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RESEARCH PAPER

Removal of roosters alters the domestic phenotype and microbial and genetic profile of hens

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Abstract

Hens are raised apart from roosters in modern poultry production, a substantial change from their natural social structure. We compared productivity, injuries, behavior, physiology, microbiome and transcriptome of hens housed with (R+) or without (R-) roosters to quantify the effects of this change in social structure. Hens were raised free-range from 70 to 280 days when 30 birds per treatment were assigned to battery cages until Day 315 (R+C vs R-C), while 30 birds per treatment remained in free-range pens (R+F vs R-F). Response to a novel environment and object, behavioral time budgets, cecum microbiome, blood composition and transcriptomic sequencing of thigh muscle and spleen were analyzed. Hens housed without roosters showed better survival, consumed less food, produced more eggs and had better feed conversion. R+F hens clustered around the rooster and were less mobile in the novel environment and object tests. R+F hens displayed the richest microbiome, and the presence of roosters resulted in differentially expressed genes related to muscle development, cellular processes, environmental information processing and immune function. Removing roosters from housed hens intensified desirable characteristics favored by domestication probably operating by deprivation of mating behavior and reduced fear, along with altered microbial and genetic function.

Keywords: chicken, domestic phenotype, fear, gut microbiome, rooster, transcriptome

Introduction

The chicken has been domesticated for thousands of years (Xiang et al., 2014), and is a critical source of animal protein. With the development of poultry genetics, nutrition, housing, management and disease control, poultry production has improved dramatically from backyard to specialized and concentrated poultry production in the last century (Al-Nasser et al., 2007). High productivity is always an important driving factor for artificial selection and optimized management. Intensive selection and breeding have resulted in many specialized chicken breeds with outstanding meat and egg production traits (Eriksson et al., 2008). Along with changes in productivity (Corr et al., 2003), other traits have also changed including gait (Duggan et al., 2015), stress tolerance (Ericsson and Jensen, 2016) and personality, such as fearfulness (Jongren et al., 2010). A reduction in fearfulness is believed to an early and necessary change for successful domestication (Agnvall et al., 2015; Jensen, 2014). Reduced fearfulness correlates with, and contributes to, increased productivity of red jungle fowl (Agnvall et al., 2015). In response to adaptation to domestic environments and rearing systems, chickens display altered morphology, behavior and physiology. Increased stress tolerance is suggested as a driving factor behind such changes, via modified endocrine and behavioral profiles (Ericsson and Jensen, 2016). Consequently, microevolution of phenotypes, including fearfulness, stress tolerance and productivity are critical for early chicken domestication.

Chickens are naturally gregarious birds that live in mixed flocks of roosters and hens. Roosters usually lead flocks by manipulating sources of food, space and sex (Collias and Collias, 1967), while hens often prefer to mate with roosters that provide more food regardless of their social rank (Pizzari, 2003). However, hens are now commonly raised apart from roosters since male aggression can reduce production and increase management cost. This change to social structure is a major departure from the structure that development from natural selection. During the intensification of the modern poultry industry, hens have adapted to living without roosters which required phenotypic changes that have been subsumed within a wider set of modification referred to as the domestic phenotype (Price, 1999). This includes changes in productivity, appearance and color, reproduction, size and behavior. The absence of males contributes to the ability of hens to achieve their domesticated phenotype, but it is unclear how this ability is underpinned by changes in gene expression and composition of gut microbial community.

In the present study, we compared hens living with (R+) or without (R-) roosters for production performance, behaviors, injuries, and blood metabolites, as well as their gut microbiome and transcriptome. Specifically, we reared free-range R+ and R- hens from Day 70-280 and then transferred some birds to a cage environment (R+C and R-C, respectively) for 35 days to test their flexibility and adaptability in coping with a stressful environment. This study evaluated phenotypic and genetic responses of hens and roosters rose together, which in modern poultry production are raised apart, under different experimental conditions. The aim was to illustrate the genetic basis of microevolution and adaptation of hens to living without roosters in the intensified modern poultry industry.

