Short- and long-term consequences of stocking density during rearing on the immune system and welfare of laying hens

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ABSTRACT Already during early life, chickens need to cope with chronic stressors that can impair their health and welfare, with stocking density being one of the most influential factors. Nevertheless, there is a gap in research on the influence of stocking density on laying hens during rearing and in the subsequent laying period. This study therefore investigated how stocking density during rearing affects the immune system and welfare of pullets, and whether effects are persistent later in life. Pullets were reared at either low (13 birds/m^2) or high (23 birds/m^2) stocking densities but in identical group sizes from wk 7 to 17. Afterward, hens were kept at the same stocking density (2.4 birds/m^2) until wk 28. Blood and tissue samples (spleen and cecal tonsils) were collected at the end of the rearing period and in the laying period. The parameters evaluated encompassed number and distribution of leukocytes and lymphocyte subsets in blood and lymphatic tissue, lymphocyte functionality, plasma corticosterone concentrations as well as behavior and physical appearance of hens. At the end of rearing, pullets kept under high stocking density had lower numbers of T lymphocytes, especially $\gamma\delta$ T cells in blood, spleen, and cecal tonsils and displayed a higher heterophil to lymphocyte ratio. These effects are mostly persistent during the laying period, although stocking density was identical at this time. Furthermore, birds from the high stocking density group showed less active behavior, more pecking behavior and worse physical appearance throughout both examination periods. In conclusion, stocking density during rearing affects pullets' immune system and behavior not only in the rearing, but also subsequently in the laying period, indicating a strong correlation between health and welfare during rearing and the laying period.

Key words: poultry, stocking density, rearing condition, welfare, immunity

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INTRODUCTION

The issue of animal welfare in poultry production is of increasing importance worldwide (Bessei, 2018). During production, chickens are confronted with a wide range of potential stressful conditions that modulate their immune system and may impair their health (Hofmann et al., 2020). The inability to cope with environmental challenges can lead to stress responses (Broom, 1991) and to the release of glucocorticoids, with corticosterone (**CORT**) being the most important in birds (Matos, 2008). An activated hypothalamic-pituitary-adrenal (**HPA**) axis is associated with changes in behavior, metabolism and the immune system − with negative consequences for welfare, especially if chronically stimulated (Matos, 2008). Glucocorticoids are described as major mediators of changes in leukocyte distribution (Stefanski and Engler 1998; Dhabhar, 2009). The exogenous administration of CORT in chickens has been shown to increase the number of circulatory heterophils and to decrease circulatory lymphocytes, resulting in an increase of heterophil to lymphocyte (**H/L**) ratio, whereas the total circulating leukocyte number decreased (Puvadolpirod and Thaxton, 2000; Shini et al., 2008a; Shini et al., 2008b; Shini and Kaiser, 2009; Mehaisen et al., 2017). One important stressor during the entire lifespan in many chicken production systems is overcrowding (Gomes et al., 2014). High stocking densities (**HSD**) may cause discomfort, frustration and stress, as natural behaviors cannot be performed (Appleby, 2004). Maximum stocking densities are consequently controlled in some countries, but the respective rules vary considerably worldwide. In the European Union, the maximum stocking density for laying hens (reproductive adult hens) is regulated by the

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Council Directive 1999/74/EC (European Union, 1999). However, there is no legislative coverage of stocking density for layer pullets during rearing prior to the period of laying in the European Union. It is known, however, that early-life experiences and rearing conditions have the potential to trigger short- and long-term effects on development, physiology and behavior in mammals and birds due to a high plasticity in the developing brain in the early life phase (Janczak and Riber, 2015; Dixon et al., 2016; Ellis and Del Giudice, 2019; Rodenburg and de Haas, 2016). So far, very few studies have examined the effects of stocking density during rearing on health and welfare in later life. It is reported that pullets reared at HSD have higher CORT levels in plasma and feathers (Eugen et al., 2019), and display more anxious behavior (Eugen et al., 2019) and feather pecking (Hansen and Braastad, 1994; Huber-Eicher and Audige, 1999; Bestman et al., 2009; Zepp et al., 2018). Interestingly, feather pecking is still higher even if hens were given more space during the laying period (Hansen and Braastad, 1994; Bestman et al., 2009). The effects of stocking density during rearing on immune parameters in layer pullets were investigated in 2 studies (Patterson and Siegel, 1998; Bozkurt et al., 2008). Bozkurt et al., (2008) reported an increased H/L ratio due to increased cage density, while Patterson and Siegel (1998) did not find any impact of stocking density on H/L ratio. However, none of the studies investigated long-lasting effects.

The aims of the present study were thus twofold: 1) to investigate the consequences of high and low stocking density during rearing on pullets' immune system and welfare parameters at the end of the rearing period, and 2) to identify possible long-term effects in the laying period when animals are kept under identical conditions. In order to obtain a comprehensive overview of the health and welfare of hens, many authors suggest measuring a wide range of different indicators (Dawkins, 2003; Blokhuis et al., 2007). The present study therefore assessed various parameters at physiological and behavioral levels. We hypothesized that pullets reared in HSD, compared to low stocking densities (**LSD**), differ with regard to number, distribution and functionality of immune cells, concentration of plasma CORT as well as with regard to behavior, and that HSD pullets show worse plumage and integument conditions. We furthermore hypothesized that some differences are still present during the laying period, even if the hens are then housed under identical stocking density.

MATERIAL AND METHODS

Experimental Design

All procedures were conducted according to the ethical and animal care guidelines and approved by the local authority Animal Ethics Committee (Regional Council Tübingen, approval number HOH 44/17 TH). The animals were kept at the farm animal research center of the University of Hohenheim (Agricultural Experimental Station, Unterer Lindenhof, Eningen, Germany).

A total of 552 one-day-old non-beak-trimmed female White Leghorn (Lohmann Selected Leghorn) chicks were purchased from a commercial hatchery (LSL Rhein-Main, Dieburg, Germany) and were randomly distributed into floor pens of 46 birds per pen. All birds were raised under identical commercial management practices (e.g., temperature, humidity, light) according to breeding standards (Lohmann Management Guide, 2017). Each pen was equipped with wood shaving litter, one bell drinker and 2 tube feeders, so the amount of feeder and drinker space per bird was kept constant in all groups. Birds had free access to feed and water throughout the study. From wk 7, each pen was additionally furnished with perches, providing 15 cm perch space per bird. The birds were housed in a windowless space that had artificial light, providing 24 h light in the first 3 d, which was then gradually reduced to 9 h light in wk 7 until the end of rearing in wk 17. Afterward, the light phase was gradually increased to 16 h light during the laying period. Room temperature was 34°C for the first 48 h and was then progressively reduced to 20°C until wk 5. Chicks received vaccinations against Marek's Disease and coccidiosis at the hatchery and against salmonellosis, Infectious Bronchitis, Newcastle Disease and Infectious Bursitis throughout the rearing period at the experimental research center via drinking water. Diet was formulated according to their age to provide all recommended nutrients and the feed was mixed on site at the experimental station.

The study was divided into 3 trials with each trial consisting of 2 replicates per stocking density. Experimental design is displayed in Figure 1. From d 1 to wk 6, all chicks were kept at identical stocking density of 18 chicks/m^2 corresponding to a pen area of 2.6 m^2 for habituation to facility. From wk 7 to wk 17 (rearing period), groups were assigned to either LSD (13 hens/m^2) or HSD (23 hens/m^2) with usable floor spaces of 3.5 or 2 m^2 respectively with equal group sizes of 46 chicks per pen. Thus, stocking density was created by floor space, not by group size in order to avoid disturbance in social stability. Thereafter, hens were kept at identical stocking density of 2.4 hens/m^2 and group size (40 hens/pen) with usable space of 16.88 m^2 until wk 28 (laying period). After arriving at the research center, all chicks were tagged with a neck band that was fixed with plastic filaments injected under the skin, bearing a number for clear identification. Gentle pecking of the tags by the pen mates was observed at a low frequency during the first days, but disappeared afterwards.

