTT GC 3′	60	266
CEP164	XM_040652181	5′ GAG GAA GCT GGC AGT CTG AG 3′	5′ GGA AAG CCA AGT CCA CAT GC 3′	60	556
LOC101751752	XM_040701751	5′ TCA ACT GCT CAG GTG TCGC3′	5′ AGA CAA CAG TGG CAT CCT GC 3′	60	453
LOC107051846	XM_015277004	5′ CCT CCT TCG AGA ACG TGT GG 3′	5′ TGT ATA GGT CAT CAT TCT TCC CG 3′	58	309
28S	JN639848	5′ CAG GTG CAG ATC TTG GTG GTA GTA 3′	5′ GCT CCC GCT GGC TTC TCC 3′	58	273

Note: Ta= annealing temperature.

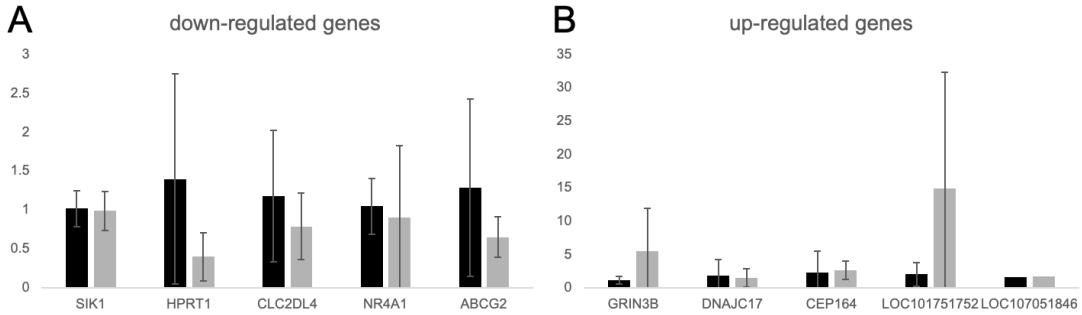

Figure 4. Real-time PCR quantification of candidate differentially-expressed genes (DEGs) of Thai Praduhangdum chickens raised under a free-range system and a conventional floor pen system identified by transcriptome. Five down-regulated (A) and five up-regulated (B) genes were tested by RT-PCR, relative to the 28S rRNA housekeeping gene, and the expressions are shown between native chickens raised in conventional floor pen (black bars, ■) and free-range (grey bars, ■) conditions. Error bars indicate standard deviation.

Table 4. The sensory evaluation score meat of Thai Praduhangdum chickens raised under a free-range system and a conventional floor pen system

Attributes	Breast meat			Leg meat		
	Conventional	Free-range	p-value	Conventional	Free-range	p-value
Appearance	3.42 ± 0.673	3.68 ± 0.768	0.011	3.72 ± 0.834	3.6 ± 0.857	0.348
Aroma/Smell	3.44 ±0.884	3.48 ±0.974	0.728	3.56 ± 0.733	3.58 ± 0.731	0.855
Taste/Flavor	3.22 ± 0.910	3.28 ± 0.970	0.595	3.62 ± 0.945	3.52 ± 0.953	0.498
Tenderness	2.7 ± 1.035	2.88 ± 0.982	0.202	3.84 ± 0.766	3.80 ± 0.808	0.761
Firmness	3.68 ± 0.978	3.54 ± 0.838	0.197	3.64 ± 0.851	3.72 ± 0.730	0.533
Juiciness	2.42 0.971	2.6 ± 0.948	0.182	3.78 ± 0.954	3.70 ± 0.814	0.591
Overall satisfaction	3.24 ± 0.771	3.24 ± 0.960	1	3.98 ± 0.750	3.9 ± 0.684	0.471

Southeast Asia, including Thailand. Thai native chicken breeds likely still possess genetic variations that are not found elsewhere in modern commercial breeds, making them valuable for preservation in future breeding programs (Mekchay *et al*., 2014; Fallahsharoudi *et al*., 2015; Yaemkong *et al*., 2024).

In terms of production performance, we found that the chicken raised in a free-range condition yielded a slightly lower production performance, as indicated by the higher feed conversion ratio. This is consistent with previous studies, which demonstrated that having access to outdoor free-range conditions negatively affected the production performance of the chicken but positively affected the resulting meat characteristics (Stadig *et al*., 2016; Xiang *et al*., 2018). This is likely due to the free-range chicken having access to more space and engaging in energy-consuming activities such as walking, running, and scratching.

