ANIMAL HEALTH AND WELL BEING

Adaptive response to a future life challenge: consequences of early-life environmental complexity in dual-purpose chicks

Chao Yan,^{†,‡} Kate Hartcher,^{||} Wen Liu,[†] Jinlong Xiao,^{†,‡} Hai Xiang,^{‡,\$}
Jikun Wang,[¶] Hao Liu,[†] Hui Zhang,[†] Jian Liu,[‡] Siyu Chen,^{‡,\$,1} Xingbo Zhao,^{†,‡,\$,1}

[†]National Engineering Laboratory for Animal Breeding, College of Animal Science and Technology, China Agricultural University, Beijing 100193, China, [‡]Guizhou Nayong Professor Workstation of China Agricultural University, Bijie 551700, China, ^{||}Centre for Animal Welfare and Ethics, the University of Queensland, Brisbane, QLD 4072, Australia, ^{\$}Guangdong Provincial Key Laboratory of Animal Molecular Design and Precise Breeding, School of Life Science and Engineering, Foshan University, Foshan 528225, China, [¶]Key Laboratory of Qinghai-Tibetan Plateau Animal Genetic Resource Reservation and Utilization, Ministry of Education, Institute of Qinghai-Tibetan Plateau, Southwest University for Nationalities, Chengdu 610041, China

¹Corresponding author: zhxb@cau.edu.cn (X. Z.); jihebuluo2015@163.com (S. C.)

Siyu Chen and Xingbo Zhao contributed equally to the manuscript

Abstract

Conditions in early life play profound and long-lasting effects on the welfare and adaptability to stress of chickens. This study aimed to explore the hypothesis that the provision of environmental complexity in early life improves birds' adaptive plasticity and ability to cope with a challenge later in life. It also tried to investigate the effect of the gut-brain axis by measuring behavior, stress hormone, gene expression, and gut microbiota. One-day-old chicks were split into 3 groups: (1) a barren environment (without enrichment items) group (BG, $n = 40$), (2) a litter materials group (LG, $n = 40$), and (3) a perches with litter materials group (PLG, $n = 40$). Then, enrichment items were removed and simulated as an environmental challenge at 31 to 53 d of age. Birds were subjected to a predator test at 42 d of age. In the environmental challenge, when compared with LG, PLG birds were characterized by decreased fearfulness, lower plasma corticosterone, improved gut microbial functions, lower relative mRNA expression of GR, and elevated mRNA expressions of stress-related genes CRH, BDNF, and NR2A in the hypothalamus (all $P < 0.05$). Unexpectedly, the opposite was true for the LG birds when compared with the BG ($P < 0.05$). Decreased plasma corticosterone and fearfulness were accompanied by altered hypothalamic gene mRNA expressions of BDNF, NR2A, GR, and CRH through the HPA axis in response to altered gut microbial compositions and functions. The findings suggest that gut microbiota may integrate fearfulness, plasma corticosterone, and gene expression in the hypothalamus to provide an insight into the gut-brain axis in chicks. In conclusion, having access to both perches and litter materials in early life allowed birds to cope better with a future challenge. Birds in perches and litter materials environment may have optimal development and adaptive plasticity through the gut-brain axis.

Key words: adaptation, chick, early-life environmental complexity, fearfulness, gene expression, gut microbiota

Abbreviations

5-HT1A	5-hydroxytryptamine (serotonin) receptor 1A
BDNF	brain-derived neurotrophic factor
CRH	corticosterone-releasing hormone
GR	glucocorticoid receptor
NR2A	N-methyl-D-aspartic acid receptor subunit 2A
OTU	operational taxonomic unit
PICRUST	Phylogenetic Investigation of Communities by Reconstruction of Unobserved State
Rdit	Relative to an Identified Distribution
TrkB	tyrosine kinase receptor B

Introduction

Barren environmental conditions generally cause complicated and long-lasting effects by reducing adaptability and increasing vulnerability to stress in birds (Elfwing et al., 2015). The environment exerts intricate effects on chickens, particularly during the sensitive early-life period affecting production, behavior, and welfare throughout the birds' whole lives (Campbell et al., 2018). Barren environments during early life give rise to a series of problems in later life including an increased risk of feather pecking, fearfulness, stereotypic behaviors (De Haas et al., 2014; Rodenburg and Haas, 2016), greater levels of abnormal hormones and gene expression (Elfwing et al., 2015). Early-life stress is also associated with an imbalance of gut microbiota in broilers (Shi et al., 2019). Environmental enrichment is thought to be "an improvement in the biological functioning of captive animals resulting from modifications to their environment" (Newberry, 1995). In recent years, studies have found positive effects of providing environmental enrichment during the early-life period on feather pecking, fearfulness, brain morphology, and neural plasticity in broilers and layer hens (Brantsæter et al., 2017; Tahamtani et al., 2018). Besides, litter materials, perches, and complex housing have been shown to increase gut microbial α diversity and improve gut microbial colonization and development, affecting the production performance of chickens (Torok et al., 2009; Chen et al., 2019). These positive changes therefore ultimately optimize the bird's adaptability and improve welfare (Riber et al., 2017, 2018).

Animals receiving environmental complexity and stimuli during early-life prepare an organism to be adaptive for the individual's future life. The "silver spoon" hypothesis stipulates that providing good conditions during early life is advantageous for fitness and performance throughout life due to having the necessary stimulation for optimal development (Pat, 2008). Another model is the mismatch theory, which means when individuals receive a simple environment during early-life, they then adapt normally to similar conditions in later life (Pat, 2008; Patrick et al., 2014). These studies explored the ability of animals' adaptation to environments, which means encountering certain environmental conditions in early life and the triggering of phenotypic neural plasticity pathways to cope with future life challenges (Van Bodegom et al., 2017). Thus, understanding early-life conditions is crucial for animal

welfare and could help to explain the phenotypic variation in populations (Goerlich et al., 2012).

Gut microbiota has an inseparable relationship with the host and has been shown to affect birds' performance, health, and welfare (Apajalahti, 2005; Oakley et al., 2015; Sugiharto, 2016). Several studies examining the so-called gut-brain axis in response to early-life stress have been conducted in rats (O'Mahony et al., 2011; Hyland et al., 2015). Studies have shown that gut microbiota can modulate changes in region-specific gene expressions in the brain (Nobuyuki et al., 2004). Further, changes in the expression of hypothalamic genes have been associated with anxiety-like behavior (Bercik et al., 2011; Cryan and Dinan, 2012). For example, changes in the expression of brain-derived neurotrophic factor (BDNF) and 5-hydroxytryptamine (serotonin) receptor 1A (5HT1A) mRNA were linked to anxiety-like behavior in germ-free mice (Bercik et al., 2011; Cryan and Dinan, 2012). Although the gut-brain axis has important effects on behavior, and gene expression, these have not yet been examined in chickens.

This study, therefore, aimed to test the hypothesis that the provision of environmental enrichment in early life improves adaptive plasticity in chickens and subsequently better prepares them for a challenge in later life. Further, we hypothesized that the environmental complexity presented in early life would impact gut microbiota, affecting behavior and gene expression. We measured a certain amount of indicators, including *in situ* behavioral traits, fearfulness, corticosterone, body condition, hypothalamic gene expressions, and gut microbiota. Our study will make recommendations to improve birds' early-life environments to affect their future welfare and adaptability to stress.

Materials and Methods

Ethical note

The experimental protocol was approved by the China Agricultural University Laboratory Animal Welfare and Animal Experimental Ethical Inspection Committee (approval number: CAU20170618-3).