Results

Production performance and product quality

Through the early rearing period before transfer of R-C and R+C birds to cages, R- hens had a similar bodyweight to R+ hen, recorded on Day 280, but produced more eggs ($p < 0.05$),

consumed less feed per day ($p < 0.05$), and displayed a much higher feed conversion rate ($p < 0.05$) (Table 1). Moreover, R- rearing produced a lower mortality rate, 5% vs 10% for R-C and R+C, respectively; Table 1). Further, R-F hens had lower drip loss from both thigh and breast muscles than R+F ($p < 0.05$; Table S1). However, no significant differences between R-F and R+F hens were found for other meat quality traits, including cooking loss, shear force, and meat color (Table S1) or egg quality indicators, including egg weight, yolk weight, yolk color, Haugh unit, and eggshell weight, thickness and strength (Table S2).

Behavioral observations

When placed in a novel environment, R- hens were bolder than R+ hens and began exploring after a short period of adaption. Particularly, R- hens moved and divided into several small groups during the first 195 sec, and after that behaved in a manner similar to behavior in their home pens. In contrast, the R+ hens clustered around the rooster and stayed frozen throughout the 10-min test. These treatment differences were evident from percentages of time spent walking, resting (standing), resting (lying), exploring and preening which occupied 11.0%, 37.5%, 11.5%, 32.5%, and 7.5% of the test for R- and 1.5%, 77.0%, 6.5%, 9.0, and 6.0% for R+ hens, respectively. Definitions for chicken behaviors are provided in Table S3. Walking, resting (standing), and exploration significantly differed between treatments ($p < 0.001$). Further, during the 10-min period after the introduction of a ball into the test arena, most R- hens showed immediate escape behavior but then settled down and quickly divided into small groups again. The behavior of the R+ hens reflected that before the introduction of the ball; after their initial avoidance, they froze in a group around the rooster. Walking (R- 14.0%, R+ 1.0%, $p < 0.001$), resting (standing) (R- 50.0%, R+ 33.5%, $p = 0.031$), resting (lying) (R- 0.5%, R+ 13.5%, $p = 0.003$), preening (R- 0.5%, R+ 6.0%, $p = 0.008$) and freezing (R- 12.5%, R+ 37.5%, $p < 0.001$) behaviors differed between R- and R+ hens but exploration (R- 17.0%, R+ 8.0%) and escape (R- 5.5%, R+ 0.5%) behaviors did not.

Except for mating behavior, the presence or absence of roosters during the rearing period (R+ or R-) did not significantly influence behavioral time budgets. However, behavioral responses to cage rearing were different between the R+C and R-C hens. After birds were transferred into cages, resting (lying) behavior was observed in 15.0% of R+C hens and only 11.3% of R-C hens during the first daytime period from 7:00 am to 7:00 pm ($p = 0.04$). This was supported by the continuous observations during the 3 h period after eating (32.4% and 14.8% of time spent lying down in R+C and R-C birds respectively; Figure 1, $p < 0.01$). No treatment differences were observed in the amount of time spent in other behaviors.

Injuries

Most free-range hens were able to walk normally (Figure 2a) and no treatment-related difference in footpad dermatitis between R+F and R-F hens was observed (Figure 2b). After the 35-day adaptation to cage rearing, R+C and R-C hens had a similar gait score and footpad dermatitis score (all $p < 0.05$; Figure 2a and 2b). Feather condition scores of head and back were higher in R+F than R-F hens ($p < 0.05$; Figure 2c) but no significant differences were found for other body areas between. The condition of feathers on the abdomen was poorer in R+C than R-C hens ($p < 0.05$, Figure 2c), but again significant differences were not found between treatments of caged birds in other body areas.

Blood metabolites

Serum creatine kinase (CK) was significantly lower in R-F than R+F ($p < 0.05$; Table 2).

Blood hemoglobin (HGB) and red blood cell count (RBC) was significantly higher in R-C than R-F ($p < 0.05$; Table 2), but no significant difference between R+C and R+F were seen. Presence or absence of roosters did not influence other serum biochemical or routine blood indicators (Table 2).

Gut microbiome

A total of 48,666-67,564 effective tags were generated for each sample (Table S4). After quality control, the tags were annotated into 1,479 operational taxonomic units (OTUs) for R-F, 1,935 for R+F, 1,692 for R-C, and 1,774 for R+C, respectively; 1203 OTUs were the core OTUs shared by all 4 treatment groups.