Our gene ontology enrichment analysis for biological terms revealed the top 5-10 identified terms (Figure 3) typically associated with chicken growth and development, such as multicellular organism development, cell differentiation, adhesion, signaling, and organ morphogenesis. This has somewhat similar results and overlaps the previous study done in Beijing You chicken. However, our GO enrichment analysis did not include the immune response, such as lymphocyte and leukocyte differentiation. Interestingly, a few GO BP enrichment terms in the lower part of the list showed biological pathways that are somewhat unexpected, such as retina development in camera-type eyes, retina morphogenesis, and regulation of appetite (Figure

3). These genes and pathways may be functionally related to the condition in which the chicken was raised (conventional vs free-range). Although surprising, one could further hypothesize that the retinal tissue developmental process, such as cellular differentiation and neurogenesis, may be affected by the chicken environmental housing condition, which influences gene expression, potentially via epigenetic regulation of key developmental genes, as shown previously in model organisms such as mouse and zebrafish (Seritrakul & Gross, 2019). Further investigation into the biological functions of these genes and pathways could reveal their roles in chicken growth and development in different rearing conditions.

In this study, the transcriptome profiling identified several genes associated with animal growth and development that are differentially expressed. A previous study identified a family of methyltransferase genes responsible for skeletal muscle development in chickens (Yang *et al*., 2019). Notably, the methyltransferase-like 21C (METTL21C) showed an increase in the Lueyang chicken raised in free-range condition compared to the caged condition. Similarly, a study conducted on Jinghai yellow native chicken also identified several genes associated with animal development and signaling pathways by transcriptome analysis, comparing the leg meat of slow-growing and fast-growing chickens (Wu *et al*., 2018). However, we did not detect the same gene expression difference in Thai native chicken in our experiments. This may be due to the difference in the chicken breeds, experimental conditions (caged, floor pen, free-range), or the difference in the blood sample and skeletal muscle tissue sampled for the gene expression analysis.

In the sensory panel evaluation, our results showed that there was largely no difference in the consumer's preference for either the leg or breast meat of chicken raised in the conventional floor pen or free-range condition, as shown by the sensory evaluation score. This indicates that the consumers are likely unable to identify the difference between the chicken meat raised in the floor pen and the free-range condition. However, the appearance score of the free-range breast meat was statistically higher, which may be due to the higher physical activities of the free-range chicken, resulting in a more attractive appearance of the meat. Our findings differ from a previous study on broiler chicken, in which the meat characteristics clearly showed a difference between free-range and industrially-raised chicken (Da Silva *et al*., 2017). Although our results showed that the meat from chicken raised in both systems is largely similar in terms of the sensory evaluation score, this does not rule out the possibility that both are different in some aspects. Alternative cooking methods that involve longer exposure to heat and water, such as stewing or braising, could bring out more flavor and are more suitable for free-range chicken meat.

In addition to a wider space, the chickens raised in the free-range condition also had access to a wider variety of natural herbaceous plants, invertebrates, and soil microorganisms. This was shown in a previous study that identified differences in production quality and gut microbial composition between chicken raised under caged and free-range conditions (Chen *et al*., 2018). Although in this study we did not strictly focus on the difference between dietary choices, as both groups of chicken were fed the same complete diet *ad libitum*, we acknowledge that the wider dietary choices that inevitably come with the free-range condition may affect the difference in their gene expression. However, the difference in chicken gut microbiome is beyond the scope of this study.

To independently verify the gene expression differences between chickens raised under floor pen and free-range conditions, we performed quantitative RT-PCR using primers for 5 genes from each of the DEGs list. Although our RT-PCR results did not show a statistically significant difference when quantified against the housekeeping gene using the delta-delta Ct method (Livak & Schmittgen, 2001), the overall expression levels showed agreement with the direction of the expression difference identified by the transcriptome analysis. For instance, among the down-regulated genes, HPRT1 and CLC2DL4 showed lower expression in the chicken raised under free-range than floor pen condition, and for up-regulated genes, GRIN3B and LOC101751752 showed higher expression in the chicken raised under free-range than floor pen condition. This may be because transcriptome profiling and RT-PCR methods are fundamentally different in terms of biochemical reactions and methods of detection. Because transcriptome profiling utilizes next-generation sequencing (NGS) technology, it could be more sensitive in detecting small differences in gene expression than RT-PCR. Unlike the previous methods of gene expression quantification, such as oligo probe-based microarrays, NGS-based transcriptome profiling does not rely on probes and has been shown to be more reliable; thus, RT-PCR verification may not be necessary (Coenye, 2021). Although the number of chickens raised and sampled in this study was limited due to the cost of transcriptome sequencing, the genes and pathways identified here can provide a starting point for future functional tests and investigation into the relationship between chicken welfare and their physiological responses at a molecular level within the chicken body.

CONCLUSION

This study identified differentially expressed genes as a result of the free-range and conventional farming conditions in Praduhangdum Thai native chicken using transcriptome analysis. Many of these genes are associated with signaling pathways related to chicken growth and development. Additionally, sensory panel evaluation of the resulting meat showed that consumers preferred the appearance of the breast meat from the free-range chicken. The candidate genes identified here can be utilized as molecular markers for assessing overall chicken welfare conditions in the animal industry.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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