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Multiomics-based analysis of key genes, metabolites and pathways unveils mechanism associated with social rank in Chickens

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ABSTRACT

Social rank plays a crucial role in shaping physiological, psychological and immune responses in animals. Our previous study found that gut microbes and short-chain fatty acids as well as brain neurotransmitters are involved with the mechanism of social hierarchy. Nevertheless, how these gut and brain metabolites interact to affect the mechanism of social hierarchy in chickens is not yet known. In this study, 40 hens were randomly divided into four groups at the age of 49 days, and social rank of each hen was determined by 6 times according to the feeding competition tests. Then hens ranked 1/2 were named High Social Rank (HSR, n = 8) and hens ranked 9/10 were named Low Social Rank (LSR, n = 8). We use multiomics to explore gut metabolites, neurotransmitters, and brain transcriptome, neurotransmitters and related gene expression in 91-day-old chickens, so as to better understand the underpinning mechanism that regulates the social hierarchy. We found that the pro-inflammatory genes were significantly lower while anti-inflammatory factor in spleen was higher in HSR chickens than in LSR chickens ($P<0.05$). Besides, seven immune-related genes were significantly different in the amygdala between HSR and LSR ($P < 0.05$). In addition, *AVP, RXFP3, VIP* and *NKX2-1* were associated with the social rank through GABAergic neurons and neuroactive ligand-receptor pathways, with the up-regulation of *5-HT* in the amygdala of LSR. Genes *SLC11A2* and *HMOX1* in ferroptosis pathway influenced the cecum metabolite l-glutamate and tyrosine. While fumaric, l-glutamic and 4-Oxoproline were found enriched in "alanine, aspartate and glutamate metabolism" and "arginine biosynthesis". In conclusion, social rank affects the immunity, in which higher ranking hens show better anti-inflammatory and lower pro-inflammatory than lower ranking hens. Genes *TAP2, PLD4, P2RX7, ALDH9A1, SLC11A2, ADM, C3, AVP, RXFP3, VIP, NKX2-1, SLC11A2* and *HMOX1* may play an important regulatory role on GABAergic neurons, neuroactive ligand-receptor and ferroptosis pathways related to neuron, immune, and stress behaviour, and in turn affects social rank. Fumaric, l-glutamic and 4-Oxoproline, may regulate social rank through the "alanine, aspartate and glutamate metabolism" and "arginine biosynthesis".

Introduction

Social hierarchy ranging from teleosts to primates, is characterized by the consistent dominance of one individual over another, which represents a fundamental principle of social organization across diverse taxa (Cordero and Sandi, 2007; Byrne and Bates, 2010). A well-organized social system allows animals to adapt to a wide range of eco-systems (Lukas and Clutton-Brock, 2013). Since the significant role of social hierarchy on individuals' survival and physical and mental health (Johnson, et al., 2012; Snyder-Mackler, et al., 2016b), extensive knowledges are accumulating on how social hierarchy influences brain function and health to understand the underlie mechanism on a broad kind of animals. In recent years, scientists have found that the social hierarchy of mice is controlled by postsynaptic AMPAR trafficking, which is regulated via a novel pathway of lncRNA AtLAS and its target syn2b (Ma, et al., 2020). Furtherly, social hierarchy is revealed to be modulated by the accelerated evolution of an Lhx2 enhancer PAS1-Lhx2 (Wang, et al., 2020). The establishment of social hierarchy relies on the organization and coordination of multiple brain regions such as the medial prefrontal cortex, hippocampus, and amygdala. Particularly, the brain neurotransmitters such as serotonin (5-HT), dopamine, oxytocin and γ-aminobutyric acid (GABA) are known to exert essential roles on

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social hierarchy (Watanabe and Yamamoto, 2015). For example, the neurons of GABAergic and serotoninergic were activated after the social failure in mice (Challis, et al., 2013). The enhancement or inhibition of 5-HT is associated with the rise or fall of social status in adult male vervet monkeys (Raleigh, et al., 1991), and the concentration of 5-HT in higher ranking monkeys was twice higher than that in lower ranking monkeys (Raleigh, et al., 1984). Besides, the dorsal raphe nucleus of dominant monkeys contains more gray matter in the brain, which contains serotoninergic and dopaminergic neurons (Noonan, et al., 2014). Notably, the level of dopamine metabolites in cerebrospinal fluid was higher in dominant monkeys than that in subdominant monkeys (Kaplan, et al., 2002).

Studies of social hierarchy on avian also have a long history, which is traced back to the pecking order in 1922 (Guhl, 1968). Nevertheless, most of the related studies focused on the behavioral traits and productive and reproductive indicators. For instance, the rooster with the highest rank had priority access to feeding (Rohwer, 1975) and crowing (Shimmura, et al., 2015). High-ranking roosters exhibited greater plasma testosterone levels and lower ejaculatory testosterone compared to their lower-ranking counterparts, indicating that social hierarchy significantly influences hormonal regulation in chickens (Lelono, et al., 2019). Dominant hens compel subordinate hens to display submissive behaviors through intimidation or aggression (Appleby, et al., 2002), and hens of higher rank produce heavier eggs than lower rank hens (Cunningham and Van Tienhoven, 1983; Lundberg and Keeling, 1999). Our previous study indicated that the different social rank in chicken contributes to changes of microbial composition, diversity and short-chain fatty acids. Dominant chickens seem to benefit from short-chain fatty acids activities and subdominant chickens profit from microbial functions (Chen, et al., 2022). Additionaly, high-ranking hens are more vigilant and have priority when foraging, and possess beneficial bacteria that use favorable substances to maintain the balance of the gut environment (Xie, et al., 2023). Furthermore, the sub-neurons GABA_LEG of GABAergic neurons and Glu3 of glutamatergic neurons are considered as main neurons mediating the social hierarchy in chickens (Chen, et al., 2024b). Nevertheless, more evidences are still needed to shed lights into better understanding of the underpinning mechanism regulating social hierarchy in chickens. Notably, increased studies have showed that the gut microbiota and metabolites may influence neurodevelopment and behavior via the neuroendocrine, neuroimmune, and central nervous systems (Arentsen, et al., 2015; Weber, et al., 2017). Likewise, secretion of corticotropin-secreting factor from the hypothalamus in chickens stimulate the anterior pituitary lobe to release adrenocorticotropic, and then prompts the adrenal glands to produce corticosteroids. Afterwards, corticosteroids affect the gastrointestinal tract by directly interacting with intestinal cells and microbiota, leading to the release of cytokines that impact the brain and modulate appetite, mood, and cognitive function (Cryan and Dinan, 2012). However, whether the gut and brain metabolites have an interaction to impact on social hierarchy is not yet known.

