



Full-Length Article

Comparative transcriptomic and meat quality responses to electrical and controlled atmosphere stunning in broiler chickens

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ARTICLE INFO

Keywords:

Broiler
Stunning
Welfare
Transcriptome
Meat quality

ABSTRACT

In the poultry industry, the slaughter process poses significant animal welfare concerns due to the substantial stress it induces in broilers. Electrical stunning (ES) and controlled atmosphere stunning (CAS) are commonly used methods to render broilers unconscious before slaughter, but both have welfare implications. This study compares ES and CAS by analyzing stress-related gene expression profiles using RNA sequencing (RNA-seq) and evaluating meat quality parameters. The results reveal distinct molecular responses to each method. Meat quality analysis shows that ES leads to higher redness and fat content, while CAS results in greater moisture content. ES induces physiological stress by causing tissue damage and activating inflammatory pathways, whereas CAS does so through hypercapnia and immune response downregulation, suggesting lower oxidative stress and reduced physiological burden. Future studies should integrate behavioral and neurophysiological assessments, tissue-specific histology, biochemical markers, and comparative evaluations of inert-gas stunning methods (e.g., argon, nitrogen) to better contextualize welfare implications. These findings provide new insights into the molecular and physiological impacts of stunning methods, contributing to the development of more humane poultry production practices.

Introduction

Global poultry meat production has surged to approximately 142 million tons annually, driven by increasing consumption rates (FAO, 2022). Broilers are among the most productive livestock due to their short production cycles and cost efficiency (Kranjac et al., 2019). To maximize production, the broiler industry relies on intensive farming methods, including high-density stocking, extensive antibiotic use, and automated slaughter processes (Ward, 1999). However, these practices expose broilers to various stressors that negatively impact their welfare. Since the late 1990s, growing awareness of the ethical concerns surrounding intensive farming has heightened interest in animal welfare (Fraser, 2008). The slaughter process is a critical stage in poultry production, as it subjects broilers to significant stress and potential pain, making it a key factor in assessing animal welfare (Grandin, 1997; Gregory and Grandin, 1998). European regulations mandate that animals be rendered unconscious before slaughter to minimize suffering

(Directive, 1993). The physiological effects of slaughter include elevated cortisol levels, increased heart rates, and metabolic changes (Debut et al., 2005; Terlouw et al., 2012). Broilers may experience pain and fear during this phase, leading to physiological stress responses that affect their welfare (Grandin, 1980). The reactions to stress in broilers significantly influence meat quality attributes, including color, pH, and water-holding capacity (WHC), which serve as indicators of stress levels during slaughter (Grandin, 1980; Health and Welfare, 2014).

Electrical stunning (ES) is the primary method used in commercial poultry farming to render broilers unconscious. This technique disrupts normal conscious activity by passing an electric current through the brain, thereby depolarizing and hyperpolarizing neurons (Terlouw et al., 2016). Despite its efficiency, ES raises significant welfare concerns. The intense muscle contractions induced by electric shock can cause pain, distress, and physical injuries such as fractures and hemorrhages (Bedanova et al., 2007). Additionally, suspending birds in shackles before stunning induces considerable stress and discomfort, as

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indicated by vocalizations and distress behaviors (Gregory and Wilkins, 1989). The effectiveness of ES also varies due to differences in electrical resistance among individual chickens, leading to inconsistent stunning outcomes (Smaldone et al., 2021; Woolley et al., 1986). Some birds may receive excessive shocks, causing unnecessary pain, while others may remain conscious at the time of slaughter (Smaldone et al., 2021; Woolley et al., 1986). These challenges underscore the urgent need for improved stunning methods to reduce stress and enhance poultry welfare during slaughter.

Controlled atmosphere stunning (CAS) has been proposed and implemented as an alternative to ES, offering the potential for improved animal welfare outcomes. CAS induces unconsciousness in poultry by causing hypercapnia and hypoxia through controlled exposure to gas mixtures, primarily carbon dioxide (CO₂) (Terlouw et al., 2016). This process suppresses neural activity and induces unconsciousness by acidifying brain cells (Martoft et al., 2002; Rodríguez et al., 2008). CAS is considered a more humane method, as it eliminates the need for live shackling prior to stunning. Studies have suggested that CAS reduces stress indicators, including cortisol levels, pH 45 minutes postmortem, and WHC (Raj et al., 1990). However, poultry possess chemical receptors that are highly sensitive to CO₂, which can trigger a painful response before they lose consciousness (Rucinke et al., 2024). Elevated CO₂ levels raise concerns regarding animal welfare, as they may induce distress behaviors such as head shaking, panting, and gasping (McKeegan et al., 2006). These findings underscore the need to evaluate stunning methods to determine the most humane approach for poultry slaughter. Among physiological indicators, blood cortisol concentration is commonly used as a biomarker of slaughter-induced stress in poultry (Terlouw et al., 2008; Zulkifli et al., 2019). However, interpreting cortisol levels in this context is challenging, as various pre-slaughter stressors, such as transport and handling, can influence cortisol concentrations (Ali et al., 2008; Nicol and Scott, 1990). Although multiple studies have investigated the relationship between cortisol levels and molecular stress responses during stunning, an essential factor in assessing animal welfare, these approaches have significant limitations due to the influence of pre-slaughter conditions. Therefore, alternative parameters are needed to assess stress responses to different stunning methods more accurately.

Understanding the molecular mechanisms underlying slaughter stress induced by different stunning methods is crucial for a comprehensive assessment of their welfare implications. Transcriptome analysis offers a powerful approach to identifying stress-related genes and elucidating the molecular pathways involved in physiological responses to stunning. RNA sequencing (RNA-seq), a next-generation sequencing (NGS) technology, enables detailed profiling of gene expression and provides insights into the molecular mechanisms activated during stress responses (Wang et al., 2009). This study compares ES and CAS by analyzing stress-related indicators to determine which method is more favorable for animal welfare. To this end, the blood transcriptome of broilers was examined both before and after slaughter (Mohr and Liew, 2007). This study aims to identify key stress-related genes and pathways associated with each stunning method while also evaluating meat quality parameters to assess the physiological effects of stress. By integrating molecular and physiological data, this study seeks to provide insights into the mechanisms through which different stunning methods impact broilers and to identify the approach that minimizes stress most effectively.

Materials and methods

Animals and sample collection

A total of 80 Cobb-500 commercial broilers were obtained from two nearby farms. Prior to the experiment, the broilers were housed in pens with *ad libitum* access to water and feed and allowed to rest for 12 hours. They were then fasted overnight prior to slaughter. After lairage, the

broilers were assigned to either the ES or CAS group ($n = 40$ per group), with broilers from one farm undergoing ES and those from the other farm undergoing CAS. The ES group was stunned using a water bath stunner that delivered a constant current of 120 mA (50 Hz sinusoidal waveform) for 4 to 5 seconds. The CAS group was rendered unconscious in a specially designed gas chamber with 80 % CO₂ (Supreme Thermal Instrument; length: 220 cm, width: 100 cm, height: 135 cm; Daegu, Korea). Following stunning, all broilers were suspended, and the carotid artery was severed with a knife, followed by a two-minute bleeding period (Fuseini et al., 2016). Whole blood samples were collected from all broilers immediately before and immediately after stunning, as ES induces a near-instantaneous loss of consciousness, whereas CAS proceeds more gradually. To minimize potential confounding effects arising from farm-specific differences, such as variations in feed, environment, and management practices, we paired each treatment group (ES or CAS) with its own pre-stunning control group from the same farm (Before-ES and Before-CAS, respectively). Consequently, transcriptomic comparisons were conducted within the same farm (for example, After-ES vs. Before-ES and After-CAS vs. Before-CAS) rather than across different farms. Both pre-stunning groups were sampled under similar untreated conditions. We intentionally avoided cross-farm comparisons, such as "After-ES vs. Before-CAS," to reduce the risk of inter-farm variability affecting the interpretation of treatment-specific effects. Carcasses were stored overnight at 2°C in a cold room. After 18 hours of chilling, all carcass samples were transported to the laboratory, where breast muscle samples were collected for meat quality analysis.

