

Immune parameters in two different laying hen strains during five production periods

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ABSTRACT During life, the number and function of immune cells change with potential consequences for immunocompetence of an organism. In laying hens, studies have primarily focused on early development of immune competence and only few have investigated systemic and lymphatic distribution of leukocyte subsets during adolescence and the egg-laying period. The present study determined the number of various leukocyte types in blood, spleen, and cecal tonsils of 10 Lohmann Brown-Classic and 10 Lohmann LSL-Classic hens per wk of life 9/10, 15/16, 23/24, 29/30, and 59/60, encompassing important production as well as developmental stages, by flow cytometry. Although immune traits differed between the 2 hen strains, identical patterns of age-related immunological changes were found. The numbers of all investigated lymphocyte types in the spleen as well as the numbers of blood $\gamma\delta$ T cells increased from wk 9/10 to 15/16. This suggests an

ongoing release of lymphocytes from primary lymphoid tissues and an influx of blood lymphocytes into the spleen due to novel pathogen encounters during adolescence. A strong decrease in the number of CTL and $\gamma\delta$ T cells and an increase in innate immune cells within blood and spleen were found between wk of life 15/16 and 23/24, covering the transition phase to egg-laying activity. Numbers of peripheral and splenic lymphocytes remained low during the egg-laying period or even further decreased, for example blood CD4⁺ T cells and splenic $\gamma\delta$ T cells. Functional assessments showed that in vitro IFN- γ production of mitogen-stimulated splenocytes was lower in wk 60. Taken together, egg-laying activity seems to alter the immune system toward a more pronounced humoral and innate immune response, with probable consequences for the immunocompetence and thus for productivity, health and welfare of the hens.

Key words: laying hen, immune system, lymphoid tissue, productive life span, age

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INTRODUCTION

Changes in number and function of immune cells within blood, lymphatic, and other tissues throughout life are described for many vertebrate species including birds (Hausmann et al., 2005; Lavoie, 2005; Lavoie et al., 2007; Koutsos and Klasing, 2014; Simon et al., 2015; Vermeulen et al., 2017; Alkie et al., 2019). The most conspicuous changes are represented by the ontogeny of the immune system during fetal development and childhood, resulting in a fully competent immune system (Dowling and Levy, 2014; Simon et al., 2015), as well as by the so-called immunosenescence leading to higher

disease susceptibility in aging individuals (Torroba and Zapata, 2003; Lavoie, 2005; Dowling and Levy, 2014; Simon et al., 2015). Moreover, age-related immunological changes potentially affecting the immunocompetence of an organism are associated with growth, sexual maturation, reproductive activity, nutrition, or seasonal alterations (Lamason et al., 2006; O'Neal and Ketterson, 2011; Koutsos and Klasing, 2014; Cheng et al., 2017; Hofmann et al., 2020). These factors also play a significant role during the productive life span of laying hens under conventional housing conditions.

In chickens, the majority of studies have focused on the early development of immune competence until wk 8 of life, an age at which chickens are defined as immunologically mature (Moticka, 1975; Peleg et al., 1985; Lowenthal et al., 1994; Bar-Shira and Friedman, 2006; van Haarlem et al., 2009; Lammers et al., 2010; Karpala et al., 2012; van Ginkel et al., 2015; Alkie et al., 2019; Schmiedeke et al., 2019; Zhang et al., 2019). Very few studies have investigated immune parameters during adolescence and laying activity (McCorkle et al., 1979;

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McCorkle and Glick, 1980; Traill et al., 1984), although laying hens are not fully grown at an age of 8 wk and, upon reaching sexual maturity, undergo a prolonged and extensive reproductive phase (Bain et al., 2016). Studies in the field of ecoimmunology reveal immunosuppression during breeding in free-living birds (ONeil and Ketterson, 2011). Likewise, an increased susceptibility to salmonellosis at the onset of laying activity was associated with remarkable changes in the proportion of immune cells and a decreased cellular immune response in chickens (Wigley et al., 2005; Johnston et al., 2012). Moreover, a recent study in turkeys showed that the proportion of lymphocytes further decreased during late laying periods (Kowalczyk et al., 2020).

Thus, there are indications that immunological changes occur during the ontogeny of laying hens especially at the onset of sexual maturity and during laying activity, but also during adolescence. These alterations in the immune system might influence the hens' immunocompetence and health with possible negative consequences for vaccination success, disease susceptibility, and need of antibiotic treatment. Animal health is not only a prerequisite for welfare, but also for high productivity and safe products, and thus for sustainable egg production. However, detailed studies on the absolute number of leukocytes and especially on their different subtypes throughout the whole production period are lacking so far.

Therefore, the present study aimed at characterizing the number and distribution of various leukocyte types in peripheral and lymphoid tissues as well as the function of lymphocytes in high-yielding laying hens during adolescence, sexual maturation and laying activity, encompassing important stages of the production cycle. Focus was put on the investigation of the systemic immune system by analysis of blood as well as lymphatic immune cell subsets in spleen and the gut-associated cecal tonsils (CT), representing important secondary lymphoid tissues in chickens (John, 1994; Casteleyn et al., 2010; Oláh et al., 2014). As strain effects on baseline immune parameters but also on immune functions are well-known (Kjærup et al., 2017; Dobner et al., 2019; Hofmann et al., 2021), all investigations were conducted using the 2 genetically distinct and high-yielding laying hen strains Lohmann Brown-Classic (LB) and Lohman LSL-Classic (LSL).

MATERIAL AND METHODS

Birds and Housing

All procedures were conducted according to the current ethical and animal care guidelines and approved by the local authority's Animal Ethics Committee (Regional Council Tübingen, approval number HOH50/17TE). A total of 50 LB and 50 LSL hens (both Lohmann Tierzucht GmbH, Cuxhaven, Germany), representing 2 distinct genetic backgrounds but equal egg-laying performance (Habig et al., 2012), from 10 nonrelated roosters per strain (thus, 5 hens per rooster per strain) were used for the present study. Hens were

obtained as hatchlings from the breeding company and kept at the farm animal research center of the University of Hohenheim (Agricultural Experimental Station, Unterer Lindenhof, Eningen, Germany) as a large flock in one group in floor pens on deep litter bedding. All animals were vaccinated against Marek's disease (day of hatching), coccidiosis (d of life 7), salmonellosis (d of life 2, wk of life 7 and 14), infectious bronchitis (day of hatching, wk of life 2, 4, 9, and 16), Newcastle disease (wk of life 3, 10, and 15), and infectious bursitis (wk of life 3). Immunological parameters were assessed at 5 periods within the hen's life, encompassing important production as well as developmental stages: During adolescence and before the onset of laying activity (wk of life 9/10 and 15/16), during early laying activity (wk of life 23/24), at peak laying activity (wk of life 29/30), and during continuing laying activity thereafter (wk of life 59/60).

Therefore, 10 hens per strain were randomly chosen with the restriction of 1 hen per rooster per period of life for assessment of immune parameters in blood, spleen, and CT as explained below. Thus, hens descending from the same 10 roosters were represented at every investigated period of life to ensure that a probable rooster effect was identical at every analyzed point in time (Figure 1). In order to exclude an influence of the slaughter process on blood immune cell numbers, blood was taken by *vena ulnaris* puncture 1 wk prior to slaughter. The temporal separation of both sampling techniques also avoided potential handling effects of the blood puncture on measurements conducted at the time point of slaughter.

As the present study was part of a larger experiment in which metabolic investigations were also conducted (Sommerfeld et al., 2020a), hens chosen for either period of life were placed individually in metabolism units

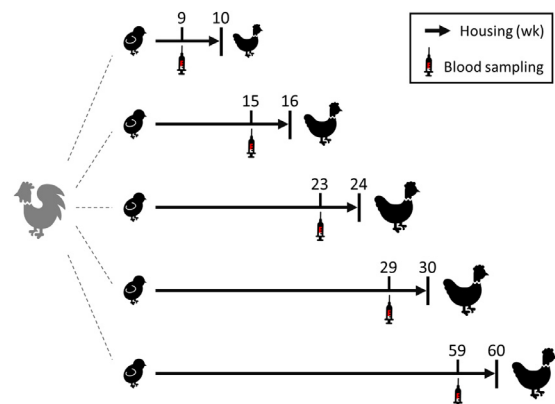


Figure 1. Study design. A total of 50 Lohmann Brown-Classic (LB) and 50 Lohmann LSL-Classic (LSL) hens from 10 nonrelated roosters per strain (= 5 hens per rooster) were used for the present study. Immunological parameters were assessed during adolescence and before onset of laying activity, during early laying activity, at peak laying activity and at continuing laying activity thereafter, encompassing important production as well as developmental stages within the hen's life. Therefore, 10 hens per strain with 1 hen per rooster were randomly chosen per production period for tissue sampling after slaughter at wk of life 10, 16, 24, 30, or 60 and preceding *vena ulnaris* puncture at wk of life 9, 15, 23, 29, or 59 respectively.