Thus, in this study we aim to investigate the transcriptomic pattern of amygdala and gene expression related to social hierarchy, gut metabolites, neurotransmitters of both the brain and gut, so as to figure out the underpinned mechanism regulating social hierarchy in chickens. Our results will provide an understanding that gut and brain metabolites regulates social hierarchy of chickens, and lead to provide new insights into social behavior traits that could be leveraged in future breeding selection strategies.

Materials and methods

Experimental design and sampling

All animals used in this study were cared and handled in accordance with the guidelines of the Animal Care Committee of Foshan University (Approval ID: FOSU#121). For the study, forty Qingyuan partridge hens with similar body weight (652.2±50.12g), from Guangdong Tiannong Food Co., Ltd., were chosen and randomly divided into four groups at the age of 49 days. Birds in each group were housed in a separate barn (1.5m × 1.5m × 2.5m) with concrete ground and no litter material. All the experimental animals were tagged using colored leg rings and given free access to drinkers and a commercial diet that was provided daily at 8:00a.m. Social rank of each hen was determined by 6 times (57, 58, 59, 76, 77, and 78 days of age, the day after the chickens began fasting at 18:00) by feeding competition tests (detailed experimental procedures refer to our previous study (Xie, et al., 2023), in which hens ranked 1/2 were named High Social Rank (HSR) group (four duplicates, n = 8) and hens ranked 9/10 were named Low Social Rank (LSR) group (four duplicates, n = 8). Notably, social rank remained stable in the later stages of the feeding tests.

Hens were slaughtered at 91 days of age. Cecum contents, spleen, amygdala and hypothalamus tissues were removed and placed in 2 mL enzyme-free EP tubes. The samples were stored at -20°C and then transported back to -80°C for storage. These samples were prepared for gene and neurotransmitter quantification, RNA sequencing and metabolomic analysis.

Gene expression

The hypothalamus, amygdala and spleen of HSR and LSR (eight in each group) were collected and preserved. In order to analyze the genes related to immune in spleen, the relative expression of interleukin 6 (*IL-6*), interleukin 8 (*IL-8*), interleukin 1β (*IL-1β*), tumor necrosis factor α (*TNF-α*), interferon gamma (*IFN-γ*), interleukin 4 (*IL-4*), and interleukin 10 (*IL-10*) were measured. To investigate the gene expression associated with stress and cognition in the hypothalamus, the relative expression of γ-aminobutyric acid (*GABA*), dopamine (*DA*), 5-hydroxytryptamine (*5-HT*), corticotropin-releasing hormone (*CRH*), glucocorticoids (*GR*) and brain-derived neurotrophic factor (*BDNF*) were quantified. In addition, the relative expression of conserved oligomeric Golgi complex subunit 4 (*COG4*), aldehyde dehydrogenase 9 family member A1 (*ALDH9A1*), family with sequence similarity 187 member A (*FAM187A*), transthyretin (*TTR*), arginine vasopressin (*AVP*), granzyme M (*GZMM*), hemoglobin subunit alpha (*HBM*), proteoglycan peptide core protein (*SRGN*), solute carrier family 22 member 6-A (*LOC103624403*), FYVE, RhoGEF and PH domain-containing protein 5 isoform X1 (*FGD5*), randomly selected from transcriptome differently expressed genes (DEGs) ($P<0.05$, $|\log_2\text{FoldChange}|>1$), were determined for validation of the transcriptome data in amygdala.

Total tissue RNA was extracted according to AG RNAex Pro RNA Extraction instructions. The RNA was reverse transcribed into cDNA using the Reverse Transcription Kit (R 323) from Nanjing Novozymes Biotechnology Co. Referring to the gene sequences published by NCBI, primer design was carried out using https://primer3.ut.ee 4.1, and then Blast comparison was performed on NCBI, and the sequences that passed the comparison were synthesised at Sangong Bioengineering Co. The synthetic primer sequences of genes are shown in Supplementary Table 1. The qRT-PCR was performed on an Applied Biosystems QuantStudio 5.0 fluorescence quantitative PCR instrument using the ChamQ Universal SYBR Quantitative Real-time Polymerase Chain Reaction (qPCR) Master Mix fluorescence quantitative special premix from Nanjing Novozymes Bioscience & Technology Co. and the internal reference gene was *GAPDH* to calculate the relative gene expression by $2^{-\Delta\Delta Ct}$.

Transcriptomics

The total RNA of amygdala (n = 8 per group) was extracted according to the instruction manual of the TRlzol Reagent (Life technologies, California, USA). RNA concentration and purity was measured using NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent

Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using Hieff NGS Ultima Dual-mode mRNA Library Prep Kit for Illumina (Yeasen Biotechnology (Shanghai) Co., Ltd.) following manufacturer's recommendations. The libraries were sequenced on an Illumina NovaSeq platform to generate 150 bp paired-end reads. The downstream data were filtered to obtain clean data, and sequence comparison was performed with the specified reference genome (*Gallus_gallus*_5.0.genome.fa) to obtain Mapped Data by HISAT2 (Kim, et al., 2015), and then the data were analyzed using the bioinformatics analysis process provided by BMKCloud, a BMK cloud platform. Differently expressed genes (DEGs) were screened using $|\log_2 FC| > 1$ and $P<0.05$.

Neurotransmitter

Target metabolites of nine neurotransmitters from both the amygdala and cecum were identified. All metabolomic data and calibration solutions were normalized to internal standards for UHPLC-MS/MS analysis. The UHPLC separation was carried out using an ACQUITY Premier (Waters) System, equipped with a Waters ACQUITY UPLC HSS T3 (100 × 2.1 mm, 1.8 μm). SCIEX Analyst Work Station Software (Version 1.6.3) and DATA DRIVEN FLOW (Version 1.0.1) was employed for MRM data processing. The signal-to-noise ratios (S/N) were used to determine the lower limits of detection (LLODs) and lower limits of quantitation (LLOQs). The LLODs and LLOQs were defined as the analyte concentrations that led to peaks with signal-to-noise ratios (S/N) of 3 and 10, respectively, according to the US FDA guideline for bioanalytical method validation. The precision of the quantitation was measured as the relative standard deviation, determined by injecting analytical replicates of a QC sample. The accuracy of quantitation was measured as the analytical recovery of the QC sample. The percent recovery was calculated as [(mean observed concentration) / (spiked concentration)] × 100%.

Untargeted metabolomics of cecum contents

Sixteen collected cecum samples were utilized for the metabolomics analysis, which was conducted by Lc-Bio Technologies Company (Hangzhou, China). A high-resolution tandem mass spectrometer Q-Exactive (Thermo Scientific UltiMate 3000 HPLC) was used to detect metabolites eluted form the column. In order to evaluate the stability of the LC-MS during the whole acquisition, a quality control sample (Pool of all samples) was acquired after every 10 samples. LC-MS raw data files were converted into mz XML format and then processed by the XCMS, CAMERA and metaX (Li, et al., 2018) toolbox implemented with the R software. PCA was performed for outlier detection and batch effects evaluation using the prepossessed data set. Quality control-based robust LOESS signal correct ion was fitted to the OC data with respect. Student *t*-tests were conducted to detect differences in metabolite concentrations between 2phenotype. The *P* value was adjusted for multiple tests using an FDR (Benjamini-Hochberg). Supervised PLS-DA was conducted through metaX to discriminate the different variables between groups. The VIP value was calculated. A VIP cut-off value of 1.0 was used to select important features.

