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Flow-cytometric analysis of circulating leukocyte populations in turkeys: Establishment of a whole blood analysis approach and investigations on possible influencing factors

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ABSTRACT

The blood cell counting methods used for diagnostic and research purposes in turkeys are, up to date, inferior to the techniques established for mammals and chickens. While microscopic counts are time consuming, previous flow cytometric approaches did not cover all blood cell types of interest due to the lack of turkey-specific markers for the different cell populations. Moreover, it is unknown to what extent the different leukocyte populations are affected by host and environmental factors including age, breed of the bird, and housing environment, respectively. In this study we established a whole blood based flow cytometric analysis method for turkeys. This method was used to determine baseline values depending on the age of the birds as well as under consideration of variations between trials and animal room effects. During three trials whole blood samples of B.U.T. 6 female turkeys were collected to analyze different leukocyte concentrations (cells/µl whole blood). In the first trial one group and in the second and third trial two groups with 22 birds/group were housed. Blood samples were collected at days one, 23, 43, 60, and 88 post hatch and concentrations of monocytes, MHC class II-positive, $CD4^+$, and $CD8^+$ lymphocytes, as well as thrombocytes and granulocytes were determined by flow cytometric analysis. Concentrations of all identified populations were not only influenced by the bird's age ($p < 0.05$), they varied also among trials ($p < 0.05$) and even for some of the populations between animal rooms within the same trial despite comparable housing and management conditions. Therefore, for the establishment of baseline values for leucocyte concentrations in whole blood effects of age and housing have to be considered. In addition, our data emphasize the importance of the establishment of baseline values for different age groups, as age had the strongest effect on the blood cell numbers in this study.

1. Introduction

Automated blood cell counts are a quick and precise tool for the assessment of an individual's immune and health status, and are widely used both in human and veterinary medicine. The current systems, however, may not differentiate between nucleated bird erythrocytes, thrombocytes, and leukocytes, making the application of blood cell population-specific antibodies obligatory in avian species. The majority of commercially available antibodies for avian species is designed for chicken, and an automated system for the analysis of whole blood samples has been established (Seliger et al., 2012). However, comparable tools for turkeys are currently not available, particularly because no pan-leukocyte marker is available for turkey cells. A suitable alternative could be the use of a commercially available mouse anti-chicken CD44 antibody that was reported to bind to most peripheral blood mononuclear cells (PBMC) of turkeys, which were isolated by density centrifugation (Lawson et al., 2001; Meyerhoff et al., 2012). In chickens, CD44 is expressed on many cell types, including B-cells, most T-cell subsets, and monocytes as specified by the manufacturer and former studies on isolated blood and lung leukocytes (Dalgaard et al., 2010; Kameka et al., 2014). However, it is not known which turkey cell types express CD44. In mice CD44-receptor expression was also observed on maturing erythrocytes (Chen et al., 2009), therefore, it needs to be clarified if anti-CD44 antibodies may also bind to turkey erythrocytes.

Due to the close phylogenetic relation between chickens and turkeys some of the chicken cell-specific antibodies may cross-react with turkey cells, but others may not (Meyerhoff et al., 2012). Cross-reaction was

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described for monoclonal anti-chicken CD4- and CD8- (Li et al., 1999) as well as anti-chicken MHC class II- antibodies (Lawson et al., 2001), which detect the homologous circulating turkey T-cell populations and monocytes as well as MHC II positive lymphocytes, respectively, isolated via density gradient centrifugation. The anti-MHC class II- antibodies were previously used for flow cytometric analysis in combination with forward and sideward scatter characteristics to identify turkey B-lymphocytes (Rubbenstroth et al., 2010). Although being developed against human cell epitopes, the mouse anti-human antibody against the CD51/61-complex (reviewed by Hartman and Duggan, 2000) also binds to chicken thrombocytes isolated by density centrifugation (Viertlboeck and Gobel, 2007). This cross-reactivity is based on high homology between chicken and human αV and β3 integrins (CD51 and CD61, respectively) forming the vitronectin-receptor on the cell surface of thrombocytes, osteoclasts, and several tumor cell types (Hartman and Duggan, 2000). A phylogenetic analysis of the β3 subunit via BLAST (Altschul et al., 1997, 2005) shows an 81% identity between chicken (XM_015299304.1) and human (NM_000212.2) cDNA-sequences while turkey (XM_010724492.2) and human share 79% identity (Zhang et al., 2000). The predicted amino acid sequences of turkey (XP_010722794.1) and human (NP_000203.2) β3 subunits show 85% homology, which is comparable to the 80% homology between chicken and human. The αV-subunit of the vitronectin-receptor of both species (chicken: NM_205439.1 → NP_990770.1, turkey: XM_010713363.2 → XP_010711665.1) show a homology of 78% for cDNA and 83% of amino acid sequences with the human sequences (NM_001144999.2 → NP_001138471.1). This homology suggests also cross reactivity of the anti-CD51/61 antibodies with turkey thrombocytes (Fig. 1), which has to be experimentally confirmed.

For the clinical evaluation of blood cell concentrations (cells/µl whole blood) or comparison of values between experiments, baseline values from healthy turkeys need to be established. Variation of hematocrit and white blood cell concentrations may be expected. Previous studies in bustards and cranes demonstrated that age (Harrison and Lightfoot, 2006) and season (Abelenda et al., 1993), respectively, may influence these parameters. Also the time of day the samples were collected had an impact on the proportions (%) of leukocyte subpopulations. This was previously evaluated via microscopic counting of blood samples from broiler chickens (Makeri et al., 2017). Therefore, the identification of non-infectious influencing factors is crucial for the subsequent discrimination between healthy and health-compromised individuals. Most studies provide only a limited insight into the dynamics of blood cell populations. Hence, systematic evaluations of influencing factors such as age or trial are needed.

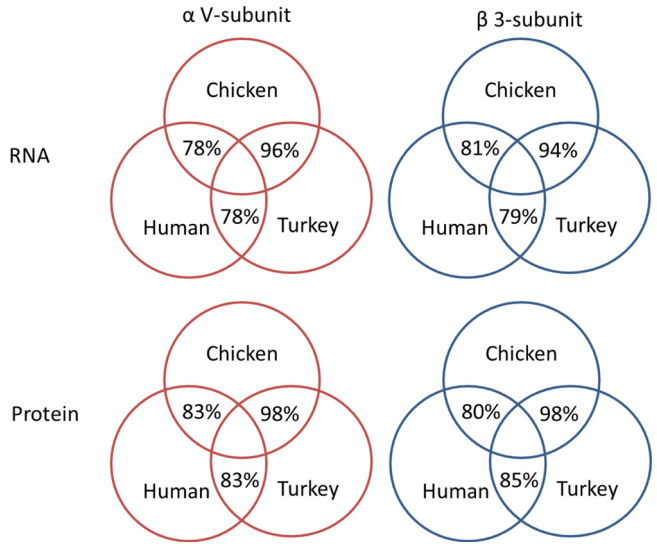

Fig. 1. Homology in percent (%) of the RNA and predicted amino acid sequences of the αV- and the β3-subunits among humans, chickens and turkeys.