Blood Sampling

Birds were habituated and trained prior to blood sampling to get used to handling. Blood was taken at the end of the rearing period (wk of life 16) and at 11 wk after during the laying period (wk of life 27) (Figure 1). In order to allow intraindividual blood sampling, 24

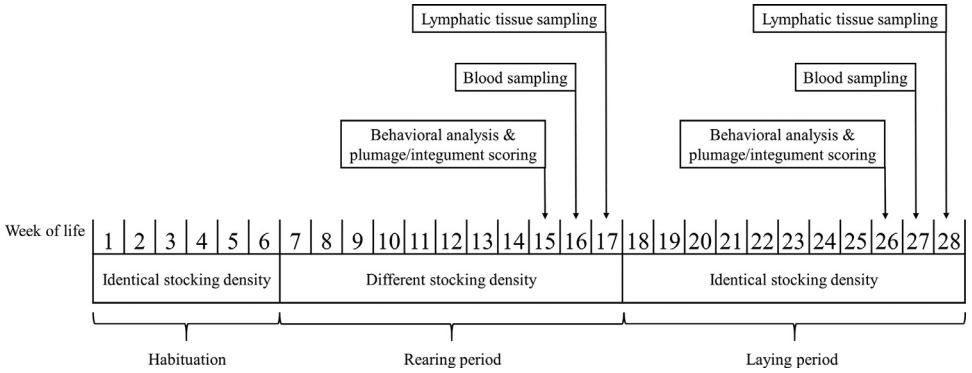

Figure 1. Scheme of experimental design. After 6 wk of habituation at identical stocking density (18 birds/m^2), chicks were assigned to either low (13 birds/m^2) or high (23 birds/m^2) stocking density at the same group size of 46 pullets per pen during rearing until wk 17. Afterward, hens were kept at identical stocking density (2.4 birds/m^2) during the laying phase until wk 28, in group sizes of 40 hens per pen. The study was divided into 3 trials, with each trial consisting of 2 replicates per stocking density.

birds per treatment and trial were sampled at the end of the rearing period and 12 birds of these 24 in the laying period, as the other 12 chickens were subjected to tissue collection at the end of the rearing period (s. below). Blood samples were collected by *vena ulnaris* puncture into 2 mL Eppendorf tubes and 5 mg/mL EDTA (Sigma Aldrich, St. Louis, MO). All samples analyzed were taken within 3 min of the hen being removed from the pen in order to avoid an acute stress response to capture and handling. Fixation of blood samples was obtained by the addition of TransFix reagent (# TFB-20-1; Caltag Medsystems Ltd., UK) according to manufacturer instruction. Stabilized blood samples were kept at room temperature and processed within 4 h after blood collection. Plasma samples were obtained by centrifuging unfixed blood samples for 15 min at 2000 × *g* and 4°C, and were stored at −20°C until measurement.

Lymphatic Tissue Sampling and Preparation

The same birds used for blood sampling were used for tissue sampling at the end of the rearing period in wk of life 17 and in the laying period in wk of life 28 (Figure 1). Per treatment, period and trial, 12 birds were sacrificed and killed by a CO_2 gas mix. Immediately after death, spleen and cecal tonsils were removed and stored for transportation on ice in PBS + 1% Fetal Bovine Serum (**FBS**) + 0.05 mg/mL Gentamycin (Biochrom, Berlin, Germany). All tissues were weighed prior to further processing. Spleen and cecal tonsils were collected, as due to the absence of encapsulated lymph nodes, induction and emergence of immune response in chicken mainly takes place in these lymphatic tissues (Jeurissen, 1991; Lillehoj and Trout, 1996), making them important immunological organs in chickens.

Lymphatic tissue was processed according to Hofmann and Schmucker (2021). In brief, the spleen was cut into pieces under sterile conditions, transferred into gentleMACS C-Tube (#130-096-334, Miltenyi Biotec, Bergisch Gladbach, Germany) containing PBS + 0.05 mg/mL Gentamycin (Biochrom, Berlin, Germany) and dissociated with a gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell suspension was then applied to a 40 μm MACS SmartStrainer (Miltenyi Biotec, Bergisch Gladbach, Germany). Flow-through was centrifuged for 10 min at 300 × *g* at 4°C and cell pellet resuspended in PBS + 1% FBS. The final volume was determined and stored on ice until further processing.

Intraepithelial lymphocytes of cecal tonsils were removed from the mucosa by shaking the tissue twice in Hanks' Balanced Salt solution (without Mg^{2+} and Ca^{2+}) supplemented by 5 mM EDTA, 5% FBS and 1 mM Dithiothreitol for 20 min at 37°C under continuous rotation. Between the two steps, tissue pieces were put onto a 40 μm MACS SmartStrainer (Miltenyi Biotec, Bergisch Gladbach, Germany), with the flow-through containing desired intraepithelial lymphocytes. Intraepithelial lymphocytes were washed twice in PBS + 1% FBS by centrifuging for 10 min at 300 × *g* at 20°C. Finally, cell pellet was resuspended in PBS + 1% FBS. The final volume of intraepithelial lymphocytes suspension was determined and stored on ice until further processing.

Flow Cytometric Analysis

Specific immune cell populations were characterized using flow cytometric analysis, followed by the no-lyse-no-wash method described by Seliger et al. (2012) with some modifications and amplifications described in detail in Hofmann and Schmucker (2021). The following antibodies were used for the discrimination of blood or tissues leukocytes: Anti-CD45-APC (# 8270-11, clone LT40), anti-Monocyte/Macrophage (# 8420-09, clone Kul01), anti-CD4-Pacblu (# 8210-26, CT-4), or -PE (# 8210-09,), anti-CD8α-FITC (# 8220-02, clone CT-8) and anti-Bu-1-FITC (# 8395-02, clone AV20) were obtained from SouthernBiotech (Birmingham, USA). Anti-CD41/61-PE (# MCA2240GA, clone 11C3) was purchased from BioRad (California, USA) and anti-TCR$\gamma\delta$-PerCP (# NBP1-28275PCP, clone TCR1) from Novus Biologicals (Colorado, USA). Mouse IgM isotype control conjugated to APC (# 0101-11, clone 11E10) was used to exclude non-specific binding of anti-CD45-

APC and to verify correct numbers of leukocytes. Measurements were performed on a BD FACSCanto II (BD Biosciences, Heidelberg, Germany) equipped with a 488 nm blue laser, 630 nm red laser and a 405 nm violet laser. Acquisition and analysis were done using BD FACSDiva Software II (BD Biosciences, Heidelberg, Germany). Absolute number of leukocytes per μL blood or g tissue was determined using BD Trucount tubes (# 340334, BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions by dividing the number of cell events by the number of bead events. They were afterward multiplied by bead concentration divided by sample volume per tube, recalculated based on sample dilution. Absolute cell numbers of leukocyte subsets were calculated by combining cell frequencies with total leukocyte counts. Immune cells were distinguished and gated by the respective combination of surface marker expression (cluster of differentiation = CD), as described below.

Blood In blood, the following immune cells were distinguished by the respective combination of surface marker expression in one staining step: total leukocytes ($CD45^+$), thrombocytes ($CD45^{dim}/CD41/61^+$), monocytes ($CD45^+/Kul01^+$), T helper (**TH**) cells ($CD45^+/CD4^+/TCR\gamma\delta^-/CD8\alpha^+$ or $CD8\alpha^-$), cytotoxic T cells (**CTL**; $CD45^+/CD4^-/TCR\gamma\delta^-/CD8\alpha^+$), $\gamma\delta$ T cells ($CD45^+/Kul01^-/CD4^-/TCR\gamma\delta^+/CD8\alpha^+$ or $CD8\alpha^-$), and B lymphocytes ($CD45^+/Kul01^-/Bu\text{-}1^+$). Heterophils were identified based on their FSC/SSC characteristics. The detailed staining and gating strategy are described in Hofmann and Schmucker (2021). In short, 20 μL of stabilized EDTA-blood were diluted with 980 μL staining buffer (PBS pH 7.4, 2% BSA, 0.1% NaN_3, 5mg/mL EDTA). Fifty μL of diluted blood sample were stained with 20 μL of the respective antibody mixture in a Trucount tube (# 340334, BD Biosciences, Heidelberg, Germany) and incubated for 45 min in the dark at room temperature. Afterward, 400 μL of staining buffer was added and the samples were kept cold and dark until measurement. Reverse pipetting was used for every step to pipette precise volumes and reduce measurement errors. At least 10.000 $CD45^+$ cell events per sample were analyzed.

Lymphatic Tissue Flow cytometric analysis of splenocytes and intraepithelial lymphocytes of cecal tonsils including gating strategy is described in detail in Hofmann and Schmucker (2021). In brief, single-cell suspensions were stained with respective antibody mixtures to discriminate all leukocytes and leukocyte subsets according to a no-lyse no-wash single-step two-tube protocol. In order to accurately distinguish live and dead cells, SYTOX Blue Dead Cell Stain (ThermoFisher Scientific, MA) was added. At least 50.000 $CD45^+$ cell events per sample for spleen and intraepithelial lymphocytes of cecal tonsils were analyzed. The following immune cells were distinguished by the respective combination of surface marker expression in tube 1) total leukocytes ($CD45^+$), TH cells ($CD45^+/CD4^+/TCR\gamma\delta^-/CD8\alpha^+$ or $CD8\alpha^-$), CTL ($CD45^+/CD4^-/TCR\gamma\delta^-/CD8\alpha^+$), $\gamma\delta$ T cells ($CD45^+/Kul01^-/CD4^-/TCR\gamma\delta^+/CD8\alpha^+$ or $CD8\alpha^-$), and in tube 2) thrombocytes ($CD45^{dim}/CD41/61^+$) (only spleen), monocytes/macrophages ($CD45^+/Kul01^+$) (only spleen), and B lymphocytes ($CD45^+/Kul01^-/Bu\text{-}1^+$).