Statistical analysis

SPSS 25.0 statistical software was used. The gene expression and neurotransmitter data were checked for normality and homogeneity of variance. If data did not meet normality assumptions, log transformation or square root transformation was applied as appropriate. After ensuring normality, one-way ANOVA after fits the normality. Graph Pad Prism 9.0 was used for graphing. All data are presented as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

Results

Related genes expression

For relative expression levels of immunity-related genes in spleen, among five pro-inflammatory related indicators *IL-8* and *IFN-γ* were significantly lower in HSR than in LSR ($P < 0.05$) (Fig. 1A), while among the two anti-inflammatory related indicators, *IL-10* was significantly higher in HSR than in LSR ($P < 0.05$) (Fig. 1B). Other immune related gene expression was not different between the two groups.

For stress- and cognition-related genes in hypothalamus, we measured the expression levels of genes *GABA, DA, 5-HT, CRH, GR* and *BDNF,* and found the relative expression of *GABA* in HSR was higher than LSR hens ($P < 0.05$) (Fig. 1C).

Relative expression of cecum and amygdala neurotransmitters

We used targeted metabolomics to measure neurotransmitters, including kynurenine, DA, 5-HT, tryptophan, tyrosine, GABA, HTR1A, α-ketoglutaric acid and glutamine in both the cecum content and amygdala. Among them, tyrosine in the cecum and 5-HT in the amygdala were significantly higher in LSR than in HSR ($P < 0.05$), while the levels of other neurotransmitters were not significantly different between the two groups (Fig.2A and B).

Cecum metabolome profiling

PLS-DA under positive and negative ion modes, the two sample points were completely separated (Fig. S1), indicating a significant change in the cecum metabolome between HSR and LSR hens. We identified 318 differential metabolites (DEMs) with $P < 0.01$, of which 262 were down-regulated and 56 were up-regulated in HSR compared with LSR hens (Fig. 3A).

As shown in Fig. 3A and B, 20 DEMs on the top 20 Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment signaling pathways such as "arginine biosynthesis", "valine, leucine and isoleucine biosynthesis", "central carbon metabolism in cancer", "alanine, aspartate and glutamate metabolism", "protein digestion and absorption", "lysine degradation", "biosynthesis of amino acids", "2-Oxocarboxylic acid metabolism", "glucosinolate biosynthesis", "ABC transporters", "metabolic pathways" were obtained. Among that, 4-Oxoproline and l-glutamic acid enriching in the "arginine biosynthesis" significantly down-regulated in the HSR hens ($P < 0.05$). In addition, fumaric acid and L-glutamic acid were down-regulated in the HSR hens, the two metabolites were enriched in alanine, aspartate, and glutamate metabolism pathways ($P < 0.05$).

Amygdala transcriptome profiling

Results showed that the values of alignment rate was above 93% between the sequencing data and the reference genome, indicating that the sequencing data was accurate and reliable. We found 221 differently expressed genes (DEGs) from LSR compared with HSR, including 160 up-regulated genes and 61 down-regulated genes (Fig. S2). We randomly selected 10 genes showing significant up- and down-regulation genes for qPCR validation (Fig. 4A). The quantitative results of qPCR showed the same trend with the transcriptome results, which proved the validity of the transcriptome data (Fig. 4B).

To further understand the biological function of DEGs in response to social rank, we performed Gene Ontology (GO) enrichment analysis and found that 221 DEGs was annotated into 33 entries, including 19 biological processes, 4 cellular components and 10 molecular functions (Fig. S3). Among the enriched GO entries, we found a high enrichment of immune-related categories, such as "adaptive immune response", "response to bacterium", "innate immune response", "positive thymic T cell selection", "alpha-beta T cell receptor complex", "intestinal immune