Meat quality

The methods used to assess meat quality were adapted from Alam et al. (Alam et al., 2024). To determine the pH of raw meat, 5 g of each sample was blended with 50 mL of distilled water using a hand blender. The pH of the resulting solution was measured using a digital pH meter (FP 20, Mettler Toledo, Greifensee, Switzerland). Each chicken breast sample was measured four times.

The surface color of raw chicken breast meat was assessed using a chroma meter (CR-400, Minolta Camera, Osaka, Japan) after allowing the meat to bloom at room temperature for approximately 40 minutes. The chroma meter was calibrated using a white plate with the following values: $Y = 86.32$, $X = 0.3165$, and $y = 0.3242$. Color values were recorded at eight different locations on both the surface and the bone side of the meat. Results were reported according to the Commission Internationale de l'Éclairage (CIE) system, including L^* (lightness), a^* (redness-greenness), and b^* (yellowness-blueness) values (Roufs, 1978).

The proximate composition of raw chicken breast meat was analyzed using a Food Scan Lab (Foss Tecator Hillerød, Denmark). Approximately 100 g of the blended sample was measured in triplicate. The WHC was determined by centrifuging 0.5 g of finely ground meat in an ultracentrifuge tube at $470 \times g$ and 4°C for 10 minutes, following sample heating at 80°C for 20 minutes. After centrifugation, the sample was left at room temperature for 10 minutes before weighing. WHC was then calculated using a standard formula.

Cooking loss (CL) was assessed by weighing raw chicken breast samples, sealing them in heat-resistant plastic bags, and heating them in a water bath until their core temperature reached 70°C. After cooking, the samples were cooled in ice water for 30 minutes, blotted dry, and reweighed to determine CL. Warner-Bratzler shear force (WBSF) was measured using an Instron Universal Testing Machine (5543, Instron Corp., USA). For each chicken breast sample, five cylindrical specimens (length: 2.0–2.5 cm, diameter: 1.25 cm) were collected along the muscle fiber direction after CL measurement.

Statistical Analysis

Meat quality data were analyzed using SAS version 9.4 (SAS Inst., Inc., Cary, NC) with the following model:

$$y = \mu + \beta_1 + \beta_2 + \epsilon$$

where y represents each meat quality parameter (e.g., pH, L^* , a^* , b^* , moisture, protein, fat, ash, collagen, WHC, cooking loss, and shear force), μ is the intercept, β_1 is the coefficient for body weight, and β_2 is the coefficient for stunning method. The error term (ϵ) accounts for unexplained residual variation in meat quality parameters due to factors not included in the model. The stunning method was treated as a fixed effect, as it was the primary experimental treatment, and its impact on meat quality parameters was assessed using the General Linear Model (GLM) procedure. Body weight was included as a covariate, as it can influence meat quality traits (Llonch et al., 2012). The mean, standard deviation, and P -value were calculated for all treatments, with statistical significance set at $P < 0.05$.

RNA extraction, library construction, and sequencing

Total RNA was extracted from blood samples using TRIzol reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions (Simms et al., 1993). RNA concentration was measured using Quant-IT RiboGreen (Invitrogen, Inc., Waltham, MA, USA). RNA integrity was assessed using the TapeStation RNA ScreenTape system (Agilent, Santa Clara, CA, USA). Only high-quality RNA samples were used for cDNA library preparation. The constructed libraries were analyzed using an Illumina HiSeq X Ten instrument (Illumina, Inc., San Diego, CA, USA). Sequencing was performed with a 150-base pair paired-end method.

NGS data processing and differentially expressed gene analyses

The quality of raw read data for each sample was assessed using FastQC v.0.11.8 (Andrews, 2010). Reads were trimmed for adaptors using Trimmomatic v.0.39, based on the quality results (Bolger et al., 2014). After trimming, the reads were rechecked with FastQC and mapped to the GRCg7b reference genome (GCA_016699485.1), sourced from the Ensembl genome browser (https://www.ensembl.org/Gallus_gallus/), using the default settings of HISAT2 v.2.1.0 (Kim et al., 2019). Raw counts for each library were calculated based on exons in the *Gallus gallus* GTF v111 (Ensembl) annotation file using the featureCounts tool from the Subread package v2.0.0 (Liao et al., 2014). Differentially expressed gene (DEG) analyses were conducted using the 'edgeR' package v4.0.16 of Bioconductor (Robinson et al., 2010), and the raw counts were normalized using the trimmed mean of M-value (TMM) method (Robinson and Oshlack, 2010). DEGs were identified with a false discovery rate (FDR) < 0.05 and absolute $\log_2$ fold change ($\log_2FC$) ≥ 1 . Multidimensional scaling (MDS) was performed using the 'limma' R package to visualize the relationships between samples (Smyth, 2005), after which the results were plotted using 'ggplot2' (Villanueva, 2019).

Functional enrichment analyses and DEG clustering

Functional annotation was performed to identify the molecular mechanisms associated with the DEGs. The DEGs were annotated with Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 (Huang et al., 2009). GO terms were classified into biological process (BP), cellular component (CC), and molecular function (MF) categories. Enriched GO terms were grouped with related terms and visualized using treemaps generated with REVIGO (Supek et al., 2011). The KEGG enrichment analyses were represented by $-\log_{10}$ P -value and fold enrichment. All data used in enrichment analyses were annotated for *Gallus gallus*. Modulations in the genes involved in the selected representative KEGG pathway were validated using the 'clusterProfiler' package in the R (Yu et al., 2012).

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was conducted using KEGG database gene sets with GSEA v4.3.3 software (Subramanian et al., 2005). The analysis utilized TMM-normalized counts for each comparison group, ranking gene sets based on their normalized enrichment scores (NES). Significant KEGG pathways ($P < 0.05$) were visualized as enrichment maps using Cytoscape v3.10.1 (Shannon et al., 2003), where connections between gene sets indicated their functional similarities.

Results

Descriptive statistics for meat quality

The descriptive statistics for the entire sample are summarized in Table 1, which presents the mean, maximum, minimum, and standard deviation (SD) values of welfare-related traits that serve as indirect indicators of stress in broilers. Table 2 displays the means and SD for each group (ES and CAS), while the results of the GLM analysis, which includes body weight as a covariate, are shown in Table 3.

A comparison between the ES and CAS groups revealed statistically significant differences in a^* , moisture, and fat content. The mean values for each stunning method were as follows: a^* (ES: 3.66, CAS: 1.77, $P < 0.01$), moisture (ES: 74.88, CAS: 75.87, $P < 0.01$), and fat (ES: 1.59, CAS: 0.97, $P < 0.01$). Among these traits, the ES group exhibited significantly higher a^* and fat levels, whereas the CAS group had a significantly higher moisture content. No statistically significant differences were observed for the other measured traits between the two stunning methods.

