

# Influence of environmental enrichment on circulating white blood cell counts and behavior of female turkeys

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**ABSTRACT** Under commercial conditions turkeys are housed in large groups in poorly structured environments. This leads to stress and subsequently to pecking and cannibalism. Environmental enrichment is suggested to reduce stress and feather pecking, thus leading to an increase of the overall flock health. However, the effect of increasing age on the use of enrichment elements and on the behavior repertoire as well as its correlation with health parameters has scarcely been studied. Therefore, our objective was to investigate the influence of environmental enrichment on the behavioral repertoire and on health parameters of turkeys. In 3 consecutive trials, female turkeys were housed up to 12 wk either in an unstructured (control group) or enriched environment (EE group) featuring elevated plateaus at different levels (“turkey tree”). Behavior parameters, clinical health, and immune parameters were determined at selected time points. The percentage of birds using the turkey tree increased with age up to 55 to 77% at 22 to 30 d post hatch (**dph**). Thereafter, the number of birds located on the turkey tree decreased to 25 to 32% at 73 to 79 dph. Feather pecking and fighting was significantly lower in the EE group compared to the control group in 2 and 3 trials, respectively ( $P < 0.05$ ). The integrity of feathers and integument, scored in the

head/neck, wing, and tail regions was repeatedly better in the EE birds compared to control birds at most investigated time points ( $P < 0.05$ ), suggesting a reduction in stress related aggression by the use of the turkey tree. Head pecking, running and flying activity, foraging, and preening were overall comparable between the EE and the control group ( $P > 0.05$ ). Humoral immunity as determined by vaccination-induced anti-Newcastle disease virus antibody titers was not affected by the turkey tree use. The flow cytometric evaluation of blood monocyte and T-lymphocyte numbers showed no repeatable difference between control and EE groups. Interestingly, compared to the control groups, EE birds displayed significantly higher numbers of circulating MHC class II<sup>+</sup> lymphocytes and lower numbers of thrombocytes at various time points compared to controls ( $P < 0.05$ ). This study provides clear evidence that environmental enrichment with plateaus not only leads to an altered behavioral repertoire but also modifies some of the investigated immune parameters, implying that EE may have a modulatory effect on turkeys’ immunity and overall fitness. Further studies are needed to understand the correlation between behavior and health parameters in birds more closely.

**Key words:** poultry, corticosterone, blood count, stress, feather pecking, elevated platforms, animal welfare

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## INTRODUCTION

Commercial turkeys are exposed to a variety of stress factors, which include social stress due to large group

sizes and high densities of birds. This may correlate with a higher incidence of birds suffering from lameness as well as head, wing or tail wounds due to increased pecking (El-Lethey et al., 2000; Marchewka et al., 2013; Marchewka et al., 2019). The signaling pathways for the mediation of stress effects include the elevation of serum corticosterone (Koolhaas et al., 1999). In comparison to beak trimmed poult, turkeys with intact beaks showed higher serum corticosterone levels (Schwean-Lardner et al., 2019), which may result in immunosuppression (Koutsos and Klasing, 2014; Shini et al., 2010). Administration of corticosterone via feed or water can

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be experimentally used to simulate stress in animals to determine stress effects (Post et al., 2003). In chickens, administration of corticosterone via feed resulted in a decrease of circulating lymphocytes, a lower antibody response against sheep red blood cells, and an increase in the number of circulating granulocytes (Gross et al., 1980). In addition, the administration of corticosterone led to decreased feed conversion ratio, reduced size of lymphoid organs, testis, and breast muscles (Gross et al., 1980). A housing system invoking less feather pecking and a reduction of the associated stress is desired to improve the birds' overall health.

Stress may be reduced by optimized management, feeding regime and housing conditions, including the implementation of environmental enrichment (EE) (Moe et al., 2010; Nazar and Marin, 2011; Dalton et al., 2013; Kjaer and Bessei, 2013). Quail provided with enrichment that encourages foraging, such as hanging bottle caps, colored wool, Velcro cylinders or structural enrichment (platforms), maintained high antibody titers against sheep red blood cells and a low heterophil/lymphocyte ratio (H/L ratio) compared to controls. This was even the case when the quail were restrained in a basket for 15 min daily as an applied stressor (Nazar and Marin, 2011) as long as enrichment was provided before and afterwards.

It is speculated that feather pecking may arise from misguided pecking desire (El-Lethey et al., 2000; Marks, 2017), therefore EE is mostly provided as an additional foraging stimuli, which includes pecking blocks, straw, or hay (Crowe and Forbes, 1999; Martrenchar et al., 2001; Kulke et al., 2017; Spindler et al., 2017). Crowe and Forbes (1999) compared four different EE approaches to influence the pecking behavior of turkeys. These included perches, straw, grain supplementation to the litter or objects. Interestingly, perches and objects were sufficient to significantly reduce the injurious pecking. The use of perches increased in weeks one to six before a drop in wk 7 post hatch was observed, which coincided with an

increase in pecking at enrichment objects (Crowe and Forbes, 1999).

We presumed environmentally enriched housing would not only affect the behavior but also physical parameters important for the overall health of the birds. The goal of this study was to determine the use of EE provided by elevated platforms ("turkey tree") over time, and its effect on body weight development, the behavior repertoire including feather and head pecking, running/flying, foraging, preening, and fighting repertoire and immune parameters in turkeys. The parameters were compared between control birds kept in a nonenriched environment, and birds housed in a room enriched with the turkey tree.

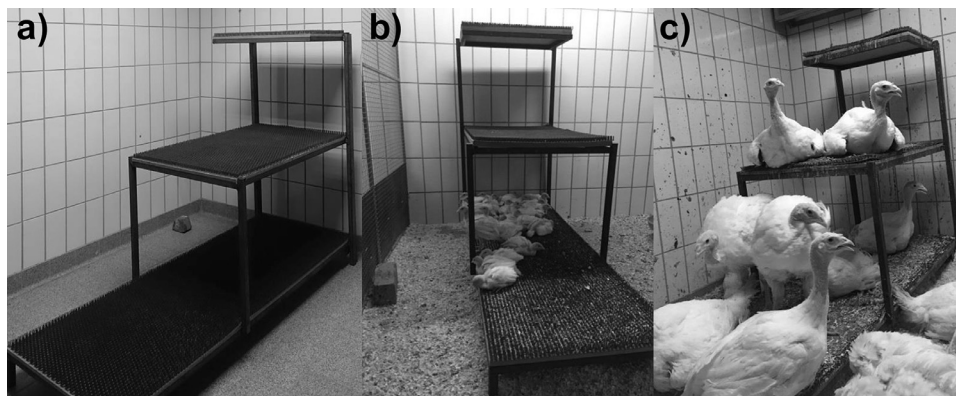
## MATERIALS AND METHODS

### Animals

Three animal trials with 44 poult/trial were conducted at the University of Veterinary Medicine, Hannover, Germany. Female 1-day-old B.U.T. 6 turkeys were either provided by Kartzfehn (Bösel, Germany) or Heidemark (Ahlhorn, Germany). All trials were approved by the Lower Saxony State Office for Consumer Protection and Food Safety (33.14-42502-04-15/1813). Handling times of the birds were kept as short as possible to reduce associated stress. The handling of the birds was performed in the dark to minimize escape behavior.