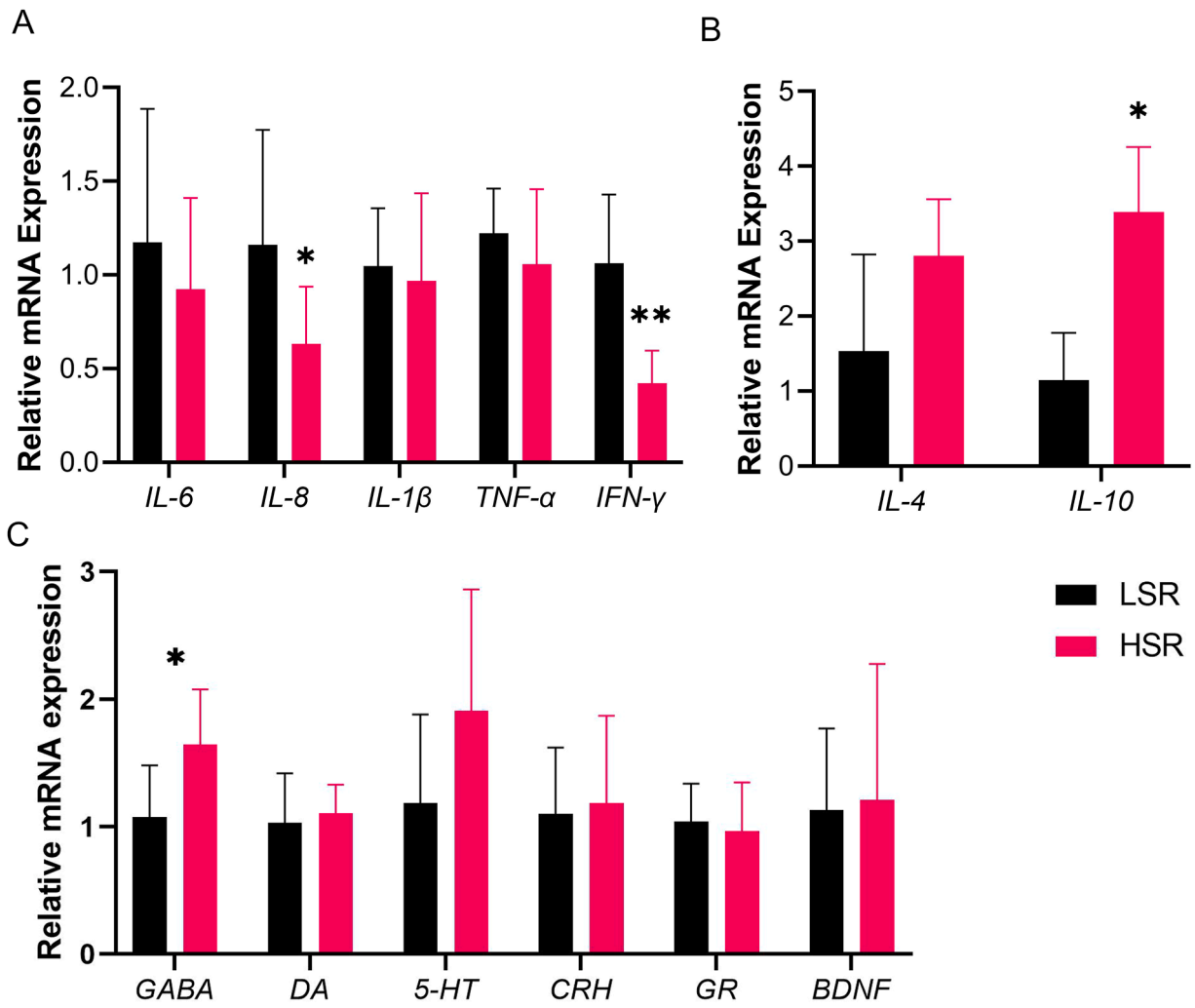

Fig. 1. Related gene Expression between high social rank (HSR) and low social rank (LSR) hens. A. The relative expression of pro-inflammatory cytocines in spleen between HSRand LSR hens. B. The relative expression of anti-inflammatory cytocines in spleen between HSR and LSR. C. The relative expression levels of stress- and cognition-related genes in hypothalamus between HSR and LSR. * Denotes significant difference between groups ($P < 0.05$). n = 8 in each group.

network for lgA production" and so on. In addition, phospholipase D4 isoform X2 (*PLD4*) and antigen peptide transporter 2 (*TAP2*) up-regulated in LSR were enriched in immune system process ($P < 0.05$). Homeobox protein Nkx-2.1 isoform X1 (*NKX2-1*), down-regulated in LSR, were annotated on the cerebral cortex GABAergic interneuron differentiation ($P < 0.05$).

Through the analysis of KEGG pathways (Fig. 4C and D), we found that vasopressin (*AVP*), vasoactive intestinal peptides (*VIP*) and relaxin-3 receptor (*RXFP3*) were significantly down-regulated, while complement component 3 (*C3*), adrenomedullin (*ADM*) significantly were up-regulated in LSR compared with HSR hens annotated on the neuropituitary hormone activity pathway ($P < 0.05$). Aldehyde dehydrogenase family 9 member A1 (*ALDH9A1*) and choline O-acetyltransferase (*CHAT*) significantly down-regulated in LSR were annotated on glycerophospholipid metabolism ($P < 0.05$). In addition, natural resistance-associated macrophage protein 2 (*SLC11A2*) and heme oxygenase 1 (*HMOX1*) down-regulated in LSR hens were annotated on the ferroptosis pathway ($P < 0.05$).

Combined analysis of amygdala transcriptome and cecum metabolome

We set up an association between amygdala transcriptomics and cecum metabolome for joint KEGG analysis. Results revealed that neuroactive ligand-receptor interaction were enriched in HSR vs LSR (Fig. 5). In this pathway, DEGs such as P2X purinoceptor 7 (*P2RX7*) and *C3* were significantly up-regulated, *RXFP3, VIP, AVP* and *ADM* were down-regulate in LSR compared with HSR hens ($P < 0.05$). In parallel, l-glutamate as one of the DEMs was involved in this pathway (Fig. 3A and 4C).

Discussion

For many decades, the mechanism of social hierarchy has been attracted great attentions since its significant roles on immune, reproductivity, evolution and etcs. Up to date, most of the related studies on poultry mainly focus on production performance, behavioral traits and resource allocation. In this study, multiomics was applied to figure out the interaction of gut and brain metabolites that regulates the social hierarchy in chickens, so as to better understand the its underpinning mechanism.

A previous study indicated that dominant animals redirect most of their energy away from immune processes and towards other physiological processes related to maintain their high status (Ambrée, et al., 2018). On the other hand, subordinate animals typically exhibit reduced viral resistance and clearance, compromised cell-mediated immunity, protracted wound healing, and heightened incidence of morbidity (Archie, et al., 2012; Cavigelli and Chaudhry, 2012). For instance, lower social ranking was correlated with elevated expression of