RNA-seq data processing and DEG profiling

Sequencing data were obtained from blood samples collected under different stunning conditions (i.e., ES and CAS; $n = 4$) and before stunning. An average of 22,297,089 paired-end reads were generated from 16 blood samples. After quality filtering, 21,640,643 reads remained, with 3.04 % of raw reads being trimmed out. The average overall alignment rate was 88.80 %, and the uniquely mapped rate was 81.56 % (Table 4).

MDS plots revealed clustering patterns for each group, indicating clear separation and similarity among the samples (Fig. 1A). DEGs were identified by comparing expression levels between groups. A total of 139 DEGs were identified for the ES vs. Control comparison (116 upregulated, 23 downregulated) and 140 for the CAS vs. Control comparison (29 upregulated, 111 downregulated) (Fig. 1B).

Table 1
Descriptive statistics of the phenotype data.

Factors	Means (n = 80)	Max ¹ (n = 80)	Min ² (n = 80)	SD ³ (n = 80)
Body weight (g)	915.45	1080.87	742.99	98.21
pH	5.97	6.39	5.48	0.23
L^*	55.39	63.28	48.90	2.87
a^*	2.72	5.26	0.13	1.05
b^*	5.06	9.04	0.79	1.63
Moisture (%)	75.37	76.93	73.94	0.60
Protein (%)	22.77	24.27	21.17	0.59
Fat (%)	1.28	2.18	0.66	0.32
Ash (%)	1.18	2.93	0.85	0.39
Collagen (%)	1.23	1.88	0.67	0.23
WHC (%)	42.28	47.77	29.98	2.67
Cooking loss (%)	11.89	20.23	6.89	2.57
Shear force (kg/cm ²)	1.35	2.81	0.72	0.40

¹ : Maximum

² : Minimum

³ : Standard deviation

Table 2
Descriptive statistics of phenotypic traits by stunning method.

Factors	ES (n = 40)				CAS (n = 40)			
	Means	Max ¹	Min ²	SD ³	Means	Max ¹	Min ²	SD ³
Body weight (g)	823.99	929.36	742.99	36.23	1006.92	1080.87	945.45	32.63
pH	5.87	6.37	5.48	0.24	6.07	6.39	5.73	0.16
<i>L</i> [*]	55.26	63.28	48.90	3.12	55.75	62.01	51.21	2.60
<i>a</i> [*]	3.43	5.26	0.77	0.79	2.00	3.59	0.13	0.74
<i>b</i> [*]	5.86	9.04	1.95	1.46	4.25	7.34	0.79	1.37
Moisture (%)	75.21	76.84	73.94	0.67	75.53	76.93	74.47	0.47
Protein (%)	22.78	24.27	21.17	0.66	22.76	24.03	21.50	0.52
Fat (%)	1.35	1.98	0.66	0.35	1.21	2.18	0.72	0.28
Ash (%)	1.33	2.93	0.86	0.50	1.03	1.26	0.85	0.08
Collagen (%)	1.25	1.88	0.67	0.25	1.21	1.66	0.82	0.21
WHC (%)	42.74	47.38	29.98	3.03	41.82	47.77	37.87	2.20
Cooking loss (%)	12.12	20.23	6.89	3.31	11.66	15.05	9.02	1.52
Shear force (kg/cm ²)	1.36	2.54	0.72	0.40	1.35	2.81	0.88	0.41

¹ : Maximum
² : Minimum
³ : Standard deviation

Table 3
Comparison of phenotype means between stunning methods.

Factors	ES (n = 40) ¹	CAS (n = 40)	P-value
pH	5.89 (0.07) ²	6.05 (0.07)	NS ³
<i>L</i> [*]	55.61 (0.98)	55.16 (0.98)	NS
<i>a</i> [*]	3.66 (0.26)	1.77 (0.26)	< 0.01
<i>b</i> [*]	5.74 (0.48)	4.37 (0.48)	NS
Moisture (%)	74.88 (0.19)	75.87 (0.19)	< 0.01
Protein (%)	22.74 (0.20)	22.80 (0.20)	NS
Fat (%)	1.59 (0.10)	0.97 (0.10)	< 0.01
Ash (%)	1.13 (0.12)	1.23 (0.12)	NS
Collagen (%)	1.23 (0.08)	1.23 (0.08)	NS
WHC (%)	43.31 (0.90)	41.25 (0.90)	NS
Cooking loss (%)	11.32 (0.87)	12.46 (0.87)	NS
Shear force (kg/cm ²)	1.36 (0.14)	1.35 (0.14)	NS

¹ : Number of animals
² : Standard error
³ : Non-significance

Functional annotations through GO and KEGG pathway enrichment analysis

Functional enrichment analyses of DEGs in each comparison group, conducted using the DAVID database, identified functional changes induced by different stunning methods. In the after-ES vs. before-ES comparison, significant GO enrichment was observed in upregulated gene pathways within the BP category, such as ‘Positive regulation of

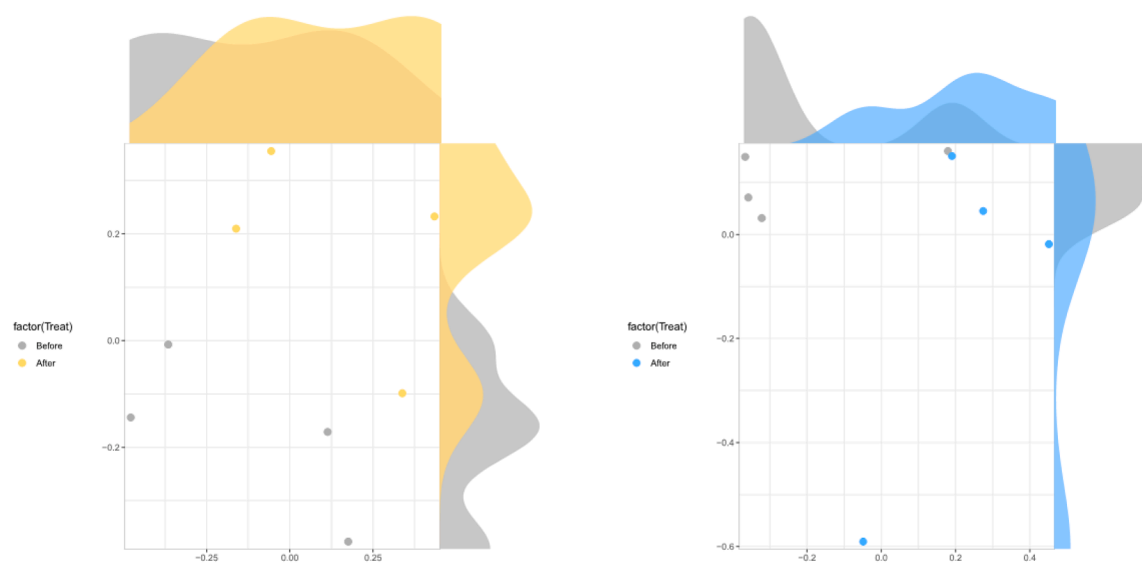
pro-T cell differentiation’ and ‘Cellular response to unfolded protein’ (Fig. 2A), in addition to CCs such as ‘TIM22 mitochondrial import inner membrane insertion complex’ (Fig. 2B) and MFs such as ‘Misfolded protein binding’ and ‘Calcium ion binding’ (Fig. 2C). For the after-CAS vs. before-CAS comparison, significant enrichment downregulated genes were observed in BPs such as ‘Immune response’ and ‘Positive regulation of macrophage cytokine production’ (Fig. 2D); CCs including ‘Lysosome’ and ‘Cell surface’ (Fig. 2E); and MFs including ‘MHC class I protein binding’ and ‘Signaling receptor activity’ (Fig. 2F).