(1 m × 1 m × 1 m) in wk 8, 14, 22, 28, and 58. Within these metabolism units, hens were provided with a wooden perch, a nest, a feeding trough, and water cups. Throughout each production period all hens were fed the same diets mixed in the certified feed mill of the Agricultural Experimental Station of the University of Hohenheim. All diets were based on corn-soybean meal and adjusted to nutritional requirements corresponding to the respective production periods. Composition of diets conformed to commercial grower/developer (wk of life 8–16), prelayer (wk of life 17–18), and layer feed (wk of life 18 to end), meeting or exceeding breeder recommendations. For detailed diet composition and feed analysis see Sommerfeld et al. (2020a). Feed and water were provided ad libitum. All hens were housed under the same light conditions, with 9 h of light from wk 9 to wk 16, subsequently gradually extended to reach 16 h of light in wk 23, which was maintained until the end of the experiment. Barn temperature was kept at 18°C to 22°C during all sampling periods and hens regularly underwent health checks. None of the hens died before the end of the experiments. One LSL hen was excluded from immunological analyses at wk of life 60 due to illness. During the single housing of hens in metabolic cages, the numbers of laid eggs per hen were counted on 9 consecutive days. Mean number (\pm SD) of laid eggs per 9 d for LB hens was 8.6 ± 0.6 at wk of life 22 to 24, 8.5 ± 0.9 at wk of life 28 to 30, and 8.4 ± 1.0 at wk of life 58 to 60, and for LSL hens 8.5 ± 0.7 at wk of life 22 to 24, 8.5 ± 0.8 at wk of life 28 to 30, and 7.8 ± 1.9 at wk of life 28 to 30.

Blood Sampling by Vena Ulnaris Puncture

For the analysis of leukocyte numbers in the blood, blood was taken from the hens by *vena ulnaris* puncture at wk 9, 15, 23, 29, or 59 (Figure 1). Blood samples were collected in 5 mg/mL EDTA (Sigma Aldrich, St. Louis, MO) within 3 min of the hen being removed from the pen. Half of the sample was fixed by the addition of TransFix reagent (#TFB-20-1; Caltag Medsystems Ltd., Buckingham, UK) according to manufacturer's instructions. Stabilized blood samples were kept at room temperature until further processing for flow cytometric analysis within 4 h after blood collection. The rest of the blood sample was used unfixed to obtain plasma by centrifuging for 15 min at $2000 \times g$ and 4°C. Plasma was stored at -20°C until analysis of corticosterone concentration (see below).

Slaughter of Hens and Sampling of Trunk Blood, Spleen, and Cecal Tonsils

In wk 10, 16, 24, 30, and 60 the hens were individually stunned with a gas mixture of 35% CO₂, 35% N₂, and 30% O₂ and killed by decapitation. Trunk blood was collected in K3 EDTA tubes (Sarstedt, Nürnbrecht, Germany) and plasma for immunoglobulin analyses was obtained at once by centrifugation for 10 min at $2,500 \times g$

and stored at -80°C until further processing. Immediately after slaughter, spleen, and CT were dissected and stored in ice-cold PBS + 1% Fetal Bovine Serum (FBS) + 0.05 mg/mL Gentamycin (Biochrom, Berlin, Germany) until further processing in the laboratory.

Tissue Sample Preparation

In order to separate immune cells from spleen and CT, lymphatic tissues were prepared as described in Hofmann and Schmucker (2021). In brief, one randomly chosen CT per hen was weighed and cut into pieces. Intraepithelial lymphocytes (IEL) were disrupted from the mucosa by shaking the CT twice in Hanks' Balanced Salt solution (without Mg²⁺ and Ca²⁺) + 5 mM EDTA and 5% FBS, supplemented by a mild detergent (1mM Dithiothreitol; DTT), under slow rotations for 20 min. Remaining tissue pieces were separated from obtained IEL by the use of 40 μm cell filters (MACS SmartStrainer, Miltenyi Biotec, Bergisch Gladbach, Germany). IEL were maintained in defined volumes of PBS + 1% FBS and stored on ice until further processing. The whole spleen was weighed and cut into pieces, which were homogenized using a gentleMACS Dissociator (Miltenyi Biotec) and gentleMACS C-Tubes (#130-096-334, Miltenyi Biotec). Homogenized spleens were passed through 40 μm cell filters (MACS SmartStrainer, Miltenyi Biotec) to clear cells from connective tissue. Flow-through was resuspended in PBS + 1% FBS after centrifugation for 10 min at $300 \times g$ and the final volume was determined. One sample of spleen cell suspensions per hen was stored on ice until further processing for immune cell numbers by flow cytometry. In order to obtain mononuclear splenic leukocytes for functional analyses, the remaining spleen cell suspension was further processed by gradient centrifugation to separate mononuclear leukocytes from erythrocytes and thrombocytes as described in Hofmann et al. (2021). The cell suspension was carefully layered onto a gradient (1.077 g/mL Biocoll separation solution, Biochrom, Berlin, Germany). After centrifugation for 12 min at $600 \times g$ at 20°C, the interphase was collected, washed in PBS + 1% FBS and resuspended in RPMI 1640 + 10% FBS. Cell numbers of mononuclear splenic leukocytes were determined using a Z2 Coulter Counter (Beckman Coulter, Krefeld, Germany). Isolated mononuclear splenic leukocytes were immediately processed in *in vitro* assays as described below.

Characterization and Enumeration of Leukocyte Subsets in Blood, Spleen, and IEL from CT

Leukocyte subsets in blood, spleen, and within IEL of CT were characterized and enumerated by flow cytometric analysis via a no-lyse-no-wash method described in detail in Seliger et al. (2012) and Hofmann and Schmucker (2021). For all analyses, the following directly labeled antibody-fluorochrome conjugates were

used: Anti-CD45-APC (# 8270-11, clone LT40, SouthernBiotech, Birmingham, AL), anti-Monocyte/Macrophage-PE (# 8420-09, clone Kul01, SouthernBiotech), anti-CD4-PACBLU or -PE (# 8210-26, # 8210-09, CT-4, SouthernBiotech), anti-CD8 α -FITC (# 8220-02, clone CT-8, SouthernBiotech), anti-Bu-1-FITC (# 8395-02, clone AV20, SouthernBiotech), anti-CD41/61-PE (# MCA2240GA, clone 11C3, BioRad, Hercules, CA), and anti-TCR $\gamma\delta$ -PerCP (# NBP1-28275PCP, clone TCR1, Novus Biologicals, Littleton, CO). Mouse IgM specific for lipopolysaccharide conjugated to APC (# 0101-11, clone 11E10, SouthernBiotech) was used as isotype control excluding nonspecific binding of anti-CD45-APC. Acquisition and analyses were performed on a BD FACSCanto II (BD Biosciences, Heidelberg, Germany) equipped with a 488 nm, 630 nm, and 405 nm laser using BD FACSDiva Software (BD Biosciences). Various immune cell types in blood and lymphatic tissues were characterized and gated as described in Hofmann and Schmucker (2021). In blood, immune cell types were distinguished in one staining step by the following surface marker expressions: total leukocytes (CD45⁺), thrombocytes (CD45^{dim}/CD41/61⁺), monocytes (CD45⁺/Kul01⁺), B cells (CD45⁺/Kul01⁻/Bu-1⁺), $\gamma\delta$ T cells (CD45⁺/Kul01⁻/CD4⁻/TCR $\gamma\delta$ ⁺/CD8 α ⁺ or CD8 α ⁻), CD4⁺ T cells (CD45⁺/CD4⁺/TCR $\gamma\delta$ ⁻/CD8 α ⁺ or CD8 α ⁻) comprising mostly T helper (TH) cells but probably also a proportion of regulatory T cells (Shanmugasundaram and Selvaraj, 2011), and cytotoxic T cells (CTL; CD45⁺/CD4⁻/TCR $\gamma\delta$ ⁻/CD8 α ⁺). The latter marker combination does not exclusively distinguish CTL from natural killer (NK) cells, which are present with only <1% in blood and spleen, but might include a proportion of up to 30% within IEL of CT (Göbel et al., 2001). To note, chickens show the occurrence of extrathymic CD4⁺CD8 α ⁺ lymphocytes (Luhtala, 1998), which were analyzed by the simultaneous use of anti-CD4 and anti-CD8 α antibodies. Similarly, varying proportions of CD8 α ⁻ and CD8 α ⁺ subsets are found among $\gamma\delta$ T cells (Luhtala, 1998), probably reflecting differences in their activation status (Pieper et al., 2008). Heterophils were identified based on their FSC/SSC characteristics.