### Experimental Setup

Female turkey poults with intact beaks were randomly assigned to 2 groups (control; EE) of 22 birds each and individually marked with wing bands. The assessable space of each group was limited to 5.4 m<sup>2</sup> in concordance with the commercial housing conditions (Verband Deutscher Putenerzeuger, 2013). In trial 1 as well as 2, each group was placed in a separate room, which differed between trials. In trial 3, both groups



**Figure 1.** Environmental enrichment object ("turkey tree") provided for the EE – group. The lowest platform was located 0.1 m above floor level and measures were 0.6 m × 1.6 m (0.96 m<sup>2</sup>). The measures of the second level were 0.6 m × 1 m (0.6 m<sup>2</sup>) and of the third 0.6 m × 0.4 m (0.24 m<sup>2</sup>). Each distance between the middle level and top as well as bottom levels was 0.6 m. All levels are covered with Astro turf for poultry (Grass Tech Solutions, Louvain-la-Neuve, Belgium). The turkey tree before the start of the animal trial (A), at 14 (B) and at 71 d post hatches (C). Photos were taken between 10 AM and 12 AM. Abbreviation: EE, enriched environment.

were placed in separate pens of the same room. All groups were managed and handled by the same staff and kept under the identical temperature program, and lighting conditions including a light period from 5 AM to 9 PM. Birds were housed on wood shavings and provided with commercial turkey feed (P0, P2, and P3-turkey phase feeding, one feeder/group, Deutsche Tiernahrung Cremer GmbH & Co. KG, Regensburg, Germany) and water ad libitum (2 drinkers/group). One group served as an EE-deprived control. The second group was housed with EE composed of a steel framework with 3 plateaus covered with Astro turf poultry XPNP (Grass Tech Solutions, Louvain-la-Neuve, Belgium) on press boards. The EE, further on referred to as “turkey tree” (Figure 1), was designed to provide sitting areas and shelter at three different levels. The first platform was located 0.1 m, the second 0.7 m and the third 1.3 m above floor level. Measures of the lowest platform were 0.6 m × 1.6 m (0.96 m<sup>2</sup>). The second level measured 0.6 m × 1 m (0.6 m<sup>2</sup>), and the third level 0.6 m × 0.4 m (0.24 m<sup>2</sup>). Since it may be unrealistic under commercial conditions to allow all birds of a flock to sit on elevated platforms at the same time, the size of the tree in this study was chosen to allow a maximum of 15 birds to sit on at the end of the trial. The maximum possible number of turkeys sitting on the levels at the end of the trials was calculated based on the estimated weight/m<sup>2</sup> of the birds using the formula  $y = 5.90x^2 + 226.64x - 435.0$ , where  $x$  is the weight of the turkey in kg and  $y$  is the area in cm<sup>2</sup> (Ellerbrock and Knierim, 2002). Birds were housed for 88 d and monitored daily for their overall clinical health. An infection with *E. coli*, diagnosed in heart, liver and spleen of an animal, which deceased on d 7, was observed in the second trial. A resistance test showed susceptibility for enrofloxacin, a medication that was approved for treatment at the time of the study. To control further disease development, both groups were treated with enrofloxacin over 5 d (8 to 12 dph, Baytril, Bayer Vital GmbH, Leverkusen, Germany, 1 mL/1 drinking water). Birds were vaccinated against Newcastle disease (ND, AviPro ND LASOTA, Lohmann Animal Health GmbH & Co. KG, Cuxhaven, Germany) on d 14, 42, and 67 with a commercially available live vaccine according to standard vaccination programs for turkeys in the field. Each bird received the same dose orally by manual inoculation. Blood samples for flow cytometric analysis of whole blood and antibody in serum by ELISAs, were taken from the wing vein at the age of 23, 43, 60, and 88 d. Time needed for blood sampling ranged from 10 to 60 min/group depending on the age of the birds, and was performed between 7:30 AM and 2 PM. The time of blood sampling was recorded individually. Both animal groups were split in half and sacrificed on two consecutive days (d 87–88, further indicated as “d 88”), to be able to manage the follow-up laboratory investigations in a timely manner. Animals were stunned via electrocution (Schermer Kleintier-Betäubungsanlage, Karl Schermer GmbH & Co KG, Ettlingen, Germany) and blood was taken during subsequent exsanguination.

## Video Surveillance and Ethograms

All stables were video recorded daily over the total housing period from 5 AM to 10 PM, covering the entire light period, dim phase and 30 min of the dark phase. Video tapes were evaluated at five selected days (trial 1: d 8, 22, 37, 51, and 84; trial 2 and 3: d 16, 30, 45, 58, and 73). On these days no distracting factors such as handling procedures occurred, so the animals were completely undisturbed despite the routine daily health control. During each day of video analysis 8 different time points such as 6, 8, 10, 12 AM as well as 2, 4, 6, and 8 PM were evaluated. To evaluate the use of the turkey tree, the location (first, second, or third turkey tree level, anywhere else in the stable) and posture (standing or sitting) of all visible birds were noted on the first frame of each video sequence of the EE group. Subsequent video sequences of both groups were evaluated via one-zero scan sampling for performed behaviors of each animal/group within a video time interval of 20 s (Naguib, 2006). “Running/flying” (fast movement in one direction while flapping the wings), “foraging” (pecking at the environment except feeders and drinkers), and “preening” (rubbing the beak against the own body) (Ellerbrock, 2000; Martin et al., 2007) episodes were quantified. To quantify potentially injurious behavior, bird encounters involving “head pecking” (quick peck of one turkey at the featherless head-region of another turkey), “feather pecking” (peck of a turkey on any body part of another turkey excluding the head), or “fighting” (turkeys facing one another accompanied by pecking movements against the other or one turkey chasing another) were counted in the same time intervals. Nine time intervals of 20 s were scanned in a row adding up to an evaluation time of three minutes per time point (Ellerbrock, 2000; Martin et al., 2007).

## Evaluation of Plumage and Integument

To extend the data on observed pecking activity, we additionally included the observation of pecking lesions to allow comparisons among the birds. Plumage condition and skin lesions were scored thrice during trial 2 (d 14, 52, and 84) and five times during trial 3 (d 14, 32, 42, 53, and 67) following a modified scheme as published previously (Ellerbrock, 2000; Schulze Bisping, 2015). To be able to relate injuries and plumage damage to data obtained in the video analysis, the bird’s body was divided into 7 regions: head, neck, back, wings, tail, legs, and breast/abdomen. Each region was scored individually for plumage and skin condition using the score system indicated in Table 1 (Ellerbrock, 2000; Schulze Bisping, 2015). Animals were weighed and individually restrained to score each part of the body. In trial 1, the animals were only weighted on d 1, 14, 30, 58, and 88 post hatches. In trials 2 and 3, weighting was conducted together with the plumage and integument scoring.