To investigate the molecular pathways affected by different stunning methods, gene expression changes within specific pathways were analyzed. In the after-ES vs. before-ES comparison, significantly enriched KEGG pathways included the ‘Mitogen-activated protein kinase (MAPK) signaling pathway’ (5 upregulated, 1 downregulated), ‘Salmonella infection’ (4 upregulated, 1 downregulated), and ‘Toll-like receptor (TLR) signaling pathway’ (3 upregulated) (Fig. 3A). Stunning with ES resulted in significant upregulation and downregulation of genes within the MAPK and TLR signaling pathways (Fig. 3B and C), with several key genes showing notable expression changes. Additionally, the TLR signaling pathway also showed significant changes in gene expression. For the after-CAS vs. before-CAS comparison, enriched KEGG pathways included ‘Phagosome’ (5 downregulated), ‘Glycerolipid metabolism’ (3 downregulated), and ‘Cell adhesion molecules’ (1 upregulated, 3 downregulated) (Fig. 4A). Under CAS, DEGs were primarily observed in the ‘Cell adhesion molecules’ and ‘Phagosome pathways’ (Fig. 4C). Genes associated with MHC class II receptor

Table 4
Summary of RNA-seq raw reads, trimming, and mapping results.

Group	Sample name	Raw data		After Trimmomatic		Trimming rate (%)	Mapping data	
		reads	%GC	reads	%GC		Uniquely mapped read (%)	Overall alignment rate (%)
Before_ES	BF_Elec_1	20,641,536	43	19,681,650	43	4.65	88.39	93.54
	BF_Elec_5	22,101,688	44	21,275,692	44	3.74	88.12	93.07
	BF_Elec_6	23,430,573	44	22,495,975	44	3.09	88.14	93.26
	BF_Elec_9	23,191,359	44	22,336,634	44	3.69	87.77	93.12
After_ES	AT_Elec_10	25,290,938	44	24,286,453	44	3.97	88.04	93.12
	AT_Elec_2	20,023,267	45	19,332,0361	45	3.45	85.57	91.23
	AT_Elec_5	23,701,663	45	22,825,636	45	3.70	87.09	92.50
	AT_Elec_9	23,032,793	45	22,216,260	45	3.55	86.34	91.86
Before_CAS	BF_Gas_12	17,915,087	43	17,565,418	43	1.95	76.94	87.15
	BF_Gas_3	21,275,752	44	20,842,630	44	2.04	77.14	86.64
	BF_Gas_5	23,173,757	43	22,722,625	43	1.95	75.68	85.75
	BF_Gas_7	25,448,878	43	24,982,144	43	1.83	73.72	84.23
After_CAS	AT_Gas_10	21,813,104	44	21,379,922	44	2.03	75.09	86.56
	AT_Gas_11	22,385,345	44	21,911,579	44	2.12	76.24	86.96
	AT_Gas_4	20,828,489	44	20,443,672	44	1.85	75.31	85.68
	AT_Gas_9	22,438,590	44	21,951,696	44	2.17	75.37	86.08

A



B

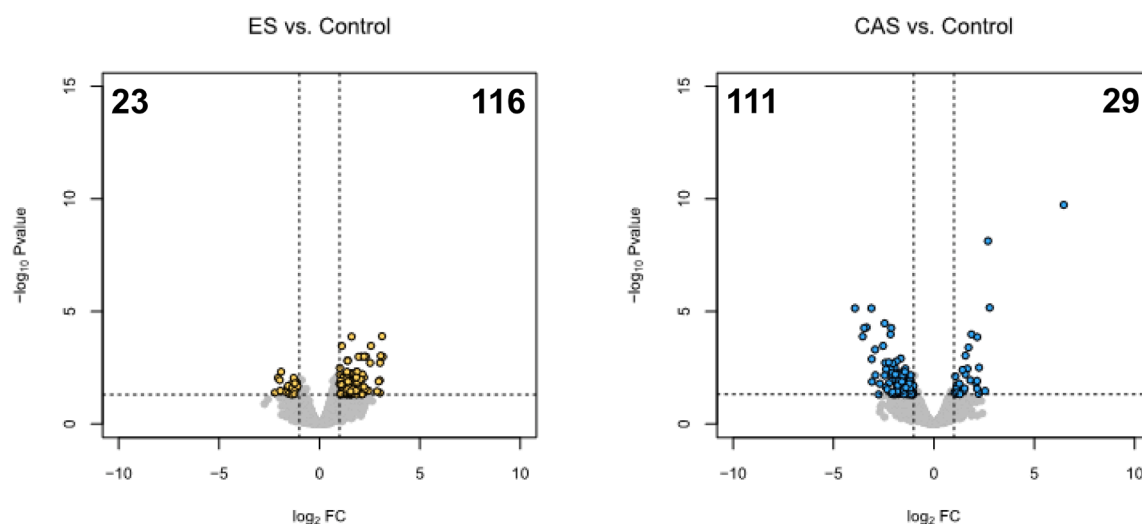


Fig. 1. RNA-seq transcriptional profiling in whole blood samples according to stunning methods. (A) Multidimensional scaling (MDS) plot, where each point represents an individual sample. Yellow (ES) and blue (CAS) dots indicate treatment (post-stunning) groups, while gray dots represent control (pre-stunning) groups. (B) Volcano plots overview of DEGs. The x and y axes display $\log_2$ fold changes (FCs) and $-\log_{10}$ P-values, respectively. Yellow and blue dots represent significantly up- and down-regulated DEGs (false discovery rate < 0.05 and $\log_2$ FC ≥ 1 ; Up, $\log_2$ FC ≤ -1 ; Down). The horizontal line represents FDR < 0.05, and the vertical lines represent absolute $\log_2$ FC ≥ 1 .

binding and TLR binding in these pathways were found to be significantly downregulated.

GSEA

GSEA was performed to assess the effects of the two stunning methods in chickens. The results in Fig. 5A and B reveal distinct pathway enrichment patterns for each method. In the ES group, inflammation-related pathways, including 'Influenza A,' 'Salmonella infection,' 'Herpes simplex virus 1 infection,' and 'TLR signaling pathway,' were significantly enriched (Fig. 5A). Additionally, ES was associated with the enrichment of cellular signaling pathways such as the 'VEGF signaling pathway,' 'C-type lectin receptor signaling pathway,' 'Regulation of

actin cytoskeleton,' and 'MAPK signaling pathway' (Fig. 5A). Similarly, CAS primarily influenced cellular signaling and structural pathways, with significant enrichment observed in 'Focal adhesion,' 'MAPK signaling pathway,' 'VEGF signaling pathway,' and 'Regulation of actin cytoskeleton' (Fig. 5B). Furthermore, pathways involved in GnRH signaling and cell cycle regulation were notably enriched in CAS, suggesting its impact on signaling and cell cycle processes (Fig. 5B). While both stunning methods affected cellular signaling pathways, the direction of gene regulation differed between the two, demonstrating distinct molecular responses to ES and CAS.