Spleenic Lymphocyte Proliferation

Isolated splenocytes were further separated by density gradient to obtain mononuclear cells for a lymphocyte transformation test. Therefore, 14 mL cell suspension was carefully layered onto a gradient (Biocoll separation solution, 1.077g/mL, Biochrom, Berlin, Germany) and centrifuged for 12 min at 600 × g at 20°C. The interphase was then collected and washed in PBS + 1% FBS by centrifuging for 10 min at 500 × g at 20°C. Finally, cell pellet was resuspended in RPMI 1640 + 10% FBS. Cell numbers were determined using a Z2 Coulter Counter (Beckman Coulter, Krefeld, Germany). Activity of spleen lymphocytes was examined in vitro using a mitogen-induced lymphocyte proliferation assay. The mitogens concanavalin A (**ConA**, Sigma Aldrich, Munich, Germany) and pokeweed mitogen (**PWM**, Sigma Aldrich, Munich, Germany) were used for stimulation of T lymphocytes as well as T- and B lymphocytes, respectively (Al-Khalifa, 2016). About 1.5 × 10^5 cells per well were transferred into 96-well round bottom cell culture plates (Neolab, Heidelberg, Germany) in triplicates per treatment, and stimulated with 10 μg/mL of the respective mitogen or left without stimulation as negative control (medium only). After an incubation time of 46 h (41°C, 5% CO_2), 0.25 μCi ^{3}H-thymidine (PerkinElmer, Rodgau, Germany) per well was added. After another 24 h of incubation, cells were harvested on glass fiber filters (Skatron, Lier, Norway) and radioactivity was evaluated by liquid scintillation analyzer (PerkinElmer, Rodgau, Germany). For each triplicate, mean of cpm was calculated and delta cpm for ConA and PWM (delta cpm = stimulated cells - unstimulated cells) generated. CV of intra-assay for delta cpm of ConA was 11% and for delta cpm of PWM 15%.

Antibody Analysis

Concentrations of total plasma IgY, IgM, and IgA were measured by ELISA in triplicates. 96-well flat-bottom microtiter plates (Thermo Scientific, Roskilde, Netherlands) were coated with 200 ng/well of either goat anti-chicken IgY Fc antibody (# A30-104-A; Bethyl Laboratories, Montgormery, TX), goat anti-chicken IgM antibody (# A30-102-A; Bethyl Laboratories, Montgormery, TX) or goat anti-chicken IgA antibody (# A30-103-A; Bethyl Laboratories, Montgormery, TX) diluted in coating buffer (15 mM $NaHCO_3$, pH 9.6) and incubated over night at 4°C. Following this, plates were blocked with 1% BSA (Roth, Karlsruhe, Germany) and incubated for 30 min at room temperature. Plasma samples were diluted (IgY 1:200,000; IgM 1:10,000; IgA 1:2,000) in a dilution buffer (50 mM Tris, 0.0027 M KCL, 0.14 M NaCl; pH 8.0),

applied to the antibody-coated plates and incubated for 1 h at room temperature. Bound plasma IgY, IgM and IgA were detected with horseradish peroxidase-labeled goat anti-chicken IgY Fc antibody (# A30-104-P; Bethyl Laboratories, Montgormery, TX), goat anti-chicken IgM antibody (# A30-102-P; Bethyl Laboratories, Montgormery, TX) or goat anti-chicken IgA antibody (# A30-103-P; Bethyl Laboratories, Montgormery, USA) diluted 1:100,000 in coating buffer, respectively. After 1 h of incubation at room temperature, tetramethylbenzidine (AppliChem, Darmstadt, Germany) was added and color formation was stopped after 20 min with 2M H_2SO_4 (Roth, Karlsruhe, Germany). Plates were washed 5 times between each step with washing buffer (50 mM Tris, 0.0027 M KCL, 0.14 M NaCl, 0.05% Tween 20; pH 8.0). A calibration curve was derived from serial dilutions of a pooled plasma sample whose IgY, IgM, and IgA concentration was determined in advance with Chicken IgG ELISA Kit (# E33-104), Chicken IgM ELISA Kit (# E33-102) and Chicken IgA ELISA Kit (# E33-103), all from Bethyl Laboratories (Montgomaery, TX). The standard curve of IgY, IgM and IgA ranged from 4.96 ng/mL to 300 ng/mL, from 7.8 ng/mL to 500 ng/mL and from 15.63 ng/mL to 1,000 ng/mL, respectively. The absorbance was measured at 450 nm and the concentration was calculated relatively to the absorbance of the calibration curve. CV of intra-assay were 4.3 % for IgY, 7.6 % for IgM and 3.7 % for IgA and CV of interassay 5.9 % for IgY, 3.2 % for IgM and 3.7 % for IgA.

Corticosterone Analysis

Plasma CORT concentrations were determined radioimmunologically in duplicate after an extraction with ethyl acetate. Fifty μL plasma was dissolved in 100 μL phosphate buffer, and 3 mL ethyl acetate (Applichem, Darmstadt, Germany) were added. The sample was mixed gently for 30 min and the aqueous phase was then frozen. Subsequently, the supernatant was transferred into a vial and the solvent evaporated. Afterward, buffer was added and the concentrations of CORT were determined by RIA. Procedural losses were determined in each assay with 2 samples spiked with ^{3}H CORT (10,000 cpm/sample). For the radioimmunological determinations, a polyclonal antibody (raised against corticosterone-3-cmo-urease; # AB1297, Merck Millipore, Billerica, MA) was used at a final dilution of 1: 77,000 in 0.1% BSA buffer. The antibody revealed a cross-reactivity of 0.67% 11-dehydrocorticosterone, 1.5% Deoxycorticosterone, <0.01% 18-OH-DOC, <0.01% Cortisone, <0.01% Cortisol and 0.2% Aldosterone. 10,000 cpm of ^{3}H CORT ([1,2,6,7-3H (N)]-corticosterone; 89.8 Ci/mmol, Perkin Elmer, Boston, MA) were added to each sample as a tracer. Separation of bound/free was performed with dextran-coated charcoal (0.05% Dextran70, Carl Roth; 0.5% NoritA, Serva Electrophoresis, Heidelberg, Germany) by centrifugation for 20 min at 2,000 × g at 4°C. Supernatants were decanted to 5 mL Ultima-Gold (Perkin Elmer, Boston, MA) to determine radioactivity (Tri-Carb 2800 TR). The standard curve was prepared in phosphate buffer and covered a range between 0.01 ng and 1 ng per test. Precision was determined with spiked samples, based on plasma with low endogenous CORT concentrations spiked with 0.5 and 1 ng CORT/mL. The mean recovery rate ranged between 79.14% and 103.17% for samples spiked with 0.5 ng/mL and 1 ng/mL. Interassay variabilities ranged between 8.2 %, 14.5 % and 17.4 % depending on endogenous CORT (3.91 ng/mL, 1.7 ng/mL and 0.83 ng/mL). Intra-assay variability for a biological sample with endogenous CORT concentration of 1.59 ng/mL was 2.42 % and for a biological sample with endogenous CORT concentration of 0.85 ng/mL was 5.97%.

Behavior Analysis

Behavior was recorded directly on four consecutive days in wk 15 and 26 from 10 am to 2 pm. Each group (2 groups per stocking density per trial) was observed for 60 min each day, balanced for observation day and time of day. The observer stood in front of the pen with a clear view of the birds for about 10 min before starting observations to accustom the birds to his or her presence. At 5-min intervals, the following behavior patterns were recorded by behavior sampling (Martin and Bateson, 2007): locomotion, resting, standing, foraging, drinking, feeding, preening and dustbathing. The mean of all scans was taken as the average number of birds in view during the observation time. Results are expressed as the percentage of birds performing the respective behavior/total number of birds observed (Reiter and Bessei, 2000). Additionally, gentle feather pecking, severe feather pecking and aggressive pecking were recorded continuously in 20-min sessions. As pecking occurs often in series in rapid succession, a new peck was only recorded if there was a break of 3 s between the pecks. Data on pecks are given as the sum of pecks over the complete observation session (Kjaer, 2002). All behavior patterns recorded are defined in Table 1.