Beta diversity analyses revealed different gut microbiome populations among the four groups of hens, where an apparent distinction between R-F and R+F hens but limited differences between R-C and R+C hens were observed (Figure 3a). Microbe species (Figure 3b) suggested that R+F hens possessed more diverse microbial populations than R-F hens and R+C and R-C hens (all $p < 0.05$). Moreover, ACE and chao1 also supported that R+F had the most abundant gut microbial population (Figure S1).

Between treatments, R-F and R+F shared 1323 OTUs, R-C and R+C shared 1395 OTUs. Likewise, R-F and R-C shared 1314 OTUs, R+F and R+C shared 1539 OTUs. Further, R+F and R-F hens differed for 20 genera and 8 species of gut microbes ($p < 0.05$), of which R+F outnumbered R-F for 19 genera and 7 species (Table 3). The microbiome of R+F and R-F hens displayed differential functions in 19 KEGG pathways ($q < 0.01$), involved mainly in metabolism, human diseases, and organismal systems. Lysine biosynthesis was the only pathway to be more highly expressed in the microbiome of R-F hens. Pathways related to immune function (such as endocytosis and Fc gamma R-mediated phagocytosis), nutrient metabolism (including of fatty acids, geraniol, carotenoids, valine, leucine and isoleucine), and gonadal hormonal level (GnRH signaling) were more highly expressed in the microbiome of R+F hens (Figure 4a). R-C and R+C differed in five genera and one species (Table 3). Carbohydrate metabolism was the only differentially expressed pathway between R-C and R+C that was more highly expressed in R+C hens ($q < 0.01$; Figure 4b).

R+F and R+C hens differed in 2 genera and 2 species, and R-F and R-C differed in 13 genera and one species. Compared to the microbiome of R+F hens, the biome of R+C hens showed downregulation of five pathways related to immune function, nutrient metabolism and human disease, and upregulation of one pathway involved with antibiotic biosynthesis ($q < 0.01$; Figure S2). No functional differences were found between R-F and R-C.

Transcriptome profiles

The transcriptome profiles of thigh muscle and spleen were determined with a total of 53,856,692-72,932,650 paired-end reads sequenced for each library (Table S5). The Pearson correlation coefficient of biological repeats from each group was higher than 0.99. Overall, free-range and caged hens with or without roosters showed different transcriptome profiles both in thigh muscle and spleen tissues (Figure S3 and Figure S4).

Between R-F and R+F, a total of 23,947 genes were analyzed and 323 differentially expressed genes (DEGs) were identified ($q < 0.05$) in thigh muscle (Table S6), and 24,484 genes were analyzed, and 805 DEGs were identified in spleen tissue (Table S6). The highest differential expression between R-F and R+F in thigh muscle tissue was observed in peroxisome proliferator-activated receptor gamma coactivator-related 1 (*PPRC1*),

mitogen-activated protein kinase kinase kinase 4 (*MAP3K4*), L-threonine dehydrogenase (*TDH*), cholesteryl ester transfer protein (*CETP*,), and forkhead box O_3 (*FOXO3*). Genes, including myosin heavy chain 1A (*MYH1A*), troponin C2 (*TNNC2*,), myosin light chain 1 (*MYL1*), and creatine kinase mitochondrial 2 (*CKMT2*) showed the highest differential expression between R-F and R+F in spleen tissue. DEGs in thigh muscle of R-F and R+F hens were enriched in eight biological processes (BP) Gene Ontology (GO) terms, of which six were related to muscle development and two to acylglycerol and neutral lipid metabolism (Figure 5a). All muscle development-related GO terms were enriched mainly by downregulation of genes in R-F hens, and the two metabolic processes related GO terms were enriched by both downregulated and upregulated genes. Further, DEGs were enriched in pathways controlling insulin signaling, insulin resistance, and FoxO signaling. The former two pathways were both up- and downregulated in R-F hens, while the FoxO signaling pathway was downregulated in R-F compared to R+F (Table 4). In spleen tissue, DEGs were enriched in 107 GO terms. All the 30 most significant GO terms were mainly enriched by downregulation genes in R-F hens, and mainly classified into developmental processes, especially cellular and vascular development, signal transduction and stimulus-response (Figure 5b). DEGs involved in cellular processes functioned in focal adhesion, cytokine-cytokine receptor interaction and endocytosis, and environmental information processing including ECM-receptor interactions, activity of cell adhesion molecules, and regulation of the actin cytoskeleton (Table 4).