Single-cell suspensions of splenocytes and IEL from CT were stained with respective antibody mixtures according to a no-lyse no-wash single-step two-tube protocol as described in Hofmann and Schmucker (2021). By doing so, total leukocytes (CD45⁺), B cells, thrombocytes (characterization in spleen only), monocytes/macrophages (characterization in spleen only), CD8 α ⁻ and CD8 α ⁺ $\gamma\delta$ T cells, CD8 α ⁻ and CD8 α ⁺ CD4⁺ T cells as well as CTL in spleen or the fraction of CTL and NK cells in CT were characterized according to the above-named marker combinations. For antibody labeling, 50 μ L of 1:50 prediluted stabilized EDTA-whole blood or 50 μ L of single-cell suspensions of splenocytes or IEL from CT were incubated with respective antibody mixtures (Hofmann and Schmucker, 2021) in a total volume of 70 μ L for 45 min at room temperature. After addition of 400 μ L PBS supplemented by 2% BSA, 0.1% NaN₃,

and 5 mg/mL EDTA, labeled cells were stored at 6°C until analysis. SYTOX Blue Dead Cell Stain (# S34857, Invitrogen, Thermo Fisher Scientific, Waltham, MA) was used for the exclusion of dead cells from splenocytes and IEL from CT with an incubation time of 10 min. For flow cytometric determination of leukocyte subsets, at least 10,000 CD45⁺ cell events per sample were analyzed for whole blood and 50,000 CD45⁺ cell events per sample for CT and splenocytes. Absolute numbers of leukocytes per μ L blood or g tissue were determined by labeling cells with antibodies directly in BD Trucount tubes (# 340334, BD Biosciences) according to the manufacturer's instructions. For the calculation of cell counts, the number of acquired cell events was divided by the number of acquired bead events and afterward multiplied by the given bead concentration, divided by the sample volume per tube, and recalculated based on sample dilution. By combining determined cell frequencies with total leukocyte counts, absolute cell numbers of particular leukocyte subsets were calculated. Total numbers of immune cells per spleen and CT were calculated by multiplying immune cell counts per g of tissue with the determined weight of the particular tissues.

Measurement of In Vitro Proliferative Capacity of Splenocytes

The activity of splenic lymphocytes was examined in vitro using a mitogen-induced lymphocyte proliferation assay as described in Hofmann et al. (2021). In detail, 1.5 \times 10⁵ cells per well of 96-well round bottom cell culture plates (Neolab, Heidelberg, Germany) were incubated with or without 10 μ g/mL of either concanavalin A (ConA, Sigma Aldrich, Munich, Germany) or pokeweed mitogen (PWM, Sigma Aldrich) in triplicates at 41°C and 5% CO₂. For detection of DNA-synthesis, 0.25 μ Ci ³H-thymidine (PerkinElmer, Rodgau, Germany) per well was added after 46 h and cells were further incubated for 24 h. Subsequently, cells were harvested on glass fiber filters (Skatron, Lier, Norway) and radioactivity was evaluated by a liquid scintillation analyzer (PerkinElmer). Mean of cpm was calculated for each triplicate and the delta cpm for ConA- and PWM-stimulated splenocytes generated (= mean cpm of stimulated cells – mean cpm of unstimulated cells). Frozen splenocytes from a separate chicken were used as biocontrol for all investigated periods of life. Delta cpm for ConA- and PWM-stimulated splenocytes across all timepoints were normalized based on this biocontrol.

Measurement of In Vitro IFN- γ Production of Splenocytes

The capacity of IFN- γ production by mononuclear splenocytes was assessed by mitogenic stimulation of 5 \times 10⁵ mononuclear splenocytes per well of a 96-well flat bottom in duplicate as described above. After 22 h of incubation at 41°C and 5% CO₂, cell culture supernatant was collected after centrifugation of 96-well plates

at $300 \times g$ for 5 min. The supernatant was frozen at -20°C until further processing for ELISA analysis. For the determination of IFN- γ amount, duplicates of cell culture supernatants were analyzed using a commercially available IFN- γ ELISA-Kit (CytoSet ELISA kit, # CAC1233; Invitrogen, ThermoFisher Scientific) according to manufacturer's recommendations. The cell culture supernatant was diluted 1:10 to 1:40 for ConA-stimulated samples, 1:10 to 1:20 for PWM-stimulated samples, and 1:10 for unstimulated samples. The dilution factor was chosen based on resulting OD in order to meet the linear range of the standard curve. Color formation by horseradish-peroxidase (HRP) mediated conversion of Tetramethylbenzidine (TMB; AppliChem, Darmstadt, Germany) was stopped after 30 min with $2\text{M H}_2\text{SO}_4$ (Roth, Karlsruhe, Germany) and absorbance was measured at 450 nm at a Power Wave X plate reader (Bio-Tek Instruments, Bad Friedrichshall, Germany). Mean of IFN- γ concentration was calculated for each duplicate and delta IFN- γ concentration for ConA- and PWM-stimulated splenocytes generated (= mean IFN- γ concentration of stimulated cells – mean IFN- γ concentration of unstimulated cells). Intra-assay variance of ELISA was 4.7% and interassay variance 11%. A pooled cell culture supernatant of stimulated splenocytes was used as biocontrol on all ELISA plates. Delta IFN- γ concentrations for ConA- and PWM-stimulated splenocytes across all plates were normalized based on this biocontrol.

Determination of Plasma IgY, IgM, and IgA Concentration

The concentration of IgY, IgM, and IgA in trunk blood plasma was determined by ELISA as described in Hofmann et al. (2021). In brief, 96-well ELISA plates (Thermo Scientific, Roskilde, the Netherlands) were coated with 20 ng/well of either goat anti-chicken IgY Fc antibody (# A30-104-A; Bethyl Laboratories, Montgomery, TX), goat anti-chicken IgM antibody (# A30-102-A; Bethyl Laboratories) or goat anti-chicken IgA antibody (# A30-103-A; Bethyl Laboratories) as capture antibodies. After blocking wells with 1% BSA (Roth), 100 μL of diluted plasma samples (1:500,000 for analysis of IgY; 1:5,000 for IgM in life period wk 12, 1:10,000 for analysis of IgM in life period wk 16, 1:20,000 for IgM in life period wk 24, 30 and 60; 1:2,000 for analysis of IgA) were applied in triplicates to the antibody-coated plates. For the detection of captured antibodies, 1:100,000 of HRP-conjugated goat antichick IgY Fc antibody (# A30-104-P; Bethyl Laboratories), goat antichick IgM antibody (# A30-102-P; Bethyl Laboratories) or goat antichick IgA antibody (# A30-103-P; Bethyl Laboratories) were used respectively. TMB (AppliChem) was added to the wells and color formation was stopped after 20 min with $2\text{M H}_2\text{SO}_4$ (Roth). Absorbance was measured at 450 nm with a Power Wave X plate reader (Bio-Tek Instruments). The concentration of either Ig was calculated relative to the absorbance of

a calibration curve derived from a pooled plasma sample with known concentrations of chicken IgY, IgM, and IgA. The concentration of respective Ig within this plasma sample was determined in advance with chicken IgG ELISA Kit (# E33-104, Bethyl Laboratories), chicken IgM ELISA Kit (# E33-102, Bethyl Laboratories), and chicken IgA ELISA Kit (# E33-103, Bethyl Laboratories). CV of intra- and interassay was 4.3% and 6.0% for IgY, 6.3% and 2.3% for IgM, and 8.6% and 3.8% for IgA, respectively.