**Table 1.** Scores used for description of plumage condition and skin lesions after Schulze Bisping (2015).

Score	Plumage condition		Skin lesion	
	Neck, back, wings	Tail	Head	Back, wings, tail
0	No feather missing	No feather missing	No injury <sup>1</sup>	No injury <sup>1</sup>
1	Single feathers missing	Few feathers missing	Injured <sup>1</sup> area <0.5 cm	Injured <sup>1</sup> area <2 cm
2	Area <2 cm is defeathered	Half of the feathers remaining	Injured <sup>1</sup> area 0.5–2 cm	Injured <sup>1</sup> area 2–8 cm
3	Area of 2–8 cm is defeathered	One third of the feathers remaining	Injured <sup>1</sup> area >2 cm	Injured <sup>1</sup> area >8 cm
4	Area >8 cm is defeathered	Less than five feathers remaining	<sup>2</sup>	<sup>2</sup>

<sup>1</sup>Injuries were defined as bleeding skin and visible hematomas or both.

<sup>2</sup>No further scores were defined in this trial as this led to the removal of the animal from the trial according to animal welfare criteria.

## Antibody Measurements by ELISA

Anti-Newcastle Disease virus antibody titers were determined using a commercially available ELISA-Kit (ProFlok NDV T, Zoetis, NJ) according to the manufacturer's instructions. Data are presented as antibody titers calculated according to the manufacturer's instruction.

## Blood Cell Counts

Whole blood cell composition was analyzed by flow cytometry. EDTA blood samples (20  $\mu$ L) were diluted 1:1,000 (trial 1) or 1:500 (trials 2, 3). Two triple stainings were applied to each blood sample. First triple staining included mouse anti-chicken CD8 $\alpha$ -Cy5 (clone 3-298, 0.63–1.25 mg/mL sample, Southern Biotech, Birmingham, AL), mouse anti-human CD51/61-FITC (clone 23C6, 0.2–0.4 mg/mL sample, Biolegend, San Diego, CA), and mouse anti-chicken MHC class II-PE (clone 2G11, 0.1 mg/mL sample, Southern Biotech). In trial 3, the mouse anti-chicken MHC II was replaced by mouse anti-chicken CD44-PE. Second triple staining included: mouse anti-chicken CD4-FITC (clone CT4, 0.05–0.07 mg/mL sample, Southern Biotech), mouse anti-chicken MHC class II-PE (clone 2G11, 0.1 mg/mL sample, Southern Biotech) and mouse anti-chicken CD8 $\alpha$ -Cy5 (clone 3-298, 0.63–1.25 mg/mL sample, Southern Biotech). In trial 3, the anti-chicken CD8 $\alpha$ -Cy5 antibody was exchanged for mouse anti-chicken CD44-APC (clone AV6, 0.08 mg/mL sample, Southern Biotech, Birmingham, AL). Cells were incubated with the antibody mix for 30 min on ice in the dark. To exclude dead cells from the counts, 5  $\mu$ L of 7-Aminoactinomycin D (7AAD, Biozol, Eching, Germany) were added to each sample. Samples were acquired and analyzed with the Accuri C6 flow cytometer (BD Sciences, Becton, Dickinson and Company, Franklin Lakes, NJ) and the FlowJo software (FlowJo Software, Tree Star, Ashland, OR), respectively. In addition to fluorescence intensities, cellular forward and sideward scatter characteristics were recorded (Seliger, 2009; Rubbenstroth et al., 2010; Seliger et al., 2012). While staining 1 and 2 both allowed the detection of CD8 T-cells and MHC II lymphocytes (trials 1 and 2) or granulocytes and CD 44 lymphocytes (trial 3), staining 1 additionally

provided information about thrombocyte numbers and staining 2 about CD4 T-cells. Results are presented as mean cell counts/mL blood per animal group and trial.

## Statistical Analysis

All behavior counts (head pecking, feather pecking, fighting, running/flying, foraging, and preening) were considered quantitative variables and analyzed by mixed model analysis. Due to different evaluation days, the first animal trial was analyzed separately. Age, group, day time, and the interactions of group and day time, group, and age, as well as group and trial were used as main factors. Plumage and integument scores were considered as qualitative variables and analyzed for each body region and animal trial separately using the Fisher's exact test. Antibody titers were compared between groups of each trial by using the Wilcoxon's rank-sum test. For each trial, blood cell counts of monocytes, thrombocytes, CD4<sup>+</sup>, CD8<sup>+</sup>, MHC class II<sup>+</sup> lymphocytes, and granulocytes were compared with general mixed models using age and group and the interaction of both as main factors. *P*-values of <0.05 were considered as significant. Because of varying parent flocks, brooders, batches of antibodies used for flow cytometry, and seasons during housing periods each trial was evaluated separately. Statistical analysis was performed using SAS, Version 7.1 (SAS Institute Inc., NC) and Statistix 10 (Analytical Software, FL).

## RESULTS

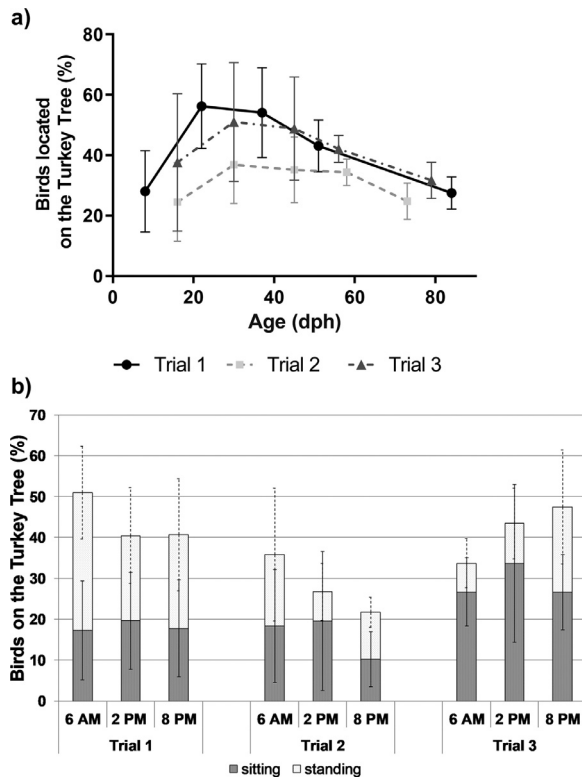
### Clinical Health and Weight Development

No clinical disease or mortality, which may have been specifically associated with EE, was observed in any of the trials. In trial 1, one bird died of injurious pecking (control group, 28 dph), and one bird was euthanized because of crop stasis (EE group, 71 dph). In trial 2, one bird died because of a bacterial infection with *E. coli*, which was confirmed at necropsy and subsequent microbiological evaluation (EE group, 7 dph). Another bird was euthanized in trial 2 because of a broken wing (EE group, 80 dph). The weight development was comparable between the groups in all trials and fit the Aviagen guidelines for B.U.T. 6 turkey hens.