In this study our first objective was to establish a standardized method for automated blood cell concentration analysis for turkeys by comparing a whole blood flow cytometric approach with the classical determination of leukocyte proportions via microscopic blood cell counts. The second objective was to determine baseline values for blood cell concentrations of female B.U.T. 6 turkeys considering the possible influence of age. Birds were raised up to 90 days of age and blood samples were collected at days one, 23, 43, 60, and 88 post hatch to determine numbers of monocytes, MHC class II-positive lymphocytes, $CD4^+$, and $CD8^+$ lymphocytes, as well as thrombocytes and granulocytes. Three trials with identical housing and management conditions were conducted to identify possible trial effects.

2. Materials and methods

2.1. Establishment of a standardized method for automated blood cell analysis for turkeys

2.1.1. Blood samples for preliminary investigations

For method adjustment, turkey blood samples from random clinical cases sacrificed for diagnostic necropsy at the Clinic for Poultry, University for Veterinary Medicine Hannover, Germany, were used. Also samples which were collected during the main animal experiment as described below were used for selected aspects. Blood was collected in ethylenediaminetetraacetic acid (EDTA) coated tubes (BD Biosciences, San Jose, CA, USA). Whole blood samples were used at a dilution of 1:500 in flow buffer (PBS with 1% bovine serum albumin (BSA) (Albumin fraction V, Carl Roth GmBH, Karlsruhe, Germany) and 0.4% EDTA (Sigma-Aldrich, St. Louis, MO, USA)). Furthermore, for selected aspects including the confirmation of antibody cross-reactivity to turkey blood cells also leukocytes were separated from erythrocytes by centrifugation at 715 × *g* for 15 min with subsequent harvest of the buffy coat cells at 1600 x *g* for 10 min. The pelleted cells were diluted in 1 ml of flow-buffer. The pelleted cells were diluted in 1 ml of flow-buffer, resulting in a cell suspension consisting mainly of leucocytes with only low proportions of erythrocytes. This suspension was used for determination of cell-specific antibody binding since low proportions of leucocyte subpopulations such as CD8 positive cells in whole blood might be mistaken for false positive staining and vice versa.

2.1.2. Flow cytometer

For the experiments the flow cytometric analyzer AccuriC6 (BD Biosciences, San Jose, CA, USA) with four different color channels was used. One channel (FL3, 670 nm) was used for life-dead staining (7-Aminoactinomycin D (7AAD) (Exbio, Vestec, Czech Republic)) to exclude false positive cells due to non-specific intracellular staining. The other three channels were used to identify different cell populations.

2.1.3. Set-up of the flow cytometric analysis

Based on the information provided in the literature (Meyerhoff et al., 2012; Seliger et al., 2012; Viertlboeck and Gobel, 2007), mouse-monoclonal antibodies were selected (Table 1) and tested to confirm their cross reactivity with turkey blood cells by flow cytometry. All antibodies were used in different combinations to optimize the analysis of whole blood samples for monocytes, lymphocytes, granulocytes, and thrombocytes. Antibodies were adjusted to the following concentrations in 1 ml of diluted blood sample: anti-CD51/61-FITC (0.2 – 0.4 µg/ml), anti-MHC class II-PE (0.1 µg/ml), and anti-CD8-Cy5 (0.63–1.25 µg/ml); anti-CD4-FITC (0.05 -0.07 µg/ml, and anti-CD44-PE or -APC (0.8 µg/ml). Samples were incubated in the dark with the mixture of three different monoclonal antibodies for at least one hour (h) at room temperature. AccuCheck Counting Beads were added for quantification following the manufacturer's recommendations (PCB100, Thermo Fisher Scientific, Waltham, MA USA). Five microliters (µl) of 7-Aminoactinomycin D (7AAD) (Exbio, Vestec, Czech Republic) solution were

Table 1
Antibodies used for flow cytometric analysis.

Antibodies	Detected cell types	Conjugated Fluoro-chrome	Reported cross-reactivity with turkey cells
Mouse anti-chicken CD4 (clone CT4)[a]	T-helper cells	FITC	(Li et al., 1999)
Mouse anti-chicken CD8 (clone 3-298)[a]	Cytotoxic T-cells	CY5	(Li et al., 1999)
Mouse anti-chicken MHC class II (clone 2G11)[a]	Monocytes, B-cells	PE	(Lawson et al., 2001)
Mouse anti-chicken CD44 (clone AV6)[a]	Nucleated cells	PE or APC	(Meyerhoff et al. 2012)
Mouse anti-human CD51/61 (clone 23C6)[b]	Thrombocytes	FITC	not described yet

[a] Southern Biotech, Birmingham, AL, USA.
[b] Biolegend, San Diego, CA, USA.

added one minute prior to flow cytometric analysis. Cells were gated by size (forward scatter, FSC) and granularity (side scatter, SSC) to exclude both cell fragments and cell aggregates. Cells stained by 7AAD were considered dead and excluded from further evaluation. Subsequently, the respective cell populations of interest were gated based on positive antibody staining, size and granularity. Cell concentrations for statistical analysis were extracted using the FlowJo software (BD Biosciences, San Jose, CA, USA). The following antibodies known to not cross-react with turkey cells were included as isotype controls: FITC-labeled mouse anti-bovine CD11b (IgG2b), mouse anti-chicken Kul1-PE (IgG11κ), mouse anti-chicken Kul1-FITC (IgG11κ), and mouse anti-chicken IgA-biotin (IgG2bκ) combined with avidin- APC.

2.1.4. Experimental design for the steps of establishment of a whole blood automated cell counting system

First antibody dilutions were optimized for triple color staining protocols. The following antibody combinations were established and the indicated cell populations detected under consideration of forward and sideward scatter characteristics: anti-CD51/61-FITC + MHC class II-PE + anti-CD8-CY5 to identify monocytes, MHC class II-positive lymphocytes, thrombocytes, as well as $CD8^+$ lymphocytes; anti-CD4-FITC + MHC class II-PE + CD8-CY5 to detect monocytes, MHC class II-positive lymphocytes as well as $CD4^+$ and $CD8^+$ lymphocytes; anti-CD44-PE + CD51/61-FITC + CD8-Cy5 to identify thrombocytes, $CD8^+$ lymphocytes as well as monocytes and granulocytes; anti-CD44-APC + MHC class II-PE + CD4-FITC to count monocytes, MHC class II-positive lymphocytes, $CD4^+$ lymphocytes as well as granulocytes.

The cell types identified with flow cytometric analysis were confirmed by cell sorting. After incubation with various antibody combinations, antibody-labelled cells were sorted via fluorescence-activated cell sorting (FACS) in a FACSAria Fusion flow cytometer (BD Biosciences, San Jose, CA, USA). Sorted cells were subsequently stained with Wright Giemsa following standard procedures and examined via light microscopy for cell morphology and purity of respective cell types. In addition concentrations evaluated by the established automated cell counting technique were converted to proportions of all counted leucocytes and compared with proportions obtained by manual microscopic blood cell counting and the intra assay variance determined. For this comparison five aliquots of blood samples from two turkeys each were prepared. Each of these aliquots were processed for manual blood cell counts and stained with either a combination of anti-CD51/61 + CD44 + CD8-antibodies (sample 1) or anti-CD4 + MHC class II + CD44-antibodies (sample 2) and specifically monocyte cell numbers were evaluated (Fig. 2: G1.3 and G4.1). For manual counts standard procedures were followed, and cells were stained with Wright-Giemsa and subsequently mounted with Roti®-Mount (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). A minimum of 100 leukocytes/sample including monocytes, lymphocytes, heterophilis, eosinophils and basophils. The percentages of lymphocytes, monocytes, heterophilic, eosinophilic and basophilic granulocytes were calculated.