Determination of Plasma Corticosterone Concentration

Variations in distribution and function of immune cells are associated with a complex interplay of multiple endocrine signaling molecules, of which the glucocorticoid corticosterone seems to have key functions with regard to environmental sensitivity and immunomodulatory capacity (O'Neal and Ketterson, 2011; Koutsos and Klasing, 2014). Thus, corticosterone concentration in blood plasma obtained by *vena ulnaris* puncture was determined by RIA after extraction with ethyl acetate. Plasma was diluted 1:3 in phosphate buffer and added to ethyl acetate (AppliChem). After freezing the aqueous phase, nonfrozen solvent supernatant was collected and subsequently evaporated under air in a vacuum dryer for 30 min at 55°C to dryness. Extracts were resuspended in phosphate buffer and analyzed in the RIA in duplicate. Losses of plasma corticosterone by extraction were determined by ^3H -Corticosterone-spiked samples ([1,2,6,7- ^3H (N)]-corticosterone; 89.8 Ci/mmol, PerkinElmer, Boston, MA; 10,000 cpm/sample). A polyclonal anticorticosteron-3-cmo-urease antibody (# AB1297, Merck Millipore, Billerica, MA) for binding of corticosterone and 10,000 cpm of ^3H -Corticosterone (PerkinElmer) as tracer was added to each sample. Separation of bound/free was performed with dextran-coated charcoal (0.05% Dextran70, Roth; 0.5% NoritA, Serva Electrophoresis, Heidelberg, Germany) by centrifugation for 20 min at $2,000 \times g$ at 4°C . Supernatants were transferred to 5 mL Ultima-Gold (PerkinElmer) to determine radioactivity (Tri-Carb 2800 TR, PerkinElmer). A standard curve covering a range between 0.01 ng and 1 ng corticosterone was prepared in phosphate buffer per test. Precision was determined by the spiking of plasma with low endogenous corticosterone concentrations with 0.5 and 1 ng corticosterone/mL. The mean recovery rate ranged between 79.14% and 103.17% for samples spiked with 0.5 ng/mL and 1 ng/mL, respectively. Cross-reactivity of the polyclonal antibody was 0.67% to 11-dehydrocorticosterone, 1.5% to deoxycorticosterone, <0.01% to 18-OH-DOC, <0.01% to cortisone, <0.01% to cortisol, and 0.2% to aldosterone. Interassay variabilities ranged between 9.3%, 6.6% and 14.7% for samples with endogenous corticosterone of 2.96 ng/mL, 1.24 ng/mL, and 0.55 ng/mL, respectively. Intra-assay variability for a biological sample with endogenous corticosterone concentration of 1.59 ng/mL was 2.42 %, and for a

biological sample with endogenous corticosterone concentration of 0.85 ng/mL 5.97 %.

Statistical Analysis

Statistical data analyses were performed with SAS Version 9.4 (SAS Institute Inc., Cary, NC), using a linear mixed model with the PROC MIXED procedure after testing residuals for normal distribution and homogeneous error variance via the graphical check of residual plots (Kozak and Piepho, 2018). Logarithmic transformations were used to stabilize variance and to meet the distribution assumption, if model assumptions were not fulfilled and results were back-transformed for presentation only. Results are presented as the LSmeans and SEM of the untransformed or retransformed data. Where needed, SEM was back-transformed using the Delta method. The individual hen was considered as the experimental unit. The following model was used:

$$Y_{ijklm} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + (\gamma\beta)_{kj} + \delta_l + \phi_m + \varepsilon_{ijklm},$$

where Y_{ijklm} = response variable, μ = overall mean, α_i = effect of strain (fixed), β_j = effect of wk of life (phrase shortened to wk within statistical analyses and display of results hereafter; fixed), $(\alpha\beta)_{ij}$ = the interaction between strain and wk (fixed), γ_k = block (random), $(\gamma\beta)_{kj}$ = the interaction between block and wk (random), δ_l = metabolism unit (random), ϕ_m = father/rooster (random), and ε_{ijklm} = residual error. Degrees of freedom were determined by the method of Kenward-Roger and variance components were estimated using the restricted maximum likelihood method. Statistical significance was declared at $P < 0.05$. In case of nonsignificant interaction ($P > 0.05$), the main effects of wk and strain were considered. In case of significance of F-tests, a Fishers LSD test was used for multiple pairwise post-hoc testing. For splenocyte proliferation data, the model included the particular well plate during analysis and for ELISA data the particular well plate during stimulation as well as during analysis as further random effects. In order to consider probable effects of duration of blood sampling on immune and endocrine parameters, sampling time was included as covariate for statistical analysis of immune parameters and corticosterone concentration determined in *vena ulnaris* blood samples. Covariables were checked for significance and were dropped from the model if they were not significant.

RESULTS

Distribution of Immune Cells in Blood and Lymphatic Tissue

The numbers of various immune cell types in blood, spleen and also CT differed between wk of life in the

investigated hens as well as between the 2 investigated strains (Figures 2–4).

Blood Leukocytes. Within phagocytic and innate immune cell types (Figure 2A), neither the number of thrombocytes, monocytes nor heterophils in blood were affected by an interaction of strain and wk. However, LB hens constantly had higher numbers of thrombocytes and heterophils in blood than LSL hens. Regarding an influence of the wk of life, thrombocyte counts in blood did not differ between wk 9, 15, and 23, but were higher at 29 wk and 59 wk compared to the 3 earlier periods ($P < 0.05$). Numbers of monocytes and heterophils in blood increased between wk 15 and 23 (monocytes: $P < 0.001$; heterophils: $P = 0.035$), stayed elevated at wk 29, and were lower at wk 59 (monocytes: $P < 0.001$; heterophils: $P < 0.001$). In contrast to heterophils, monocyte numbers in blood were higher at wk 15 compared to wk 9 ($P = 0.02$). Within adaptive immune cells (Figure 2B), the interaction of strain and wk affected the number of B cells, $CD4^+$ T cells and $\gamma\delta$ T cells, whereas CTL were affected either by wk or strain. In general, the various T cell types showed a decrease in numbers in blood between wk 15 and 59. While CTL showed a sharp drop at wk 23 ($P < 0.001$ for wk 15 vs. wk 23) and stayed low in number thereafter, the numbers of $CD4^+$ T cells and $\gamma\delta$ T cells decreased gradually from wk 15 until wk 59 in both strains (wk 15 vs. wk 59 with $P < 0.001$ for both strains). Different from CTL, the numbers of $\gamma\delta$ T cells in blood in both strains and the numbers of $CD4^+$ T cells in LSL hens increased between wk 9 and 15 ($\gamma\delta$ T cells: $P < 0.001$ for LB and LSL; $CD4^+$ T cells in LSL hens: $P = 0.002$). This effect can also be seen in the numbers of B cells of LSL hens ($P < 0.001$; Figure 2B), displaying an interaction effect between the strains ($P = 0.001$ for LSL vs. LB hens at wk 9), as LB hens do not show any variation in the number of B cells in blood. From wk 15 until wk 59, the number of B cells in blood stayed unchanged in LSL hens as well. Notably, the course of blood $CD4^+$ and $\gamma\delta$ T cells is formed by the $CD8\alpha^-$ part of both T cell subsets, also reflecting the influence of the interaction of strain and wk (Supplementary Fig. S1). In contrast, $CD8\alpha^+ CD4^+$ T cells were not influenced by an interaction of strain and wk, but showed a decrease at wk 23 and an increase in wk 59 in both strains ($P < 0.01$ wk 23 vs. wk 15 or wk 59). $CD8\alpha^+ \gamma\delta$ T cells were found in similar numbers in wk 9, 15, and 23, and decreased in number at wk 29 ($P < 0.05$ vs. all other weeks) and wk 59 ($P < 0.001$ vs. all other wk; Supplementary Fig. S1). The alterations in innate and adaptive leukocytes also resulted in an increase of the heterophil-to-lymphocyte (H:L) ratio in whole blood at wk 23 ($P = 0.009$ wk 15 vs. wk 23; Figure 2C), which was unchanged thereafter until wk 59. In general, LB hens displayed higher H:L ratios than LSL hens (Figure 2C).