## Perching Behavior

Slight variations between trials were observed not only in the overall use of the turkey tree but also in the distribution of birds sitting or standing on the tree at different times during the day. The lowest level was already used on d 1. The middle and upper level were used from 16 dph onward. At the end of the trials the turkeys weighted 8.5 (trial 2) – 9.9 (trial 1) kg. Therefore, the highest level could have been used by about 2 turkeys (9% of the group), the second level by five (22.7%) and the lowest level by eight to nine (36.3–40.9%) turkeys. Utilization of the turkey tree increased from about 20 to 40% at the beginning of the trial starting at 8 dph up to a peak of 55 to 77% at 22 to 30 dph (Figure 2A). A maximum of 8 birds were located on the middle level (37 dph) and 7 on the upper level (22 dph). Thereafter, the mean number of birds located on the whole tree decreased continuously to 5.5 to 7 birds (25–32% of the group) at the end of the trials. Three representable time points were chosen to compare the activity in the morning (6 AM), the middle of the day (2 PM) and evening (8 PM). No clear trend with regard to more sitting or more standing was found in two trials at most timepoints. The number of sitting birds on the tree were similar for all compared time points (Figure 2B). The numbers of birds standing on the tree varied more among the time points compared to birds sitting on the



**Figure 2.** Use of the turkey tree quantified by one-zero scan sampling at 8 time points/evaluation day. (A) Percentage of birds sitting or standing on the tree at different day times. For each day, time points (6 AM, 2 PM, 8 PM) of all age groups per trial were combined (means  $\pm$  SD). No statistical comparison of trials was done due to differing evaluation days in the 3 animal trials. (B) Average percentage of birds located on the tree at days post hatch (dph) (means  $\pm$  SD).  $n = 22$  birds / group.

tree. In trial 1, more birds stood on the tree than sat on it, and at 6 AM more birds were located on the tree than at 2 PM and 8 PM. In trials 2 and 3 the tree was used mainly for sitting. In trial 2 the use of the tree decreased with day time, while in trial 3 the usage increased over the day. Altogether, the tree was well accepted and used until the end of the housing period.

## Behavior

An overall low feather pecking activity was observed in all trials in both groups. Comparing all evaluated days, the peak of feather pecking activity was reached in all trials between 30 and 45 dph with an average of 0.1 to 0.4 feather pecking activities observed per bird within three minutes (Figure 3A). However, the statistical effect of age was not significant (trial 1:  $P = 0.35$ ;  $F = 1.13$ ; trial 2–3:  $P = 0.28$ ;  $F = 1.29$ ). In all trials, the overall feather pecking activity was significantly higher in the control group in comparison to the EE group (trial 1:  $P = 0.02$ ;  $F = 5.44$  (data not shown); trials 2–3:  $P = 0.01$ ;  $F = 7.04$ ; Figure 4) by looking at the summary of all investigated time points.

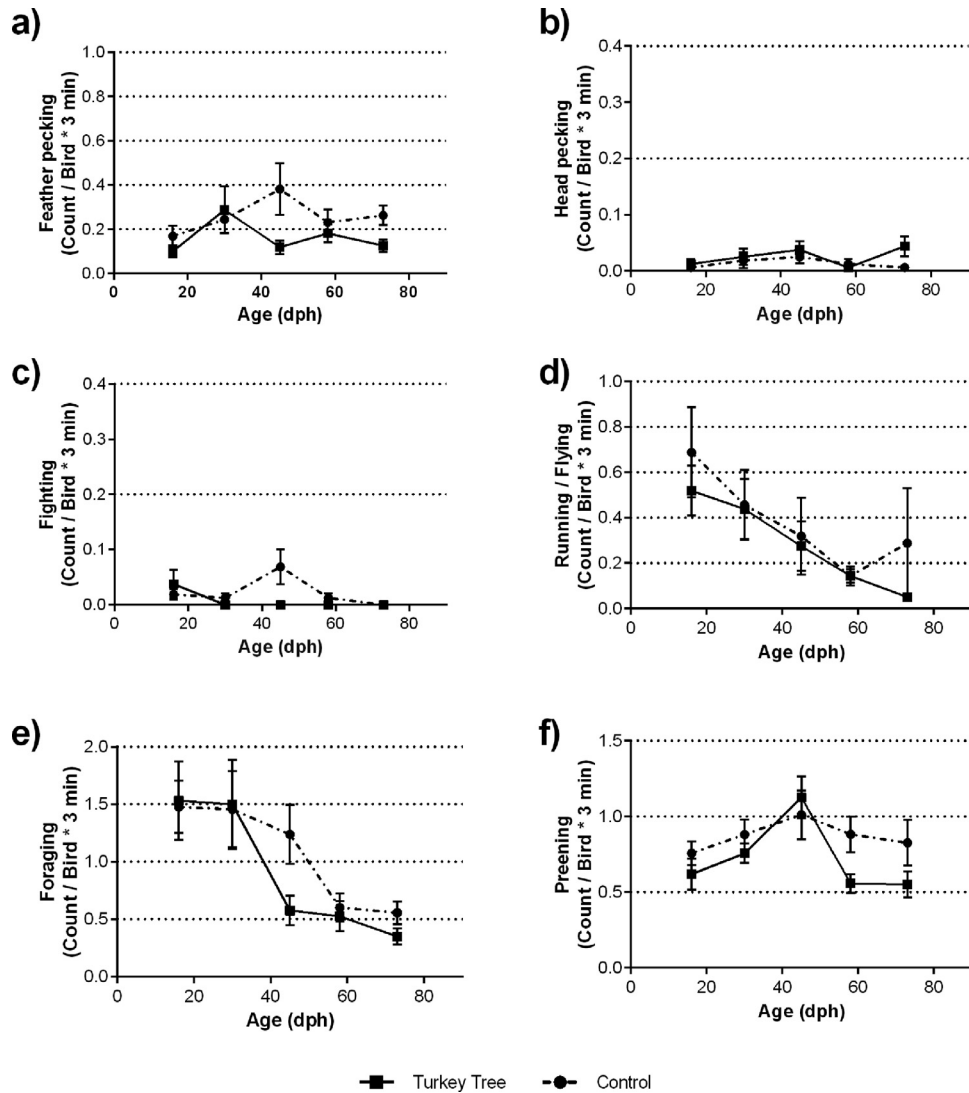
Head pecking activity was low and comparable between trials, the investigated time points, and groups (trial 1:  $P = 0.67$ ;  $F = 0.2$ ; trials 2–3:  $P = 0.19$ ;  $F = 1.7$ ; Figure 3B, Figure 4). A peak of head pecking activity was reached either at 51 dph (trial 1) or between 73 and 79 dph (trials 2 and 3) with an average of 0.02 to 0.07 pecking activities observed per bird within 3 min.

Fighting activity was low in all trials and was mainly observed in the control group. Age did not influence the frequency of fighting (trial 1:  $P = 0.78$ ;  $F = 0.4$ ; trial 2–3:  $P = 0.11$ ;  $F = 1.9$ ; Figure 3C) but showed a significant interaction with the group (trial 2:  $P = 0.049$ ;  $F = 2.5$ ) due to the fact that almost all counted fights took place in the control group on 45 dph.