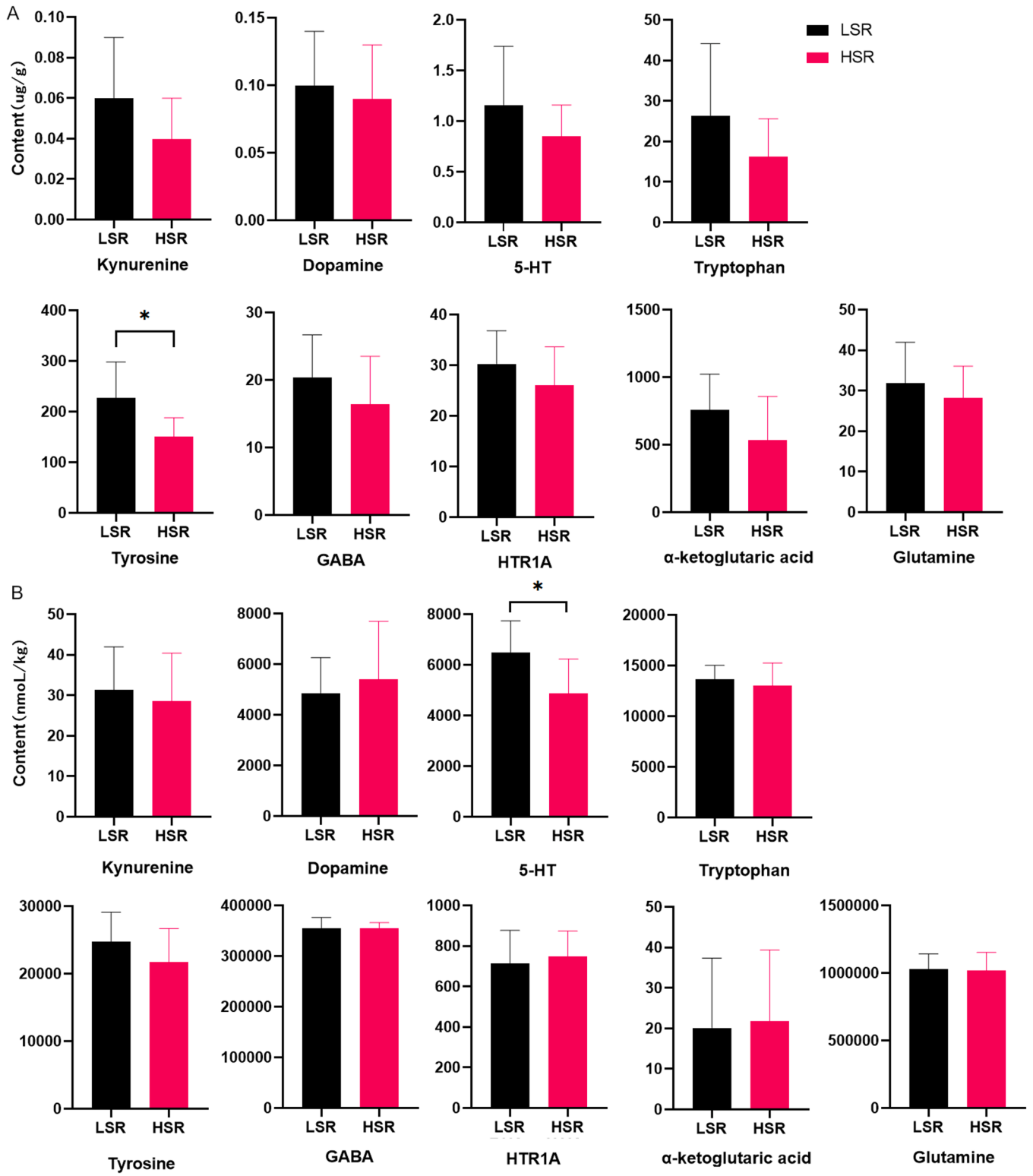

Fig. 2. The relative expression of cecum and amygdala neurotransmitters between high social rank (HSR) and low social rank (LSR) hens. A. Cecum neurotransmitters between HSR and LSR hens. B. Amygdala neurotransmitters between HSR hens and LSR hens. * Denotes significant difference between groups ($P < 0.05$). n = 8 in each group.

pro-inflammatory cytokine genes, such as *IL-6* and *IL-1β*, in blood leukocytes of rhesus macaques (Snyder-Mackler, et al., 2016a). In this study, the relative expression of pro-inflammatory factors *IL-8* and *IFN-γ* in the spleen were significantly lower, while the anti-inflammatory factor *IL-10* was significantly higher in HSR than in LSR hens. Furthermore, we found that several immune-related DEGs in amygdala from transcriptome. The *ADM* and *C3,* important components of the complement system, were significantly up-regulated in LSR hens, which were involved in inflammatory responses and immune activation (Csölle, et al., 2013; Hu, et al., 2017). The upregulation of *ADM* and *C3* in low-ranking hens suggests that hyperactivation of the immune system may be triggered by chronic stress, which is closely related to long-term

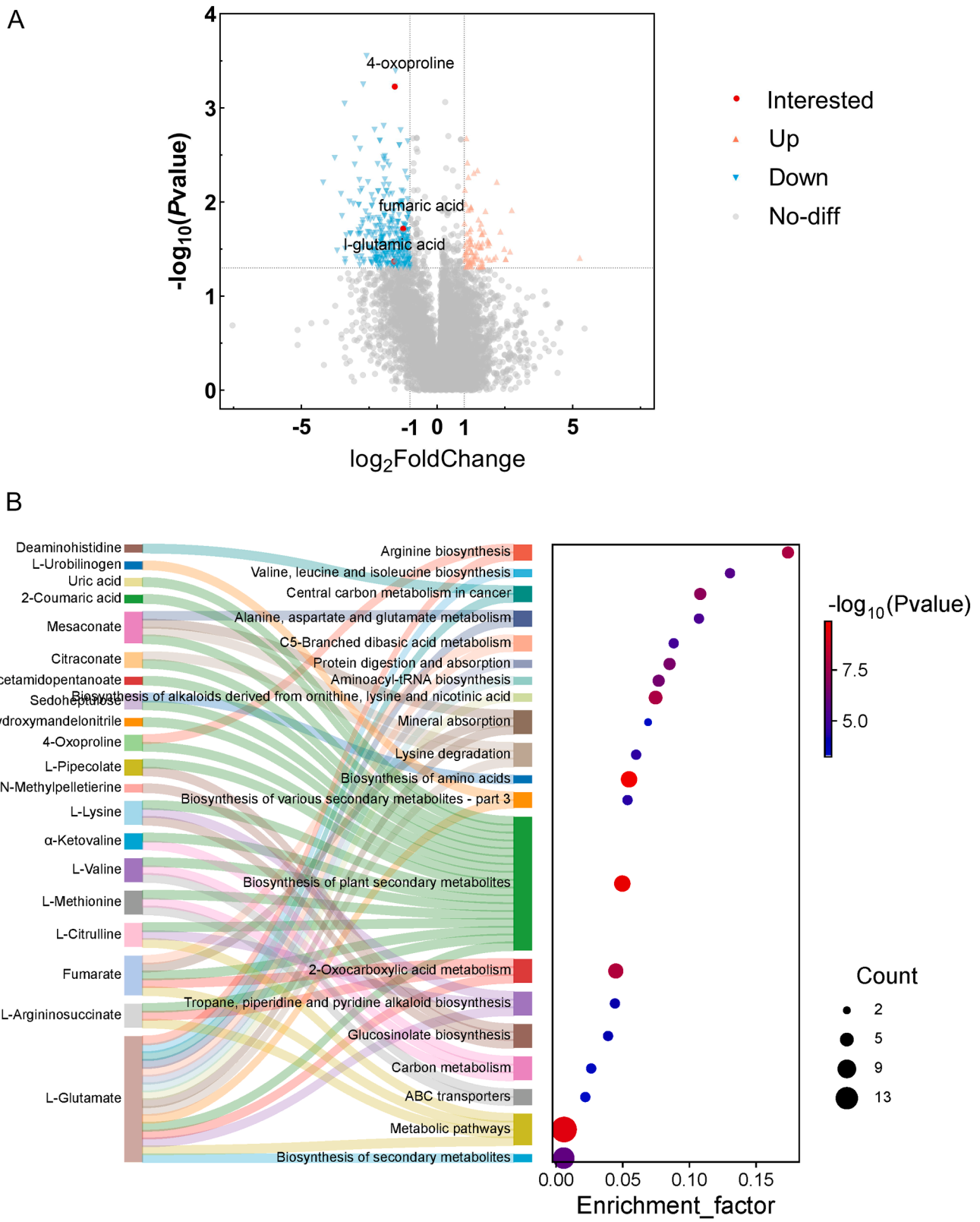

Fig. 3. Untargeted metabolomics in cecum between high social rank (HSR) and low social rank (LSR) hens. A. Volcano of differentially expressed metabolites in cecum between HSR and LSR hens. Values of log2FoldChange >1 indicate higher levels in LSR hens relative to HSR hens, while values of log_2FoldChange<1 indicate higher levels in the LSR hens relative to the HSR hens. B. KEGG enrichment analysis of differentially expressed metabolites in cecum by HSR vs LSR. n = 8 in each group.