Splenic Leukocytes. No interactive effects of strain and wk were found for the number of the various leukocyte types in the spleen (Figure 3). However, strain effects revealed higher total numbers of macrophages and $\gamma\delta$ T cells in the spleen of LSL hens and higher total numbers of B cells per spleen of LB hens (Figure 3A, B).

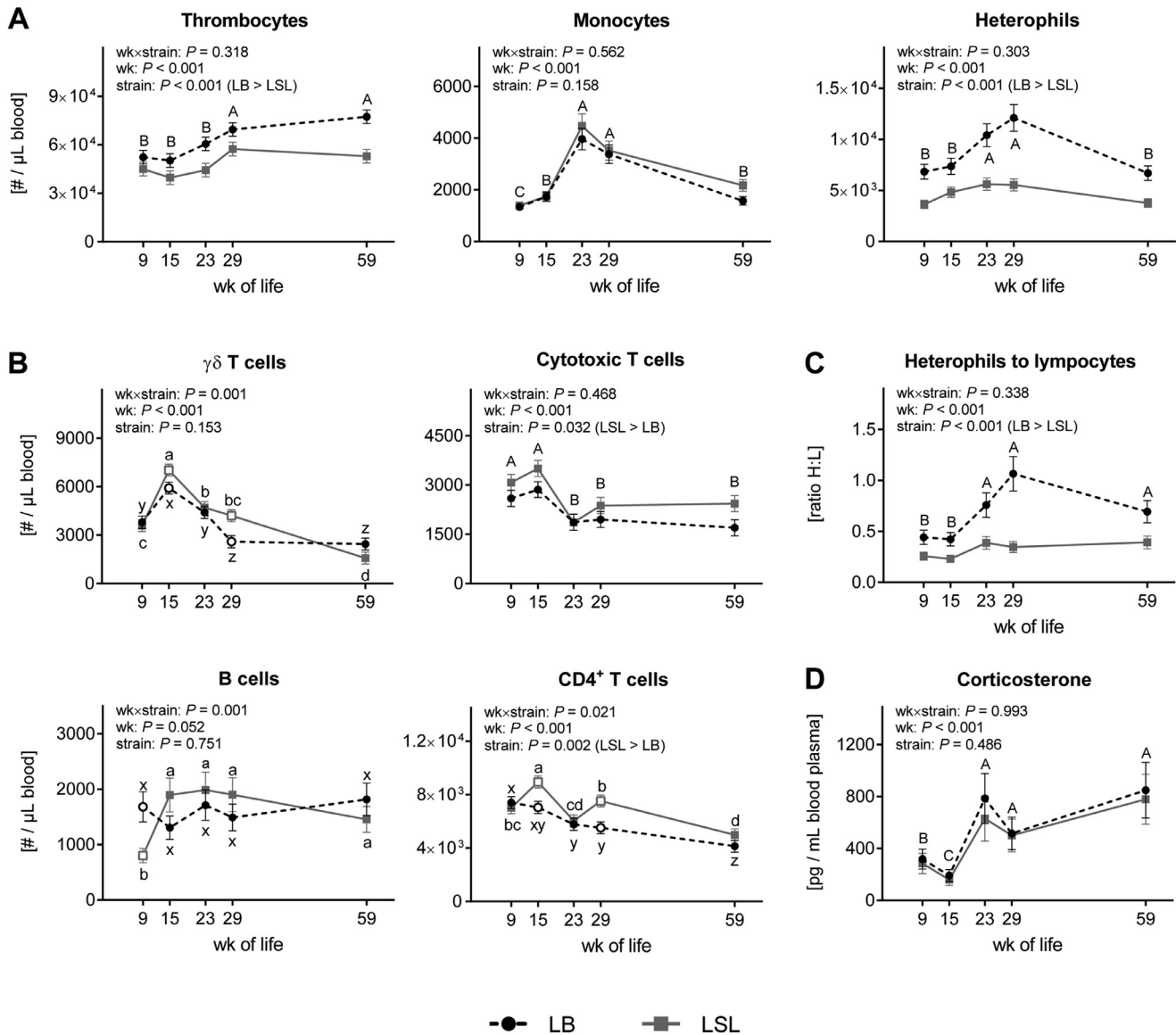


Figure 2. Absolute counts of leukocyte subsets, ratio of heterophils to lymphocytes, and corticosterone concentration in whole blood of Lohman LSL-Classical (LSL) and Lohman Brown-Classical (LB) hens at different weeks of life. EDTA blood was drawn by *vena ulnaris* puncture in wk 9, 15, 23, 29, and 59. Cell count of various leukocytes types was determined by flow cytometry and corticosterone concentration by RIA. (A) Innate immune cells, (B) lymphocyte subsets, (C) ratio of heterophil numbers to numbers of lymphocytes, (D) corticosterone concentration in plasma. LSmeans \pm SEM of $n = 10$ hens per wk and strain. Black circles depict counts of LB hens, gray squares depict counts of LSL hens. Results of statistical analysis of the main effects wk and strain as well as their interaction (wk \times strain) are shown in each diagram. In case of interaction of wk and strain: Means within a strain missing a common lower case (LSL: a-c; LB: x-z) differ in regard of wk within same strain ($P < 0.05$) and means depicted by open symbols differ in regard of strain at particular wk ($P < 0.05$). Different uppercases depict differences ($P < 0.05$) of the main effect wk irrespective of strain in case of missing interaction.

When analyzing the number of leukocytes per g of spleen, strain effects showed higher concentrations of CTL, CD4⁺ T cells, $\gamma\delta$ T cells, macrophages and thrombocytes per g of spleen in LSL hens and no difference in the concentration of B cells per g of spleen between both strains. In consequence, spleens of LB hens were significantly heavier than spleens of LSL hens (Supplementary Fig. S2). Regarding effects of wk, the total number of thrombocytes per spleen were found to steadily increase with advancing age ($P < 0.001$ for effect of wk; Figure 3A), whereas macrophages showed an increase in numbers between wk 16 and 24 ($P < 0.001$) after their number had decreased between wk 10 and 16 ($P = 0.002$). Like in blood, the total number of splenic $\gamma\delta$ T cells showed an increase from wk 10 to 16 ($P <$

0.001; Figure 3B), which was also found for splenic CTL ($P < 0.001$) and CD4⁺ T cells ($P < 0.001$). Again, numbers of CTL decreased at wk 24 (wk 16 vs. wk 24: $P < 0.001$) and stayed unchanged thereafter. However, cell numbers of splenic CTL during laying activity at wk 24, 30, and 60 stayed higher compared to young hens in wk 10 ($P < 0.001$). In contrast, numbers of $\gamma\delta$ T cells in spleen gradually decreased from wk 16 until wk 30 (wk 16 vs. wk 24: $P = 0.014$; wk 24 vs. wk 30: $P = 0.003$) and stayed low until wk 60 with cell numbers comparable to wk 10. In contrast to blood, splenic CD8 α^+ $\gamma\delta$ T cells outweighed the number of splenic CD8 α^- $\gamma\delta$ T cells and both subsets showed almost similar courses (Supplementary Fig. S3). Total numbers of CD4⁺ T cells in spleen increased from wk 10 to 16 ($P < 0.001$), but in contrast