Running and flying, which were counted as one activity, was low and decreased with the birds' age in all trials (trial 1:  $P < 0.0001$ ;  $F = 16.7$ ; trials 2–3:  $P = 0.01$ ;  $F = 4.1$ ; Figure 3D). During trials 2 and 3, significantly more running and flying activity was observed at 6 AM compared to most other time points ( $P = 0.01$ ;  $F = 2.7$ ) (data not shown). Running and flying activity was comparable between both groups (trial 1:  $P = 0.63$ ;  $F = 0.2$ ; trial 2–3:  $P = 0.26$ ;  $F = 1.3$ ; Figure 4).

The most commonly observed behavior was foraging (up to 5.2 times/ bird within 3 min, Figure 3E, Figure 4). We found an age-related decrease in the foraging activity during all trials (trial 1:  $P = 0.0002$ ;  $F = 6.8$ ; trials 2–3:  $P < 0.0001$ ;  $F = 11.7$ ; Figure 4). No clear influence of EE on foraging activity was observed. In trial 1, the EE group showed more foraging activity ( $P = 0.043$ ;  $F = 4.3$ ), in trials 2–3 this effect was not detected ( $P = 0.17$ ;  $F = 1.9$ ; Figure 3E).

Preening activity was counted up to 2.2 times/bird within 3 min. This activity was comparable between the EE and control group in trial 1 ( $P = 0.42$ ;  $F = 0.67$ ; Figure 4), but higher in the control group in comparison



**Figure 3.** Influence of EE on the behavior repertoire of turkeys at different time points (summary of trials 2 and 3). In trials 2 and 3 we investigated: feather pecking (A), head pecking (B), fighting (C), running/flying (D), foraging (E), and preening (F). Presented are mean counts  $\pm$  SEM assessed via one-zero scan sampling at 5 different days (16, 30, 45, 58, and 73 d post hatches). Dots represent means, which are calculated of the number of activities/bird of all total of eight time points/day.

to the EE group in trials 2 and 3 ( $P = 0.03$ ;  $F = 4.64$ ). In trials 2–3 preening increased up to day 45 (trial 2) or 56 (trial 3) and decreased thereafter (trials 2–3:  $P = 0.002$ ;  $F = 4.36$ ).

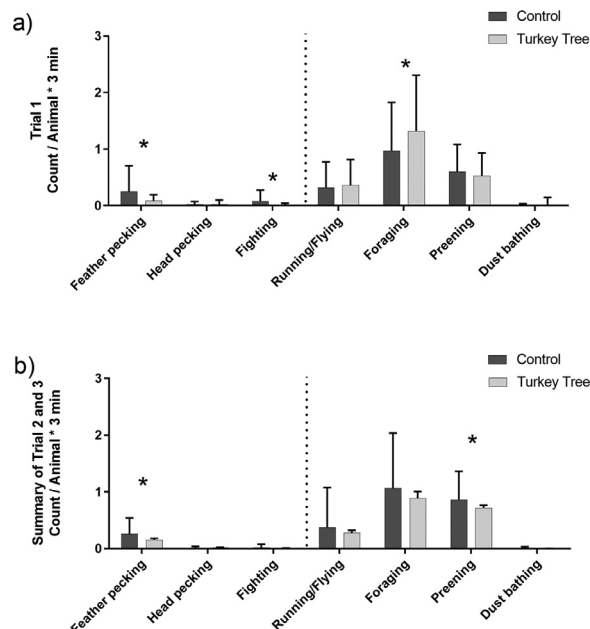
### Plumage, Integument

Feather loss was scored in the neck-, wing-, and tail-region at three time points during trial 2 and at 5 time points during trial 3 (Figure 5, Figure S1). In trial 2, the control group showed significantly higher plumage scores ( $P < 0.05$ ) in the wing region at 88 dph compared to the enrichment group (Figure S1c). In trial 3, this was observed at 3 time points in the neck region and wing region as well as at 4 time points in the tail region (Figure 5). At 14 dph in trial 2, EE birds showed a significantly higher feather score in the neck region compared to the control group.

The control group showed higher injury scores in comparison to the EE group (trials 2 and 3) in various body regions at different time points in birds older than 14 dph (trial 3) and 52 dph (trial 2) ( $P < 0.05$ ). A higher injury score was observed in the head region of the EE birds in comparison to the control group at 88 dph (trial 2) and at 14 dph (trial 3) (Figure 5, Figure S1).

### Antibody Titers

In all 3 trials, ND-live vaccination induced a detectable seroconversion. No repeatable differences were observed between the groups within the trials. Booster vaccination induced an increase in antibody levels at subsequent time points of serum collection in all groups (Figure 6).



**Figure 4.** Influence of EE on the behavior repertoire of turkeys. Behaviors were counted via one-zero scan sampling and divided by the number of observed birds to calculate the average number of observations/bird in 3 minutes. Presented are the behavior counts/bird of the EE-group (turkey tree) compared to the control group, evaluated within the first (A), second (B) and third animal trial. Bars represent means calculated for all evaluated days (trial 1: 8, 22, 37, 51, and 84 dph; trial 2 and 3: 16, 30, 45, 58, and 73 dph) over all 8 time points and divided by the number of animals observed in the video sequence to obtain an average count/bird. Pecking behaviors are presented left of the dotted line, noninjurious behaviors on the right. Error bars indicate standard deviation. Asterisks indicate significant differences between the groups by 3-factorial ANOVA ( $P < 0.05$ ). Abbreviation: EE, enriched environment.

### Flow Cytometric Analysis of Different Circulating Immune Cell Populations

Whole blood cell counts were determined at 23, 43, 60, and 88 d post hatch. Overall blood thrombocyte numbers were highest in trial 2 (Figure 7C). Numbers increased with age up to 43 dph (trial 3) and 60 dph (trial 1). Compared to EE birds, control birds showed significantly higher thrombocyte counts at 43 dph ( $P < 0.03$ , trial 2 and 3) and 60 dph ( $P < 0.0001$ , trial 1).

Blood monocyte numbers varied between trials with highest numbers in trials 2 and 3 (up to 2,908 cells/ $\mu\text{L}$ ) compared to trial 1. Age significantly affected the monocyte numbers in trial 3. Concentrations increased up to 43 dph. Afterward, a continuous decrease until 88 dph was observed. Significant differences between EE and control birds were detected at 23 dph ( $P = 0.01$ , trial 1) and 43 dph ( $P = 0.02$ , trial 3) but not at other time points or trial 2 (Figure 7D).

MHC class II<sup>+</sup> lymphocyte numbers increased up to 43 (trial 1, 2) or 60 dph (trial 3) with a subsequent decrease in all trials (Figure 8). The EE groups showed significantly higher counts compared to the control birds at 23 ( $P = 0.04$ , trial 2), 60 ( $P = 0.047$ , trial 3), and 88 dph ( $P = 0.002$ , trial 1;  $P = 0.003$ , trial 3).