2.2. Acquisition of automated blood cell evaluation for baseline establishment

2.2.1. Animals

Three animal trials were conducted over a time period of three years (2015–2017). The first trial was conducted in autumn (September – November), the second during summer (July-September) and the third in spring (April – June). For each trial one-day old female turkey poults (B.U.T. 6) were obtained from either of two commercial breeders (Kartzfehn, Bösel, Germany and Heidemark, Ahlhorn, Germany). These animals were housed in pens of 5.8 m with 22 birds/pen in one (first trial) or two isolation rooms (second and third trial) in the facilities of the University for Veterinary Medicine Hannover, Clinic for Poultry, according to the guidelines for turkey management in Germany from 2013. In each trial different rooms within the facility were used. The animal's health status was monitored daily by visual examination of the behavior and posture of the birds. For welfare reasons, injured birds were separated within the same room in an isolation pen and kept under the same environmental conditions until they could return to their group again. All birds in each trial were provided with water and commercial feed ad libitum, and were housed on wood shavings without natural light. An 8 h (hour) light – 16 h dark - lighting program was applied from day two on. All animals were vaccinated orally with the recommended dose of a commercially available live Newcastle disease (at days 14, 42, and 67 post hatch (dph). All animal experiments were authorized by the Lower Saxony State Office for Consumer Protection and Food Safety (33.14-42502-04-15/1813).

2.2.2. Experimental design

To establish baseline values for leukocyte analysis using the adapted protocol for automated cell counting three animal trials were conducted. Each trial lasted 88 days starting the day of hatch. At one, 23, 43 and 88 dph EDTA-blood samples were collected either by exsanguination at days 1 and 88 or for the other time points via puncturing the brachial vein. The blood hematocrit was determined following standard procedures. Blood samples were diluted with PBS at a ratio of 1:500 (first trial) or 1:250 (second and third trial). Two multicolor staining protocols were applied to aliquots of each sample: in trial 1 and 2: anti-CD51/61-FITC + MHC class II-PE + CD8-Cy5 or anti-CD4-FITC + MHC class II-PE + CD8-Cy5 in concentrations as indicated under 2.1.2. In trial 3 anti-CD51/61-FITC + CD44-PE + CD8-Cy5 or anti-CD4-FITC + MHC class II-PE + CD44-APC in concentrations as indicated in 2.1.2. Automated cell counting was conducted by the method as described under 2.1.2.

2.3. Statistical analysis

All datasets were tested for normal distribution and analyzed accordingly. Hence, all three trials were compared for age-related and trial-related influences as class effects on blood cell concentrations using mixed model analysis (generalized linear mixed models), using age, trial and the interaction of both as fixed effects. The comparison between the two groups within the second and third trial was done with mixed model analysis (generalized linear mixed models), using age,

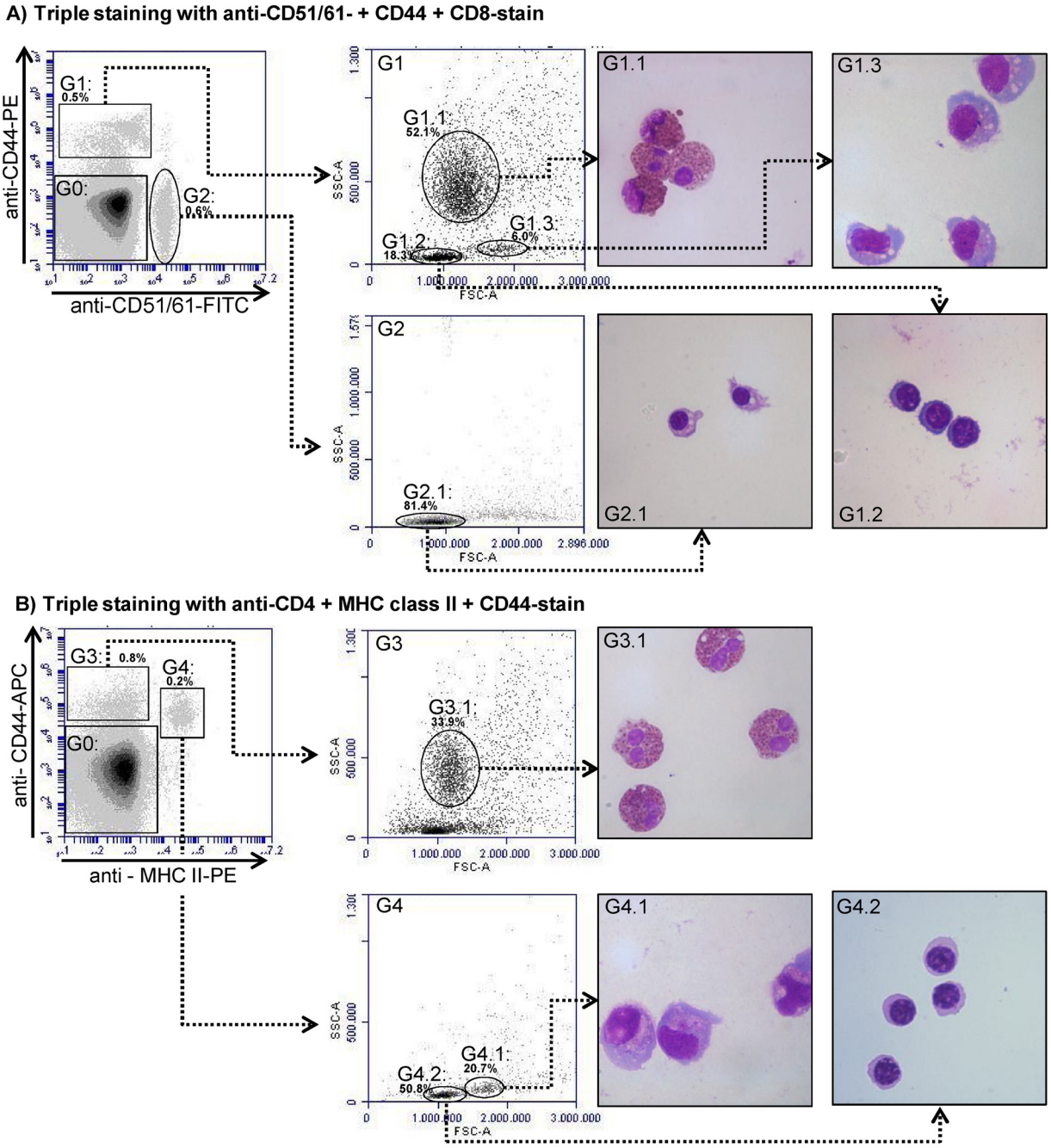

Fig. 2. Gating procedure and identified cell populations after flow cytometric acquisition of diluted turkey blood samples. EDTA-whole blood samples were diluted 1:250 in flow buffer. A) Cells were triple-stained with anti-CD15/61-FITC, anti-CD44-PE, and anti CD8-Cy5. G0: All unstained cells (in A) mainly erythrocytes, in B) mainly erythrocytes and thrombocytes, G1: $CD44^+$ cells, G1.1: granulocytes, G1.2: lymphocytes, G1.3: monocytes, G2: CD51/61+ cells, G2.1: thrombocytes. B) Cells were triple-stained with anti-MHC class II-PE, anti-CD4-FITC, and anti CD44-APC antibodies. G3: $CD44^+$ cells, G3.1: granulocytes, G4: MHC class II^+ and $CD44^+$ cells, G4.1: monocytes, G4.2: lymphocytes. Magnification = 1000×.

animal room and the interaction of both as fixed effects. Values of $p < 0.05$ were considered as significant. Numbers of monocytes as identified by size, granularity as well as binding of anti-MHC class II or alternatively CD44 antibodies were compared by using the Wilcoxon's two-sample test. Statistical analysis was done with SAS, Version 7.1 (SAS Institute Inc., NC, USA) and Statistix 10 (Analytical Software, FL, USA).