Chicks and treatments

The trial was performed at an organic farm at Nayong County, Bijie City, Guizhou Province, China. One hundred and twenty newly hatched female Weining chicks, a Chinese dual-purpose native breed, provided by Guizhou Nayong Yuanshengmuye Ltd were used. They were sourced from a breeding farm 2 hr drive from the farm. Chicks were separated into 3 different early-life environmental groups ($n = 40$) for brooding from post-hatching to 31 d of age. The 3 treatment groups were: (1) a barren environment (without enrichment items) group (BG); (2) a litter materials (wood shavings and sand) group (LG); and (3) a perches with litter materials (wood shavings and sand) group (PLG). The LG had access to 2 substrates: a plate of sand ($1 \times 1 \text{ m}^2$) and wood shavings ($1 \times 1 \text{ m}^2$), which were replaced daily. The PLG was provided with 2 perches (length: 2 m; diameter: 0.05 m) plus the same litter materials as the LG. Each group was kept in a pen measuring $2 \times 2 \times 1.2 \text{ m}$, and 0.5 m high above the ground, with wire flooring and 2 drinkers and feeders per group. All enrichment materials were removed from 32 d of age. All treatment groups had a heat lamp to guarantee the temperature exceeded 32°C ($\pm 1^\circ\text{C}$) during brood to 16 d of age. The lighting schedule was 16 hr light: 8 hr dark. All chicks

were fed a commercial starter feed for the first 31 d and then a grower feed (New Hope Group, Chengdu, Sichuan, China) to the end of the experiment at 53 d. The birds survived in the BG ($n = 38$), LG ($n = 38$), and PLG ($n = 40$) until the end of the study. The experimental design is shown in Figure 1.

Body weight

The body weights of all chicks in the 3 groups were measured at 21, 28, 35, 42, and 49 d of age.

In situ behavior observations

When birds were 7, 14, 21, and 28 d of age, behaviors in all animals were observed by video in their home pens over a 2-hr period (8:30 to 10:30 a.m.) using a binary scoring system at 1 min intervals and recording whether a behavior was present or absent at that point in time. Behaviors parameters that were recorded included “aggression” (a bird attacking or fighting another bird with claws or the beak without picking feathers), “preening” (combing and picking at the feathers with the tip of the beak for cleaning and tidying), “feather pecking” (vigorous pecking the feathers of another bird with the beak), and “stereotypic behavior” (making an invariant, repetitive, and purposeless motions such as pacing). The observations were recorded by the same person throughout the experiment.

Novel arena test

Novel arena tests are well-validated behavioral tests performed on individual birds to assess variations in exploration and general vigilance (Favati et al., 2015). The motivational states behind the test are considered to be fear and anxiety in response to social isolation and perceived potential danger in the novel open area. At 31 d (when the treatments were removed and all birds exposed to the barren environment), 15 randomly selected chicks from each treatment group were transported (<5 min) to the test room in a dark basket. The chicks were allowed 30 min to acclimatize to the basket to reduce and standardize the stress responses before testing. The test was recorded by video for 15 min. The occurrence of “foraging” (pecking at the ground), “vigilance” (appearing alert, with the head looking around above a horizontal plane), and “exploration” (transitions across the

imaginary lines, for specific information see [Supplementary Figure S1](#)) were analyzed by a scanning method at 10 s intervals, where those behaviors were recorded as present or absent at that time point.

Predator test

A predator model was used to investigate the difference in fearfulness between individual birds (Favati et al., 2015). All birds were familiarized with live worms (200 g for each group per day) as a highly valued food resource, which was provided alongside the regular feed from 35 to 42 d in their home pens. At 42 d, food and water were restricted from 18:00 hours the previous day before the test. Twenty randomly selected chicks from each treatment group were placed in a 4 m L × 4 m W × 1.2 m H test arena simultaneously. Two polyvinyl chloride material hawks (0.11 m L × 0.27 m W × 0.07 m H) were used as predator models to test the fear response. Regular feed was placed in 1 corner of the arena, and regular feed with live worms was placed in the opposite corner where 2 hawk models were placed 0.50 m vertically above the feed. The hawks' vocalizations were played 3 times at 0 to 1 min, 6 to 7 min, and 11 to 12 min during the test period. The test was observed and recorded by video. The immediate behavior reactions in response to the “predator” were measured for each bird by using a scale from 0 to 3, where 3 represented the highest level of fear (Favati et al., 2015; Table 1). “Predator vigilance” in response to the hawk models was also recorded for 5 min after the vocalizations of the hawk were played using the same method for “vigilance” in the novel arena test (where vigilance was recorded where the bird appeared alert, with the head looking around above a horizontal plane).

Feather condition and footpad dermatitis

At 52 d of age, feather condition and footpad dermatitis were measured as indicators of animal health. Six parts of the body including the head, breast, back, wing, vent, and abdomen were evaluated using a 5-point scoring system (Wechsler and Huber-Eicher, 1998). Score 0 = no damage, 1 = some feather damage but no denuded area, 2 = denuded area up to 3 × 3 cm, 3 = denuded area more than 3 × 3 cm, 4 = completely denuded. A 5-point scoring system was also used to evaluate footpad dermatitis

Figure 1. Experimental roadmap description.

(Martrenchar et al., 2002) where scores 0 = no lesions, 1 = lesions on either of the footpads covering up to 5%, 2 = lesions covering up to 5% to 25%, 3 = lesions covering up to 25% to 50%, and 4 = lesions >50%.

Plasma corticosterone concentration

At 53 d of age, 10 randomly selected birds in each treatment were humanely euthanized. Blood samples were collected immediately and put into anticoagulation tubes, 4000 × g centrifuged for 5 min at 4 °C and then stored in 1.5 mL tubes at –20 °C for 1 mo to prepare the subsequent detection. Corticosterone concentrations (ng/mL) were then measured using the commercial assay chick ELISA kit (ColorfulGene, JYM0062Ch). The intra- and interassay coefficient of variation are 0.036 to 0.089 and 0.104 to 0.248, respectively.

Gene expression of the hypothalamus

After the slaughter at 53 d, tissue from the hypothalamus was immediately collected from 5 of the euthanized birds in each group and stored in dry ice and then at –80 °C for 1 mo until further processing. To investigate the relative expression of genes, high-quality total RNA was isolated using the RNeasy Mini-Extraction kit (Aidlab, RN2802, China) according to the manufacturer's protocols. The purity of RNA was the ratio of OD 260/OD 280 (a measure of protein contamination) in 2.0 to 2.2, of which 2.2 represented high-quality RNA. The RNA integrity was analyzed by the method of agarose gel electrophoresis, of which the quantity of 28S rRNA was twice than 18S rRNA. Then each qualified RNA sample was reversed the transcript to cDNA using TRUEscript RT MasterMIX (Aidlab, PC5801, China) in a

20-μL volume, 16 μL containing 1000 ng RNA template in RNase-free water, and 4 μL 5 × TRUE RT MasterMix, following programs: 42 °C for 10 min for RT-qPCR analyses. The relative mRNA expression of stress-related genes included corticosterone-releasing hormone (CRH, a hypothalamic peptide in the control of HPA axis), glucocorticoid receptor (GR, known as a stress-related indicator), brain-derived neurotrophic factor (BDNF, a key neurotrophin linked in neural growth and survival), tyrosine kinase receptor B (TrkB, high-affinity receptor for brain-derived neurotrophic factor), N-methyl-D-aspartic acid (NMDA) receptor subunit 2A (NR2A, as an indicator of synaptic development and plasticity), and 5-hydroxytryptamine (serotonin) receptor 1A (5HT1A, involved in the serotonin receptor to emotion and behavior) were measured through RT-qPCR. Gene-specific complementary primers were designed according to their gene sequences (Table 2). The glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was selected as a reference gene (Borowska et al., 2016). RT-qPCR was conducted on qTOWER 2.2 touch (Analytik Jena, Germany) in a 20-μL volume containing 10 μL SYBR×Premix Ex Taq (Aidlab, PC3302, China), 0.5 μL of each forward and reverse primer (10 μM), 1 μL of cDNA and 8 μL ddH₂O according to the following program: 95 °C for 3 min; 95 °C for 10 s, annealing (temperature see in Table 1) for 20 s and 72 °C for 20 s for 40 cycles. Each amplification was performed for 3 control replicates, and 3 case replicates. The amplification efficiencies were close to 100%, using the 2^{-ΔΔCt} method for calculating the relative gene expression levels of a sample.