Between R-C and R+C, a total of 28,626 genes were analyzed in thigh muscle and 489 DEGs were identified, while the same genes were analyzed in the spleen, but no DEGs identified (Table S6). Eighty-four GO terms in thigh muscle were enriched between R-C and R+C. All of these GO terms were enriched by upregulation of genes in R-C, and most were related to the regulation of metabolic or biosynthetic processes (Figure 6). DEGs were enriched for the FoxO signaling pathway, which was downregulated in R-C hens (Table 4).

Comparison of R-F and R-C hens identified 412 DEGs in thigh muscle and 51 in spleen tissue, and comparison of R+F and R+C identified 559 DEGs in thigh muscle and 21 in spleen (Table S6). Between R-C and R-F, most enriched GO terms in thigh muscle were upregulated in R-C. Among the 30 most significant GO terms, only the immune function related GO term (cytokine binding) was mainly enriched by downregulated genes in R-C, otherwise negative cell apoptotic and death process, negative catabolic processes and stimulus responses related GO terms were mainly enriched by upregulated genes in R-C hens (Figure S5). Further, four KEGG pathways were enriched in thigh muscle tissue between R-C and R-F hens (Table 4), of which genes controlling proteasome and ribosome biogenesis in eukaryotes were upregulated, and the immune function related pathway of phagosome activity was downregulated in R-C hens. In spleen tissue, no GO terms were enriched by the 51 DEGs between R-C and R-F, and no KEGG pathways were enriched between these 2 groups (Table 4). Even though DEGs were present in thigh muscle between R+C and R+F, no significant GO terms were enriched. These DEGs were enriched in four KEGG pathways (Table 4), of which R+C birds showed upregulation of the proteasome pathway and downregulation in immune function related pathways (phagosome and focal adhesion activity) and a related developmental pathway (dorso-ventral axis formation). In spleen tissue, the DEGs between R+C and R+F showed low differential expression and lacked biological function.

Discussion

Hens are typically raised apart from roosters in modern poultry production, even in free-range rearing, to minimize costs. This situation is a substantial departure from their natural social grouping and eliminates certain aspects of natural behavior. Our study examined the impacts of removing roosters on domesticated phenotype and microbial and genetic profiles of hens.

Hens living without roosters showed a higher feed conversion rate, consuming less food and producing more eggs. Both thigh and breast meat showed higher water-holding capacity, which justified the practice of keeping hens separate from roosters. Previous studies show that chicken domestication has resulted in higher egg production (Roth and Lind, 2013), basal metabolic rate, and feed efficiency (Agnvall et al., 2014; Jackson and Diamond, 1996; Schutz et al., 2004). The findings from our study indicate that housing hens in the absence of roosters allows them to more closely achieve this domesticated phenotype. KEGG pathways relative to metabolic processes, such as carbohydrate, fatty acid and glutathione metabolism of the microbiome, and acylglycerol, neutral lipid and insulin metabolism pathway of the tissue transcriptome, are upregulated in hens with roosters, which likely explains higher feed consumption and lower production of R+F hens.

A motivational state behind the response toward a novel environment is fear or anxiety (Favati et al., 2016; Forkman et al., 2007), which can be observed as altered vigilance patterns. Novel environment and novel object tests of this study suggest that R+ hens were more fearful than R- hens. Domestic chickens are considerably less fearful than their red jungle fowl ancestors (Agnvall et al., 2012; Belteky et al., 2016; Campler et al., 2009), an important aspect of chicken domestication (Agnvall et al., 2015; Jensen, 2014). Moreover, our study showed that higher production performance is achieved with less fearful hens raised in the absence of roosters, providing further evidence for the positive correlation between reduced fearfulness and increased productivity (Agnvall et al., 2015). From this perspective, hens allowed to live with roosters experience a more natural social structure but exhibit a more natural, heightened level of neophobia and the removal of roosters may have been a key component in the domestication process leading to substantial effects on behavioral patterns.

No discernable behavioral differences were observed between R+F and R-F hens in their home pen, R+F hens were able to express mating behavior, which was evidenced by the poorer feather condition on the head and back in R+F than R-F birds. Specifically, the gonadal hormonal pathway, GnRH signaling, was highly expressed in the microbiome of R+F hens, again suggesting the impact of mating behavior due to the existence of roosters. Further, a higher level of serum CK in R+F than R-F hens probably reflects tissue damage from active behaviors such as mating (Hicks et al., 2017) and stress (Branciari et al., 2009; Marchi et al., 2012) in hens living with roosters.