3. Results

3.1. Establishment of a flow cytometric based method for the analysis of whole blood samples of turkeys

A combination of different antibodies was used to detect different immune cell populations in turkey whole blood samples by flow cytometric analysis. By differentiation of antibody-labelled cells based on their forward and sideward scatter characteristics the following cell populations were identified: dead cells, erythrocytes, thrombocytes, monocytes, and lymphocytes, which could be further differentiated into

CD4$^+$, CD8$^+$ and MHC class II$^+$ cells. The mouse anti-chicken CD44 antibodies cross-reacted with turkey blood cells and in combination with the applied gating strategies allowed the detection of monocytes, granulocytes and lymphocytes and could therefore be used as a pan-leukocyte-like marker (Fig. 2). The mouse anti-human CD51/61 antibodies cross-reacted with turkey thrombocytes (Fig. 2).

The detected cell types were confirmed by fluorescence-activated cell sorting and subsequent microscopical evaluation of their morphological characteristics after Wright Giemsa staining (Fig. 2, Pendl, 2008). The CD44$^+$ population (Fig. 2, G1) consisted of heterophils (Fig. 2, G1.1), lymphocytes (Fig. 2, G1.2), and the G1.3 cells. The latter included 80% monocytes, but also 9.2% dead cells, 4.8% erythrocytes, 6% lymphocytes in 500 counted cells. The CD51/61$^+$ population was confirmed to consist of thrombocytes (Fig. 2, G2.1). Double stained MHC class II$^+$ and CD44$^+$ cells separated in two subpopulations by size and granularity, which included lymphocytes (Fig. 2, G4.2) and monocytes (Fig. 2, G4.1). The MHC class II-negative CD44$^+$ cells (Fig. 2, G3) consisted almost entirely of granulocytes (G3.1), but also few lymphocytes and monocytes (not shown).

Overall, monocyte cell numbers either detected by the anti-MHC class-II or by the anti-CD44-antibodies and subsequent gating were comparable (Suppl. Fig. 2). Slight variations were observed at three of five investigated time points with significant less MHC class II + cells compared to the CD44 + cells confirming the microscopical analysis of the G1.3 cells as indicated above.

3.2. Age, trial and animal room as influencing factors on blood cell concentrations in turkeys

Three trials with female turkeys were conducted with one (first trial) or two animal rooms (second and third trial) with 22 birds each, and whole blood samples were collected at different days post hatch and subsequently analyzed by automated cell counting. No signs of clinical diseases were observed during all three trials. In all three trials, blood cell concentrations changed between different age groups and increased up to 43–60 dph (Fig. 3). Monocyte concentrations showed a parabolic course with peak concentrations at 43 days post hatch. The most significant increase of MHC class II lymphocyte concentrations was observed between day one and 23 post hatch. CD4 + lymphocyte concentrations increased almost linearly up to day 60 post hatch. Thrombocyte concentrations also showed a parabolic development with increasing age with a peak at day 60 post hatch. Granulocyte concentrations were only evaluated in the third trial. Also these concentrations increased over time up to 8074 cells/µl on day 60. Between days 60 and 88 post hatch granulocyte concentrations showed a significant decrease ($p = 0.047$) again (Fig. 4).

The strongest effect of age was observed for CD4$^+$ and MHC class II$^+$ lymphocytes with F values of 81.96 and 51.6, respectively, while the age effect was intermediate for monocytes and thrombocytes with F values of 24.73 and 39.6 respectively, and the lowest for CD8$^+$ lymphocytes (F-value of 22.7).

The overall changes in concentrations over time were comparable between trials for all investigated cell types. But the absolute cell concentrations varied for most cell types significantly between trials ($p < 0.05$, Suppl. Table 1). The highest concentrations were observed for monocytes, thrombocytes, MHC class II$^+$ and CD4$^+$ lymphocytes in trial 2 or 3 and the lowest in the first trial, while this trend was reversed for CD8$^+$ lymphocytes (Fig. 3).

While trial effects were visible for all cell types, animal room effects, as evaluated in the second and third trial, were only detected in the third trial. At 43 days post hatch significant differences in cell concentrations were observed between animal rooms of the third trial for all lymphocytes populations, while CD44$^+$ granulocytes were comparable between the two animal rooms (Fig. 4). At all other time points, evaluated concentrations for all cell populations were comparable between animal rooms in the second trial as well as the third (data not shown).

The variation of blood cell concentrations between age groups and trials indicates that these factors have to be considered for the establishment of base line values for turkeys. Suggested ranges of base line values are presented in Table 2.

4. Discussion

The objective of this study was to establish a rapid and high precision tool for blood cell counts for turkeys corresponding to the method used for chicken (Seliger et al., 2012). Although the antibodies available to detect specifically turkey cell populations, are restricted, a combination of cross-reacting antibodies suitable for the identification of different immune cell populations was identified in this study. Our method allowed the differentiation of monocytes, three subgroups of lymphocytes, granulocytes, and thrombocytes when antibody staining was combined with a suitable gating strategy. Proportions of leucocytes calculated from the evaluated concentrations were comparable to proportions evaluated by manual counting of blood smears. Results were highly reproducible. This matches findings in chicken (Seliger et al., 2012).

Due to the lack of a turkey specific B-cell marker, B-cells were detected by MHC class II-expression in combination with gating on size and granularity to allow differentiation from monocytes. A differentiation of B-cells from other MHC class II$^+$ lymphocytes is not possible using the mentioned antibodies.

Based on sequence analysis it was hypothesized that the mouse anti-human CD51/61 monoclonal antibody may also bind to turkey thrombocytes as well as chicken thrombocytes (Viertlboeck and Gobel, 2007). In this study, we confirmed the cross-reactivity of this antibody with turkey thrombocytes. The total of 37,385 - 77,666 thrombocytes/µl whole blood of 60-day old turkeys correspond nicely to values determined for chicken, ranging from 45,000–72,000 cells/µl (Seliger, 2009). The anti-CD44-antibody was selected as a possible candidate for a panleukocyte-marker, since CD44 was reported to be expressed on most peripheral blood mononuclear cells in turkeys (Lawson et al., 2001; Meyerhoff et al., 2012). Vachon et al. (2006) demonstrated the detection of CD44 on mouse leukocytes, and indicated its role in phagocytosis. However, anti-CD44 antibodies may also bind to subpopulations of other cell types. CD44–expression was demonstrated on murine erythrocytes, but expression declined with increasing age (Chen et al., 2009). In our study, about 98.6% of the cells of whole blood samples were CD44-negative. These results provide circumstantial evidence that turkey erythrocytes are negative for CD44. Nevertheless, some false positive staining of erythrocytes may be detected. In our study we identified low percentages of CD44$^+$ erythrocytes contaminating the CD44$^+$ monocyte population, which might be either due to the expression of CD44 by younger erythrocytes (Chen et al., 2009) or a less defined separation of positive-stained and negative cell populations in the analysis steps.