Gut microbiota

At 53 d, cecum contents from the 10 euthanized birds were also immediately collected. These were stored in dry ice and then at –80 °C until further processing. The cecum microbiota was measured and analyzed by the 16S rDNA. Total bacterial genomic DNA samples were extracted using the Fast DNA SPIN extraction kits (MP Biomedicals, Santa Ana, CA). The quantity and quality of extracted DNAs were measured and agarose gel electrophoresis, respectively. The full-length of V3–V4 hypervariable region from the bacterial 16S rRNA operon was amplified from cecal DNA using a universal primer set, 16S-338F:(5'-ACTCTACGGGAGGAGCA-3') and 16S-806R:(5'-GGACTACHVGGGTWTCTAAT-3'), individually barcoded (Zhou et al., 2016). The high-quality DNA was used to construct a library, and the library was then used for sequencing. Sequencing of the

Table 1. Scores of the immediate behavioral reaction of chicken to the predator test

Score	Description of behavioral reaction
0	No visible response at all
1	The bird lifts its head once and then immediately returns to previous behavior
2	The bird lifts its head and looks around and/or rapidly walks for more than 3 s or freezes for 3 to 5 s
3	The bird runs or attempts to fly away or freezes for more than 5 s

Table 2. Primers used for RT-qPCR analysis

Genes	GenBank accession number	Primer sequences, 5' to 3'	T _m , °C	Size, bp
GAPDH	AF047874.1	F: GAAGGCTGGGGCTCATCTG R: CAGTTGGTGGTGACGATG	60	150
CRH	NM 001123031.1	F: TCTCCCTGGACCTGACTTTC R: GCCTCACTTCCCGATGATT	58	119
GR	NM 001037826.1	F: TGGGAACGCTCAACCTTTC R: GCTGCCACAAGTAAGAACCACATA	58	184
BDNF	NM 001031616.1	F: ACATCACTGGCGGACACTTT R: GTTACCCACTCGCTTGTGCT	61	275
TrkB	NM 205231.1	F: TCCCAAAGTGGGACTTACC R: GAGCACCAGGACACATTA	58	125
NR2A	XM 025155259.1	F: CATCTTTGCCACTACGGG R: TCAGCCACAGGGTTTCTAAC	58	129
5HT1A	NM 001170528.1	F: GGTGCTGAACAAGTGGAATCTG R: AAGAAGCCGATGAGCCAGGT	58	207

full-length V3–V4 16S rRNA PCR product using Illumina Miseq PE300 platform was generated with millions of reads up to 300 × 2 bp in length.

The bioinformatics analysis was carried out with sequencing data. The raw data were filtered to eliminate the adapter pollution and low quality to obtain clean reads using UCHIME (v4.2.40). The paired-end reads were merged the fragments by FLASH v1.2.11, which generated the consensus sequence. The high-quality paired-end reads were combined to tags based on overlaps. The tags were clustered into operational taxonomic unit (OTU) with a 97% sequence similarity threshold by scripts of software USEARCH (v7.0.1090). OTU representative sequences were taxonomically classified using Ribosomal Database Project (RDP) Classifier v.2.2 trained on the Greengenes database, using 0.6 confidence values as cutoff.

To measure the relative abundance of gut microbiota, we conducted a heat map using the package “pheatmap” of software R (v3.1.1) reflecting the hierarchical clustering of samples. Based on the OTUs information, the relative abundance of taxonomic ranks, α diversity, and β diversity were analyzed through bioinformatics. α diversity was used to explore within-group sample diversity (Schloss et al., 2009). The indexes of Observed species, Chao, Ace, Shannon's diversity, Simpson's diversity, and Good's coverage were applied to analyze α diversity. Higher index numbers represent higher α diversity. Anosm analysis is a nonparametric test to test whether the differences between groups (2 or more groups) are significantly greater than the differences within groups to determine if setting groups is logical. When R is between $(-1, 1)$ and R is >0 , this indicates a significant difference between groups. When R is <0 , it indicates that the difference within the group is greater than the difference between groups. The reliability of statistical analysis is represented by P , and $P < 0.05$ indicated statistical significance. β diversity was applied for evaluating differences of groups in species complexity, which was done by software QIIME (v1.80). One of the β diversity methods, principal component analysis (PCA), was used to construct 2D graphs to summarize contributors mainly responsible for the differences of OTUs composition in different groups. The package “ade4” of software R (v3.1.1) was taken to conduct in the step.

The functional capabilities of microbial communities were predicted by PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved State; Qiong et al., 2007), which was used in the Human Microbiome Project and predicts the abundance of gene families in host-associated and environmental communities. PICRUSt was carried out to identify significant KEGG differences between the functional potential of the microbial communities.

Statistical analysis

Body weight met the assumptions for parametric analysis after checking for normality and homogeneity of variance and analyzed using a 2-way ANOVA followed by post-hoc testing in IBM SPSS Statistics 21. Corticosterone concentration and gene expressions were checked for normality and homogeneity of variance test and analyzed using a 1-way ANOVA. The behavioral data did not meet the assumptions for parametric analysis, and nonparametrical methods were therefore conducted. The Kruskal–Wallis test was used for *in situ* behavior observations, the novel arena test, the predator test's vigilance, and the α diversity. The Wilcoxon test was used to analyze the gut microbial composition and function. The relative to an identified distribution (Ridit) analysis was used to assess the fear score in response to the predator test, feather condition, and footpad dermatitis. All values with $P < 0.05$ were regarded as statistically significant.

Results

Body weight

The body weight of the BG birds was heavier than in the LG (average 22.64 and 22.70 g) at 21 and 28 d of age, and heavier than the PLG birds (average 16.39 g) at 21 d ($P < 0.05$) but not 28 d (Table 3). No significant difference was observed between the 3 groups between 35 and 49 d (Table 3). There was no interaction between day and treatment group.

Behavioral traits

Aggression in the LG was higher the 58.52% (percent difference) than in the PLG ($P < 0.05$), while there was no difference in BG compared with the 2 other groups (Figure 2a). There were pairwise differences between treatments for preening, where birds in the BG performed the least preening, followed by LG (10.94% compared with BG) and PLG (74.06% and 55.02% compared with BG and LG, respectively; $P < 0.05$; Figure 2a). Feather pecking was higher in BG (40.05% and 43.78%) than LG and PLG ($P < 0.05$), while there was no difference between LG and PLG (Figure 2a). Stereotypic behavior was higher in BG (225.60% and 925.93%) than in the LG and PLG ($P < 0.05$), and higher in LG (184.38%) compared with PLG ($P < 0.05$; Figure 2a).

Response to the novel arena test

Birds in the BG showed higher vigilance than those reared in the LG (57.45%, percent difference) and PLG (109.43%; $P < 0.05$), while the responses of LG compared with PLG did not differ (Figure 2b). The incidence of exploration and foraging behaviors were not different among the groups (Figure 2b).

Table 3. Body weight at different age of each group

Body weight ¹	21 d	28 d	35 d	42 d	49 d
BG	195.14 ± 4.90 ^a	315.14 ± 7.92 ^a	431.62 ± 12.52	511.94 ± 13.73	643.61 ± 19.59
LG	175.50 ± 6.76 ^b	287.44 ± 10.07 ^b	412.82 ± 14.71	472.56 ± 16.46	594.47 ± 24.33
PLG	178.75 ± 4.60 ^b	292.00 ± 7.67 ^{ab}	407.50 ± 11.04	495.64 ± 13.31	593.61 ± 22.87
P-value	0.033	0.060	0.387	0.168	0.204
Day × treatment interaction	0.913				

¹BG, barren environment group; LG, litter materials group; PLG, perch with litter materials group.

Values with different small letter superscripts mean a statistical difference in the same column ($P < 0.05$) in the same column.

Figure 2. Behavioral traits. The effects of environmental complexity during early-life (a) aggression, preening, feather pecking, and stereotypic behavior; (b) exploration, foraging, and vigilance in the novel arena test. In the later barren environment, (c) the response to the predator and predator vigilance in the predator test. Differing superscripts represent statistical differences.

Response to the predator test

The responses to the predator vocalizations were higher in the LG than those of the PLG and BG birds ($P < 0.05$), while PLG showed no difference with BG (Figure 2c). Moreover, birds reared in the PLG exhibited reduced predator vigilance compared with both BG (27.31%, percent difference) and LG (32.91%) ($P < 0.05$), while there was no difference between LG and BG (Figure 2c).

Plasma corticosterone concentrations

Corticosterone concentrations were higher in LG (34.56%, percent difference) and BG (60.28%) than in PLG, as LG (19.10%) higher than BG ($P < 0.05$; Figure 3).