In a natural environment, red jungle fowl societies consist of a dominant male and female that must deal with competition for food, space and sex within the group, but also external pressures, including predators such as foxes and hawks (Smith and Johnson, 2012). Males likely play a major role in managing these challenges, and this role may be reflected in our study. R+ hens show more fear in the novel environment and object test, but they also show more preening behavior, which could indicate greater confidence when encountering fear (Zimmerman et al., 2011). This confidence could be that the presence of the rooster is a

source of social support. However, preening is also displayed in hens as a displacement behavior during periods of motivational conflict and frustration (Kuhne et al., 2013). Thus, it is difficult to establish whether the occurrence of more preening in the presence of a male indicates greater or lesser ability to cope with novelty.

In the test of flexibility to stress, both R-C and R+C hens are likely to be frustrated by constraints imposed by a barren cage environment (Janczak and Riber, 2015; Okpokho and Craig, 1987). Nevertheless, the larger amount of resting seen in the R+C birds at the beginning of test compared to the R-C hens might indicate that the experience was more problematic for birds previously housed with a rooster. After 35 days adaptation to the stress environment, blood HGB and RBC of hens housed without roosters changed, but these parameters in hens housed with roosters did not. The level of HGB and RBC was similar in R+F and R-F birds, and the increase seen for hens housed without roosters might suggest that they found the transition to cages to be more problematic than birds previously housed with roosters. This finding seems consistent with the result that serum CK level was lower in R+C than R+F but higher in R-C than R-F. Housing in a mixed-sex group apparently increased the flexibility of the R+ hens and prepared them for a dramatic change in environment. Domestication effects were found in chickens for corticosterone response to restraint as well as to recovery patterns, showing increased stress tolerance in chicken domestication (Ericsson et al., 2014). Also, the differential transcription profile between R-F and R+F identified the candidate gene, creatine kinase mitochondrial 2 (*CKMT2*), and GO terms associated with negative cell apoptotic and death processes, catabolic processes, response to stimulus pathways and ribosome biogenesis were also identified, suggesting again that the transition to cages had less impact on birds that had previously been housed with roosters.

Previous studies revealed that stress-induced accumulation of cells in the spleen and thymus modulate immune response (Sasaguri et al., 2017), which provided evidence for a positive effect of roosters on chicken fitness and health. This finding also implies upregulation of immune function pathways in hens living with roosters. Upregulation of cytokine-cytokine receptor interactions in R+F hens is also associated with heightened capacity of the immune system to respond to stress (Pedro et al., 2017). Meanwhile, the higher abundance of *Stenotrophomonas*, an emerging opportunistic human pathogen (Looney et al., 2009), was observed in the microbiome of hens with roosters. Expression of pathways associated with human disease was also observed in R+F hens, which could be a driving factor for activating immune systems of hens living with roosters.

Stress is known to alter gut microbiome diversity in mice (Bailey et al., 2010) and laying hens (Zhang et al., 2017) and to affect gene expression in chicken brain and spleen (Elfwing et al., 2015; Van Goor et al., 2017). Cage rearing eliminated some normal behavior and resulted in poorer gait scores and feather conditions, as well as a decrease in microbiome diversity and the regulation of gene expression and microbial function (Chen et al., 2019; Chen et al., 2018). When transferred to cage rearing, both R+C and R-C hens showed a reduction in the abundance of several genera of bacteria compared with R+F and R-F hens, confirming that cage rearing was a source of stress. After adapting to the cage environment, the new microbiome reflected species capable of tolerating more stressful host housing. R+C hens showed a reduction in microbial abundance to R+F hens, comparable to hens that remained free-range without roosters (R-F). This result illustrates the significant effect that

the presence of roosters has on the chicken gut microbiome population. Substantial DEGs were found between caged and free-range hens, and upregulation of pathogenic pathways was found in both caged R-C and R+C hens as compared to their free-range comparisons. Also, upregulation of microbial and transcriptome functions, such as metabolic and biosynthetic between R-C and R+C, was identical to the comparison of R-F and R+F, suggesting a prolonged impact of roosters on chicken gut microbiome and gene expression.