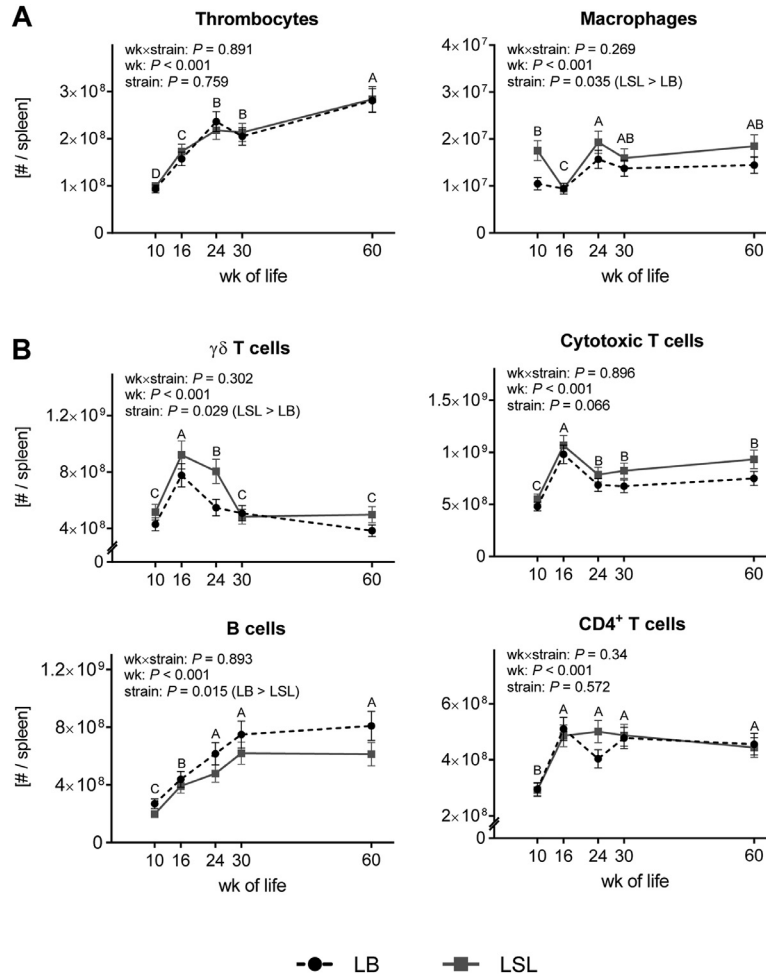


Figure 3. Absolute counts of leukocyte subsets in spleen of Lohman LSL-Classical (LSL) and Lohman Brown-Classical (LB) hens at different weeks of life. Splensens were dissected after slaughter in wk 10, 16, 24, 30, and 60 and cell count of various immune cell types was determined within splenocytes by flow cytometry. (A) Innate immune cells, (B) lymphocyte subsets. LSmeans \pm SEM of $n = 10$ hens per wk and strain, except $n = 9$ LSL hens at wk 60. Black circles depict counts of LB hens, gray squares depict counts of LSL hens. Results of statistical analysis of the main effects wk and strain as well as their interaction (wk \times strain) are shown in each diagram. Different uppercases depict differences ($P < 0.05$) of the main effect wk irrespective of strain due to missing interaction.

to CD4⁺ T cells in blood, showed no variation in total numbers thereafter. Again, the course of CD4⁺ was formed by the CD8 α ⁻ part of this T cell subset, whereas splenic CD8 α ⁺ CD4⁺ T cells showed a temporary increase in numbers at wk 16 and 24 in both strains, and high numbers at wk 60 in LSL hens. The number of B cells gradually increased from wk 10 to 24 (wk 10 vs. wk 16: $P < 0.001$; wk 16 vs. wk 24: $P = 0.048$) and stayed high until wk 60.

IEL from CT. Within IEL from CT, the interaction of strain and wk affected the number of $\gamma\delta$ T cells, CD4⁺ T cells and B cells. Whereas, $\gamma\delta$ T cells in LB hens showed an increase in number between wk 16 and 24 ($P = 0.007$) and a constant level thereafter (Figure 4), $\gamma\delta$ T cells in LSL hens increased between wk 10 and 16 ($P = 0.002$), followed by a decrease at wk 24 ($P < 0.001$), after which the number of cells went up again (wk 24 vs. wk 30: $P < 0.001$). In LB hens, CD4⁺ T cells decreased in number between wk 10 and 16 ($P = 0.024$) and strongly increased in number at wk 24 ($P < 0.001$). CD4⁺ T cells among IEL of CT in LSL hens showed almost no age-related variations, but lower numbers in wk 60 compared to LB hens ($P = 0.001$). Like in spleen,

CD4⁺ T cells were mostly of the CD8 α ⁻ phenotype, whereas $\gamma\delta$ T cells were mainly comprised of the CD8 α ⁺ expressing subset (Supplementary Fig. S4). Numbers of B cells among IEL of CT increased between wk 16 and 24 in both hen strains (LB: $P < 0.001$; LSL: $P = 0.002$) and reached highest numbers at wk 30 and 60. For LB hens, however, the numbers of B cells were lower in wk 16 compared to wk 10 ($P < 0.001$), whereas no differences in cell numbers of B cells at the respective wk of life were found among IEL of CT from LSL hens. The fraction of CTL and NK cells were not influenced by an interaction of wk and strain, and similar numbers within IEL from CT were found for both hen strains. Numbers of CTL and NK cells stayed unchanged from wk 10 to 24, increased at wk 30 (wk 24 vs. wk 30: $P = 0.002$), but decreased again in wk 60 (wk 30 vs. wk 60: $P = 0.013$).

Immunoglobulin Concentration in Plasma

As a measure of in vivo activity of the immune system, we assessed the concentration of total IgY, IgA, and IgM in blood plasma. All 3 Ig classes were affected by an

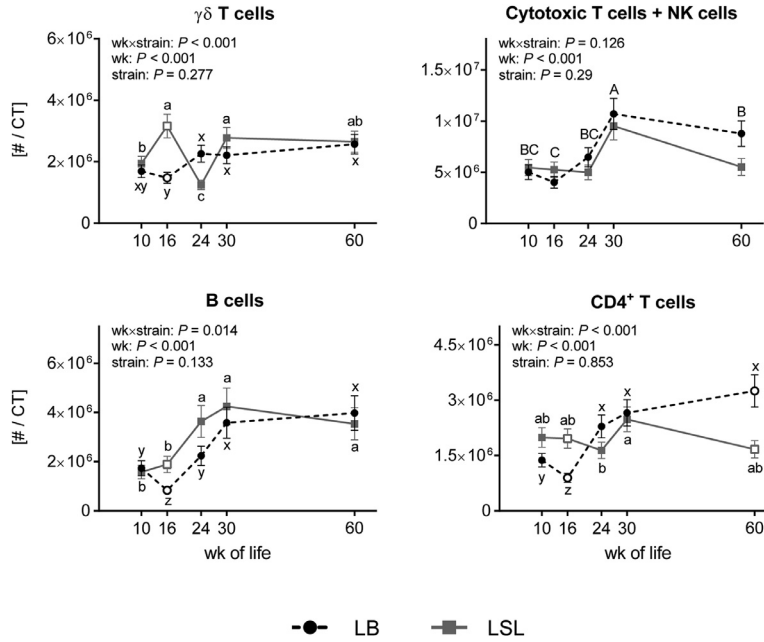


Figure 4. Absolute counts of lymphocyte subsets within intraepithelial lymphocytes from cecal tonsils of Lohman LSL-Classical (LSL) and Lohman Brown-Classical (LB) hens at different weeks of life. Intraepithelial lymphocytes were isolated from mucosa of cecal tonsils after slaughter in wk 10, 16, 24, 30, and 60 and cell count of various lymphocyte types was determined by flow cytometry. LSmeans \pm SEM of $n = 10$ hens per wk and strain, except $n = 9$ LSL hens at wk 60. Black circles depict counts of LB hens, gray squares depict counts of LSL hens. Results of statistical analysis of the main effects wk and strain as well as their interaction (wk \times strain) are shown in each diagram. In case of interaction effects of wk and strain: Means within a strain missing a common lower case (LSL: a-c; LB: x-z) differ in regard of wk within same strain ($P < 0.05$) and means depicted by open symbols differ in regard of strain at particular wk ($P < 0.05$). Different uppercases depict differences ($P < 0.05$) of the main effect wk irrespective of strain in case of missing interaction.

interaction of wk and strain (Figure 5). These interactions resulted from strain-dependent differences in the magnitude of age-related variation, whereas the course of Ig concentrations between both strains did not generally differ. In this regard, LSL hens had higher plasma concentrations of IgM than LB hens at wk 16 ($P = 0.004$), but LB hens showed higher concentrations of IgA at wk 24 ($P = 0.011$) and 60 ($P = 0.046$) and of IgY at wk 10 ($P = 0.002$) and 24 ($P < 0.001$) than LSL

hens at the corresponding wk. In general, IgM plasma concentration steadily increased until wk 30 in both strains and stayed high at 60 wk. Whereas LSL hens only showed marginal changes in IgA plasma concentrations, with higher concentrations at 24 wk compared to wk 60 ($P = 0.011$), plasma concentration of IgA strongly increased in LB hens at wk 24 compared to wk 16 ($P < 0.001$). Plasma concentration of IgY was higher at wk 16 compared to wk 10 in LSL hens.