Feather condition and footpad dermatitis

Birds in the BG had the poorest feather condition on their wings than the enriched groups ($P < 0.05$; Figure 4). There were no differences in condition on other body parts including the head, neck, back, vent, breast, and abdomen feathers (Figure 4). There was also no difference in footpad dermatitis between groups (Figure 4).

Gene expression in the hypothalamus

The relative mRNA expression of GR was higher in LG (1.06 and 2.60, times) than BG and PLG ($P < 0.05$), while PLG and BG showed no difference (Figure 5b). PLG and BG had higher relative mRNA expressions of CRH (5.73 and 4.66; $P < 0.05$; Figure 5a), BDNF (4.83 and 1.57; $P < 0.05$; Figure 5c), and NR2A (1.64 and 1.19; $P < 0.05$;

Figure 3. Plasma corticosterone levels. Differing superscripts represent statistical differences.

BG, barren environment group; LG, litter materials group; PLG, perch with litter materials group.

Figure 5e) than LG. The relative mRNA expression of *TrkB* in LG was lower than PLG (0.52; $P < 0.05$), while BG did not differ from LG and PLG (Figure 5d). No difference was found between treatments in *5HT1A* expression (Figure 5f). There was no difference between PLG and BG among those genes (Figure 5).

Gut microbiota

Gut microbial community profiles

Cecum samples were analyzed for gut microbiota by the sequence of 16S rDNA. The average pair number of reads was

Figure 4. Scores of feather condition and footpad dermatitis. Differing superscripts represent a statistical difference.

81248 ± 184. Due to paired-end reads overlapping, high-quality reads were combined with a total of 1 538 486 tags. After removing the primer sequences, a total of 1 492 305 tags were obtained, with 74 615 tags per sample on average. Filtered tags were clustered at 97% similarity and generated the number of OTUs among BG, LG, and PLG.

The α diversity of the gut microbiota was higher in the BG than the LG analyzed by the indexes of Observed species, Chao, and Ace ($P < 0.05$), while the Shannon, Simpson, and Good's coverage index analysis found no difference (Table 4). Those indexes showed no difference in PLG compared with BG and LG (Table 4). There were greater differences among groups than within groups among BG, LG, and PLG ($R = 0.353$, $P = 1e-04$; Figure 6a), indicating the reliability of these samplings. Beta diversity was presented in the PCA plot and caused a unique structural change of gut microbiota in the BG compared with the LG and PLG, while the LG and PLG had a similar microbial structure (Figure 6b).

Gut microbial composition

The relative abundance of microbiota at the genus level is shown in Table 5. *Mucispirillum*, AF12, and unclassified microbiota were higher, while *Sphaerochaeta*, *Spirochaeta*, and *Brachyspira* were lower in LG than BG ($P < 0.05$; Table 5). When PLG was compared with BG, the relative abundance of *Deftuviitalea*, *Lactobacillus*, and *Mucispirillum* were higher, and the relative abundance of *Rikenella*, *Prevotella*, and *Brachyspira* were lower ($P < 0.05$; Table 5). The microbial composition showed no difference between the LG and PLG at the genus level. The relative abundance of the top 35 dominant genus-level bacteria was examined and shown on the heat map reflecting the hierarchical clustering of samples. The dominant genera were different between the 3 groups (Figure 6c). The dominant genera in BG were *Bacteroides*, *Desulfovibrio*, *Phascolarctobacterium*, and *Prevotella*; in LG, they were *Fusobacterium*, *Mucispirillum*, *Megamonas*, and *Faecalibacterium*; and in PLG, they were *Parabacteroides*, *Lactobacillus*, and *Ruminococcus*.

Gut microbial function

Histidine metabolism, oxidative phosphorylation, ribosome biogenesis, and thiamin metabolism were all upregulated in LG was compared with BG, while pentose and glucuronate interconversions, and transcription machinery were downregulated ($P < 0.05$; Supplementary Table S1). When PLG was compared with BG, lipopolysaccharide biosynthesis, and lipopolysaccharide biosynthesis proteins were lower ($P < 0.05$; Supplementary Table S1). Pentose and glucuronate interconversions, signal transduction mechanisms, and transcription factors pathways were higher in PLG birds compared with LG birds, while oxidative phosphorylation, ribosome biogenesis, thiamin metabolism, lipopolysaccharide biosynthesis, and lipopolysaccharide biosynthesis proteins were lower ($P < 0.05$; Supplementary Table S1).

Discussion

When exposed to early-life environmental complexities, body weights of the birds from the PLG and LG were lower than BG at 21 d of age, which does not support the previous research showing that perches or litter have no effects on body weight (Martrenchar et al., 2000; Aksit et al., 2017). It was, however, consistent with findings that broilers reared with bedding had lower body weights (Riber et al., 2018). Thus, the reason for body weight difference is unknown and complicated in various environments. In particular, birds in the PLG and LG exhibited lower rates of feather pecking and stereotypic behaviors than the BG birds. This is in line with several studies on the topic, illustrating that higher environmental complexity and the provision of environmental enrichment in early life contribute to lower rates of feather pecking later in life (Tahamtani et al., 2016; Campbell et al., 2018), and that birds with barren conditions during early life were afflicted with stress and abnormal behaviors (Riber et al., 2018). Results indicated that the higher the level of complexity in a treatment group, the more preening

Figure 5. The relative mRNA expressions. (a) CRH, (b) GR, (c) BDNF, (d) TrkB, (e) NR2A, and (f) HTR1A in the hypothalamus at 53 d. Differing superscripts represent a statistical difference.

(a comfort behavior) (Delius, 1988; Riber et al., 2018) was observed in the PLG and LG birds. Further, stereotypic behaviors and aggression were higher in LG than PLG birds, demonstrating the advantage of the provision of perches in early life. The novel arena test is one of the measure reactions to evaluate a trait of fearfulness. PLG and LG birds showed lower levels of vigilance in the novel arena test. This is in agreement with previous studies found that access to environmental enrichment in the form of colored drawings and objects (Jones and Waddington, 1992), as well as the provision of litter (Brantsæter et al., 2017), reduced fearfulness in birds. Additionally, the lower levels of fearfulness in the PLG may have contributed to lower rates of feather pecking seen in this group (Rodenburg et al., 2013; Hartcher et al., 2016). These findings of behavioral traits during early life are supported by the previous reviews, which suggested that perches and litter materials condition exerted positive effects on behavior and biological function (Campbell et al., 2018; Riber et al., 2018).

In the future environmental challenge, body weight was no difference between treatment groups at 35 and 49 d of age. Combined with this study and other studies (Martrenchar et al., 2000; Aksit et al., 2017; Riber et al., 2018), the law of body weight influenced by environments needs to be further investigated. Fearfulness was evaluated by the predator test in later life. PLG birds from the more complex environment generally exhibited lower levels of fearfulness (lower responses to the predator and predator vigilance) in the predator test. These findings support previous studies showed that birds with the most enrichment had the lowest fearfulness (Jones and Waddington, 1992; Brantsæter et al., 2017; Tahamtani et al., 2018). Meanwhile, they also had the lowest concentrations of stress hormone corticosterone, and a downregulation of GR, a stress-related gene, in the hypothalamus. Previous studies have indicated that corticosterone responses were decreased in enriched environments (Fairhurst et al., 2011) and evoked in stress (Feltenstein et al., 2003). The result indicating a downregulation of GR in the hypothalamus of PLG birds is in line with studies on other species which found that enriched environments inhibit the hippocampal GR expression in mice (Lin et al., 2011) and conversely, that exposure to early-life stress increases GR mRNA

expression in the pituitary gland of mammals (Zimmer and Spencer, 2014). Particularly, the hypothalamus is a regulator in the central brain region associated with fearfulness and stress through the HPA axis. In this study, the CRH mRNA expression was upregulated in PLG compared with LG birds. The CRH mRNA expression has been shown to increase when exposed to stress (Hatalski et al., 2000). Since stress responses can be adaptive or maladaptive (Herman et al., 2016), a negative feedback mechanism may have mediated the expression of CRH through the HPA axis, thus decreasing corticosterone concentrations and GR mRNA expression in PLG birds to allow these birds to effectively adapt to their subsequently barren environments in contrast with their LG counterparts (Dallman et al., 1992; Mizoguchi et al., 2001). These results suggest that PLG birds may have had the lowest levels of fear and stress response.