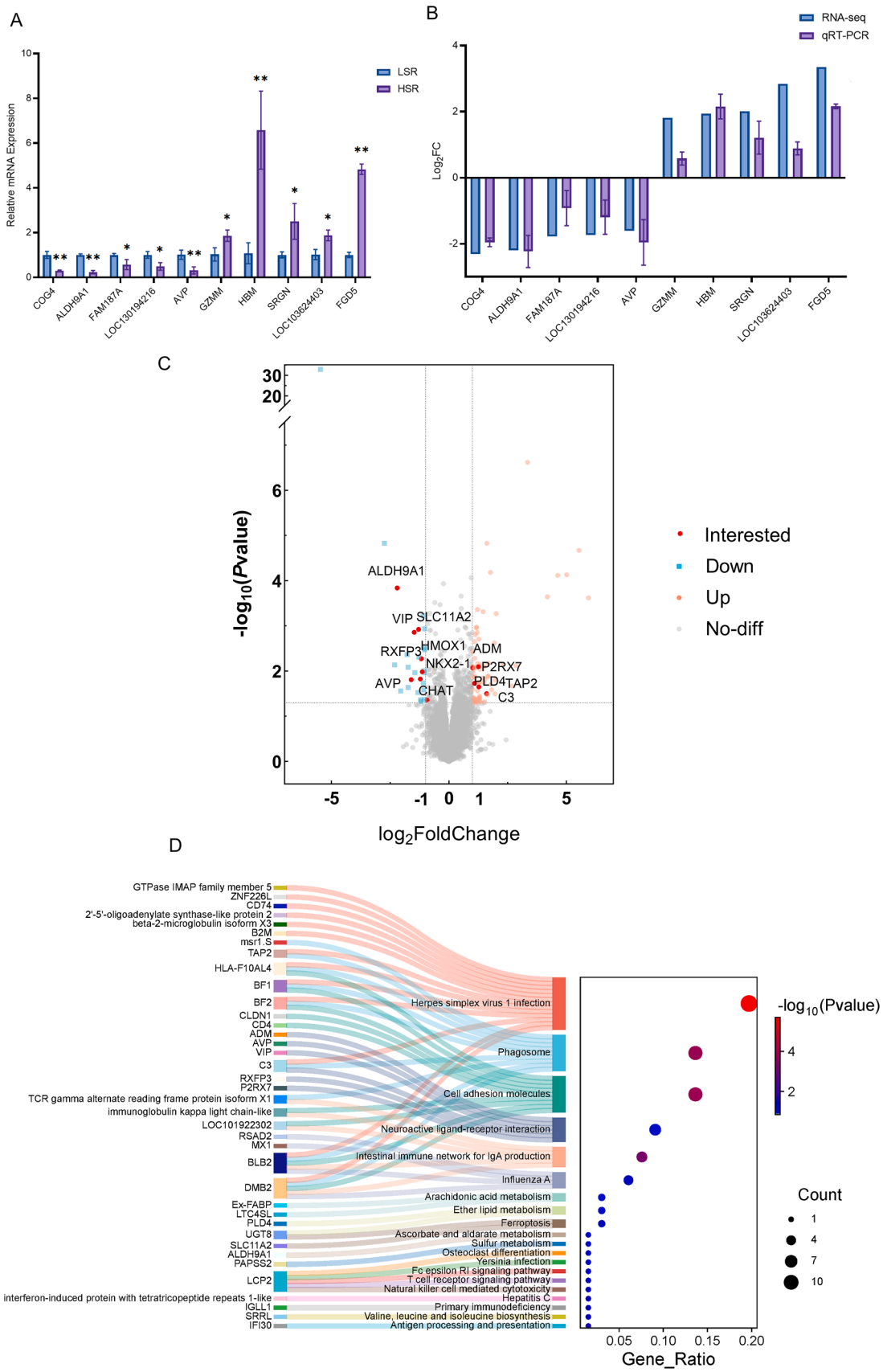

Fig. 4. Transcriptome profiling in Amygdala between high social rank (HSR) and low social rank (LSR) hens. A. PCR validation of amygdala transcriptome. B. Volcano of differentially expressed metabolites in cecum between HSR and LSR hens. Values of log2FoldChange >1 indicate higher levels in HSR relative to LSR hens, while values of log_2FoldChange<1 indicate higher levels in the HSR hens relative to the LSR hens. C. KEGG enrichment analysis of differentially expressed metabolites in cecum between HSR and LSR hens. n = 8 in each group.

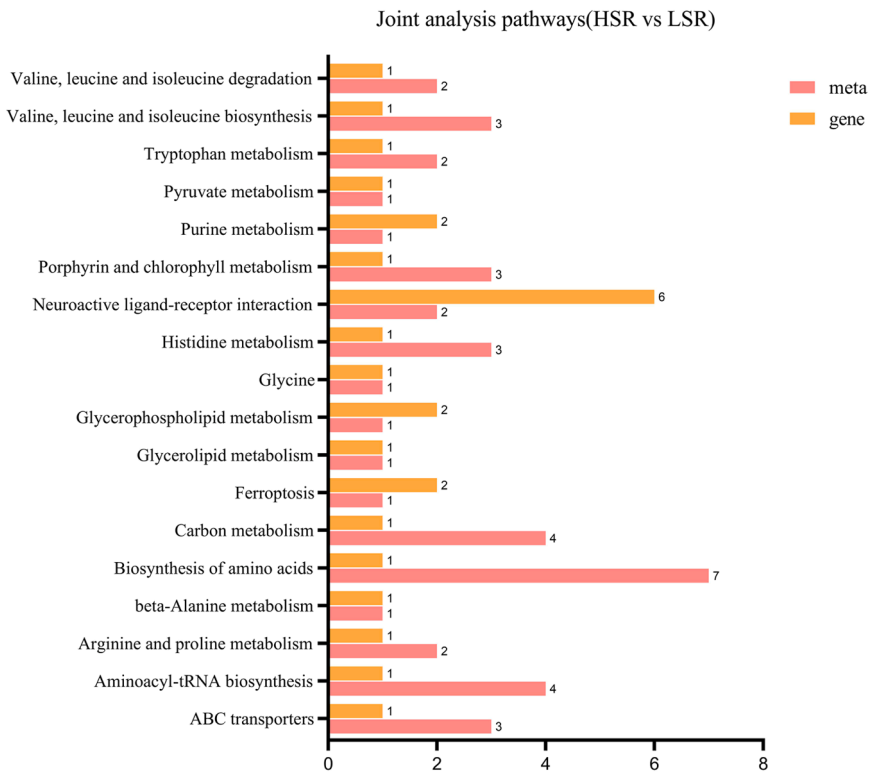

Fig. 5. KEGG pathways collectively enriched by amygdala transcriptome and gut metabolome between high social rank (HSR) and low social rank (LSR) hens. Histograms of DEGs and DEMs enriched in the same KEGG pathway from HSR vs LSR. n = 8 in each group.