Plumage and Integument Scoring

Plumage and integument scoring were done individually for 20 hens per group at 15 and 26 wk of age. A modified scoring system (Knierim et al., 2016) was used ranging from 1 to 3 for scoring plumage condition (neck, back, breast, vent, wing), tail feather condition as well as plumage pollution and skin and leg lesions. Lesions of comb and beak were scored with 1 to 2. Higher scores represent a worse condition. Feather damage at a young age may look different and may not be as severe as in adult life, as pullets molt their feathers 3 times during rearing. As a consequence, earlier damage will be repaired by this natural process (Bestman et al., 2009). We, therefore, used 2 different scoring systems for pullets and adult laying hens. A detailed description of the scoring system can be found in Table 2.

Table 1. Ethogram of recorded behavior.

Behavior	Description
Locomotion	Progressively moving forward by walking or flying with no other activity
Resting	Laying or sitting on the ground or perch with tucked up legs and no other activity
Standing	Upright position on the ground or perch, legs extended with no activity
Foraging	Pecking or scratching, by moving litter with the feet at the ground with head lowered to the ground
Drinking	Beak inside the drinker
Feeding	Beak inside the feeder
Preening	Running beak through feathers
Dustbathing	Lying on the ground and tossing litter onto its back/wing
Gentle feather pecking	Pecking at another bird's plumage without pulling or removing feathers
Severe feather pecking	Pecking at another bird's plumage with pulling or removing feathers
Aggressive pecking	Pecking at the head or comb of another bird

Statistical Analysis

All statistical analyses were performed with SAS Version 9.4 (SAS Institute Inc., Cary, NC), denoting $P < 0.05$ as being significant and denoting $0.05 \leq P < 0.1$ as a tendency. Immunological parameters, CORT concentrations as well as behavior and scoring of plumage and integument were analyzed using a linear mixed model with the PROC MIXED procedure after testing residuals for normal distribution and homogeneous error variance via graphical check of residual plots (Kozak and Piepho, 2018). If model assumptions were not fulfilled, logarithmic transformations were used to stabilize variance and meet the distribution assumption. In this case, results were back-transformed for presentation only. Standard errors were back-transformed using the Delta method. Degrees of freedom were determined by the method of Kenward-Roger and variance components were estimated using the restricted maximum likelihood method. The linear mixed model included stocking density (LSD or HSD), period (rearing period or laying period) and their interactions as well as trial as fixed effects. Stocking density and stocking density × period effects were considered to assess short- and long-term effects of stocking density. In case of no significance ($P > 0.05$) of interactions (stocking density × period), the main effects (stocking density) were considered. In case of significance of F-tests, a Fishers LSD test was used for multiple pairwise post-hoc testing. For all parameters, group and pen effects were used as random effects. As observations were taken from the same hens in both periods, the model should account for the repeated

Table 2. Description of the scoring method used to evaluate integument and plumage of hens at the end of the rearing period and in the laying period.

Score	Rearing period	Laying period
Plumage pollution		
1	Dirt-particle-free plumage, stain on ≤ 2 of body parts	Dirt-particle-free plumage, stain on ≤ 2 of body parts
2	Dirt-particle-free plumage, stain on ≥ 3 of body parts	Dirt-particle-free plumage, stain on ≥ 3 of body parts
3	Visible and tactile dirt particles with or without stain	Visible and tactile dirt particles with or without stain
Plumage condition		
1	Complete plumage without feather losses or damaged feathers	Complete plumage without feather losses or damaged feathers
2	No wing feather loss with ≤ 2 damaged wing feathers or bald patch < 1 cm diameter	No wing feather loss with ≤ 2 damaged wing feathers and/or bald patch < 5 cm diameter
3	Wing feather losses and/or ≥ 3 damaged wing feathers and/or bald patch ≥ 1 cm diameter	Wing feather losses and/or ≥ 3 damaged wing feathers and/or bald patch ≥ 5 cm diameter
Tail feather condition		
1	No feather losses or damaged feathers	No feather losses or damaged feathers
2	≤ 2 damaged feathers and/or 1 missing feather	≤ 3 damaged feathers and/or 1 missing feather
3	≥ 3 damaged feathers and/or > 1 missing feather	≥ 4 damaged feathers and/or > 1 missing feather
Skin lesions		
1	No lesions	No lesions
2	Only blood-filled quills	Blood-filled quills and/or ≤ 2 fresh or crusted lesions < 1 cm diameter
3	Any fresh or crusted lesion	≥ 3 fresh or crusted lesions < 1 cm diameter or ≥ 1 fresh or crusted lesions ≥ 1 cm diameter
Comb lesions		
1	No or ≤ 1 crusted lesion	No or ≤ 4 crusted lesion
2	> 1 crusted lesions	≥ 5 crusted lesions
Beak lesions		
1	No fresh or crusted lesion	No fresh or crusted lesion
2	Any fresh or crusted lesion	Any fresh or crusted lesion
Foot lesions		
1	No lesions, no ulceration or swelling	No lesions, no ulceration or swelling
2	≤ 1 fresh or crusted lesion and/or ulceration without swelling	≤ 1 fresh or crusted lesion and/or ulceration without swelling
3	≥ 2 fresh or crusted lesion or missing claw and/or ulceration with swelling	≥ 2 fresh or crusted lesion or missing claw and/or ulceration with swelling

measures structure of the data. The model therefore allows for period-specific variances for group, pen and error effects and for a possible correlation between effects from the same group, pen or hen, respectively. For lymphatic tissue, date of slaughter and for behavioral parameters, observation day and observed hour were included. In order to eliminate body weight, order of sampling and duration of blood sampling in immune and endocrine parameters, data were statistically corrected by including body weight prior to the start of the rearing period, order of sampling for blood and lymphatic tissue as well as sampling time for blood as covariates. Covariables were checked for significance and were dropped from the model in case they were not significant.

The results are presented as LSmeans with their SEM. Furthermore, *P*-values for F tests of stocking density main effects and stocking density × period interaction effects are shown.

Feather pecking was analyzed by generalized mixed models of the PROC GLIMMIX procedure. The linear predictor in the generalized linear model was analogous to the expected value in the mixed model above. A poisson distribution was assumed and thus a log-link was used. Means were back-transformed using the inverse link function for presentation purposes only. As above, group and pen were used as random effects. As observations were taken at different hours and days, both effects were included into the model assuming random effects. Results are presented as frequencies.

RESULTS

Effects of Stocking Density on Number and Functionality of Immune Cells in Blood and Lymphatic Tissue

In order to evaluate immune status, we determined absolute numbers of leukocytes and leukocyte subsets in whole blood and spleen, as well as intraepithelial lymphocytes of cecal tonsils together with functional measurements. Mixed linear model analysis revealed effects for stocking density ($P < 0.05$), indicating short- and long-term effects of stocking density during rearing on the number of certain immune cells in blood (Table 3) and spleen (Table 4). Moreover, there was a significant interaction of stocking density × period ($P < 0.05$) for cecal tonsils (Table 5), indicating short-term effects.

Pullets reared at HSD compared to LSD had lower numbers of lymphocytes in blood at the end of the rearing period as well in the laying period ($P = 0.0002$), although both groups were kept at the same stocking density after rearing. The effect on lymphocytes was caused by lower numbers of T lymphocytes in the HSD groups ($P = 0.0002$), especially of the $\gamma\delta$ T cell subset ($P < 0.0001$) at the end of the rearing period as well as in the laying period. In-depth analyses of $\gamma\delta$ T cells showed that this effect was especially attributed to CD8α^+ $\gamma\delta$ T cells ($P = 0.007$), whereas CD8α^- $\gamma\delta$ T cells were not affected ($P > 0.05$). All other immune cell populations investigated did not differ between pullets reared at HSD or LSD (Table 3). The lower lymphocyte numbers in pullets reared at HSD resulted in a higher H/L ratio at the end of the rearing period and well into the laying period ($P = 0.024$) when compared to pullets reared at LSD.

Absolute and relative weights (organ weight/body weight) of lymphoid organs were not different between pullets reared at either LSD or HSD ($P > 0.05$) (Tables 4 and 5).