The relative mRNA expressions of BDNF, a key neurotrophin linked in neural growth and survival, and NR2A, an indicator of synaptic development and plasticity, in PLG were upregulated compared with LG birds. It has been shown that enriched environments increase BDNF in the mouse cerebellum (Vazquez-Sanroman et al., 2013) and elevate NR2A expression in the forebrain (Tang et al., 2001), which may explain the results of BDNF and NR2A in PLG birds. The expression of TrkB as a high-affinity receptor for BDNF was higher in PLG than LG. Briefly, birds reared in PLG during early life that were subsequently exposed to the barren condition appear to have a more adaptive response in hypothalamic gene expression.

The heat map showed that the gut microbiota of the PLG was differentiated from the LG and BG, which demonstrated that the gut microbiota was influenced by the provision of perches and litter materials for the chicks. The relative abundance of *Lactobacillus* was higher in PLG than BG. As the previous study showed that *Lactobacillus* species restored heat-stress and maintained the natural stability of the gut microbiota in chicks (Dalloul et al., 2003). One of the core microbiota—*Prevotella*, associated with feed efficiency—was lower in PLG than BG (Singh et al., 2012). This may imply positive effects on production performance in BG birds, although feed efficiency was not recorded. Lipopolysaccharides serve as an immunomodulatory bacterial cell wall component (Cryan and Dinan, 2012) and are

Table 4. Alpha diversity among 3 groups¹

Alpha diversity	Observed species	Chao	Ace	Shannon	Simpson	Good's coverage index
BG	483.90 ± 29.27 (19.60) ^a	553.54 ± 31.60 (19.40) ^a	547.40 ± 31.71 (19.90) ^a	3.88 ± 0.11	0.06 ± 0.01	1.00 ± 0.00
LG	428.10 ± 23.10 (9.15) ^b	489.30 ± 25.39 (9.10) ^b	486.10 ± 25.73 (9.80) ^b	3.74 ± 0.11	0.07 ± 0.01	1.00 ± 0.00
PLG	492.70 ± 9.29 (17.75) ^{ab}	558.53 ± 9.97 (18.00) ^{ab}	547.85 ± 10.33 (16.80) ^{ab}	3.80 ± 0.09	0.07 ± 0.01	1.00 ± 0.00
P-value	0.018	0.018	0.032	0.442	0.308	0.259

¹BG, barren environment group; LG, litter materials group; PLG, perch with litter materials group.

Values with different small letter superscripts mean a statistical difference ($P < 0.05$) in the same column. The rank is given in brackets.

¹BG, barren environment group; LG, litter materials group; PLG, perch with litter materials group.

activated as part of the stress response; lipopolysaccharide biosynthesis proteins pathways were lower in PLG than LG and BG, another positive effect of the early-life more enriched environment suggested by PLG.

Surprisingly, α diversity was higher in BG than LG. α diversity, one of “gut microbiota barometers”, is of importance for humans and animals. Stress broadly reduces α diversity and decreases the relative abundance of beneficial bacteria in mice (Bailey et al., 2011), chickens (Chen et al., 2019), and dairy cows (Chen et al., 2018). Evidence strongly indicates that animals exposed to more complex and positive environments have higher α diversity than those in barren environments, such as in free-range vs. caged hens (Huang et al., 2018; Chen et al., 2019). The reason why α diversity increased in BG than LG will be described below. In addition, compared to LG, pentose and glucuronate interconversions were higher, while ribosome biogenesis, oxidative phosphorylation, and thiamin metabolism were lower in PLG and BG.

From what-above discussed, in situ behavior, fearfulness, stress-related hormone and genes expression, feather condition, as well as gut microbiota were the greater in the PLG birds. These may be explained by the fact that PLG birds received good stimulation during early life, which appeared to prepare chicks for dealing with the future challenge in later life (Van Bodegom et al., 2017). This is also supported by the “silver spoon” effect that appropriate stimulation in early life is conducive to optimal development and adaptations in later life (Pat, 2008). On the contrary, LG chicks received only litter materials and therefore a less complex environment than PLG. When enrichment was removed, BG birds did not experience this as a new stressor or change in environment, and the BG birds were well equipped to cope with this environmental challenge than LG birds. Overall, our findings of LG birds may be explained by the match–match theory that insufficient environmental stimuli in early life and environmental mismatching with the environment later in life is not conducive to adaptive plasticity to later life challenge (Pat, 2008). Further, we can infer that, due to environmental matching, BG birds had lower plasma corticosterone and higher alpha diversity than LG birds, and displayed no difference hypothalamic gene expression compared to PLG birds. However, birds in the BG had the lowest feather condition on their wings than the other 2 groups.

The implication of the gut-brain axis in chicks

The modulation of gut microbiota has critical effects on regulating the development of behavior, brain, and immune response. This involves the HPA axis, immune, neural, and endocrine pathways through the gut-brain axis (Hughes and Sperandio, 2008; Cryan and Dinan, 2012). For example, the function of corticosterone through the HPA axis plays an essential role in regulating the stress response, which can cross the blood–brain barrier, influence the cross-talk between the brain and gut, and therefore affect behavior (Cryan and Dinan, 2012; Van Bodegom et al., 2017). Notably, germ-free animals were directly used to assess the role of gut microbiota on all aspects of physiology. For example, germ-free mice exposed to mild restraint stress-induced elevated plasma corticosterone through the HPA axis compared with control mice with a normal composition of gut microbiota (Nobuyuki et al., 2004). Besides, stress and the associated HPA axis can influence the gut microbial composition (Cryan and Dinan, 2012). In our study, this may be verified by the decreased plasma corticosterone concentrations seen in the PLG birds compared with LG birds, and the increased CRH mRNA expression in the

Figure 6. Gut microbiota. (a) Anova analysis showed the differences between groups are significantly greater than the differences within groups. (b) Principal components analysis (PCA) analysis β diversity of gut microbial communities. (c) The heat map was reflecting the hierarchical clustering of samples. Differing superscripts represent statistical significance ($P < 0.05$).

Table 5. The relative abundance of gut microbiota in the genus level among 3 groups

Genus ¹	BG, %	LG, %	P-value
<i>Mucispirillum</i>	0.178 ± 0.236	1.499 ± 1.222	0.009
AF12	0.004 ± 0.004	0.029 ± 0.017	0.011
<i>Spirochaeta</i>	0.195 ± 0.340	0.000 ± 0.000	0.013
<i>Sphaerochaeta</i>	0.194 ± 0.309	0.014 ± 0.045	0.019
<i>Brachyspira</i>	0.036 ± 0.044	0.000 ± 0.000	0.013
Unclassified	45.466 ± 11.456	30.277 ± 7.821	0.015
	BG	PLG	
<i>Prevotella</i>	1.522 ± 1.175	0.226 ± 0.368	0.048
<i>Lactobacillus</i>	0.199 ± 0.268	3.132 ± 7.748	0.048
<i>Mucispirillum</i>	0.178 ± 0.236	0.653 ± 0.565	0.048
<i>Rikenella</i>	0.044 ± 0.058	0.000 ± 0.000	0.048
<i>Brachyspira</i>	0.036 ± 0.044	0.001 ± 0.003	0.048
<i>DeFluviitalea</i>	0.001 ± 0.002	0.007 ± 0.006	0.048

$P < 0.05$ means a statistical difference in the same rank.
¹BG, barren environment group; LG, litter materials group.

hypothalamus through the HPA axis in response to altered gut microbial compositions and functions. Accordingly, we only infer that the composition of gut microbiota may have a role in regulating the behavior and brain through the HPA axis. Indeed, health benefits and higher performance are attributed to the balance of gut microbial compositional signature and a disruption of this balance confers the abnormal development (Cryan and O'Mahony, 2011).

The HPA axis as one of the communication pathways plays a critical role in the gut-brain axis. In our study, the lower expression of GR and lower fearfulness in the PLG compared with the LG may have been a response to the gut-brain axis. The results may correspond to a review that showed the absence of GR disrupted the HPA-controlled hormonal response to stress and decreased anxiety in mice (Tronche et al., 1999). Besides, it is worth noting that the GR expression's regulation as a critical role in affecting the gut-brain axis (Wiley et al., 2016) may be implicated in the GR expression and gut microbiota alterations of PLG birds.