It is known that the CD44-receptor in humans is mainly expressed on antigen-primed T- and B-cells but to a lesser extent on naïve lymphocytes (Camp et al., 1991). Consistent with these findings, the populations of monocytes, MHC class II$^+$, CD4$^+$ and CD8$^+$ lymphocytes showed a proportion of 32.5–47.5, 28.0–57.5, 0.6 − 0.8 and 9.7–18.4 % of CD44-negative cells. We may speculate that the CD44-negative cloud not only consists of erythrocytes but possibly also of naïve stages of lymphocytes. Therefore, a combination of CD44 and monocyte- or lymphocyte-markers is recommended to produce a more precise blood cell concentrations for whole blood samples of turkeys.

The application of a staining protocol with anti-CD44 as well as anti-MHC class II- antibodies allowed a reliable detection of monocytes. Concentrations may be less variable with this double staining approach compared to a staining with only one of the antibodies, although concentrations were fairly comparable based on our investigations. Furthermore, the use of anti-CD44 antibodies in combination with

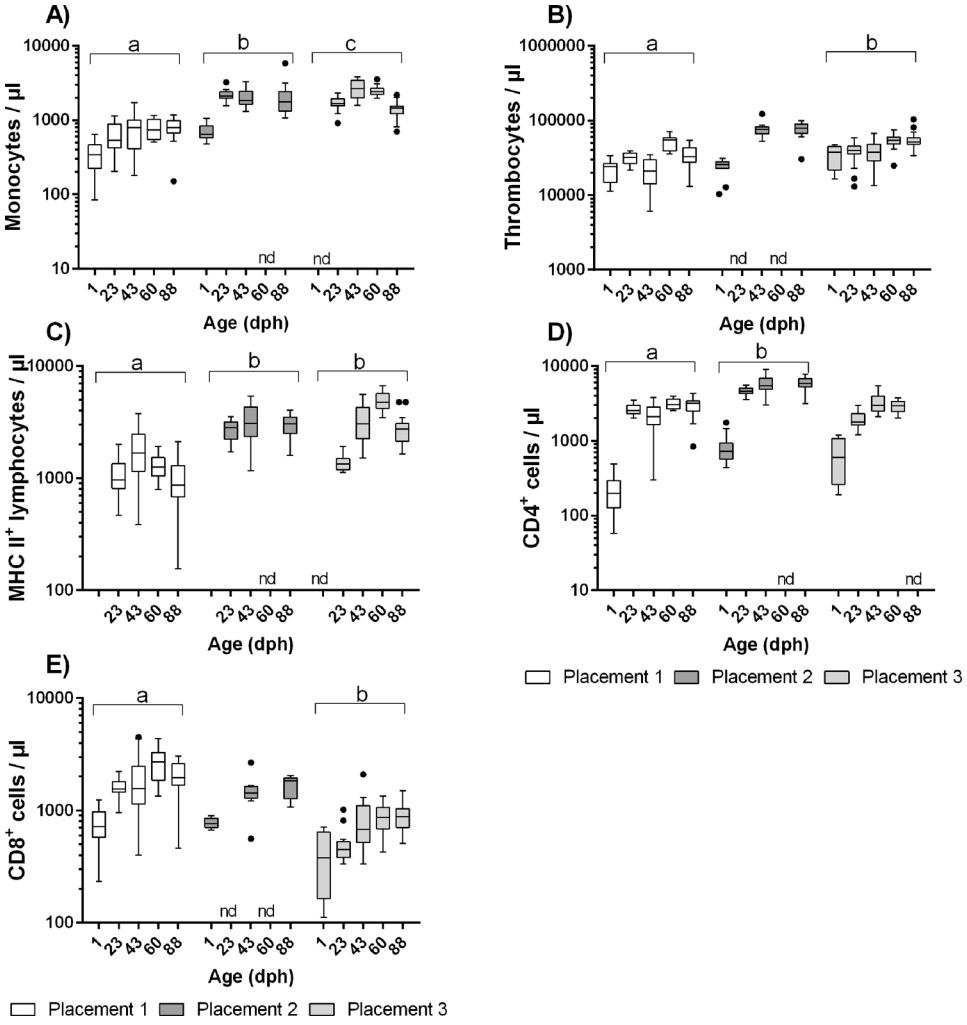

Fig. 3. Comparison of whole blood cell concentrations among all three trials. Concentrations of cells/populations are presented in Tukey box-plots (box includes second and third quartiles, horizontal line displays median, whiskers include values within 1.5 interquartile range, dots represent outliers). nd = not done. Significant differences among trials due to mixed models including age and trial as main factors for each cell type are indicated by letters ($p < 0.05$). A) monocytes, B) thrombocytes, C) MHC class II^+ lymphocytes, D) $CD4^+$ lymphocytes and E) $CD8^+$ lymphocytes. n = 22 birds/trial (animals of group 1 of the second and third trial were included).

forward and sideward scatter characteristic allowed for the first time the identification of turkey granulocytes, as shown in the third trial. This newly established automated whole blood counting method, combining different combinations of antibodies and gating strategies, opens up new possibilities now for research and clinical evaluation of turkey blood cell populations.

Generally blood cell concentrations may differ if different methods are applied. These may include microscopic based counting of leukocytes in blood smears, flow cytometric analysis of leukocyte proportions in suspensions of isolated leucocytes or leucocyte concentrations in whole blood. Values may also vary between laboratories and animal species, which has to be considered for the comparisons of our results with previously published data. We used the established flow cytometric analysis method to compare concentrations of leukocytes in whole blood between different age groups of female turkeys during a total of three trials, when birds were housed either in only one (first trial) or two different animal rooms (second and third trial). Age was clearly identified as an influencing factor. While for most cell types concentrations increased over time in all trials, peak concentrations at the respective time points varied between trials significantly ($P < 0.05$). This variation was observed for all cell types. Animal room effects were less clear and only observed in one trial at one time point. The reason for this variation at this time point is not clear, clinical disease or additional stress was not recorded, but certainly a subclinical event cannot be excluded, which may have modified cell numbers in one animal room but not the other. Overall, by considering age and trial as influencing factors, we suggest base line values for whole blood cell concentrations for different age groups of female turkeys.

Interestingly, monocyte concentrations did not vary significantly in chickens between one and 51 days post hatch (Seliger et al., 2012), which was not confirmed for turkeys in our study. The concentrations of MHC class II^+ monocytes increased between one and 43 days post hatch in turkeys (Fig. 3), suggesting species specific differences in the development of the immune system (Fairbrother and O'Loughlin, 1990).

Thrombocyte numbers 100x microscopic fields of mallard blood smears increased from day five until day 18 post hatch, and did not show significant changes thereafter (Fairbrother and O'Loughlin, 1990). These results are in agreement with our findings in turkeys, where we detected a clear increase in thrombocyte numbers between 1–23 dph and 43–88 dph.