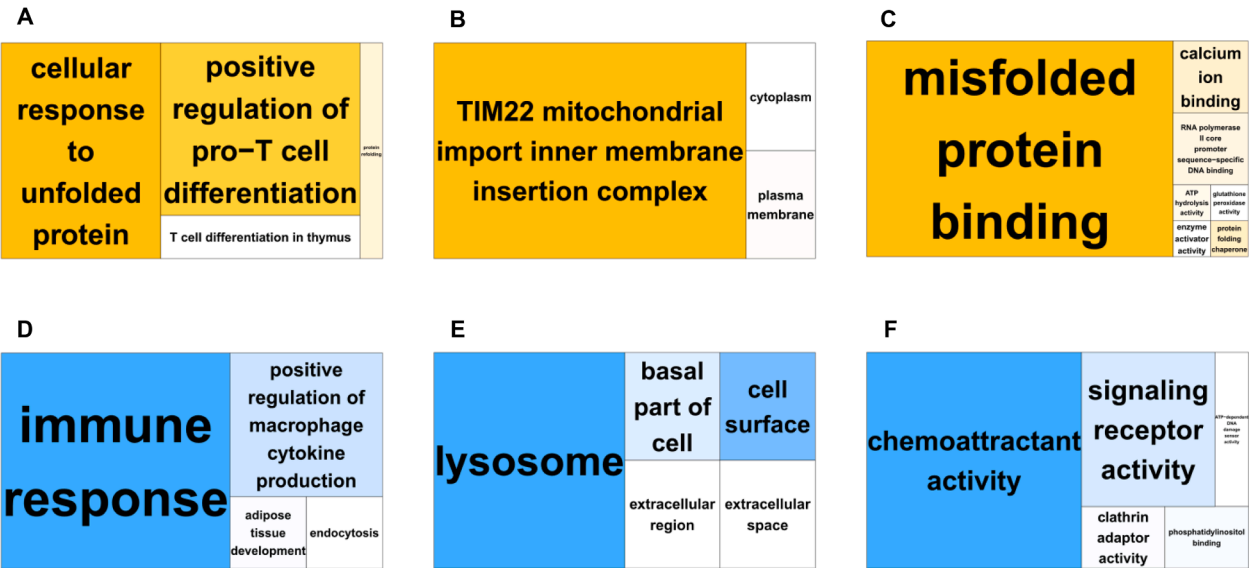


Fig. 2. Functional enrichment of the DEGs. (A), (B), and (C) display GO treemaps for biological process (BP), cellular component (CC), and molecular function (MF) terms in the comparison between post-ES and pre-ES. (D), (E), and (F) show GO treemaps for BP, CC, and MF terms in the comparison between post-CAS and pre-CAS. Each rectangle in the treemaps represents the most significant GO terms, with the size of each rectangle reflecting the number of GO terms included, and the color intensity corresponding to the $-\log_{10} P$ -values.

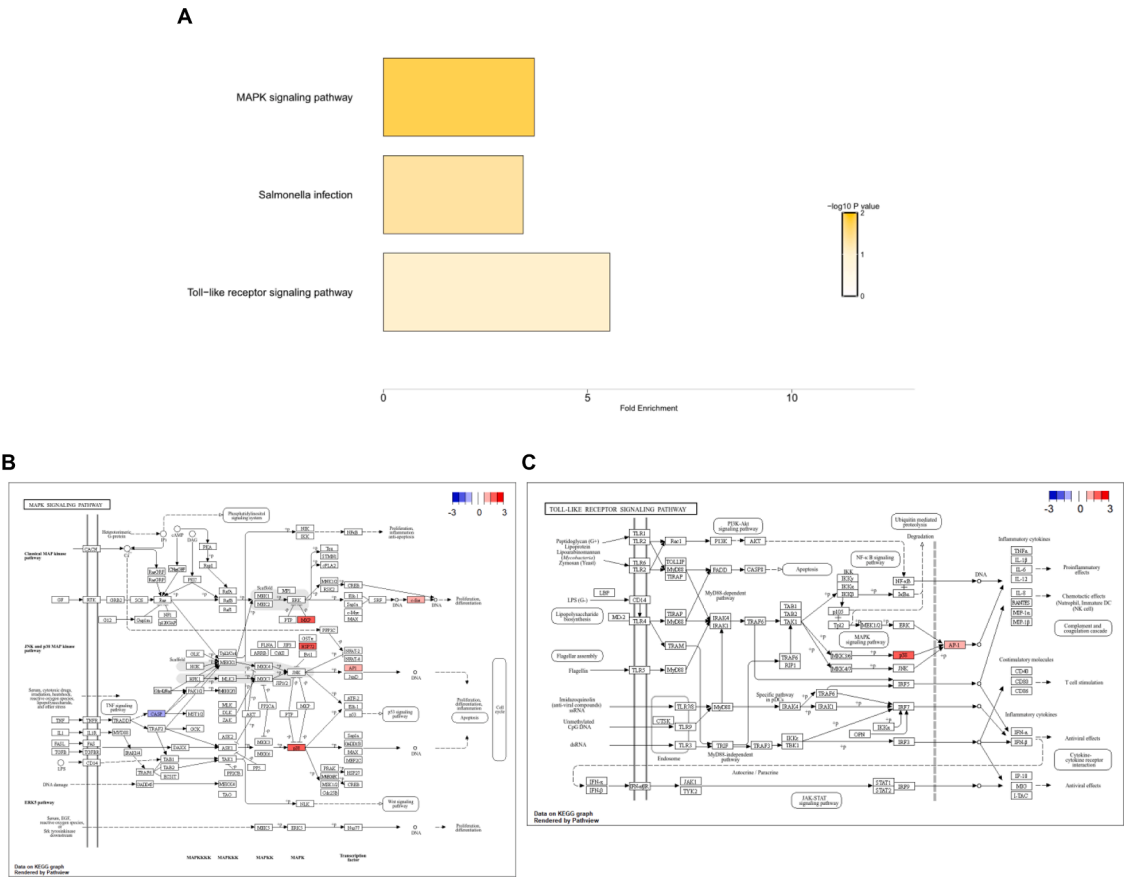


Fig. 3. Significantly enriched pathways in the ES group. (A) KEGG pathway enrichment analysis for DEGs, illustrating enriched pathways for the comparison groups based on $-\log_{10} P$ -values and fold enrichment. Significantly enriched pathways include the “MAPK signaling pathway,” “Salmonella infection,” and “Toll-like receptor signaling pathway.” (B) ClusterProfiler results for the ES group, focusing on the “MAPK signaling pathway.” (C) ClusterProfiler results for the ES group, highlighting the “Toll-like receptor signaling pathway.” In both (B) and (C), red and blue rectangles represent up- and down-regulated genes, respectively.