immune-inflammatory responses. The up-regulation of *TAP2* in LSR may affect the regulation of splenic immune factors by enhancing the antigen-presentation process and promoting the activation of T-cells and the immune response. Up-regulation of *TAP2* enhances the expression of MHC class I molecules, which in turn increases the antigen processing and presentation capacity and helps to enhance the body's immune surveillance against external pathogens (Park, et al., 2017). Meanwhile, the up-regulation of *PLD4* and *P2RX7* is associated with release of pro-inflammatory factors, such as IL-8 and IFN-γ. This is probably activated by the NF-κB pathway and P2X receptor signalling pathway, which in turn enhances the immune response of chickens (Akizuki, et al., 2019; Shokoples, et al., 2023). On the other hand, down-regulation of *ALDH9A1* in LSR associated with tryptophan metabolism may reduce immune tolerance in the gut-spleen axis, which in turn affects the regulation of splenic immune factors (Chen, et al., 2024a). Down-regulation of *SLC11A2* in LSR is associated with impaired transport of iron ions and thus the suppression of splenic immune responses (Robertson, et al., 2024). We hypothesized that *TAP2, PLD4*, and *P2RX7* in amygdala of high-ranking hens may promote spleen pro-inflammatory function by activating the NF-κB pathway, the TCR signaling pathway, and the P2X receptor signaling pathways and promoting the expression of pro-inflammatory factors. Whiles, the down-regulation of *ALDH9A1*, and *SLC11A2* in amygdala of high-ranking hens may reduce the intensity of the immune response by suppressing immune cell function and immune tolerance pathways. These findings suggest that hens of high social rank may exhibit a more regulated immune response with reduced excessive pro-inflammatory responses.

For the amygdala trancriptome, ferroptosis pathway, as a mode of cell death induced by iron overload and oxidative stress, directly affecting the function of the immune and stress response, was significantly enriched (Zhang, et al., 2022). Our study suggested that low ranking hens may lead to iron accumulation and oxidative damage in the amygdala by down-regulating the expression of *SLC11A2* and *HMOX1*, thereby activating ferroptosis pathway. Meanwhile, glutamate, as a major excitatory neurotransmitter in the central nervous system, plays a crucial role in regulating neural activity, and shapes neural circuits and intracellular signaling, which was down-regulated in the cecum of HSR hens. This is somehow supported by our previous study that low ratio of glutamate neuron clusters was found in the amygdala of high-ranking roosters than low-ranking roosters (Chen et al., 2024b). Glutamate is involved in the regulation of intracellular calcium influx through its receptors and enhances intracellular calcium concentrations, including through activation of N-methyl-D-aspartate (NMDA) receptors. When the glutamate system is dysregulated, intracellular calcium ion concentration rises, leading to increased oxidative stress, which in turn contributes to iron accumulation and activation of the ferroptosis (Xie, et al., 2022). In addition, differences in cecum neurotransmitters may reflect the indirect effects of social rank on ferroptosis with higher concentration of cecum tyrosine in HSR than that LSR. Previous studies indicate that l-tyrosine and l-tryptophan can inhibit cell death via ferroptosis. This suggests a potential role for tyrosine in modulating the ferroptosis, which may regulate the stress resilience of the nervous system and subsequently influence the immune functionality of social ranks in chickens (Liu, et al., 2023; Zeitler, et al., 2021). Down-regulation of *SLC11A2* and *HMOX1* in low-ranking hens may lead to an imbalance in iron metabolism and further activation of the ferroptosis by enhancing oxidative stress, which in turn affects the function of glutamate system. A previous study has shown a close interaction between iron overload and dysfunction of the glutamate system, which may exacerbate the activation of the ferroptosis (Zhong, et al., 2024). We speculated that high ranking individuals maintain a balance between iron homeostasis and antioxidant capacity by more effectively regulating the expression of *SLC11A2* and *HMOX1*, preventing from ferroptosis, and consequently maintaining normal immune function and a lower stress response. Accordingly, individuals of low social rank may suffer from inefficient immune function and enhanced stress response due to activation of the ferroptosis pathway and dysregulation of the

glutamatergic system.

Basing on the integrated analyze of differentially expressed genes and metabolites in the amygdala and cecum, we found that neuroactive ligand-receptor interaction pathway was significantly enriched. This pathway was involved with the interaction of multiple neurotransmitter receptors and played a key role in stress response, immune regulation and emotion management (Su, et al., 2024). Our results showed that genes related to emotion and stress response, such as *RXFP3, VIP and AVP* was down-regulated in the amygdala of LSR hens. Notably, these genes were all projected onto GABAergic neurons (Liu, et al., 2013; Ben-Ari, 2018; Mineur, et al., 2022; Tanaka, et al., 2005). The GABAergic regulatory control of VTA dopaminergic neurons projecting to the NAc for social rank attainment (van der Kooij, et al., 2018). *RXFP3* is highly concentrated in brain regions involved in stress response and anxiety-like behaviour (Watanabe, et al., 2011). Intracerebroventricular infusion of a selective *RXFP3* agonist activates paraventricular hypothalamic nucleus corticotropin-releasing factor neurons and the HPA axis (Watanabe, et al., 2011), and modulates depressive-like behaviours and attenuates stress-induced behaviours in mouse (Ryan, et al., 2013). *VIP* as a neuropeptide, its down-regulation is often associated with mood disorders such as anxiety and depression (Rotzinger, et al., 2010). In low-ranking hens, down-regulation of *VIP* may impair emotion regulation, resulting in individuals being more susceptible to environmental stress and negative emotions such as anxiety and depression. Meanwhile, down-regulation of *RXFP3* may also affect the ability to regulate emotions and behaviors, further exacerbating stress responses in low-ranking individuals. *AVP* is another important neuropeptide widely involved in stress response, emotion regulation, and maintenance of water-salt balance. A decrease of *AVP* is strongly associated with reduced stress tolerance and emotional problems such as depression (Smith, et al., 2014). Down-regulation of *AVP* in low-ranking hens may be a manifestation of chronic stress and emotional instability, further affecting the mood and behavior. Furthermore, compared with HSR hens, we found the expression of 5-HT in LSR hens was significantly reduced in amygdala. The 5-HT, a critical neurotransmitter, has a profound impact on the stress susceptibility and emotional stability (Blanchard, et al., 1991; Hashimoto, et al., 1999; Winberg and Lepage, 1998). The risen of 5-HT in LSR hens may indicate their exposure to chronic stress, which could result in heightened neurotransmitter activity.