In former studies, no significant changes were observed in the proportion of $CD4^+$ as well as $CD8^+$ cells in isolated peripheral turkey blood mononuclear cells comparing birds at the age of 21 and 56 days post hatch by flow cytometric analysis (Suresh et al., 1993). On the other hand, a steady increase of $CD4^+$ as well as CD8+ cell concentrations was detected by flow cytometric analysis of chicken whole blood collected between 20 to 110 days post hatch (Burgess and Davison, 1999). Our data coincides with the whole blood analysis of chickens suggesting a possible effect of the method using whole blood versus isolated leukocytes, when possible age effects may be more difficult to detect. In chicken whole blood samples initially low concentrations of B-cells were observed, which increased from 21 to 28 dph, remained on a plateau until 49–51 dph (Burgess and Davison,

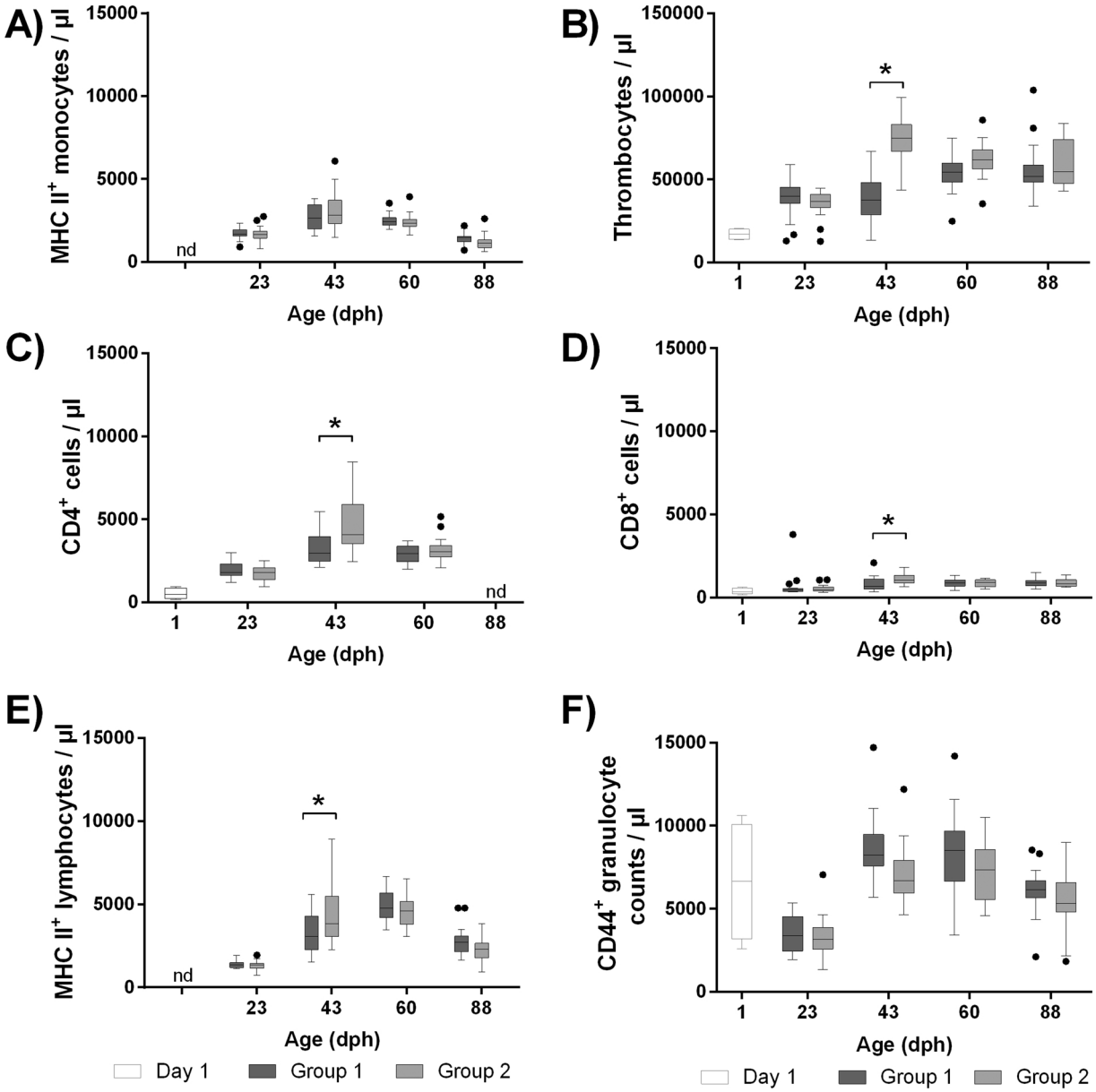

Fig. 4. Comparison of different leukocyte concentrations between animal rooms within the third trial. Cells were differentiated by different gating schemes into MHC class II^+ monocytes (A), thrombocytes (B), $CD4^+$ cells (C), $CD8^+$ cells (D), MHC class II^+ lymphocytes (E), and $CD44^+$ granulocytes (F). Average cell concentrations for all birds/animal room (n = 22/animal room) are presented in Tukey box-plots (box includes second and third quartiles, horizontal line displays median, whiskers include values within 1.5 interquartile range, dots represent outliers). Asterisks indicates significant differences between the two groups ($p < 0.05$). dph = days post hatch. Day 1 is presented as one group, as the birds had not been separated at this time point into the two animal rooms.

Table 2
Baseline values for turkey blood cell subpopulation concentrations per µl whole blood.

	Age (dph)					
Cell type	1	23	43	60	88	Total range of all investigated age groups
MHC class II^+ lymphocytes[a]	208 – 2606	657 – 3386	1028 – 5598	1139 – 6305	711 – 4518	468 – 5428
$CD4^+$ T-cells[a]	69 – 1418	1199 – 5270	1565 – 7608	2089 – 7284	2215 – 7768	249 – 6940
$CD8^+$ T-cells[a]	234 – 1106	337 – 1916	420 – 2679	541 – 3237	619 – 2699	382 – 2647
MHC class II^+ monocytes[a]	182 – 1373	377 – 2548	401 – 3784	678 – 3070	667 – 2603	351 – 3224
$CD51/61^+$ thrombocytes[a]	11,273 – 33,283	20,100 – 46,274	13,565 – 92,096	37,385 – 77,666	27,661 – 91,658	14,750 – 85,206
$CD44^+$ granulocytes[b]	2610 – 10,631	1960 – 5,267	5361 – 11,047	5256 – 11,586	2171 – 8,546	2162 – 11,002
$CD44^+$ lymphocytes[b]	830 – 6203	2809 – 5522	6495 – 14,419	7829 – 12,416	7021 – 12,383	3242 – 12,897

[a] Values were obtained from 66 animals (group 1 of trials 1–3).
[b] Values were obtained from 44 animals (group 1 and 2 of the third trial). 5% and 95% percentiles are calculated considering all animals of all trials.