Living with or without roosters also shaped the structure of gut microbiome and transcriptome profiles. Hens living with roosters showed increased alpha diversity in gut microbial structure and highly expressed most microbial functions including cellular, metabolic and human disease processes and organismal system pathways. The only ascendant bacterium in R-F, *Faecalibacterium prausnitzii*, is considered as an important bacterial indicator of a healthy gut, which improves hepatic health, and decreases adipose tissue inflammation (Munukka et al., 2017). Further, living with or without roosters leads to DEGs that triggers GO related to muscle development, cell processes, and environmental information processing. Pathways involved in developmental processes, including focal adhesion, were upregulated in R+F hens and play important roles in cellular responses to oxidative stress and longevity (Lehtinen et al., 2006). ECM-receptor interactions, and cell adhesion molecules are involved in critical cell functions (Lee et al., 2013) and embryonic development (Roy and Bandyopadhyay, 2014) and were also upregulated in R+F hens. Therefore, upregulation developmental-related GO terms and KEGG pathways represent molecular changes in response to living with roosters.

Intensive selective breeding programs have improved egg production in both jungle fowl and laying hens (Moreng and Avens, 1985; Romanov and Weigend, 2001), along with behavioral and physiological changes (Weeks and Nicol, 2006). Housing hens in the absence of roosters is the basis of modern poultry production that promotes the domestication phenotype by preventing mating behavior and reducing fear. Our study demonstrates that achievement of this phenotype requires housing without roosters, since their presence alters productivity and behavior, and is underpinned by changes in gut microbiome and the transcriptome.

Materials and Methods

Chickens

The experimental protocols and animal care were approved by the China Agricultural University Laboratory Animal Welfare and Animal Experimental Ethical Inspection board (approval number: CAU20151205-5). The study used dual-purpose native Beijing You Chickens. At Day 0, 10 thousand female chicks were randomly collected from a supplier and reared in a brooder house. At Day 70, 100 hens were selected at random and assigned to a free-range rearing unit with 10 age-matched roosters (R+). Another 100 hens were also selected at random were housed in an identical unit but without roosters (R-). The 10 selected roosters were from the same hatching group and reared in a brooder house with selected hens. At Day 280, 30 random R- and R+ hens were transferred into a cage system creating R+C and R-C groups for 35 days. Meanwhile, 30 R- and R+ hens remained until Day 315 in the free-range rearing units, creating R-F and R+F groups. Three roosters remained with R+F hens in their rearing unit. Rearing conditions and dietary program are described in our previous study (Xiang et al., 2018) in detail.

Production performance and product quality measurement

From Day 140 until Day 280 (before R+C and R-C birds were moved to cages), production performance was recorded daily on all 100 hens per rearing treatment. Parameters recorded, included feed consumption, egg number, soft-shell egg number, egg weight, and mortality. At Day 314, all fresh eggs from R+F and R-F groups were collected to measure egg quality using an egg multi-tester EMT-5200 (Robotmation, Tokyo, Japan). At Day 315, all hens were slaughtered. Thigh muscle (quadriceps femoris muscle) and pectoral muscle were collected from 10 randomly selected hens of each free-range group (R+F, R-F) to measure meat quality.

Behavioral response to novel environment and object

Novel environment tests measured exploratory behavior and general vigilance of birds (Favati et al., 2014). At Day 279, 11 (10 hens + 1 rooster) and 10 birds from R+ and R-, respectively, were randomly chosen to experience a novel environment and novel object. First, chickens were placed as a group in a novel 4 m x 4 m concrete-floored and walled environment in a building located 500 m away from the home house. After 10 min a football was suddenly thrown into the enclosure and responses of the group of chickens was continuously recorded by video for 20 min (10 min for the novel environment test and 10 min for the novel object test). Behaviors during the tests were analyzed in 30 s time bins and included walking, resting (standing), resting (lying), exploration, preening, freezing, and escape.

Behavioral time budgets

Hen behavior was recorded with a video camera for all the R+ and R- hens at Day 278. Behaviors included walking, resting (standing), resting (lying), exploration, preening, freezing, escape, drinking, eating, fighting, pecking and mating (defined in Table S3). On Day 280, the behavior of R+C and R-C birds transferred to cages during the first daytime period after the transfer was also recorded with a video camera. These behaviors were recorded by scan sampling every 10 min from 7 am to 7 pm and by continuous observations in the time-window of 3 h after morning feeding. Observations were performed by the same experimenter throughout the study. Behaviors separated by more than 5 min were regarded as 2 separate events during continuous observations.