Increased hypothalamic gene expressions of BDNF and NR2A mRNA were accompanied by decreased fearfulness in the PLG and BG compared with LG in our study, as well as increased transcription machinery and transcription factors pathways of gut microbiota. Growing evidence indicates that gut microbiota can modulate neurodevelopmental gene expression in the brain. One study showed that increased BDNF and decreased 5-HT1A mRNA expression in the hippocampus and decreased NR2B mRNA expressions in the amygdala reduced anxiety-like behavior (Neufeld et al., 2011). A higher BDNF mRNA expression in this study is therefore likely to be accompanied by reduced anxiety (Bercik et al., 2011; Cryan and Dinan, 2012). The specific microbiota regulating the behavior and brain probably involves multiple mechanisms at the molecular level and has not been fully unraveled.

The gut-brain axis could realize the communication of host-microbiota through neurotransmitters (e.g., histamines and histidine metabolism; Cryan and Dinan, 2012). The thiamin metabolism pathway was upregulated in LG compared with PLG and BG, while the histidine metabolism was downregulated in LG than compared with BG. Those amino-related metabolisms could serve as neurotransmitters donors to realize host-microbiota communication (Cryan and Dinan, 2012; Neis et al., 2015). Signal transduction mechanisms were upregulated in PLG than LG, and lipopolysaccharide biosynthesis, and lipopolysaccharide biosynthesis proteins pathways (lipopolysaccharide as immune contributors on the presence of the bacterial cell wall) in PLG were lower than LG and BG, which possibly implicate the communication through neural, endocrine, and immune pathways in the gut-brain axis (Cryan and Dinan, 2012). As a whole, gut microbiota could modulate the bioactive molecules (hormones, gene expressions, and microbial metabolites) to realize host-microbiota interactions (David and Vanessa, 2008; Cryan and Dinan, 2012). Our findings are preliminary, and further investigation is required to explore the causal relationship about how gut microbiomes affecting behavior, hormone, and gene expression in chickens.

Conclusions

Birds with perches and litter materials enriched environment during early life resulted in altered gut microbiota, accompanied by decreased fearfulness, reduced plasma corticosterone, lower relative expression of GR mRNA, and higher relative mRNA expression of CRH, BDNF, and NR2A in the hypothalamus coped better with the challenge. The findings suggest that gut microbiota may integrate fearfulness, plasma corticosterone, and gene expression in the hypothalamus to provide an insight into the gut-brain axis in chicks. Thus, the provision of both perches and litter materials in the early-life period of chicks appears to be beneficial for optimal development and adaptive plasticity.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

Data availability

The datasets used to support this paper's conclusions are contained within the article and its supplementary

materials. The raw sequencing data for the gut microbiome were deposited in the National Center for Biotechnology Information (PRJNA588517) and released after the publication of this article.

Acknowledgements

We express our sincere gratitude to the staff from Guizhou Nayong Yuanshengmuye Ltd., Bijie, 553300, Guizhou, China, for the support during the study and for providing the Weining chicks. We sincerely appreciate the help from Mr. Rong He and all persons in Qianlong organic farm for the supply of the study. This work was funded by the Joint Projects of Guizhou Nayong Professor Workstation (number: 201705510410352), the Joint Fund of Basic and Applied Basic Research Fund of Guangdong Province (number: 2019A1515110598), and the Guangdong Provincial Key Laboratory of Animal Molecular Design and Precise Breeding (number: 2019B030301010).

Authors' Contributions

X.Z. obtained the funding; C.Y., S.C., and X.Z. designed this project; C.Y., S.C., J.X., H.X., J.W., H.Z., H.L., and J.L. performed the experiment; C.Y., W.L., and S.C. analyzed and interpreted the data; C.Y., K.H., S.C., and X.Z. drafted and revised the manuscript. All authors came to an agreement for publication.

Conflict of interest statement

The authors declare no real or perceived conflicts of interest.

Literature Cited

- Aksit, M., Z. Kacamakli Yardim, and S. Yalcin. 2017. Environmental enrichment influences on broiler performance and meat quality: Effect of light source and providing perches. *Eur. Poultry Sci.* **81**. doi: [10.1399/eps.2017.1182](https://doi.org/10.1399/eps.2017.1182).
- Apajalahti, J. 2005. Comparative gut microflora, metabolic challenges, and potential opportunities. *J. Appl. Poultry Res.* **14**(14):444–453. doi: [10.1093/japr/14.2.444](https://doi.org/10.1093/japr/14.2.444).
- Bailey, M. T., S. E. Dowd, J. D. Galley, A. R. Hufnagle, R. G. Allen, and M. Lyte. 2011. Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. *Brain. Behav. Immun.* **25**:397–407. doi: [10.1016/j.bbi.2010.10.023](https://doi.org/10.1016/j.bbi.2010.10.023).
- Bercik, P., E. Denou, J. Collins, W. Jackson, J. Lu, J. Jury, Y. Deng, P. Blennerhassett, J. Macri, K. D. McCoy, et al. 2011. The intestinal microbiota affect central levels of brain-derived neurotrophic factor and behavior in mice. *Gastroenterology* **141**:599–609, 609.e1. doi: [10.1053/j.gastro.2011.04.052](https://doi.org/10.1053/j.gastro.2011.04.052).
- van Bodegom, M., J. R. Homberg, and M. J. A. G. Henckens. 2017. Modulation of the hypothalamic-pituitary-adrenal axis by early life stress exposure. *Front. Cell. Neurosci.* **11**:87. doi: [10.3389/fncel.2017.00087](https://doi.org/10.3389/fncel.2017.00087).
- Borowska, D., L. Rothwell, R. A. Bailey, K. Watson, and P. Kaiser. 2016. Identification of stable reference genes for quantitative PCR in cells derived from chicken lymphoid organs. *Vet. Immunol. Immunopathol.* **170**:20–24. doi: [10.1016/j.vetimm.2016.01.001](https://doi.org/10.1016/j.vetimm.2016.01.001).
- Brantsæter, M., F. M. Tahamtani, J. Nordgreen, E. Sandberg, T. B. Hansen, T. B. Rodenburg, R. O. Moe, and A. M. Janczak. 2017. Access to litter during rearing and environmental enrichment during production reduce fearfulness in adult laying hens. *Appl. Anim. Behav. Sci.* **189**:49–56. doi: [10.1016/j.applanim.2017.01.008](https://doi.org/10.1016/j.applanim.2017.01.008).