Circulating CD4<sup>+</sup> lymphocyte numbers were lowest in trial 1 (max. of 3,045 cells/ $\mu\text{L}$  at 88 dph) and highest in trial 2 (up to 5,662 cells/ $\mu\text{L}$ , 88 dph). Cell numbers were significantly affected by age in all 3 trials ( $P < 0.0001$ ). In trials 1 and 2, numbers increased with age. In trial 3, the peak was reached at 43 dph and thereafter numbers decreased again. CD4<sup>+</sup> lymphocyte numbers were significantly higher in the control group on 43 ( $P < 0.0001$ , trial 2) and 88 dph ( $P = 0.04$ , trial 2) and lower at 60 dph ( $P = 0.04$ , trial 3) compared to the EE group ( $P < 0.05$ ; Figure 8). At the other time points cell numbers were comparable between the groups.

CD8<sup>+</sup> blood lymphocyte numbers were highest in trial 1 (Figure 8C) and increased with age in all 3 trials up to 43 to 60 dph. EE and control birds showed no repeatable significant differences in means or variation pattern ( $P > 0.05$ ).

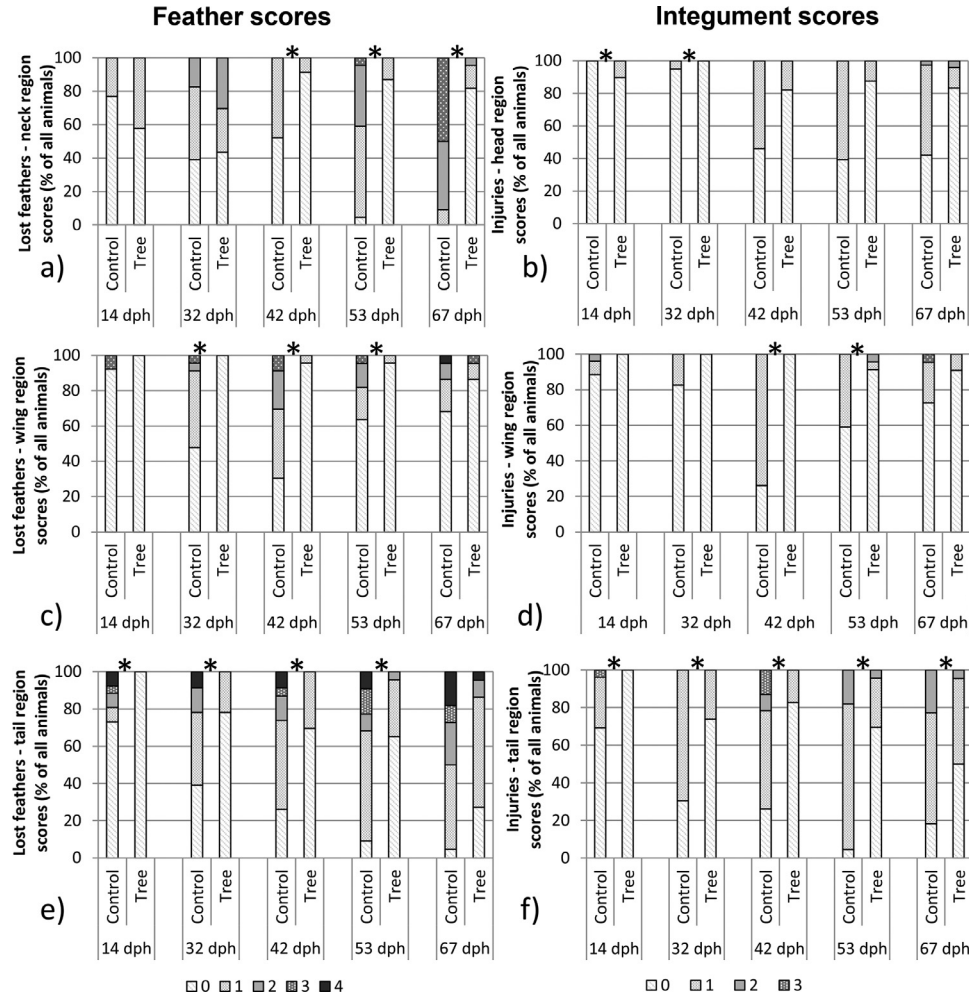
## DISCUSSION

### Clinical Health and Weight Development

In this study, we examined if environmental enrichment affects physiological parameters as well as the behavior of turkeys. Therefore, we housed 2 groups of 22 turkeys, one of which was provided with a 3-level turkey tree, and recorded bird behavior, condition of feathers and integument, Newcastle Disease antibody titers after vaccination as well as blood cell counts. The trial was repeated three times. The used EE did not have any negative effect on turkey health or performance. In contrast to particulate EE, such as straw, wood or other biological substances, which pose the risk of additional microbial exposure (Moe et al., 2010), the turkey tree allows easy cleaning and disinfection between the trials due to material characteristics. The AstroTurf did allow safe footing and sitting. One downside we observed was an increasing amount of feces accumulating on the surface during the course of the trials. This might have affected the food pad condition which was not accessed in this experiment but has to be considered for further development of such EE structures (Youssef et al., 2010; Wu and Hocking, 2011; de Jong et al., 2014). Overall, the design of the turkey tree has to be further improved to meet higher hygiene standards and to allow better cleaning during the fattening phase.

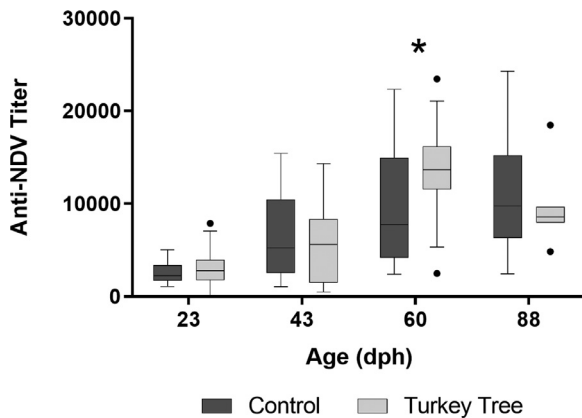
### Perching Behavior

Sixteen days post hatch the longest primary feathers exceed 5 cm in length and the secondaries 3.5 cm, enabling the poults to reach the higher levels (Leopold, 1943). The early use of the turkey tree clearly indicates that the EE was attractive for all age groups and was frequently used as soon as the birds developed the necessary body size or primal feathers to reach the respective levels. The decrease in the use of the turkey tree over the fattening phase might be due to increasing body weight associated with reduced perching activity (Crowe and Forbes, 1999; Martrenchar et al., 2001). Yet, an age-related general decrease in activity, how is



**Figure 5.** Effect of EE on feather and integument scores (trial 3). Feather scores (A, C, E) and integument scores (B, D, F) for the neck (A), head (B), wing (c, d) and tail (E, F). Scores are pattern-coded with darker patterns indicating more severe losses or lesions. Detailed criteria for scores are described in Table 1. Asterisks indicate significant differences between the control group and the EE group by Fischer's exact test ( $P < 0.05$ ).  $n = 22$  birds/group. Abbreviation: EE, enriched environment.

was described in former studies, could also be responsible for the decline in perching, as we observed also other changes in behavior with increasing age during our



**Figure 6.** Anti-NDV antibody titers after vaccination of control and EE turkeys (trial one as a representative trial). Birds were ND-vaccinated orally at d 14, 42, and 67 post hatch. Serum samples were collected at indicated days post hatch (dph) and antibody titers were determined by ELISA (Tukey box-plots, dots represent outliers). Asterisks indicate significant differences by Wilcoxon's rank-sum test ( $P < 0.05$ ) between the control and enrichment (turkey tree) group. Abbreviation: EE, enriched environment.