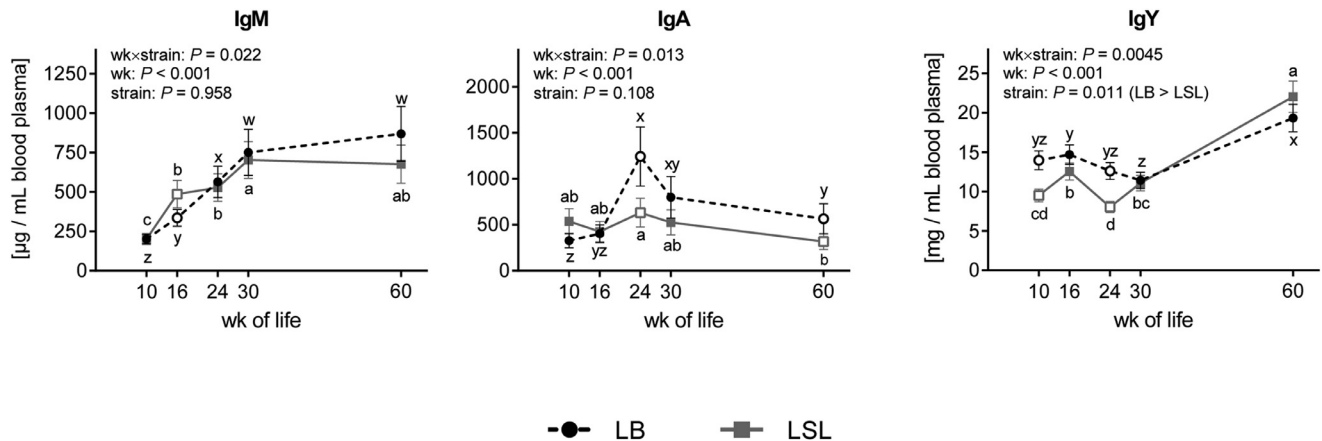


Figure 5. Antibody concentration in blood of Lohman LSL-Classical (LSL) and Lohman Brown-Classical (LB) hens at different weeks of life. Trunk blood was harvested after slaughter in wk 10, 16, 24, 30, and 60 and plasma concentration of IgM, IgA and IgY was determined by ELISA. LSmeans \pm SEM of $n = 10$ hens per strain and week at wk 10, 16 and 24; $n = 9$ LB hens at wk 24 and 30 and LSL hens at wk 24; $n = 8$ LSL hens at wk 30 and 60. Black circles depict counts of LB hens, gray squares depict counts of LSL hens. Results of statistical analysis of the main effects wk and strain as well as their interaction (wk \times strain) are shown in each diagram. In case of interaction effects of wk and strain: Means within a strain missing a common lower case (LSL: a-c; LB: w-z) differ in regard of wk within same strain ($P < 0.05$) and means depicted by open symbols differ in regard of strain at particular wk ($P < 0.05$).

Table 1. Mitogen-induced proliferation and IFN- γ production of splenocytes from hens of 2 different strains at different weeks of life.

Parameter	Strain	Wk of life ¹					SEM ¹	<i>P</i> -values		
		10	16	24	30	60		wk \times strain	wk ⁶	strain
ConA-induced IFN- γ (pg/mL) ⁴	LSL ²	8377	3776	11850	12201	8580	5510	0.818	0.086	<0.001 LSL > LB
	LB ³	3066	619	1292	1899	1281				
PWM-induced IFN- γ (pg/mL) ⁴	LSL	5044	2399	7344	6076	3771	1165	0.875	<0.001	0.003 LSL > LB
	LB	2731	1466	4672	2688	1745				
	BC	D	A	AB	CD					
ConA-induced proliferation (delta cpm) ⁵	LSL	542	399	482	534	618	575	0.063	0.334	<0.001 LSL > LB
	LB	137	77	81	64	243				
PWM-induced proliferation (delta cpm) ⁵	LSL	389	217	269	226	266	131	0.254	0.044	<0.001 LSL > LB
	LB	268	146	186	121	95				
	A	B	AB	AB	AB					

¹Data are given as LSmean \pm pooled SEM of $n = 10$ hens per wk and strain, except of LSL hens at wk 60 with $n = 9$.

²Lohmann LSL-Classic.

³Lohmann Brown-Classic.

⁴Normalized to ELISA control (pooled plasma sample measured additionally on each ELISA plate).

⁵Normalized based on biocontrol (frozen splenocytes from one individual hen analyzed simultaneously at each investigated wk).

⁶Different uppercase letters indicate main effect of wk ($P < 0.05$; multiple pairwise post-hoc testing by Fishers LSD test).

Between wk 16 and 24 in LSL ($P < 0.001$) and until wk 30 in LB hens ($P = 0.032$), plasma concentration of IgY decreased, but increased at wk 60 in both strains (both: $P < 0.001$).

In Vitro Functionality of Splenocytes

Neither the capacity of splenocytes to proliferate nor to produce IFN- γ in response to an in vitro mitogenic challenge was influenced by an interaction of strain and wk (Table 1). However, splenocytes from LSL hens consistently showed higher rates of mitogen-induced proliferation as well as higher concentrations of IFN- γ in supernatant than LB hens, irrespective of the used mitogen. Regarding age-related effects, the PWM-induced proliferation of splenocytes was lower at wk 16 compared to wk 10 ($P = 0.029$) and increased to intermediate levels thereafter. PWM-induced IFN- γ production decreased between wk 10 and 16 ($P = 0.002$), subsequently reached the highest levels in wk 24 (wk 16 vs. 24: $P < 0.001$; wk 10 vs. 24: $P = 0.034$) and decreased gradually thereafter until wk 60 (wk 24 vs. 60: $P < 0.001$).

Plasma Corticosterone Concentration

The concentration of corticosterone in plasma was not influenced by an interaction of strain and wk, nor by an effect of strain. In both strains, plasma corticosterone concentration decreased between wk 9 and 15 ($P = 0.039$), but subsequently greatly increased at wk 23 ($P < 0.001$) and stayed elevated until wk 59 (Figure 2D).

DISCUSSION

High-yielding laying hens start their reproductive and egg-laying activity at an age of 18 to 20 wk, reach their growth plateau around 20 to 26 wk, and peak in egg production at 26 to 30 wk, which also held true for the LB and LSL hens of the present study (Sommerfeld et al.,

2020b; Lohmann Tierzucht, 2021). According to generally accepted definitions (Sipos, 2019), the hens of the present study were thus considered adult at an age of 20 wk, and adolescent from wk 8 to 20. The present study shows that both particular life periods are associated with changes in number and function of peripheral and lymphoid immune cells in LB and LSL hens.

In both strains, a similar pattern of immunological change was evident, with the few strain-dependent variations that occurred mainly reflecting differences in the magnitude of effect. The present study also shows substantial immunological differences between LB and LSL hens with respect to immune cell numbers and in vitro functionality of splenocytes. The differences point to a generally higher innate and humoral immune response in LB hens and to a more pronounced cellular arm of the immune system in LSL hens. These results are in line with a recent study by Hofmann et al. (2021).

Variation of Immune Parameters during Adolescence

Within blood, numbers of B and T cells increased from wk 9 to 15 in the hens of the present study. This result is consistent with a study by Burgess and Davison (1999), who also showed that the numbers of B and T cells in blood increased between wk 10 and 15 in laying hens. Interestingly, avian $\gamma\delta$ T cells are found to divide into subsets distinguished by CD8 α expression, with CD8⁺ $\gamma\delta$ T cells displaying a more differentiated phenotype than the CD8⁻ population (Pieper et al., 2008). In the present study, the higher numbers of blood $\gamma\delta$ T cells in wk 15 resulted from an increase in CD8⁻ $\gamma\delta$ T cells, which supports the suggestion that newly-matured B and T cells are still released from primary lymphoid tissues in the growing pullet. Studies show that the involution of the primary lymphoid organs, bursa of Fabricius and thymus, only starts in the late adolescence or early adulthood of chickens (Ciriaco et al., 2003).