The number of $\gamma\delta$ T cells ($P = 0.002$) was also lower in the spleen at the end of rearing and in the laying period of pullets reared at HSD compared to LSD (Table 4). Again, this effect was due to reduced numbers of CD8α^+ $\gamma\delta$ T cells ($P = 0.002$). In addition, the number of CD8α^+ TH cells ($P = 0.075$) tended to be lower in the spleen in both periods in the HSD group. All other

Table 3. Short- (rearing period) and long-term (laying period) effects of stocking density during rearing on number of immune cells [#/μL] and heterophil to lymphocyte ratio in whole blood.[1]

	Rearing period		Laying period		*P* value	
Parameter	LSD[2]	HSD[3]	LSD[2]	HSD[3]	Stocking density	Stocking density × period
Leukocytes[4]	46,034 ± 3,302	44,068 ± 3,148	35,797 ± 2,666	35,118 ± 2,762	0.199	0.616
Thrombocytes	50,957 ± 2,732	50,103 ± 2,851	49,549 ± 3,056	49,758 ± 3,137	0.910	0.854
Monocytes[4]	1,839 ± 196	1,867 ± 195	2,664 ± 298	2,968 ± 368	0.350	0.479
Heterophils[4] (H)	1,973 ± 105	2,101 ± 116	3,444 ± 320	5,231 ± 436	0.198	0.656
T lymphocytes[4]	21,625 ± 549[a]	20,548 ± 494[b]	13,153 ± 373[a]	11,443 ± 406[b]	0.0002	0.078
T helper cells[4]	8,605 ± 701	8,349 ± 677	5,830 ± 474	5,231 ± 436	0.110	0.339
$\gamma\delta$ T cells[4]	8,634 ± 354[a]	7,819 ± 313[b]	4,345 ± 220[a]	3,447 ± 199[b]	< 0.0001	0.071
CD8α^+ $\gamma\delta$ T cells[4]	3,228 ± 1,540[a]	2,566 ± 1,223[b]	360 ± 173[a]	289 ± 139[b]	0.007	0.955
CD8α^- $\gamma\delta$ T cells[4]	3,211 ± 1,108	3,137 ± 1,086	3,730 ± 1,308	2,937 ± 1,041	0.222	0.311
Cytotoxic T cells[4]	4,075 ± 270	3,977 ± 289	2,868 ± 204	2,605 ± 198	0.117	0.341
B lymphocytes[4]	3,203 ± 220	3,222 ± 230	2,619 ± 209	2,210 ± 202	0.306	0.275
Lymphocytes[4] (L)	25,176 ± 653[a]	24,342 ± 581[b]	15,988 ± 481[a]	13,966 ± 517[b]	0.002	0.058
H/L ratio[4]	0.078 ± 0.004[b]	0.086 ± 0.005[a]	0.214 ± 0.021[b]	0.282 ± 0.037[b]	0.024	0.284

[a,b]Values in row with no common superscript letter are significantly different within the respective period ($P < 0.05$).

[1]Data are shown as LSmeans ± SEM based on n = 72 observations per treatment at the end of the rearing period and n = 35−36 during the laying period.

[2]LSD, low stocking density during rearing (13 birds/m^2).

[3]HSD, high stocking density during rearing (23 birds/m^2).

[4]Data that required logarithmic transformation are reported on the original scale after back transformation.

Table 4. Short- (rearing period) and long-term (laying period) effects of stocking density during rearing on number of immune cells [#×10^6/g] in spleen.[1]

	Rearing period		Laying period		*P* value	
Parameter	LSD[2]	HSD[3]	LSD[2]	HSD[3]	Stocking density	Stocking density × period
Leukocytes[4]	1,580 ± 66.4	1,485 ± 61.7	1,630 ± 54.8	1,570 ± 54.2	0.130	0.709
Thrombocytes	114 ± 5.86	105 ± 5.34	126 ± 5.56	126 ± 5.78	0.392	0.347
Monocytes[4]	5.23± 1	5 ± 1.20	9.84 ± 2.22	10.1 ± 2.30	0.931	0.617
T lymphocytes[4]	1,174 ± 52.4	1,109 ± 48.8	1,090 ± 38.0	1,030 ± 36.9	0.094	0.999
T helper cells[4]	257 ± 12.0	251 ± 11.6	264 ± 8.48	260 ± 8.63	0.621	0.880
CD8α^+ T helper cells[4]	14.3 ± 1.10	11.8 ± 0.89	14.2 ± 0.90	12.8 ± 0.85	0.075	0.534
CD8α^- T helper cells[4]	242 ± 11.3	239 ± 11.0	248 ± 8.34	246 ± 8.54	0.765	0.915
$\gamma\delta$ T cells[4]	453 ± 24.5[a]	398 ± 21.4[b]	309 ± 15.1[a]	278 ± 13.9[b]	0.002	0.757
CD8α^+ $\gamma\delta$ T cells[4]	299 ± 14.2[a]	265 ± 12.4[b]	199 ± 7.94[a]	170 ± 7.09[b]	0.002	0.706
CD8α^- $\gamma\delta$ T cells[4]	145 ± 15.5	127± 13.5	105 ± 10.9	103 ± 10.9	0.227	0.235
Cytotoxic T cells[4]	457 ± 24.8	454 ± 24.4	507 ± 24.0	483 ± 23.4	0.471	0.592
B lymphocytes[4]	258 ± 24.6	242 ± 23.0	430 ± 39.3	421 ± 39.0	0.448	0.700
Lymphocytes[4]	1,448 ± 60.5	1,362 ± 56.2	1,533 ± 51.2	1,471 ± 50.5	0.121	0.764
Spleen weight (g)	2.30 ± 0.06	2.37 ± 0.06	1.72 ± 0.05	1.70 ± 0.05	0.592	0.407
Spleen weight (%)	0.193 ± 0.006	0.197 ± 0.006	0.104 ± 0.005	0.109 ± 0.005	0.290	0.978

[a,b]Values in row with no common superscript letter are significantly different within the respective period ($P < 0.05$).
[1]Data are shown as LSmeans ± SEM based on 35–36 observations per treatment and period.
[2]LSD, low stocking density during rearing (13 brids/m^2).
[3]HSD, high stocking density during rearing (23 birds/m^2).
[4]Data that required logarithmic transformation are reported on the original scale after back transformation.

Table 5. Short- (rearing period) and long-term (laying period) effects of stocking density during rearing on number of immune cells [#×10^5/g] in intraepithelial lymphocytes of cecal tonsils.[1]

	Rearing period		Laying period		*P* value	
Parameter	LSD[2]	HSD[3]	LSD[2]	HSD[3]	Stocking density	Stocking density × period
Leukocytes[4]	841 ± 112	765 ± 105	530 ± 68.3	540 ± 71.3	0.472	0.291
T lymphocytes[4]	569 ± 73.9	514 ± 69.7	327 ± 43.9	350 ± 45.7	0.775	0.144
T helper cells[4]	144 ± 20.3	123 ± 17.9	93.4 ± 13.6	91.6 ± 13.7	0.355	0.379
CD8α^+ T helper cells[4]	56.8± 8.96	46.1 ± 7.29	36.5 ± 6.43	36.2 ± 5.78	0.587	0.379
CD8α^- T helper cells[4]	74.5 ± 16.2	72.2 ± 15.8	46.2 ± 9.98	47.3 ± 10.6	0.968	0.727
$\gamma\delta$ T cells[4]	185 ± 10.5[a]	157 ± 10.2[b]	74.4 ± 5.18[ab]	82.8 ± 5.78[ab]	0.630	0.027
CD8α^+ $\gamma\delta$ T cells[4]	119 ± 8.24	102 ± 6.92	40.9 ± 3.01	42.31 ± 3.44	0.406	0.185
CD8α^- $\gamma\delta$ T cells[4]	60.8 ± 4.02[a]	52.27 ± 4.71[b]	33.9 ± 2.71[ab]	37.1 ± 3.52[ab]	0.864	0.036
Cytotoxic T cells[4]	232 ± 38.0	228 ± 38.2	150 ± 24.4	170 ± 27.9	0.486	0.288
B lymphocytes[4]	153 ± 31.9	143 ± 30.6	106 ± 22.1	95.3 ± 21.0	0.390	0.844
Lymphocytes[4]	735 ± 110	664 ± 102	444 ± 68.3	463 ± 69.9	0.668	0.236
Cecal tonsil weight (g)	0.662 ± 0.033	0.649 ± 0.033	0.635 ± 0.019	0.634 ± 0.019	0.816	0.814
Cecal tonsil weight (%)	0.055 ± 0.003	0.054 ± 0.002	0.039 ± 0.002	0.040 ± 0.002	0.929	0.456

[a,b]Values in row with no common superscript letter are significantly different within the respective period ($P < 0.05$).
[1]Data are shown as LSmeans ± SEM based on 29–36 observations per treatment and period.
[2]LSD, low stocking density during rearing (13 brids/m^2).
[3]HSD, high stocking density during rearing (23 birds/m^2).
[4]Data that required logarithmic transformation are reported on the original scale after back transformation.

immune cells did not differ between pullets reared at HSD or LSD ($P > 0.05$).