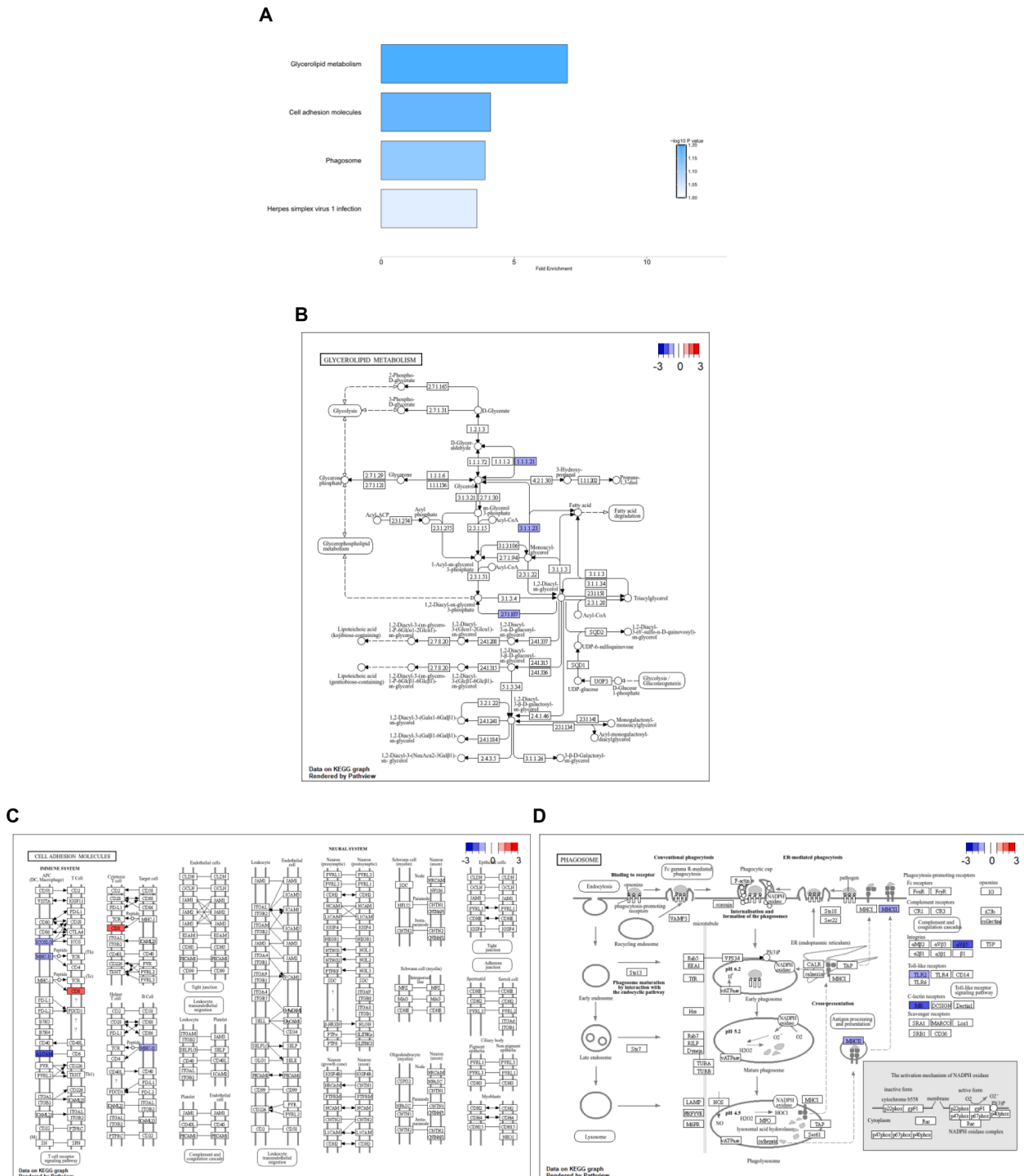


Fig. 4. Significantly enriched pathways in the CAS group. (A) KEGG pathway enrichment analysis for DEGs, showing enriched pathways for the comparison groups based on $-\log_{10} P$ -values and fold enrichment. Significantly enriched pathways include “Glycerolipid metabolism,” “Cell adhesion molecules,” “Phagosome,” and “Herpes simplex virus 1 infection.” (B) KEGG pathway map for “Glycerolipid metabolism,” illustrating gene expression changes in the CAS group. (C) KEGG pathway map for “Cell adhesion molecules,” illustrating gene expression changes in the CAS group. (D) KEGG pathway map for “Phagosome,” illustrating gene expression changes in the CAS group. In (B), (C), and (D), red and blue rectangles represent up- and down-regulated genes, respectively.

Discussion

The stunning process prior to slaughter can impact animal welfare, and meat quality data provide valuable insights into the stress experienced by broilers. ES, the most commonly used stunning method for broilers, has been associated with tissue damage, including electrical burns and capillary rupture, suggesting that it may induce considerable

stress (Grandin, 2007; Gregory, 2005). Additionally, broilers are highly sensitive to CO₂ exposure, which can influence their physiological responses to stunning methods such as CAS (Gerritzen et al., 2004; Rucinke et al., 2024). While whole blood RNA samples have limitations in detecting tissue-specific responses, they may offer a practical advantage in capturing systemic physiological stress, which was the primary focus of this study. Whole-blood transcriptomic profiles may