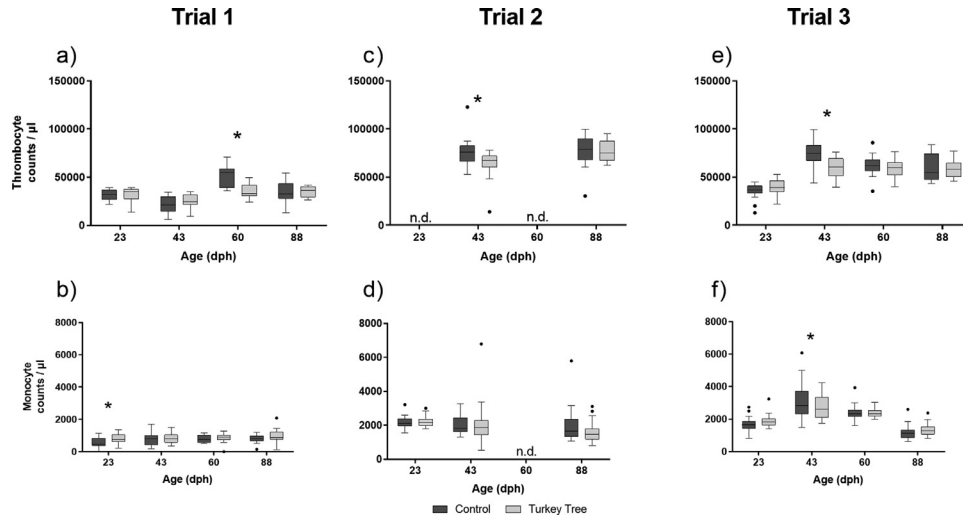
study (Martrenchar et al., 1999; Busayi et al., 2006; Marchewka et al., 2013).

The formula from Ellerbrock and Knierim (2002) to determine space requirements/bird was developed for standing male turkeys. Since the birds in our trial were female and the tree was used for sitting or standing, this calculation can only provide a rough reference for space requirements/bird on the tree. Even though the space on the turkey tree was sufficient to provide enough space for 15 birds at the end of the trials, and even more birds earlier in the fattening period, not all birds may have liked to move up to the higher levels if those are occupied by other birds to avoid aggressive encounters (Buchwalder and Huber-Eicher, 2004). More planimetric studies in association with age and body weight of the birds may help to optimize the space provided on elevated platforms (Spindler et al., 2016; Kulke et al., 2017).

## Behavior

Feather pecking activity was low in our study. In trial 2, significantly more feather pecking was observed

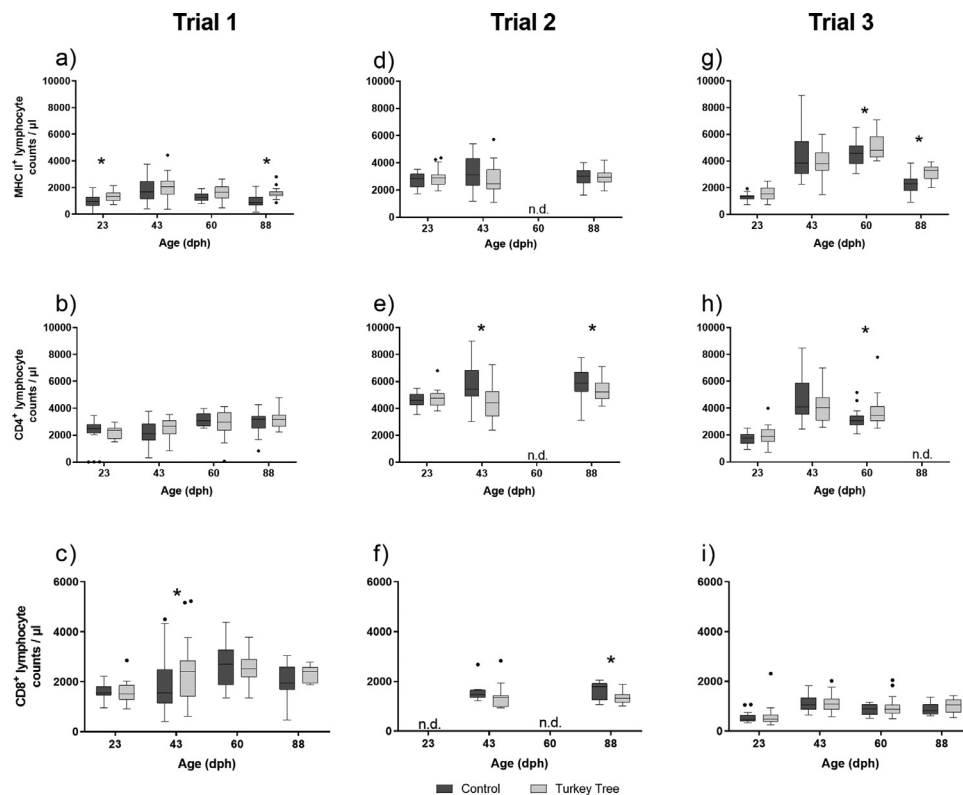




**Figure 7.** Effect of EE on circulating immune cell populations in turkeys. Thrombocyte (A, C, E) and Monocyte (B, D, F) counts determined in whole blood of turkeys in trial 1 (A, B), trial 2 (C, D), and trial 3 (E, F). Data presented in Tukey box-plots (box includes second and third quartiles, horizontal line displays median, whiskers include values within 1.5 interquartile range, dots represent outliers). Asterisks indicate significant differences between the EE group and control group (general mixed models,  $P < 0.05$ ). Abbreviations: EE, enriched environment; n.d., not done.

compared to trial 3 ( $P < 0.0001$ ;  $F = 17.96$ ). We may speculate that variations may occur among trials due to season, individual interactions within the flock, genotype and parent flock possibly associated with epigenetic effects. The data of this study suggests that the use of EE may lead to a decreased frequency of feather pecking and subsequently feather and integument lesions. This is in agreement with former studies in turkeys, which

showed less feather pecking and lesions if provided with enrichment objects (Crowe and Forbes, 1999; Martrenchar et al., 2001; Glatz and Rodda, 2013). The turkey tree aimed to allow affected birds to back away from the aggression. The AstroTurf may have also provided additional foraging stimuli, which has to be investigated further. The middle and upper level of the tree also provided additional 0.84 m<sup>2</sup> of space in the



**Figure 8.** Effect of EE on circulating lymphocyte populations of EE and control birds. Comparisons of MHC class II<sup>+</sup> (A, D, G), CD4<sup>+</sup> (B, E, H) and CD8<sup>+</sup> (C, F, I) lymphocyte counts during trial 1, trial 2, and trial 3 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 indicate significant differences due to general mixed models between the EE group and control group ( $P < 0.05$ ). Abbreviations: EE, enriched environment; n.d., not done.

enrichment group in comparison to the control group. However, it was shown that the housing space, respectively density of birds, had little effect on the behaviour of fattening turkeys (Martrenchar et al., 1999; Hafez et al., 2016). Therefore, we may speculate that the reduction of pecking is either achieved by a back out option for the victims or a general reduction in stress, due to more activity of the birds.