The number of all investigated lymphocyte types in spleen, which represents an important secondary lymphoid organ in avian species, increased from wk 10 to 16 in the chickens of the present study. Similarly, the frequency of splenic T cells was shown to increase until wk 11 to 15 in a study by [Johnston et al. \(2012\)](#) conducted in LB hens. In spleen, both $CD8\alpha^-$ and $CD8\alpha^+ \gamma\delta$ T cells increased during the growing phase of the pullets of the present study, which again points to an ongoing influx of newly-matured and naive lymphocytes from the blood to the spleen. At the same time, the increase of splenic $CD8\alpha^+ \gamma\delta$ T cells suggests a high number of primary adaptive immune reactions to novel antigens, leading to differentiation of T and B cells during this production period. The steady increase in plasma IgM concentrations, which is indicative of *in vivo* primary adaptive immune responses, also supports this view. In this regard, most pathogen encounters during adolescence should still be of a novel nature ([Simon et al., 2015](#)). Moreover, vaccinations of hens carried out within this period, like those routinely applied under conventional housing conditions, also contribute to a repeated triggering of adaptive immune responses.

In contrast to the spleen, the number of lymphocytes within CT, representing the largest lymphoid aggregate of organized avian gut-associated lymphoid tissue (GALT), does not generally increase from wk of life 10 to 16 in the chickens of the present study, but shows a heterogeneous course of particular IEL subsets. This pattern most probably reflects local variations in antigen burden rather than a general response to novel pathogen encounters within the organized GALT during adolescence. Whether this pattern also holds true for the diffuse GALT remains to be investigated.

Unlike lymphocytes, the number of splenic macrophages was lower in wk 16 compared to wk 10 of the hens' life. Macrophages express growth hormone (GH) receptors, suggesting a role of this hormone in macrophage activity ([O'Neill and Ketterson, 2011](#)). The avian spleen, which is relevant for the development of macrophages ([John, 1994](#)), also represents a target tissue of GH. A decline in GH concentrations, which was found in female chickens around an age of 12 wk in a study by [Scanes et al. \(1984\)](#), might thus have contributed to the decrease in splenic macrophages found in the present study. In contrast, the numbers of blood monocytes were higher in wk 15 compared to wk 9, indicating that hematopoiesis of mononuclear phagocytes *per se* is not affected.

Taken together, our data strongly suggest that the development of the immune system and an increase in adaptive immune responses is an ongoing process in growing and adolescent hens. Adaptive immune responses like lymphocyte proliferation or antibody production, as well as phagocytic activity of antigen-presenting cells are assumed to be very energy-demanding ([O'Neal and Ketterson, 2011](#)). Management, and in particular feeding protocols should therefore consider high energy demands for both growth and immunological processes during this production period, as the development of a fully

functional immune system is a prerequisite for health and high performance in laying hens.

Effect of Laying Activity on Immune Parameters

The most prominent changes in the number of immune cells within blood and spleen were found between wk of life 15/16 and 23/24, a crucial period with the onset of laying activity and the transition from adolescence to adulthood. These immunological changes encompassed a strong decrease in the number of lymphocytes bearing cytolytic capacities, and an increase in innate immune cells. Furthermore, these modulations did not reverse to prelaying values but stayed low or even further decreased during peak laying activity. Similarly, earlier observations in chickens found decreased frequencies of splenic T cells associated with the onset of laying activity ([Wigley et al., 2005](#); [Johnston et al., 2012](#)). Moreover, a recent study in turkeys showed that frequencies of T cells in blood and spleen did not differ at early or peak laying activity ([Kowalczyk et al., 2020](#)). To our knowledge, the present study is the first investigating the course of immune parameters at different life periods covering both the transitional phase around the onset of laying and the following phase of laying activity in chicken. From the present data, it can be assumed that the increase in innate immune cells and the decrease in cytolytic lymphocytes cause a shift in immune regulation toward more pronounced innate and humoral adaptive immune responses in the egg-laying hens. This assumption is also underlined by the unchanged or even increasing numbers of B cells found in the hens of the present study. As a consequence of the reduced cellular adaptive immunity, the capability of the hens to raise adequate immune responses might be impaired during egg laying. The alterations in distribution of immune cells at the onset of laying activity in laying hens found by [Wigley et al. \(2005\)](#) and [Johnston et al. \(2012\)](#) were shown to be associated with an increased susceptibility to salmonellosis. In addition, infectious diseases were shown to account for up to 12% of deaths in egg-laying hens in a recent study by [Herwig et al. \(2021\)](#). Interestingly, modulations in immune parameters causing diminished immunocompetence also occur during reproduction in many wild-living avian species and are supposed to result from a trade-off between highly energy-consuming reproductive activity and immune functions ([Martin et al., 2007](#); [O'Neil and Ketterson, 2011](#)). As the hens of the present study were provided with adequate energy supply, the observed modulations in immune parameters might represent an evolutionary conserved trait as has been described for various seasonal animals ([Weil and Nelson, 2001](#)).

Regarding potential underlying mechanisms, the transition to egg laying activity under productive management is accompanied by distinct adjustments in housing conditions coinciding with or causing various physiological changes in the hens. Within these adjustments,

adaptations in diet formulation to meet energy and nutritional demands for egg laying as well as changes in lighting conditions to induce egg laying activity in the seasonally reproducing chickens are most prominent. Modulations of the immune system related to nutritional adaptations have already been described, and feed ingredients may directly or indirectly affect immune cells (Koutsos and Klasing, 2014). In this respect, various nutrients shift adaptive immune responses toward pronounced humoral immunity in mammals beyond the mere effect of deficient vs. sufficient nutrition (Koutsos and Klasing, 2014). Regarding changes in lighting conditions, photoperiodic signals (referring to the duration of light per 24 h) are well-known to affect abundance of various hormones with immunomodulating capacities in seasonal animals (Weil and Nelson, 2001). As a consequence, sexual steroid hormones may at least trigger some of the immunomodulatory effects found in the present study, as both estrogen and testosterone are shown to vary with seasonal breeding and to alter immune cell numbers and function (ONeal and Ketterson, 2011; Koutsos and Klasing, 2014). However, experimental application of sexual steroid hormones in birds led to inconsistent immunological effects and point to the fact that these hormones act in close interrelation with other endocrine mediators, foremost glucocorticoids (ONeal and Ketterson, 2011; Koutsos and Klasing, 2014; Wigley et al., 2014). Glucocorticoids are well-described mediators of photoperiodic effects on immune cells (Martin et al., 2007; ONeal and Ketterson, 2011; Koutsos and Klasing, 2014) and diurnal concentrations of immunosuppressive glucocorticoids (Hakim, 1988) are found to increase under long-day conditions (Guerrero and Reiter, 2002). The increase of plasma corticosterone concentrations in the reproductively active hens of the present study matches these observations and fits in with the observed effects in immune cell numbers.

The productive life span in conventional production systems is much shorter than the natural life expectancy of hens (Bain et al., 2016). Accordingly, hens should not have experienced immunosenescence. However, *in vitro* IFN- γ production of PWM-stimulated splenocytes as well as numbers of blood monocytes, heterophils and CD4⁺ T cells were lower at wk 60 compared to early and peak laying activity. Similarly, studies by McCorkle et al. (1979) revealed a lower graft-versus-host response of splenocytes from hens aged 12 and 18 mo compared to hens aged 6 mo. Kowalczyk et al. (2020) also found lower T cell numbers at the end of the laying activity in turkeys compared to peak laying. Thus, a prolonged intensive reproductive phase might additionally challenge the immunocompetence of laying hens, presumably by high metabolic costs associated with egg laying (Vezina, 2003), irrespective of classical senescence effects. Correspondingly, Sherwin et al. (2010) found an increase in mortality rates of laying hens from wk 16 to 25 until wk 66 to 72.

With respect to the findings of the present study, management practices should consider the likely

decrease in immunocompetence in laying hens during the whole egg-laying period. Thus, housing factors with a potential to further suppress or challenge immune functions, such as environmental and social stress or strong pathogenic load should be avoided with the aim to reduce the use of antibiotics and prevent premature deaths. Moreover, breeding programs should consider the probable trade-off between highly energy demanding physiological process such as egg laying and immunocompetence with regard to productivity, health, and welfare of laying hens.

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DISCLOSURES

Authors declare they have no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.psj.2021.101408](https://doi.org/10.1016/j.psj.2021.101408).

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