- Campbell, D. L. M., H. E. N. De, and C. Lee. 2018. A review of environmental enrichment for laying hens during rearing in relation to their behavioral and physiological development. *Poult Sci.* 98:9–28. doi: [10.3382/ps/pey319](https://doi.org/10.3382/ps/pey319).
- Chen, S., J. Wang, D. Peng, G. Li, J. Chen, and X. Gu. 2018. Exposure to heat-stress environment affects the physiology, circulation levels of cytokines, and microbiome in dairy cows. *Sci. Rep.* 8(1):14606. doi: [10.1038/s41598-018-32886-1](https://doi.org/10.1038/s41598-018-32886-1).
- Chen, S., H. Xiang, H. Zhang, X. Zhu, D. Wang, J. Wang, T. Yin, L. Liu, M. Kong, H. Li, and X. Zhao. 2019. Rearing system causes changes of behavior, microbiome, and gene expression of chickens. *Poult Sci.* 0:1–12. doi: [10.3382/ps/pez140](https://doi.org/10.3382/ps/pez140).
- Cryan, J. F., and T. G. Dinan. 2012. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat. Rev. Neurosci.* 13:701–712. doi: [10.1038/nrn3346](https://doi.org/10.1038/nrn3346).
- Cryan, J. F., and S. M. O'Mahony. 2011. The microbiome-gut-brain axis: from bowel to behavior. *Neurogastroenterol. Motil.* 23: 187–192. doi: [10.1111/j.1365-2982.2010.01664.x](https://doi.org/10.1111/j.1365-2982.2010.01664.x).
- Dallman, M. F., S. F. Akana, K. A. Scribner, M. J. Bradbury, C. D. Walker, A. M. Strack, and C. S. Cascio. 1992. Stress, feedback and facilitation in the hypothalamo-pituitary-adrenal axis. *J. Neuroendocrinol.* 4:517–526. doi: [10.1111/j.1365-2826.1992.tb00200.x](https://doi.org/10.1111/j.1365-2826.1992.tb00200.x).
- Dalloul, R. A., H. S. Lillehoj, T. A. Shellem, and J. A. Doerr. 2003. Enhanced mucosal immunity against *Eimeria acervulina* in broilers fed a *Lactobacillus*-based probiotic. *Poult. Sci.* 82: 62–66. doi: [10.1093/ps/82.1.62](https://doi.org/10.1093/ps/82.1.62).
- David, T. H., and S. Vanessa. 2008. Inter-kingdom signalling: communication between bacteria and their hosts. *Nat. Rev. Microbiol.* 6(2):111–120. doi: [10.1038/nrmicro1836](https://doi.org/10.1038/nrmicro1836).
- De Haas, E. N., B. J. Elizabeth, K. Bas, T. G. G. Groothuis, and R. T. Bas. 2014. Parents and early life environment affect behavioral development of laying hen chickens. *PLoS One.* 9(6):e90577. doi: [10.1371/journal.pone.0090577](https://doi.org/10.1371/journal.pone.0090577).
- Delius, J. D. 1988. Preening and associated comfort behavior in birds. *Ann. N. Y. Acad. Sci.* 525:40–55. doi: [10.1111/j.1749-6632.1988.tb38594.x](https://doi.org/10.1111/j.1749-6632.1988.tb38594.x).
- Elfving, M., D. Nätt, V. C. Goerlich-Jansson, M. Persson, J. Hjelm, and P. Jensen. 2015. Early stress causes sex-specific, life-long changes in behaviour, levels of gonadal hormones, and gene expression in chickens. *PLoS One* 10:e0125808. doi: [10.1371/journal.pone.0125808](https://doi.org/10.1371/journal.pone.0125808).
- Fairhurst, G. D., M. D. Frey, J. F. Reichert, I. Szelest, D. M. Kelly, and G. R. Bortolotti. 2011. Does environmental enrichment reduce stress? An integrated measure of corticosterone from feathers provides a novel perspective. *PLoS One* 6:e17663. doi: [10.1371/journal.pone.0017663](https://doi.org/10.1371/journal.pone.0017663).
- Favati, A., J. Zidar, H. Thorpe, P. Jensen, and H. Løvlie. 2015. The ontogeny of personality traits in the red junglefowl, *Gallus gallus*. *Behav. Ecol.* 27(2):484–493. doi: [10.1093/beheco/arv177](https://doi.org/10.1093/beheco/arv177).
- Feltenstein, M. W., L. C. Lambdin, H. E. Webb, J. E. Warnick, S. I. Khan, I. A. Khan, E. O. Acevedo, and K. J. Sufka. 2003. Corticosterone response in the chick separation-stress paradigm. *Physiol. Behav.* 78:489–493. doi: [10.1016/s0031-9384\(03\)00030-1](https://doi.org/10.1016/s0031-9384(03)00030-1).
- Goerlich, V. C., D. Nätt, M. Elfving, B. Macdonald, and P. Jensen. 2012. Transgenerational effects of early experience on behavioral, hormonal and gene expression responses to acute stress in the precocial chicken. *Horm. Behav.* 61:711–718. doi: [10.1016/j.yhbeh.2012.03.006](https://doi.org/10.1016/j.yhbeh.2012.03.006).
- Hartcher, K. M., S. J. Wilkinson, P. H. Hemsworth, and G. M. Cronin. 2016. Severe feather-pecking in non-cage laying hens and some associated and predisposing factors: a review. *Worlds Poultry Sci. J.* 72:103–114. doi: [10.1017/s0043933915002469](https://doi.org/10.1017/s0043933915002469).
- Hatalski, C. G., K. L. Brunson, B. Tantanubutr, Y. Chen, and T. Z. Baram. 2000. Neuronal activity and stress differentially regulate hippocampal and hypothalamic corticotropin-releasing hormone expression in the immature rat. *Neuroscience* 101:571–580. doi: [10.1016/s0306-4522\(00\)00386-9](https://doi.org/10.1016/s0306-4522(00)00386-9).
- Herman, J. P., J. M. McKlveen, S. Ghosal, B. Kopp, A. Wulsin, R. Makinson, J. Scheimann, and B. Myers. 2016. Regulation of the hypothalamic-pituitary-adrenocortical stress response. *Compr. Physiol.* 6:603–621. doi: [10.1002/cphy.c150015](https://doi.org/10.1002/cphy.c150015).
- Huang, P., Y. Zhang, K. Xiao, F. Jiang, H. Wang, D. Tang, D. Liu, B. Liu, Y. Liu, X. He, et al. 2018. The chicken gut metagenome and the modulatory effects of plant-derived benzylisoquinoline alkaloids. *Microbiome* 6:211. doi: [10.1186/s40168-018-0590-5](https://doi.org/10.1186/s40168-018-0590-5).
- Hughes, D. T., and V. Sperandio. 2008. Inter-kingdom signalling: communication between bacteria and their hosts. *Nat. Rev. Microbiol.* 6:111–120. doi: [10.1038/nrmicro1836](https://doi.org/10.1038/nrmicro1836).
- Hyland, N. P., S. M. O'Mahony, D. O'Malley, C. M. O'Mahony, T. G. Dinan, and J. F. Cryan. 2015. Early-life stress selectively affects gastrointestinal but not behavioral responses in a genetic model of brain-gut axis dysfunction. *Neurogastroenterol. Motil.* 27:105–113. doi: [10.1111/nmo.12486](https://doi.org/10.1111/nmo.12486).
- Jones, R. B., and D. Waddington. 1992. Modification of fear in domestic chicks, *Gallus gallus domesticus*, via regular handling and early environmental enrichment. *Anim. Behav.* 43(6):1021–1033. doi: [10.1016/s0003-3472\(06\)80015-1](https://doi.org/10.1016/s0003-3472(06)80015-1).
- Lin, E. J., E. Choi, X. Liu, A. Martin, and M. J. During. 2011. Environmental enrichment exerts sex-specific effects on emotionality in C57BL/6J mice. *Behav. Brain Res.* 216:349–357. doi: [10.1016/j.bbr.2010.08.019](https://doi.org/10.1016/j.bbr.2010.08.019).
- Martrenchar, A., E. Boilletot, D. Huonnic, and F. Pol. 2002. Risk factors for foot-pad dermatitis in chicken and turkey broilers in France. *Prev. Vet. Med.* 52(3–4):213–226. doi: [10.1016/s0167-5877\(01\)00259-8](https://doi.org/10.1016/s0167-5877(01)00259-8).
- Martrenchar, A., D. Huonnic, J. P. Cotte, E. Boilletot, and J. P. Morisse. 2000. Influence of stocking density, artificial dusk and group size on the perching behaviour of broilers. *Br. Poult. Sci.* 41:125–130. doi: [10.1080/713654921](https://doi.org/10.1080/713654921).
- Mizoguchi, K., M. Yuzurihara, A. Ishige, H. Sasaki, D. H. Chui, and T. Tabira. 2001. Chronic stress differentially regulates glucocorticoid negative feedback response in rats. *Psychoneuroendocrinology* 26:443–459. doi: [10.1016/s0306-4530\(01\)00004-x](https://doi.org/10.1016/s0306-4530(01)00004-x).
- Neis, E. P., C. H. Dejong, and S. S. Rensen. 2015. The role of microbial amino acid metabolism in host metabolism. *Nutrients* 7:2930–2946. doi: [10.3390/nu7042930](https://doi.org/10.3390/nu7042930).
- Neufeld, K. M., N. Kang, J. Bienenstock, and J. A. Foster. 2011. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterol. Motil.* 23:255–64. doi: [10.1111/j.1365-2982.2010.01620.x](https://doi.org/10.1111/j.1365-2982.2010.01620.x).
- Newberry, R. C. 1995. Environmental Enrichment - Increasing the Biological Relevance of Captive Environments. *Appl. Anim. Behav. Sci.* 44(2–4):229–243. doi: [10.1016/0168-1591\(95\)00616-Z](https://doi.org/10.1016/0168-1591(95)00616-Z).
- Nobuyuki, S., C. Yoichi, A. Yuji, S. Junko, O. Naomi, Y. Xiaonian, K. Chiharu, and K. Yasuhiro. 2004. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *J. Physiol.* 558(1):263–275. doi: [10.1113/jphysiol.2004.063388](https://doi.org/10.1113/jphysiol.2004.063388).
- O'Mahony, S. M., N. P. Hyland, T. G. Dinan, and J. F. Cryan. 2011. Maternal separation as a model of brain-gut axis dysfunction. *Psychopharmacology (Berlin)*. 214:71–88. doi: [10.1007/s00213-010-2010-9](https://doi.org/10.1007/s00213-010-2010-9).
- Oakley, B., H. Lillehoj, M. Kogut, W. Kim, J. Maurer, A. Pedrosa, M. Lee, S. Collett, T. Johnson, and N. Cox. 2015. The chicken gastrointestinal microbiome. *FEMS Microbiol. Lett.* 360(2):100–112. doi: [10.1111/1574-6968.12608](https://doi.org/10.1111/1574-6968.12608).
- Pat, M. 2008. Early growth conditions, phenotypic development and environmental change. *Philos. Trans. Biol. Sci.* 363(1497):1635–1645. doi: [10.1098/rstb.2007.0011](https://doi.org/10.1098/rstb.2007.0011).
- Patrick, B., G. Peter, and H. Mark. 2014. The biology of developmental plasticity and the predictive adaptive response hypothesis. *J. Physiol.* 592(11):2357–2368. doi: [10.1113/jphysiol.2014.271460](https://doi.org/10.1113/jphysiol.2014.271460).
- Qiong, W., G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences

- into the new bacterial taxonomy. *Applied & Environmental Microbiology*. 73(16):5261. doi: [10.1128/aem.00062-07](https://doi.org/10.1128/aem.00062-07).
- Riber, A. B., I. C. de Jong, H. A. van de Weerd, and S. Steenfeldt. 2017. Environmental Enrichment for Broiler Breeders: An Undeveloped Field. *Front. Vet. Sci.* 4:86. doi: [10.3389/fvets.2017.00086](https://doi.org/10.3389/fvets.2017.00086).
- Riber, A. B., H. A. van de Weerd, I. C. de Jong, and S. Steenfeldt. 2018. Review of environmental enrichment for broiler chickens. *Poult. Sci.* 97:378–396. doi: [10.3382/ps/pex344](https://doi.org/10.3382/ps/pex344).
- Rodenburg, T. B., and E. N. D. Haas. 2016. Of nature and nurture: the role of genetics and environment in behavioural development of laying hens. *Curr. Opin. Behav. Sci.* 7:91–94. doi: [10.1016/j.cobeha.2015.12.007](https://doi.org/10.1016/j.cobeha.2015.12.007).
- Rodenburg, T. B., M. M. V. Krimpen, I. C. De Jong, E. N. D. De Haas, M. S. Kops, B. J. Riedstra, R. E. Nordquist, J. P. Wagenaar, M. Bestman, and C. J. Nicol. 2013. The prevention and control of feather pecking in laying hens: identifying the underlying principles. *World's Poultry Sci. J.* 69:361–373. doi: [10.1017/S0043933913000354](https://doi.org/10.1017/S0043933913000354).
- Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, et al. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75:7537–7541. doi: [10.1128/AEM.01541-09](https://doi.org/10.1128/AEM.01541-09).
- Shi, D., L. Bai, Q. Qu, S. Zhou, M. Yang, S. Guo, Q. Li, and C. Liu. 2019. Impact of gut microbiota structure in heat-stressed broilers. *Poult. Sci.* 98:2405–2413. doi: [10.3382/ps/pez026](https://doi.org/10.3382/ps/pez026).
- Singh, K. M., T. Shah, S. Deshpande, S. J. Jakhesara, P. G. Koringa, D. N. Rank, and C. G. Joshi. 2012. High through put 16S rRNA gene-based pyrosequencing analysis of the fecal microbiota of high FCR and low FCR broiler growers. *Mol. Biol. Rep.* 39:10595–10602. doi: [10.1007/s11033-012-1947-7](https://doi.org/10.1007/s11033-012-1947-7).
- Sugiharto, S. 2016. Role of nutraceuticals in gut health and growth performance of poultry. *J. Saudi Soc. Agric. Sci.* 15(2):99–111. doi: [10.1016/j.jssas.2014.06.001](https://doi.org/10.1016/j.jssas.2014.06.001).
- Tahamtani, F. M., M. Brantsæter, J. Nordgreen, E. Sandberg, T. B. Hansen, A. Nødtvedt, T. B. Rodenburg, R. O. Moe, and A. M. Janczak. 2016. Effects of litter provision during early rearing and environmental enrichment during the production phase on feather pecking and feather damage in laying hens. *Poult. Sci.* 95:2747–2756. doi: [10.3382/ps/pew265](https://doi.org/10.3382/ps/pew265).
- Tahamtani, F. M., I. J. Pedersen, C. Toinon, and A. B. Riber. 2018. Effects of environmental complexity on fearfulness and learning ability in fast growing broiler chickens. *Appl. Anim. Behav. Sci.* 207:49–56. doi: [10.1016/j.applanim.2018.04.005](https://doi.org/10.1016/j.applanim.2018.04.005).
- Tang, Y. P., H. Wang, R. Feng, M. Kyin, and J. Z. Tsien. 2001. Differential effects of enrichment on learning and memory function in NR2B transgenic mice. *Neuropharmacology* 41: 779–790. doi: [10.1016/S0028-3908\(01\)00122-8](https://doi.org/10.1016/S0028-3908(01)00122-8).
- Torok, V. A., R. J. Hughes, K. Ophel-Keller, M. Ali, and R. Macalpine. 2009. Influence of different litter materials on cecal microbiota colonization in broiler chickens. *Poult. Sci.* 88:2474–2481. doi: [10.3382/ps.2008-00381](https://doi.org/10.3382/ps.2008-00381).
- Tronche, F., C. Kellendonk, O. Kretz, P. Gass, K. Anlag, P. C. Orban, R. Bock, R. Klein, and G. Schütz. 1999. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat. Genet.* 23:99–103. doi: [10.1038/12703](https://doi.org/10.1038/12703).
- Vazquez-Sanroman, D., C. Sanchis-Segura, R. Toledo, M. E. Hernandez, J. Manzo, and M. Miquel. 2013. The effects of enriched environment on BDNF expression in the mouse cerebellum depending on the length of exposure. *Behav. Brain Res.* 243:118–128. doi: [10.1016/j.bbr.2012.12.047](https://doi.org/10.1016/j.bbr.2012.12.047).
- Wang, Q., G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73:5261–5267. doi: [10.1128/AEM.00062-07](https://doi.org/10.1128/AEM.00062-07).
- Wechsler, B., and B. Huber-Eicher. 1998. The effect of foraging material and perch height on feather pecking and feather damage in laying hens. *Appl. Anim. Behav. Sci.* 58(1–2):131–141. doi: [10.1016/S0168-1591\(97\)00137-8](https://doi.org/10.1016/S0168-1591(97)00137-8).
- Wiley, J. W., G. A. Higgins, and B. D. Athey. 2016. Stress and glucocorticoid receptor transcriptional programming in time and space: Implications for the brain-gut axis. *Neurogastroenterol. Motil.* 28:12–25. doi: [10.1111/nmo.12706](https://doi.org/10.1111/nmo.12706).
- Zhou, X., X. Jiang, C. Yang, B. Ma, C. Lei, C. Xu, A. Zhang, X. Yang, Q. Xiong, P. Zhang, et al. 2016. Cecal microbiota of Tibetan Chickens from five geographic regions were determined by 16S rRNA sequencing. *Microbiologyopen* 5:753–762. doi: [10.1002/mbo3.367](https://doi.org/10.1002/mbo3.367).
- Zimmer, C., and K. A. Spencer. 2014. Modifications of glucocorticoid receptors mRNA expression in the hypothalamic-pituitary-adrenal axis in response to early-life stress in female Japanese quail. *J. Neuroendocrinol.* 26:853–860. doi: [10.1111/jne.12228](https://doi.org/10.1111/jne.12228).