In cecal tonsils (Table 5), the interaction of stocking density × period was significant ($P = 0.027$) for $\gamma\delta$ T cells. Post-hoc analyses revealed lower numbers of $\gamma\delta$ T cells in the HSD group at the end of the rearing period ($P = 0.043$). However, this effect did not stretch into the laying period ($P = 0.246$). The interaction of stocking density × period was also significant for CD8α^- $\gamma\delta$ T cells ($P = 0.036$), with no differences found after post-hoc testing ($P > 0.05$). All other immune cells in the cecal tonsil did not differ between pullets reared at HSD or LSD ($P > 0.05$).

Effects of Stocking Density on Functionality of Lymphocytes

A significant interaction of stocking density × period was observed ($P = 0.004$) for plasma IgM concentration (Table 6). Post-hoc analyses showed higher concentrations of IgM in plasma of pullets reared at HSD compared to LSD at the end of the rearing period ($P = 0.041$), but no long-lasting effects in the laying period ($P = 0.678$). The splenic lymphocyte proliferation using mitogen ConA and PWM as well as plasma concentration of IgY and IgA was not affected by stocking density ($P > 0.05$).

Table 6. Short- (rearing period) and long-term (laying period) effects of stocking density during rearing on in vitro splenic lymphocyte proliferation using the mitogen ConcanavalinA and Pokeweed mitogen as well as plasma antibody and corticosterone concentration.[1]

	Rearing period		Laying period		*P* value	
Parameter	LSD[2]	HSD[3]	LSD[2]	HSD[3]	Stocking density	Stocking density × period
Lymphocyte proliferation (Δcpm)						
ConcanavalinA[4]	847.4 ± 148.5	765.7 ± 134.2	1142 ± 200.9	1099 ± 194.7	0.537	0.779
Pokeweed mitogen[4]	569.5 ± 102.5	476.8 ± 85.79	1130 ± 196.9	1128 ± 197.0	0.353	0.137
Antibody concentration						
IgY[4], mg/mL	13.46 ± 1.12	15.45 ± 1.29	12.28 ± 1.09	13.71 ± 1.22	0.140	0.757
IgM[4], μg/mL	558.4 ± 50.52^{b}	671.8 ± 64.15^{a}	816.3 ± 70.17^{ab}	791.2 ± 73.40^{ab}	0.289	0.004
IgA[4], μg/mL	500.9 ± 53.45	491 ± 56.72	455 ± 49.39	457 ± 55.48	0.914	0.846
Corticosterone concentration (pg/ml)[4]	221.9 ± 36.90	212.2 ± 34.80	692.9 ± 126.7	819.3 ± 150.6	0.695	0.366

^{a,b}Values in row with no common superscript letter are significantly different within the respective period ($P < 0.05$).
[1]Data are shown as LSmeans ± SEM based on n = 72 observations per treatment for antibody and corticotserone concentrations at the end of the rearing period and n = 35−36 during the laying period and based on 36 observations per treatment and period for lymphocyte proliferation.
[2]LSD, low stocking density during rearing (13 brids/m^2).
[3]HSD, high stocking density during rearing (23 birds/m^2).
[4]Data that required logarithmic transformation are reported on the original scale after back-transformation.

Effects of Stocking Density on Corticosterone Concentration in Plasma

LSmeans of CORT concentration (Table 6) did not differ between pullets reared at HSD or LSD at the end of the rearing period and in the laying period ($P > 0.05$).

Effects of Stocking Density on Behavior

Stocking density had a strong influence on pullets' behavior at the end of the rearing period (Table 7). The mixed linear model revealed a significant interaction between stocking density × period for locomotion ($P < 0.001$), resting ($P = 0.025$), preening ($P = 0.004$), and foraging ($P = 0.001$). Post-hoc testing showed less locomotion ($P < 0.001$), preening ($P < 0.001$), and foraging ($P < 0.001$) at the end of the rearing period in pullets reared at HSD compared to LSD. This effect was still evident for preening ($P = 0.044$) and foraging ($P < 0.001$) but not for locomotion ($P = 0.941$) in the laying period. Resting was shown to be similar at the end of the rearing period ($P = 0.705$) but different in the laying period, with hens reared at HSD showing less resting ($P < 0.001$). The stocking density main effect was significant for feeding ($P = 0.049$) and standing ($P < 0.001$), indicating short- and long-term effects of stocking density during rearing. Birds reared at HSD fed and stood more compared to birds reared at LSD at the end of rearing period and still during laying period. No effects on drinking and dustbathing were observed ($P > 0.05$).

Furthermore, differences in pecking behavior were observed (Figure 2). The generalized mixed model revealed no interaction of stocking density × period effects ($P > 0.05$). However, the significant effect of stocking density indicates short-term effects of stocking density during rearing that extend into the laying period. Birds reared at HSD showed more gentle feather pecking ($P < 0.001$), more aggressive pecking ($P < 0.001$) and more severe feather pecking ($P < 0.001$) than laying hens of the LSD group at the end of the rearing period and during laying period as well.

Table 7. Short- (rearing period) and long-term (laying period) effects of stocking density during rearing on behavior.[1]

	Rearing period		Laying period		*P* value	
Parameter	LSD[2]	HSD[3]	LSD[2]	HSD[3]	Stocking density	Stocking density × period
Locomotion[4], %	1.79 ± 0.31^{a}	0.37 ± 0.07^{b}	8.98 ± 1.26^{ab}	9.02 ± 1.26^{ab}	<0.0001	<0.0001
Resting[4], %	23.9 ± 1.45^{ab}	24.6 ± 1.48^{ab}	20.6 ± 0.97^{a}	16.5 ± 0.82^{b}	0.077	0.025
Standing[4], %	30.7 ± 2.34^{b}	46.6 ± 2.74^{a}	13.0 ± 1.25^{b}	22.5 ± 1.94^{a}	<0.0001	0.937
Preening[4], %	17.5 ± 1.91^{a}	9.75 ± 1.17^{b}	22.0 ± 1.94^{a}	18.1 ± 1.68^{b}	<0.0001	0.004
Foraging[4], %	12.2 ± 1.00^{a}	6.55 ± 0.58^{b}	15.6 ± 1.11^{a}	12.6 ± 0.92^{b}	<0.0001	0.001
Dustbathing[4], %	0.84 ± 0.46	0.44 ± 0.24	1.86 ± 1.00	1.71 ± 0.91	0.295	0.225
Feeding[4], %	7.99 ± 0.64^{b}	8.75 ± 0.69^{a}	11.7 ± 0.85^{b}	13.2 ± 0.94^{a}	0.049	0.683
Drinking[4], %	0.99 ± 0.18	1.00 ± 0.18	2.44 ± 0.32	3.37 ± 0.44	0.311	0.212

^{a,b}Values in row with no common superscript letter are significantly different within the respective period ($P < 0.05$).
[1]Data are shown as LSmeans ± SEM based on 6 observations per treatment and period.
[2]LSD, low stocking density during rearing (13 brids/m^2).
[3]HSD, high stocking density during rearing (23 birds/m^2).
[4]Data that required logarithic transformation are reported on the original scale after back-transformation.