Injuries

On Day 314, all 30 hens in each R+F, R-F, R+C and R-C group were scored for gait, footpad dermatitis and feather condition with evaluation systems described in our previous study (Xiang et al., 2018).

Blood metabolites

At Day 315, blood was collected from all birds (R+F, R-F, R+C, R-C) and the hematological profile was assessed using ~1 ml fresh blood in automated hematology analyzer HF-3800 (Healife, China). A total of 7 ml blood was immediately centrifuged at 6,000 × g for 10 min under room temperature (25 ℃-30 ℃) to obtain serum. Serum indicators including CK, inorganic phosphorus (P) and immunoglobulins (IgA, IgG and IgM) were measured with a semi-automatic biochemical analyzer HF-800B (Healife, China).

Gut microbiome population analyses

At Day 315, cecum contents were collected from 10 randomly selected hens per group (R+F, R-F, R+C, R-C) for gut microbiome analyses. DNA was extracted with a QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany). The V4 region of 16S rDNA was

amplified using a 515f/806r primer set. Libraries were generated using a TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's recommendations. Sequencing was conducted on an Illumina HiSeq2500 platform. Paired-end reads were merged using FLASH v1.2.7 (Magoc and Salzberg, 2011). Chimeric sequences were removed using the UCHIME algorithm (Edgar et al., 2011). Quality filtering of the raw tags was performed by QIIME v1.7.0 (Caporaso et al., 2010). OTUs were assigned using Uparse v7.0.1001 (Edgar, 2013) with a 97% similarity threshold. Taxonomy annotation was performed by comparing sequences to the GreenGene Database.

Microbes were analyzed at kingdom, phylum, class, order, family, genus and species levels. Observed species, one alpha diversity analysis, was processed by analysis of variance (ANOVA) with the Duncan post-hoc test. Another two alpha diversity indices, ACE and chao1, were analyzed to show microbiome diversity among treatments. Beta diversity was evaluated by calculation of both unweighted and weighted Unifrac distances and visualized by Non-Metric Multidimensional Scaling. All values with $p < 0.05$ were regarded as statistically significant. Functional profiles of the microbiome were predicted using PICRUSTs (http://huttenhower.sph.harvard.edu/galaxy). Results were analyzed with a two-sided Welch's t-test, and the KEGG pathways with Storey FDR corrected *p*-value (*q*-value) < 0.05 were depicted using STAMP software (Parks et al., 2014).

Transcriptome sequencing

At Day 315, 5 g of right thigh muscle and the right upper part of the spleen were collected from all hens of each group (R+F, R-F, R+C, R-C) for transcriptome sequencing. Total RNA of each sample was isolated independently using TRIzol reagent (Invitrogen, San Diego, CA, USA). After quality control, RNA samples from the same tissue of the same group were randomly pooled into three equidensity solutions and submitted for library preparation using a NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, Beverly, MA, USA). All libraries were then subjected processed with the Illumina Hiseq platform to generate paired-end 150 base pair reads.

After quality control, reads were mapped to the *Gallus_gallus*-5.0 (Ensembl release 86), used as the reference genome and gene model annotation file. The index of the reference genome was built using Bowtie v2.2.3 (Langmead and Salzberg, 2012). Paired-end clean reads were aligned to the reference genome using TopHat v2.0.12 (Trapnell et al., 2009) with 5% mismatches. Gene-level read counts were quantified using the HTSeq v0.6.1 Python tool. Differential expression analysis was performed using the DESeq R package v1.18.0 (Anders and Huber, 2010). Resulting *P*-values were adjusted using the Benjamini and Hochberg's approach (*q*-value) for controlling the false discovery rate. Genes with *q*-value < 0.05 were regarded as differentially expressed. GO enrichment was implemented by the online KOBAS 3.0 database (http://kobas.cbi.pku.edu.cn/) and GO terms with corrected *p*-value (*q*-value) < 0.05 were considered enriched. DAVID 6.8 software (https://david.ncifcrf.gov/) was used for KEGG pathway analyses using $p < 0.05$ as the statistical criterion.