Head pecking is considered an aggressive interaction (Moinard et al., 2001). However, aggressive interactions including head pecking and fighting and the resulting injuries may be less frequent in smaller flocks since the social hierarchy is more easily established if birds are familiar with all flock mates (Sherwin and Kelland, 1998; Buchwalder and Huber-Eicher, 2003; Buchwalder and Huber-Eicher, 2005; Marchewka et al., 2013). Our finding of no significant influence of EE on head pecking is supported by an earlier study, which found no impact of straw, metal objects, and perches on aggressive pecking in female turkeys (Martrenchar et al., 2001). At this point, we cannot exclude that under commercial conditions in bigger flocks, EE might reduce stress-associated aggressive encounters (Buchwalder and Huber-Eicher, 2003; Buchwalder and Huber-Eicher, 2005; Glatz and Rodda, 2013). The low head pecking rates in smaller flocks also explain our findings of very little injuries in the head region (Sherwin and Kelland, 1998; Moinard et al., 2001). In most cases, we noted more severe feather loss or injuries in the control group in comparison to the EE group (Crowe and Forbes, 1999). Only at 14 dph in the third trial and at 88 dph in the second trial a higher lesion score was observed in the EE group in comparison to the control group ( $P < 0.05$ ). We may speculate that the stress due to a more complex environment at the beginning of the trial and competition for the limited space on the tree at the end of the trial may have promoted aggressive encounters.

In earlier investigations, age was identified as an influential factor on feather pecking as well as the occurrence of subsequent lesions in turkeys (Crowe and Forbes, 1999; Martrenchar et al., 2001; Marchewka et al., 2013; Schulze Bisping, 2015). In turkey hens housed in groups of 1,200 birds, fighting increased significantly up to an age of 8 wk and decreased thereafter, while head pecking was not influenced by age (Schulze Bisping, 2015). In our study, age effects were not detected because of the overall low pecking and fighting rate. In our study, running and flying, as well as preening decreased together with the use of the turkey tree. This was partially observed before, Schulze Bisping (2015) reported a decrease in foraging after 4 wk in turkey hens, but an increase in preening in older birds up to an age of 10 to 12 wk.

### **Antibody Titers**

We did not observe a repeatable impact of EE on antibody development after ND-live vaccination. Other studies provided variable results (Huff et al., 2005;

Nazar and Marin, 2011), which might be due to differences in study design and the impact of additional stressors or factors on antibody development. These include frequent exchange of EE within a growing cycle, frequency of disturbances in the animal rooms, the group sizes, and the type of used EE (Huff et al., 2005; Berk et al., 2018).

### **Flow Cytometric Analysis of Different Circulating Immune Cell Populations**

All lymphocyte numbers were affected by age, as observed in various other studies (Dos Santos Schmidt et al., 2009; Seliger et al., 2012). Overall, detected numbers of circulating monocytes, MHC class II<sup>+</sup> lymphocytes, CD4<sup>+</sup> and CD8<sup>+</sup> T-cells as well as thrombocytes match previous studies conducted in turkeys or other avian species (Bounous et al., 2000; Huff et al., 2005; Shini et al., 2010; Seliger et al., 2012; Lindenwald et al., 2019). We did not detect a significant impact of EE on monocyte numbers, which confirms former findings. Novel objects did not change neither 21 d old broiler chickens' nor 8 wk old turkeys' monocyte blood counts (Huff et al., 2003; Altan et al., 2013).

We cannot fully exclude that the bacterial infection, although only detected in one dead bird, in the second trial or its treatment affected the lymphocyte counts in this trial of both groups. But overall, variations in cell counts were also observed between the first and third trial, therefore, other factors besides bacterial infection may lead to trial to trial variation including the season or epigenetic effects of the parent flock (Berghof et al., 2013; Valdebenito et al., 2021). Further studies are needed to identify additional influencing factors on immune cell counts and functions. At various time points, numbers of MHC class II<sup>+</sup> lymphocytes, which are suggested to include mainly B-cells (Paramithiotis and Ratcliffe, 1993) but also CD4<sup>+</sup> T cells subpopulations, were significantly higher in the EE group compared to the control group ( $P < 0.05$ ). These findings match a previous study in quail, which documented a higher percentage of lymphocytes in peripheral blood if birds were housed with elevated platforms and pecking enrichment (Nazar and Marin, 2011). While the overall increase in circulating MHC class II<sup>+</sup> lymphocytes correlated with age as well as rising ND-antibody levels in both groups. Interestingly, the elevated number of MHC class II<sup>+</sup> lymphocytes in the tree-group compared to the controls was not reflected in higher NDV-antibody titers. Also previous studies in other animal species indicated that there may not be always a correlation between peripheral B-cell numbers and antibody body levels after vaccination against various other pathogens (Amanna et al., 2007). In addition, the number of circulating NDV-specific B cells may be too low within the total number of affected circulating B cells to be detected by changes in the NDV-antibody levels in the ELISA. Changes in B memory cell numbers may last longer than detected changes in the antibody response (Hartley et al., 2020), and therefore, the selected blood

sampling time points may have been not suited to detect differences.

CD8<sup>+</sup> lymphocytes were decreased in the EE group in comparison to the control group in trial 2 ( $P < 0.05$ ). At this point, we cannot exclude the impact of an early infection in trial 2 on circulating lymphocyte numbers (Latimer et al., 1988; Berndt and Methner, 2001), as also one bird died of a bacterial infection at 7 dph.

Interestingly our study provides for the first time evidence that EE may affect numbers of circulating thrombocytes. These were significantly lower at various time points in the EE group compared to the control birds suggesting that either stress reduction and/or more space or activity may impact circulating immune cells. The significance of the elevation in circulating thrombocyte numbers in the control group for the general health status of the bird has to be elucidated in future examinations.

Overall, we demonstrated that the turkey tree is attractive for growing female turkeys and may help to reduce stress and subsequent aggressive encounters. This may positively impact the health status of the bird. Further studies are needed to understand the mechanism behind these beneficial effects and to optimize the turkey tree with respect to necessary space per bird and material composition. In addition to the descriptive evaluation of immune cells numbers functional test have to be conducted to understand more about the effect of stress on the immune system of turkeys (Dhabhar, 2014). This should not only be done in the context of enrichment but also in relation to other management parameters to eventually improve stress intervention strategies.

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## DISCLOSURES

The authors declare no conflict of interests.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.psj.2021.101360](https://doi.org/10.1016/j.psj.2021.101360).

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