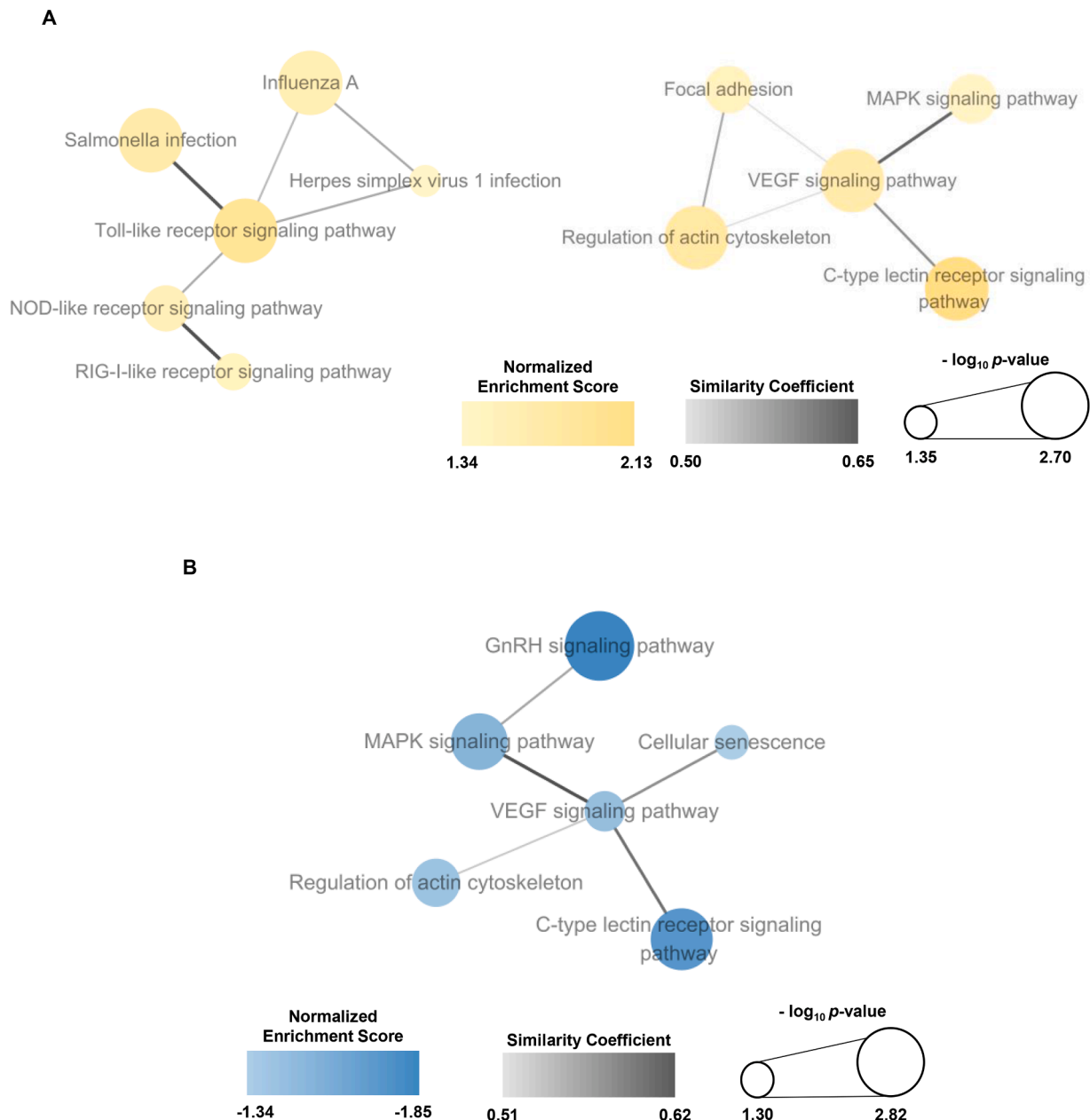


Fig. 5. GSEA network using the KEGG database. Cytoscape was used to visualize the results of the functional enrichment analysis derived from GSEA. Nodes represent enriched KEGG pathways, with the color of each node indicating the normalized enrichment score (NES) and the size reflecting the $-\log_{10}P$ -value. The thickness of the edges corresponds to the overlap coefficient, representing the number of overlapping genes between pathways. The transparency of the edges indicates the similarity between pathways based on shared gene sets. (A) after-ES vs. before-ES. (B) after-CAS vs. before-CAS.

reflect integrated stress responses, as blood interacts with various organs during circulation. However, we acknowledge that this approach may not fully capture localized molecular mechanisms within specific tissues such as the brain, muscle, or endocrine organs. Therefore, it is necessary to conduct further studies that integrate tissue-based transcriptomic and biochemical analyses. These studies would complement systemic whole-blood findings and provide tissue-specific mechanistic insights.

Previous studies have primarily assessed stress responses through behavioral indicators (e.g., panting and wing flapping), physiological markers (e.g., stress hormones and blood metabolites), and meat quality parameters (e.g., color, pH, and blood spots) (Bedanova et al., 2007; Chloupek et al., 2009; Ericsson et al., 2014). Among these endpoints, meat quality traits serve as indirect stress indicators and may reflect physiological changes resulting from pre-slaughter handling and stunning methods (Sandercock et al., 2001; Sardi et al., 2020). However,

variations in stress measurements depending on the chosen indicators can lead to inconsistencies in evaluating the effects of slaughter methods on animal welfare (Hänsch et al., 2009). While some studies suggest that CAS improves welfare outcomes, others report no significant reduction in stress compared to ES (Kang and Sams, 1999; Nicolau et al., 2015; Riggs et al., 2023). These conflicting findings highlight the need for further research into the physiological and molecular effects of stunning methods to determine the most humane approach. In this study, body weight was used as a covariate to assess phenotypic differences between stunning methods (Llonch et al., 2012). Given that body weight influences the required current for ES (Smaldone et al., 2021; Woolley et al., 1986) and the gas concentration for CAS (Guijarro et al., 2020), accounting for this factor helps mitigate confounding variables when evaluating stress responses.

A comparable number of DEGs were identified in both the ES and

CAS stunning methods. However, ES predominantly exhibited upregulated genes, whereas CAS showed an overall downregulation of gene expression (Fig. 1B). This suggests that ES may induce an acute physiological stress response, leading to increased expression of genes associated with cellular stress and inflammation. In contrast, CAS appears to suppress metabolic activity, potentially indicating a lower physiological stress burden compared to ES. The absence of overlapping DEGs between ES and CAS further suggests that these stunning methods may elicit distinct biological responses in broilers. While the current study focused on physiological and molecular evaluations, it is important to emphasize that the practical application of stunning methods in commercial settings also depends on their economic feasibility. Although a formal cost-benefit analysis was not included in this study, future research should incorporate a comprehensive assessment of economic factors. This assessment could address aspects such as equipment investment, labor efficiency, throughput, and the impact on product yield and grading. Collecting these data would provide a more comprehensive basis for evaluating the effectiveness of electrical and gas stunning methods in poultry processing systems.

Glutamate-mediated excitotoxicity and its effects on meat characteristics under electrical stunning

This study highlights the involvement of the 'TIMM22 mitochondrial import inner membrane insertion complex' and 'Calcium ion binding' in ES, as identified through functional annotation analysis (Fig. 2B and C). These findings indicate significant pathways related to the cellular response to unfolded proteins and the binding of misfolded proteins, which include upregulated genes from the heat shock protein (HSP) 70 family, such as *HSPA2*, *HSPA5*, and *HSPA8*. These findings might indicate the engagement of cytoprotective mechanisms potentially related to glutamate-mediated excitotoxicity during ES.

The cerebral cortex, which plays a role in consciousness, has a high concentration of (N-methyl-D-aspartate) NMDA receptors that utilize glutamate as their neurotransmitter (Heresco-Levy and Javitt, 1998). ES has been reported to stimulate the nervous system and increase glutamate release (Cook et al., 1995), which can subsequently overactivate NMDA receptors involved in excitotoxic processes (Cook et al., 1995; Mody and MacDonald, 1995). This process triggers downstream effects such as reactive oxygen species (ROS) generation and mitochondrial dysfunction due to elevated calcium ion levels (Mira and Cerpa, 2021; Venditti et al., 2013). The simultaneous upregulation of HSP70 family genes may indicate an intrinsic protective mechanism against excitotoxic damage (Mokrushin et al., 2005). Previous research has shown that activation of HSP70 can mitigate injury caused by glutamate (Ayala and Tapia, 2008). These findings suggest that ES may induce metabolic and oxidative stress, leading to mitochondrial vulnerability (Abramov and Duchen, 2008; Nicholls, 2004). Additionally, glutamate release during ES may contribute to ROS formation, which can promote lipid peroxidation and influence meat color (Atlante et al., 2001; Min and Ahn, 2005; Zhang et al., 2013). Metabolic and oxidative stress can impair enzyme activity and increase membrane permeability, potentially affecting the WHC of meat (Xing et al., 2019). Although this study found no significant differences in WHC between ES and CAS, a substantial difference in moisture content was observed. Since pH is closely linked to meat quality traits, including color and WHC in broilers (Qiao et al., 2001), its variation may contribute to differences in meat characteristics. A significant difference in pH between ES and CAS was observed 45 minutes post-slaughter, though this difference was no longer evident after 24 hours (da Silva-Buzanello et al., 2018). Given that proximate composition and WHC were measured 18 hours post-slaughter in this study, the timing of the measurements may have influenced the observed discrepancies. These findings suggest that ES induces increased glutamate secretion in broilers, which may impact moisture content, meat color, and flavor, ultimately contributing to metabolic and oxidative stress. A potential limitation of this study is that our

conclusions regarding excitotoxicity are primarily based on transcriptomic patterns rather than direct biochemical measurements. We did not assess biochemical markers such as glutamate concentrations, intracellular calcium levels, or HSP70 protein expression. Additionally, we cannot exclude the possibility that the observed induction of the HSP70 family reflects non-specific injury-related stress (e.g., thermal or mechanical microdamage) secondary to electrical stunning, rather than glutamate-mediated excitotoxicity. Therefore, we suggest that future studies incorporate targeted biochemical validations to confirm the presence and extent of excitotoxic responses following stunning.

p38 MAPK-mediated inflammatory response induced by ES

ES resulted in a significantly higher a^* (redness) value compared to CAS (Table 3). This could be due to tissue and cellular damage, including capillary rupture, which has been previously reported as a consequence of ES and can lead to visible blood spots (Gregory and Wilkins, 1989). The increased redness observed in ES-treated broilers could be attributed to intense muscle contractions during stunning, which may cause microhemorrhages. Additionally, ES accelerates glycogen breakdown, which may lead to a rapid decline in pH. This pH drop disrupts oxygen binding to myoglobin in muscle tissue, can prevent the oxidation of oxymyoglobin to metmyoglobin and thereby increasing redness (Hayat et al., 2024; Xu et al., 2011).