1999; Seliger et al., 2012), followed by a transient drop at day 70 (Burgess and Davison, 1999; Seliger et al., 2012). This data are in agreement with our study showing only low numbers of MHC class II^+ lymphocyte, suggested to be mainly B-lymphocytes, early after hatch and an increase with peak numbers between 43 (first trial) and 60 (third trial) days post hatch, followed by a subsequent drop. Mallard leukocytes were evaluated via microscopic counts, and an increase in the proportions of granulocytes, was observed between days 42 and 60 post hatch (Fairbrother and O'Loughlin, 1990). Also, in our study we detected an increase in granulocyte concentrations in turkeys between days 43 and 60 post hatch, which suggests more evolutionary conserved pattern in the development of granulocyte concentrations with increasing age (Maxwell and Robertson, 1998).

The variations in concentrations of all investigated cell populations between trials suggest that the environment may have a significant influence (Richter et al., 2009). In our study we used feed from the same feed mill, the same lighting, and temperature protocol as well as the same hybrid line of female turkeys in all three trials. Therefore, other influencing factors contributing to these variations may need to be considered. Some variation between the first two trails and the last trial may due to the different sources of birds, as these were obtained from different hatcheries. Despite indoor housing, the season may have influenced cell concentrations, as seasonal effects were shown for many animal species (reviewed by Nelson, 2004; Nelson and Demas, 1996) including poultry (Dawkins et al., 2004). The magnitude of immune reactions in free-living skylarks decreased during summer and increased during winter (Hahn et al., 2015). Only few studies investigated the seasonal patterns in captive animals housed in constant environments, and these focused more on hormonal changes such as corticosterone or melatonin (Nelson and Drazen, 2000; Piesiewicz et al., 2012; Romero and Remage-Healey, 2000), and not on blood cell concentrations, even though these hormones are suggested to modulate immune functions. Chickens examined in the northern hemisphere, showed a reduced proliferation rate of peripheral blood leukocytes in August, October and December compared to the time between February to April (Sander, 1995). In our second (July - September) and third trial (April – June), an increase in CD8+ and CD4+ T-cell concentrations was observed from spring to summer, matching the higher leukocyte proliferation ratesin animals housed under constant environmental conditions (Sander, 1995). Under natural conditions the opposite trend was found (Moller et al., 2003). Our results also match observations made in studies of humans, where a seasonal pattern was shown, including an increase in the numbers of $CD4^+$ cell concentrations during spring, summer, and late autumn, while a decrease of lymphocyte concentrations was observed during winter (Broadbent, 2011). Therefore, the variations between trials in this study may at least be partially based on seasonal influences, which have to be investigated further.

Other factors, which may have contributed to trial variations can be method related as different antibody batches and varying antibody-concentrations were used between the first trial and the second and third trials. These variations often cannot fully be avoided especially if repeat experiments are part of a study, which may take place at different time points. Therefore, it is important to consider this variation for the establishment of baseline values and the subsequent interpretation of data.

Another factor influencing the total concentration of cells is the administered gating strategy during the flow cytometric analysis of the data. Manually drawn gates are mainly influenced by the examiner's intuition and cannot be considered a standardized method (Lo et al., 2008). In this study though the same examiner analysed the samples, although slight variations between trials cannot be excluded. Changes of cell size and granularity due to age of the birds did not allow a common gate for all samples at all time points. But the same gate was applied to samples of one age group, which subsequently may have led to more variation between birds and a higher standard deviation.

Considering possible influencing factors including age and variations between trials, we suggest for each investigated cell population an age-related baseline value range. These are the first baseline values available for flow cytometric evaluation of leucocyte concentrations in whole blood sample of turkeys. The so far published cell concentrations are based on samples collected from four month old wild turkeys and were determined microscopically as well as via hemocytometric counting (Bounous et al., 2000). In our study, in 88-day old turkeys an average of 1465 monocytes/µl blood with a range of 667–2603 monocytes/µl was determined, which is nearly in agreement with the formerly published range of 0–3900 monocytes/µl, yet our range is narrower. The similar appearance of monocytes and lymphocytes may lead to false negative counts microscopically. For granulocytes, we detected lower values with 2171–8546 cells/µl as compared to previously published concentrations of 4000 – 27,600 granulocytes/µl (Bounous et al., 2000). Differences in age (dos Santos Schmidt et al., 2009) or method may be responsible for these findings.

Studies in wild turkeys determined 4200 – 34,300 lymphocytes/µl whole blood, while in our trials the range of total lymphocytes was narrower with 7021 - 12,383 cells/µl (Bounous et al., 2000). Narrower ranges in monocyte and lymphocyte concentrations might be due to less individual variation of inbred B.U.T. 6 turkeys in comparison to wild turkeys (*Meleagris gallopavo silvestris*) or lower standard deviation in concentrations obtained by flow cytometry compared to manual counted proportions as also demonstrated in this investigation.

No information on turkey thrombocyte numbers in whole blood had been published so far. About 37,000 ± 1200 thrombocytes/µl were detected by flow cytometry in chicken whole blood samples (Seliger, 2009), and a range from 3000 to 33,000 cells/µl was published based on microscopic counting (Samour, 2008). With our method we determined a range of 14,750 – 85,206 thrombocytes/µl suggesting a broader range for turkeys considering all investigated age groups.

Overall, we successfully established an automated blood cell counting method in this study, which allowed the identification of baseline values for concentrations of circulating granulocytes, monocytes, thrombocytes, $CD4^+$ and $CD8^+$ T-cell populations as well as MHC class II^+ lymphocytes in healthy female B.U.T. 6 turkeys. This study clearly identified an age-related influence on cell concentrations, and additionally suggests further influencing non-infectious factors based on the detected variations between trials. More experiments need to be conducted to confirm possible seasonal patterns under in-house housing conditions. Our data may help to evaluate health conditions of turkeys under field conditions in the future. It has to be considered that the established baseline values are based on samples from female turkeys of a specific hybrid line. Genotype and gender effects (reviewed by Nunn et al., 2009) cannot be excluded. Previously it was demonstrated that proportions of lymphocyte and basophils as determined microscopically for bronze turkeys varied between male and female birds (dos Santos Schmidt et al., 2009). Therefore, also male birds as well as other genotypes have to be investigated in the future, and eventually baseline values be adjusted.

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Declaration of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.03.006.

References

Abelenda, M., Nava, M.P., Fernandez, A., Alonso, J.A., Alonso, J.C., Munozpulido, R., Bautista, L.M., Puerta, M.L., 1993. Blood values of common cranes (Grus-Grus) by age and season. Comp. Biochem. Phys. A 104, 575–578.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Altschul, S.F., Wootton, J.C., Gertz, E.M., Agarwala, R., Morgulis, A., Schaffer, A.A., Yu, Y.K., 2005. Protein database searches using compositionally adjusted substitution matrices. FEBS J. 272, 5101–5109.

Bounous, D.I., Wyatt, R.D., Gibbs, P.S., Kilburn, J., Quist, C.F., 2000. Normal hematologic and serum biochemical reference intervals for juvenile wild turkeys. J. Wildl. Dis. 36, 393–396.

Broadbent, S., 2011. Seasonal changes in haematology, lymphocyte transferrin receptors and intracellular iron in Ironman triathletes and untrained men. Eur. J. Appl. Physiol. 111, 93–100.

Burgess, S.C., Davison, T.F., 1999. Counting absolute numbers of specific leukocyte subpopulations in avian whole blood using a single-step flow cytometric technique: comparison of two inbred lines of chickens. J. Immunol. Methods 227, 169–176.