In addition, fumaric acid and l-glutamic acid were significantly down-regulated in the HSR hens. Both metabolites were associated with the “ianine, aspartate, and glutamate metabolism”, a crucial metabolic pathway involved in the synthesis and metabolism of neurotransmitters. L-glutamate and aspartate serve dual roles in energy metabolism and modulation of brain function and behavioral responses via the gut-brain axis (Tsurugizawa, et al., 2009). L-glutamic is essential for regulating neurotransmission, cognitive function, and stress in response to the gut-brain axis, which the intestinal microenvironment substantially influences brain function and behaviuor (Popoli, et al., 2012). Dysregulation of l-glutamic can impact both central and peripheral nervous system functions, as well as neuronal transmission, by modulating the pathway "ianine, aspartate, and glutamate metabolism” of the gut (Koda, et al., 2023; Li, et al., 2020). Such modulation can affect the secretion of functional neurotransmitters and facilitate synaptic communication. The decreased gut l-glutamate of HSR hens may indicate a diminished glutamate activity, potentially contributing to the regulation of neural excitability and a mitigation of heightened or anxious behavioral responses (Frank, et al., 2023). The down-regulation of l-glutamate in individuals with high social rank might affect to alleviate neural excitability and mitigate anxiety or depressive symptoms, consequently enhancing cognitive performance and stress responsiveness (Falkenberg, et al., 2014). Fumaric acid, a key metabolite in the tricarboxylic acid cycle, is vital for cellular energy production, and its down-regulation is probably linked to a reduction in oxidative stress, providing cellular protection and contributing to the preservation of neurological health in higher social rank hens (Khalaf, et al., 2024; Shavakandi, et al., 2022). This down-regulation may represent an adaptive mechanism aimed at mitigating oxidative stress, thereby sustaining cognitive and emotional stability and facilitating improvements in learning and memory capabilities by alleviating the burden on the nervous system (Liu, et al., 2015). As DEMs, the significant down-regulation of fumaric acid and l-glutamic acid may reflect unique metabolic adaptive mechanisms in high social rank animals in response to social and environmental stressors. Typically, high ranking animals experience lower stress levels and exhibit enhanced learning capacity, as compared to those lower ranking individuals. Therefore, the metabolic regulation of these pathways in the gut may represent physiological adaptations to environmental stress, influencing individual social rank dynamics. Besides, 4-Oxoproline and l-glutamic acid exhibited significant down-regulation in the HSR hens. These two metabolites, were found to be enriched in the KEGG pathway "l-arginine biosynthesis”. L-arginine serves as a crucial amino acid in both human and animal physiology, participating extensively in processes such as protein synthesis, nitrogen homeostasis, and vasodilation (Paul and Ekambaram, 2011). The regulation of the l-arginine biosynthesis is vital for mediating stress responses, energy metabolism, learning capabilities, and cognitive functions (Gupta, et al., 2012). The decreased biosynthesis of l-arginine in high social rank individuals may mitigate excessive oxidative stress by limiting the overproduction of nitric oxide, thereby alleviating chronic stress levels (Kennedy, et al., 1994). The down-regulation of 4-Oxoproline and l-glutamic acid in the intestines of high-ranking hens may facilitate the regulation of stress levels, energy metabolism, and cognitive and learning functions, potentially via modulation of l-arginine synthesis. By optimizing the metabolism of l-arginine, individuals of higher social rank may contribute to the attenuation of chronic stress effects, thereby enhancing their adaptive and cognitive responses to environmental challenges.

Our study underscores a multifaceted relationship among immune responses, neurotransmitters, and metabolites in the modulation of social hierarchy in chickens. Hens classified as higher-ranking show enhanced anti-inflammatory level, thereby promoting greater resilience to stress. Immune-related genes, including *TAP2, PLD4, P2RX7, ALDH9A1, SLC11A2, ADM*, and *C3*, are involved in neural regulation. Neural genes such as *AVP, VIP, RXFP3*, and *NKX2-1* contribute to these interactions. These genes modulate neurotransmitter activity through “GABAergic neural”, “neuroactive ligand-receptor” and glutamate metabolism. Additionally, genes *SLC11A2* and *HMOX1* in the “ferroptosis” pathway plays a crucial role in regulating metabolites such as l-glutamate and tyrosine, which are critical to stress response and thus social hierarchy. Essential metabolites, including fumaric acid, l-glutamic acid, and 4-Oxoproline, exert their effects through ianine, aspartate and glutamate metabolis and l-arginene pathways, establishing connections among stress, cognitive function, and social ranking. Collectively, these elements harmonize to establish immune equilibrium, neural regulation, and metabolic adaptation, ultimately shaping the social hierarchy in chickens (as described in Fig 6).

Conclusion

In conclusion, our study reveals that immune responses, neurotransmitters, and metabolites interact to shape social hierarchy in chickens. Hens exhibiting higher social rank demonstrated a more robust anti-inflammatory response and enhanced stress recovery, while key immune-associated genes, such as *TAP2, PLD4, P2RX7, ALDH9A1, SLC11A2, ADM*, and *C3*, along with neurogenic genes like *AVP, VIP, RXFP3*, and *NKX2-1*, interacted through the “GABAergic neural", “neuroactive ligand-receptor” and glutamate metabolism to modulate neurotransmitter activity, ultimately leading to a similar outcome. Furthermore, *SLC11A2* and *HMOX1* regulate metabolites, fumaric acid and l-glutamic acid which take part in ferroptosis critical to stress, cognition, sequentially induce the social rank. Our results shed light into

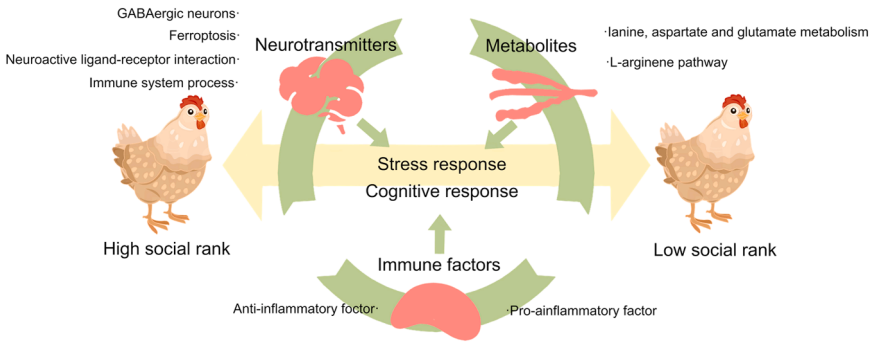

Fig 6. Simplified diagram of the mechanism of social rank in chickens.

the developing strategies to regulate genetic breeding and welfare in poultry. However, the underpinned mechanisms among these parameters still needs to be further explore.

Ethics approval and consent to participate

All animals used in this study were cared and handled in accordance with the guidelines of the Animal Care Committee of Foshan University (Approval ID: FOSU#121).

Availability of data and materials

Transcriptomic data of amylgala have been deposited in NCBI Sequence Read Archive (SRA) database under accession number PRJNA1199152. Un-targeted metabolite data of cecum have been desposited in National Genomics Data Center (NGDC) Omix database under accession number PRJCA033716.

Declaration of competing interest

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.105192.

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