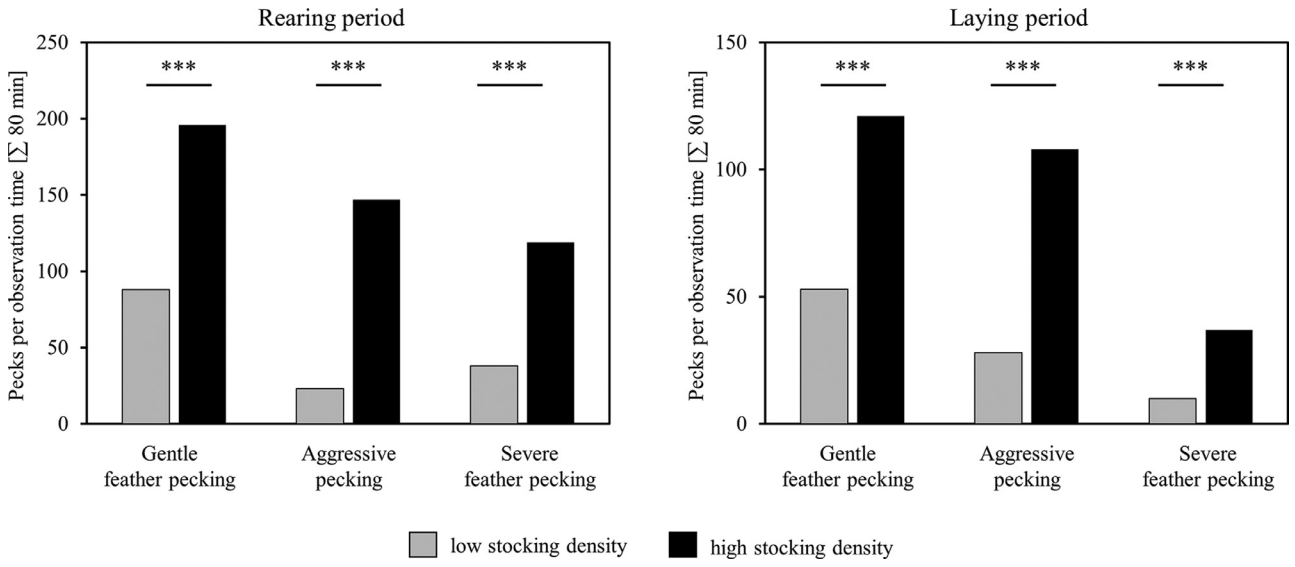

Figure 2. Short- (rearing period) and long-term (laying period) effects of stocking density (low stocking density: 13 bird/m^2, high stocking density: 23 birds/m^2) during rearing on the total count of gentle feather pecks, aggressive pecks and severe feather pecks during the observation time (on 4 consecutive days, 20 min each per pen, based on 6 observations per treatment and examined period). Asterisks indicate differences between stocking densities (*** $P < 0.001$).

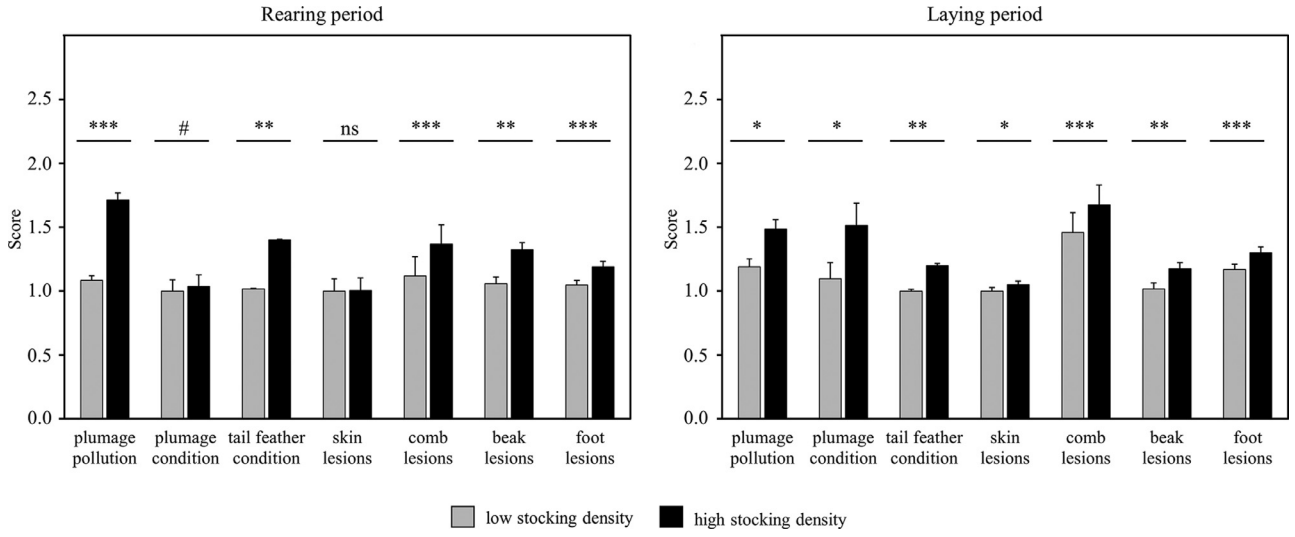

Figure 3. Short- (rearing period) and long-term (laying period) effects of stocking density (low stocking density: 13 bird/m^2, high stocking density: 23 birds/m^2) during rearing on the plumage and integument scores (high scores indicate worse condition). Data are presented as LSmeans + SEM based on 60 observations per treatment and period. Asterisks indicate significant differences between low and high stocking density: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, # $P < 0.10$, ns = not significant.

Effects of Stocking Density on Physical Appearance

The study showed differences in plumage and integument condition between pullets reared at HSD and LSD at the end of the rearing period and during the laying period (Figure 3).

The linear mixed model analyses showed significances for the interaction of stocking density × period effects for plumage pollution ($P = 0.033$), plumage condition ($P = 0.029$), skin lesions ($P = 0.053$) and beak lesions (0.047). Post-hoc testing revealed higher scores for plumage pollution ($P < 0.001$), plumage condition ($P = 0.081$) and beak lesions in the HSD group compared to the LSD group at the end of rearing period, and higher scores for plumage pollution ($P = 0.012$), plumage condition ($P = 0.026$), skin lesions ($P = 0.029$), and beak lesions ($P = 0.005$) in the laying period. The stocking density effect was significant for tail feather condition ($P = 0.002$), comb lesions ($P < 0.001$) and foot lesions ($P < 0.001$), indicating short- and long-term effects of stocking density. Pullets reared in HSD had higher scores, indicating worse condition, for tail feather condition, comb lesions, and foot lesions at the end of the rearing period and during the laying period.

DISCUSSION

We assessed short- and long-term effects of stocking density during rearing on health and welfare parameters in laying hens by combining immunological, endocrine and behavioral measurements. The results indicate that early-life experiences associated with HSD during rearing have long-term consequences that stretch well into the laying period.

Effects of Stocking Density on Number and Distribution of Immune Cells in Blood and Lymphatic Tissue

Stocking density during rearing had short- and long-term effects on the immune system of layer pullets. Leukocytes and their subsets are a main part of the immune system, and changes in number may reveal the overall state of health and the immune system's functional ability. We found that pullets reared at HSD had lower numbers of total lymphocytes, especially $\gamma\delta$ T cells, in blood, spleen, and cecal tonsils at the end of the rearing period. Our study was the first to show an influence of housing or stressors in general on $\gamma\delta$ T cells in chickens so far. Unlike humans and mice, and similar to other farm animals, $\gamma\delta$ T cells comprise a large subset of T lymphocytes in the circulation with a frequency of up to 50% in chickens (Holderness et al., 2013). $\gamma\delta$ T cells were shown to be involved during infection with salmonella (Berndt et al., 2006; Pieper et al., 2011), Marek's disease virus (Kano et al., 2009; Laursen et al., 2018), infectious bronchitis virus (Nii et al., 2015) and Eimeria (Choi and Lillehoj, 2000; Hong et al., 2006). These diseases are of vast economic importance in poultry production, which suggests that reduced numbers of those cells may increase susceptibility and the clinical course of those diseases. The reason for the lower numbers of $\gamma\delta$ T cells are not clear yet, although altered migration patterns (Dhabhar, 2002; Stefanski et al., 2003), apoptosis (Tarcic et al., 1998; Flint et al., 2005; Girardot et al., 2017; Zhang et al., 2008) or reduced production/release could certainly play a role. The reduced numbers of T lymphocytes in blood and spleen may mirror a redistribution of immune cells from the circulation into other organs, for example, the gastrointestinal tract or the skin. However, the simultaneously reduced number of $\gamma\delta$ T cells in blood, spleen and cecal tonsils points to a more systemic loss of $\gamma\delta$ T cells by apoptosis or reduced production/release. A very notable finding is that the number of $\gamma\delta$ T cells in blood and spleen in hens reared at HSD was still lower in the laying period. This indicates a long-lasting effect of rearing conditions in later life.

Lower lymphocyte numbers and higher H/L ratios in pullets reared at HSD could classically be interpreted as a regular result of stress-induced immunomodulation (Stefanski and Engler, 1998; Engler et al., 2004; Bozkurt et al., 2008; Matur et al., 2015; Kang et al., 2018; Schalk et al., 2018). Although our results agree with a previous study with layer pullets (Patterson and Siegel, 1998), the lack of an effect on heterophils appears harder to fit into this concept. Immunomodulatory sex steroids (Leitner et al., 1996; al-Afaleq and Homeida, 1998; Barua et al., 1998; Barua et al., 2000) may possibly prevent stress-induced heterophilia, as substantial sex-specific differences in the immunological stress response exist. For example, female rats − in contrast to male rats − do not show an increase in granulocytes due to social stressors (Stefanski and Grüner, 2006). This assumption could be fueled by the fact that the immune status of hens in the present study was assessed at a time when substantial changes in sex hormone concentrations take place (sexual maturity/starting to lay eggs) (Williams and Sharp, 1977; Elnagar et al., 2002; Biswas et al., 2010).