Statistical analyses

Mean±standard deviation was calculated for all data. Data on production performance, blood metabolites and product quality were checked for normality and homogeneity of variance. Normally distributed data were analyzed by one-way ANOVA and Duncan post-hoc test was used to analyze differences among groups when significance ($p < 0.05$) was detected

in SPSS 23 (IBM, Armonk, NY, USA). For data not normally distributed, Kruskal–Wallis H and post-hoc tests and Mann-Whitney tests were conducted in SPSS 23 (IBM, Armonk, NY, USA). The Wilcoxon test was used to analyze behavior patterns, time budgets, gait scores, footpad dermatitis and injuries in SAS 9.2 (SAS Inst. Inc., Cary, NC, USA). All values with $p < 0.05$ were regarded as statistically significant.

Compliance and Ethics

Authors declare no conflicts of interest, financial or otherwise.

Figure Legends

Figure 1. Comparison of behavior patterns between the caged hens living without (R-C) and with (R+C) roosters. The value of the vertical axis indicates the proportion of time budget for different behavior.

Figure 2. Injuries of hens from different groups. (a) Gait scores. (b) Footpad dermatitis scores. (c) Feather condition scores. R-F, hens in free-range without roosters; R+F, hens in free-range with roosters; R-C, caged hens from R- rearing pens; R+C, caged hens from R+ rearing pens. The value of the vertical axis indicates the proportion of injury scores for different groups. The number of observations (n) = 30 per group.

Figure 3. Gut microbiome structure. (a) Beta diversity by Non-Metric Multidimensional Scaling. (b) The gut microbiome richness based upon the number of observed species. R-F, hens in free-range without roosters; R+F, hens in free-range with roosters; R-C, caged hens from R- rearing pens; R+C, caged hens from R+ rearing pens. The number of observations (n) = 10 per group.

Figure 4. Differential functions of the gut microbiome between hens living with or without roosters. (a) Different functions between R-F and R+F hens. (b) Differences between R-C and R+C hens. The abbreviation C indicates the pathway catalog of Cellular Processes; E, Environmental Information Processing; H, Human Diseases; M, Metabolism; O, Organismal Systems; and U, unclassified pathways. R-F, hens in free-range without roosters; R+F, hens in free-range with roosters; R-C, caged hens from R- rearing pens; R+C, caged hens from R+ rearing pens.

Figure 5. The most differentiated GO terms between hens living without (R-F) and with (R+F) roosters. Figure (a) shows data from thigh muscle and (b) from spleen tissue. Upregulated/downregulated genes are in R-F birds as compared to R+F birds. The abbreviation BP indicates the GO term catalog of Biological Processes; CC, Cellular Components; and MF, Molecular Function.

Figure 6. The most differentiated GO terms between the R-C and R+C hens in thigh muscle. R-C, caged hens from R- rearing pens; R+C, caged hens from R+ rearing pens. The upregulated/downregulated genes are in R-C as compared to R+C. The abbreviation BP indicates the GO term catalog of Biological Processes; CC, Cellular Components; and MF, Molecular Function.

Figure S1. The gut microbiome alpha diversity indices of ACE (upper) and chao1 (lower). R-F, hens in free-range without roosters; R+F, hens in free-range with roosters; R-C, caged hens from R- rearing pens; R+C, caged hens from R+ rearing pens. The number of observations (n) = 10 per group.

Figure S2. Differential functions of the gut microbiome between R+F and R+C hens. The

abbreviation C indicates the pathway catalog of Cellular Processes; E, Environmental Information Processing; H, Human Diseases; M, Metabolism; O, Organismal Systems; and U, unclassified pathways. R+F, hens in free-range with roosters; R+C, caged hens from R+ rearing pens.

Figure S3. The expression pattern for each subcluster of DEGs cluster in thigh muscle between hens living without and with roosters.

Figure S4. The expression pattern for each subcluster of DEGs cluster in spleen between hens living without and with roosters.

Figure S5. GO terms with highest differential expression between the R-C and R-F hens in thigh muscle. R-F, hens in free-range without roosters; R-C, caged hens from R- rearing pens. The upregulated/downregulated genes are in R-C as compared to R-F. The abbreviation BP indicates the GO term catalog of Biological Processes; CC, Cellular Components; and MF, Molecular Function.