Camp, R.L., Kraus, T.A., Birkeland, M.L., Puré, E., 1991. High levels of CD44 expression distinguish virgin from antigen-primed B cells. J. Exp. Med. 173, 763–766.

Chen, K., Liu, J., Heck, S., Chasis, J.A., An, X., Mohandas, N., 2009. Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. Proc. Natl. Acad. Sci. 106, 17413–17418.

Dalgaard, T.S., Norup, L.R., Pedersen, A.R., Handberg, K.J., Jorgensen, P.H., Juul-Madsen, H.R., 2010. Flow cytometric assessment of chicken T cell-mediated immune responses after Newcastle disease virus vaccination and challenge. Vaccine 28, 4506–4514.

Dawkins, M.S., Donnelly, C.A., Jones, T.A., 2004. Chicken welfare is influenced more by housing conditions than by stocking density. Nature 427, 342.

dos Santos Schmidt, E.M., Paulillo34, A.C., Martins25, G.R.V., Lapera25, I.M., Testi, A.J.P., Junior, L.N., Denadai26, J., Fagliari, J.J., FAPESP-Brazil, B., 2009. Hematology of the bronze turkey (Meleagris gallopavo): variations with age and gender. Int. J. Poult. Sci. 8, 752–754.

Fairbrother, A., O'Loughlin, D., 1990. Differential white blood cell values of the mallard (Anas platyrhynchos) across different ages and reproductive states. J. Wildl. Dis. 26, 78–82.

Hahn, T.P., Brazeal, K.R., Schultz, E.M., Chmura, H.E., Cornelius, J.M., Watts, H.E., MacDougall-Shackleton, S.A., 2015. Chapter 36 - Annual Schedules A2 - Scanes, Colin G Sturkie's Avian Physiology, sixth edition. Academic Press, San Diego, pp. 847–867.

Harrison, G.J., Lightfoot, T., 2006. Chapter 22 – Diagnostic Value of Hematology Clinical Avian Medicine. Spix Publishing, pp. 587–609.

Hartman, G.D., Duggan, M.E., 2000. Alpha(v)beta(3) integrin antagonists as inhibitors of bone resorption. Expert Opin. Investig. Drugs 9, 1281–1291.

Kameka, A.M., Haddadi, S., Jamaldeen, F.J., Moinul, P., He, X.T., Nawazdeen, F.H.P., Bonfield, S., Sharif, S., Van Rooijen, N., Abdul-Careem, M.F., 2014. Clodronate treatment significantly depletes macrophages in chickens. Can. J. Vet. Res. 78, 274–282.

Lawson, S., Rothwell, L., Lambrecht, B., Howes, K., Venugopal, K., Kaiser, P., 2001. Turkey and chicken interferon-gamma, which share high sequence identity, are biologically cross-reactive. Dev. Comp. Immunol. 25, 69–82.

Li, Z., Nestor, K.E., Saif, Y.M., Fan, Z., Luhtala, M., Vainio, O., 1999. Cross-reactive anti-chicken CD4 and CD8 monoclonal antibodies suggest polymorphism of the turkey CD8alpha molecule. Poult. Sci. 78, 1526–1531.

Lo, K., Brinkman, R.R., Gottardo, R., 2008. Automated gating of flow cytometry data via robust model-based clustering. Cytometry A 73, 321–332.

Makeri, H.K., Ayo, J.O., Aluwong, T., Minka, N.S., 2017. Daily rhythms of blood parameters in broiler chickens reared under tropical climate conditions. J. Circadian Rhythms 15.

Maxwell, M., Robertson, G., 1998. The avian heterophil leukocyte: a review. Worlds Poult. Sci. J. 54, 155–178.

Meyerhoff, R.R., Ali, R.A., Liu, K., Huang, G.Q., Koci, M.D., 2012. Comprehensive analysis of commercially available mouse antichicken monoclonal antibodies for cross-reactivity with peripheral blood leukocytes from commercial turkeys. Poult. Sci. 91, 383–392.

Moller, A.P., Erritzoe, J., Saino, N., 2003. Seasonal changes in immune response and parasite impact on hosts. Am. Nat. 161, 657–671.

Nelson, R.J., 2004. Seasonal immune function and sickness responses. Trends Immunol. 25, 187–192.

Nelson, R.J., Demas, G.E., 1996. Seasonal changes in immune function. Q. Rev. Biol. 71, 511–548.

Nelson, R.J., Drazen, D.L., 2000. Melatonin mediates seasonal changes in immune function. Ann. N. Y. Acad. Sci. 917, 404–415.

Nunn, C.L., Lindenfors, P., Pursall, E.R., Rolff, J., 2009. On sexual dimorphism in immune function. Philos. Trans. R. Soc. Lond. B Biol. Sci. 364, 61–69.

Piesiewicz, A., Kedzierska, U., Podobas, E., Adamska, I., Zuzewicz, K., Majewski, P., 2012. Season-dependent postembryonic maturation of the diurnal rhythm of melatonin biosynthesis in the chicken pineal gland. Chronobiol. Int. 29, 1227–1238.

Richter, S.H., Garner, J.P., Würbel, H., 2009. Environmental standardization: cure or cause of poor reproducibility in animal experiments? Nat. Methods 6, 257.

Romero, L.M., Remage-Healey, L., 2000. Daily and seasonal variation in response to stress in captive starlings (Sturnus vulgaris): corticosterone. Gen. Comp. Endocrinol. 119, 52–59.

Samour, J., 2008. Hematology analyses. Chapter 3. Avian Medicine. Mosby/Elsevier, London, pp. 46–48.

Sander, I., 1995. Untersuchungen zum Leukozytentransformationstest unter Verwendung von 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazoliumbromid (MTT) an peripheren Blutleukozyten des Huhnes (Gallus gallus domesticus). Tierärztliche Hochschule Hannover, Hannover.

Seliger, C., 2009. Entwicklung eines durchflusszytometrischen Verfahrens zur Bestimmung der Gesamtleukozytenzahl und Thrombozytenzahl sowie zur Leukozytendifferenzierung beim Huhn. Ludwig-Maximilians-Universität, München.

Seliger, C., Schaerer, B., Kohn, M., Pendl, H., Weigend, S., Kaspers, B., Hartle, S., 2012. A rapid high-precision flow cytometry based technique for total white blood cell counting in chickens. Vet. Immunol. Immunopathol. 145, 86–99.

Suresh, M., Sharma, J.M., Belzer, S.W., 1993. Studies on lymphocyte subpopulations and the effect of age on immune competence in turkeys. Dev. Comp. Immunol. 17, 525–535.

Vachon, E., Martin, R., Plumb, J., Kwok, V., Vandivier, R.W., Glogauer, M., Kapus, A., Wang, X., Chow, C.-W., Grinstein, S., Downey, G.P., 2006. CD44 is a phagocytic receptor. Blood 107, 4149–4158.

Viertlboeck, B.C., Gobel, T.W., 2007. Chicken thrombocytes express the CD51/CD61 integrin. Vet. Immunol. Immunopathol. 119, 137–141.

Zhang, Z., Schwartz, S., Wagner, L., Miller, W., 2000. A greedy algorithm for aligning DNA sequences. J. Comput. Biol. 7, 203–214.