We did not find an effect of stocking density on basal plasma IgY and IgA concentrations. This is in line with Patterson and Siegel (1998) and Bozkurt et al. (2008), who did also not report any effect of stocking density on antibody concentration in layer pullets in response to vaccines or after sheep red blood cell immunization. We also did not find differences in lymphocyte proliferation in the present study, indicating that stocking density does not affect functionality of T and B lymphocytes in general. However, as avian $\gamma\delta$ T cells do not respond well to mitogens (Chen et al., 1994), conclusions on the functionality of this cell type cannot be drawn yet. Future investigations could use proliferation assays on a single cell level to check whether particular T lymphocyte subsets are inhibited in proliferative capacity.

Effects of Stocking Density on Corticosterone Concentration

In contrast to some other studies with adult laying hens (Mashaly et al., 1984; Cheng et al., 2003; Onbasilar and Aksoy, 2005; Mirfendereski and Jahanian, 2015; Kang et al., 2016; Kang et al., 2018), we did not find higher blood CORT concentrations in our study with pullets reared at HSD compared to LSD. One should, however, not necessarily conclude that the HPA-axis was not affected by rearing pullets at HSD. First, plasma glucocorticoid levels in the present study represent snapshots of blood CORT concentrations rather than representing glucocorticoid release over time. It is also possible that corticosteroid-binding globulin was lower in birds of the HSD group, resulting in higher levels of free, biologically active CORT without altering total plasma CORT. This effect has been observed in a variety of species before (Stefanski, 2000; Breuner and Orchinik, 2002). It should also be considered that hens may have become accustomed to the limited space available to them over time. Repeated exposure to the same stressor can cause a decrease in the responsiveness of the HPA-axis to stress (Pitman et al., 1988; Pignatelli et al., 2000), possibly due to alterations in the negative-feedback inhibition (Jaferi et al., 2003). Moreover, some studies suggest that early-life stress alters responsiveness rather than basal HPA-activity

(Love and Williams, 2008), and thus differences in glucocorticoid concentrations become only evident if exposed to acute stress (Hayward et al., 2006; Marasco et al., 2012).

Effects of Stocking Density on Behavior

HSD hens showed less active behavior such as locomotion, foraging and preening, and a higher frequency of pecking behavior as pullets and still later as laying hens. Restricted behavior and high frequencies of feather pecking are associated with reduced welfare (Bracke and Hopster, 2006; Rodenburg et al., 2013). On the other hand, higher intensity, duration and incidence of foraging behavior indicate better poultry welfare and might prevent incidences of feather pecking (Bracke and Hopster, 2006). It is very likely that the decrease in activity behavior is related to physical confinement. However, factors that are only indirectly related to stocking density may also play a role, including litter quality and temperature. HSD conditions are detrimental to litter quality and increase litter temperature, both factors which were shown to result in inhibited scratching and running activity (Blokhuis and van der Haar, 1990). Blokhuis and van der Haar (1989) suggest that feather pecking is redirected ground pecking, and that experiences in early life regarding the validation of the ground as an incentive for pecking, may exert a significant influence in pecking preferences in later life (Blokhuis and van der Haar, 1989). In a HSD situation, conspecifics will display a higher proportion of stimuli relative to e.g. the litter area compared to a lower density situation. Redirection of ground pecking happens when the incentive value of the ground is low, compared with the incentive value of other peckable substrates, like e.g. conspecifics (Hansen and Braastad, 1994). The animals' earlier experience with a material is of importance for its incentive value (Blokhuis and van der Haar, 1989), which might explain why hens still showed more pecking behavior during the laying period although stocking density was identical at that time. In addition, we can assume that the mechanisms of social transmission play a more prominent role in HSD with more conspecific coping feather pecking than in LSD (Bestman et al., 2009). Our study is in line with other studies (Hansen and Braastad, 1994; Huber-Eicher and Audige, 1999; Bestman et al., 2009; Zepp et al., 2018) confirming the view that early-life experiences modify and strengthen behavioral patterns, perceptions and preferences throughout life.

Effects of Stocking Density on Physical Appearance

Our study showed worse plumage and integument conditions at the end of the rearing period and later in the laying period in birds of the HSD group compared to the LSD group. Plumage and integument conditions are regarded as an important indicator of animal health and behavior (Campe et al., 2018). Our results are similar to another study by Hansen and Braastad, where birds also showed better plumage conditions at LSD during the rearing period and well into in laying period (Hansen and Braastad, 1994). The main reason for physical damage is believed to be feather pecking (Campe et al., 2018). The removal of feathers is painful and therefore a worse plumage condition may reflect poor welfare (Gentle and Hunter, 1991). Indeed, plumage conditions worsen with an increasing rate of severe feather pecking (Zepp et al., 2018), which has also been observed in our study. Feather damage during the laying period is reported to be less when feather pecking has not started during rearing (Bestman et al., 2009; Gilani et al., 2013; Haas et al., 2014). This is further supported by the observation that increased feather damage at the end of the rearing period is associated with an earlier onset of severe feather damage during the laying period (Drake et al., 2010).

Long-Term Effects of Early Environment

Besides short-term effects of the rearing environment, we also found long-lasting differences in immune and welfare parameters that persisted well into the laying period − a time period stretching to at least 11 wk after the experimental period. It is well-documented that pre- and postnatal environmental and management conditions have short-term but also long-term effects on behavior, mortality and physical development in chickens (reviewed in Janczak and Riber, 2015; Dixon et al., 2016; Campbell et al., 2019). However, detailed investigations of acute and persisting effects of the rearing period on the immune system in chickens are missing, although we already know that early-life programming of the immune system via nutrition and intestinal microbiota modulation is possible (reviewed in Taha-Abdelaziz et al., 2018; Rubio, 2019).

But why and how has the early-life environment the potential to affect the later life? During the early-life period, the organism is most sensitive to environmental conditions due to the high plasticity of the brain (Rodenburg and de Haas, 2016). Through experiences during early development, the brain and the immune system adapt to stimuli that are specific to the individual's unique environment (Danese and Lewis, 2017). In many cases, the impact of stressful early-life experiences on the later life is thought to be mediated via modifications to the functioning of the HPA-axis (Moisiadis and Matthews, 2014). Glucocorticoids are necessary for normal brain development, but exposure to ongoing excessive levels is detrimental and results in a dysregulation of negative feedback control of glucocorticoid secretion and can alter sensitivity to stressful stimuli (Maccari et al., 2003). There is increasing evidence that long-term effects of stressful challenges are at least partly a consequence of reprogramming the gene expression profile in the brain and other central organs, mediated by epigenetic modifications (Jensen, 2014). Studies in humans and rodents (Moisiadis and Matthews, 2014)

but also in birds (Ahmed et al., 2014; Zimmer and Spencer, 2014) have demonstrated that early-life stress leaves epigenetic marks on the DNA that have been linked to long-term changes in gene expression.

However, whether epigenetic modification in stress and immune systems also play a role in the present study needs to be evaluated in future experiments.

Based on the results of the present study, a stocking density of 13 chicks/m^2 would be preferable compared to a stocking density of 23 chicks/m^2. Similar results were also obtained by other authors who suggested appropriate stocking densities of 10 to 15 chicks/m^2 and 11 to 14 chicks/m^2 during rearing, respectively (Spindler et al., 2013; Krause and Schrader, 2019). However, it should be kept in mind that the present study design neither exactly mimics housing conditions and social environments of hens in conventional housing settings, nor alternative housing systems where birds are kept in much larger groups which might result in different social behavior and organization of chickens (Pagel and Dawkins, 1997; Keeling et al., 2003). Nevertheless, the results of the experimentally controlled setting of the present study clearly show that stocking density during rearing modulates the immune system and impairs welfare of laying hens both in the short- and in long-term respect. This aspect should be considered in future studies with the aim to optimize guidelines for the housing of hens.

Conclusion

In summary, the results of this study suggest that HSD during rearing are challenging for the immune system and the welfare of laying hens. The lower numbers of lymphocytes, especially $\gamma\delta$ T cells, as well as the higher H/L ratio suggest a stress-induced immunomodulation with possible consequences for the birds' health. The higher incidence of severe feather pecking and poorer plumage and integument condition in the HSD group indicates impaired welfare. Moreover, we demonstrated for the first time that these stress-related alterations in immune cell numbers manifest in the laying period and therefore highlight the significance of early-life conditions for the immune competence throughout the whole production cycle. Our results can serve as a basis for future recommendations on implementing optimized housing environments and management strategies into commercial farming environments.

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DISCLOSURES

The authors declare that there is no conflict of interest.

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