These injuries and acute stress responses may trigger pathways involved in cellular repair and inflammation. Our analysis identified the TLR and MAPK signaling pathways, both of which mediate inflammatory responses in damaged tissue (Fig. 3). We further identified enriched pathways associated with ES-related vascular repair, including C-type lectin receptor signaling, VEGF signaling, and actin cytoskeleton regulation, with upregulated genes such as *TLR4*, *TLR1A*, and *NFKB1* (Fig. 5A). Taken together, these results could reflect early inflammatory activation.

Our results suggest that p38 MAPK might play a key role in regulating cellular stress responses, including differentiation and apoptosis. Previous studies have reported that p38 MAPK is activated by stress signals and inflammatory cytokines (Correa and Eales, 2012; Zarubin and Han, 2005), which aligns with our observations (Fig. 3B and C). Moreover, ROS generated during ES may contribute to oxidative stress, further activating the p38 MAPK pathway (Mohanty et al., 2010; Son et al., 2011). The activation of this pathway is indicative of the physiological stress experienced by broilers before slaughter, including heat stress, tissue damage, and oxidative stress, making it could be a negative indicator of animal welfare (Correa and Eales, 2012; Obata et al., 2000; Zarubin and Han, 2005). The p38 MAPK pathway is linked to key physiological processes such as metabolism, protein degradation, and inflammation (Kyriakis and Avruch, 2001; McClung et al., 2010). Its activation can lead to increased protein degradation and altered muscle fiber integrity, potentially affecting meat tenderness (Ma et al., 2024). These findings suggest that early cellular stress and inflammatory signaling are associated with ES. However, because whole-blood transcriptomes and a^* are indirect indicators, these interpretations should be approached with caution. Given that these stress responses can directly impact animal welfare, further research is necessary to evaluate stunning methods that minimize physiological distress. This research should include tissue-specific analyses and the examination of biochemical markers such as cytokines and ROS.

Hypercapnia and immune suppression under CAS

Under normal conditions, the blood pH of broiler chickens is approximately 7.4. However, hypercapnia induced by CO₂ stunning can lower blood pH to below 6.7, leading to gradual unconsciousness (Gerritzen et al., 2006). This acidic shift has been reported to modulate inflammatory and immunosuppressive effects by downregulating nuclear factor kappa-light-chain-enhancer of activated B cells activators,

such as tumor necrosis factor (TNF) and TLRs (Shigemura et al., 2020; Wang et al., 2010). Consistent with these findings, our pathway enrichment analysis revealed a downregulation of immune-related functions under CAS conditions, including reduced expression of TNF and TLRs (Figs. 2D and 4). Unlike ES, which triggers inflammatory responses associated with acute stress, CAS displayed anti-inflammatory signaling, suggesting that it might induce less physiological stress.

In addition to immune modulation, hypercapnia under CAS could affect cellular function and metabolic activity by disrupting mitochondrial metabolism. Our study identified the downregulation of pathways related to energy metabolism (Fig. 4A and B). Since whole-blood RNA primarily originates from immune cells, these alterations may be associated with changes in immune cell activity (Fig. 2D). The hypothalamic-pituitary-gonadal (HPG) axis, which regulates reproduction, growth, and metabolism, is sensitive to changes in pH and hypoxia (Liu et al., 2020; Wilson et al., 2018) and was also downregulated under CAS conditions (Fig. 5B). This suggests that a widespread reduction in energy metabolism may influence adenosine triphosphate consumption, potentially slowing the decline of pH levels in postmortem muscle tissue.

ES directly affects muscle tissue, leading to a rapid pH decline due to accelerated glycogen depletion. In contrast, CO₂ stunning results in a more gradual pH decrease. Previous studies have reported significant differences in pH levels between electrical and CO₂ stunning methods during the first 45 minutes postmortem. However, these differences diminish over time and become negligible by 24 hours postmortem (da Silva-Buzanello et al., 2018). Since pH was measured 18 hours postmortem in this study, any initial differences may have already balanced out. Although we observed downregulation of selected energy metabolism-related genes (e.g., within glycerolipid metabolism), these transcriptomic signals are indirect; therefore, follow-up studies that directly compare early postmortem pH trajectories and biochemical indices of energy metabolism (GnRH levels, ATP content, glycolysis/TCA activity) are needed. Additionally, the effects of CAS are likely driven more by hypercapnia induced by elevated CO₂ levels than by hypoxia. To better assess the impact of different stunning methods on animal welfare, further research should investigate the effects and underlying mechanisms of inert gas stunning methods (such as argon and nitrogen) as alternatives to CO₂.

Conclusion

This study highlights the differing effects of ES and CAS on broiler welfare. The activation of inflammatory pathways in ES suggests that it might induce various physiological stresses, including excitotoxicity, oxidative stress, and tissue damage, potentially imposing a physiological stress. The physiological consequences of ES, such as oxidative stress, intense muscle contractions, and capillary ruptures, may contribute to increased discomfort in broilers. The inhibition of immune-related pathways and the presence of hypercapnia in CAS suggest a reduction in inflammatory responses and oxidative stress, potentially resulting in lower physiological stress.

From a physiological transcriptomic and meat-quality perspective, CAS showed patterns compatible with a lower systemic stress burden. Our evaluation primarily relied on whole-blood transcriptomics, which offers only indirect evidence. Therefore, these findings should be interpreted with caution. Future studies could integrate validated behavioral observations, tissue-level histology, and biochemical markers of inflammation and cellular energy metabolism. This comprehensive approach will enable a more thorough assessment of stunning methods and help clarify their welfare implications for broilers.

Ethics statements

The study was approved by the Institutional Animal Care and Welfare Committee of the National Institute of Animal Science, Rural

Development Administration, Republic of Korea (approval No. NIAS-2023-0631).

Data availability

The raw RNA-seq data supporting the findings of this study have been submitted to the NCBI Sequence Read Archive and are available under the BioProject accession number PRJNA1265288 <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1265288>. All other relevant data are available from the corresponding author upon request.

CRediT authorship contribution statement

Chang-Hyeon Ham: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Jung Hwan Jeon:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Data curation. **Seung-Hoon Lee:** Writing – review & editing, Validation, Methodology. **Seok-Won Lim:** Writing – review & editing, Validation, Investigation. **Chiwoong Lim:** Methodology. **Min-Jae Jang:** Writing – review & editing. **Byeonghwi Lim:** Writing – review & editing. **Min-Ki Seok:** Methodology. **Se Jin Lim:** Resources, Investigation. **Chan Ho Kim:** Resources, Investigation. **Jun-Mo Kim:** Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Disclosures

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was funded with the support of the Cooperative Research Program for Agriculture Science and Technology Development (Project No. RS-2021-RD010100), Rural Development Administration, Republic of Korea and the Chung-Ang University Graduate Research Scholarship in 2025.

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