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Chicken lines divergently selected on feather pecking differ in immune characteristics

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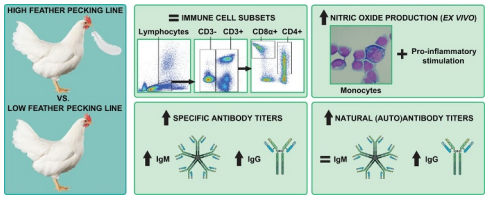
GRAPHICAL ABSTRACT



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ABSTRACT

It is crucial to identify whether relations between immune characteristics and damaging behaviors in production animals exist, as these behaviors reduce animal welfare and productivity. Feather pecking (FP) is a damaging behavior in chickens, which involves hens pecking and pulling at feathers of conspecifics. To further identify relationships between the immune system and FP we characterized high FP (HFP) and low FP (LFP) selection lines with regard to nitric oxide (NO) production by monocytes, specific antibody (SpAb) titers, natural (auto) antibody (N(A)Ab) titers and immune cell subsets. NO production by monocytes was measured as indicator for innate pro-inflammatory immune functioning, SpAb titers were measured as part of the adaptive immune system and N(A)Ab titers were measured as they play an essential role in both innate and adaptive immunity. Immune cell subsets were measured to identify whether differences in immune characteristics were reflected by differences in the relative abundance of immune cell subsets.

Divergent selection on FP affected NO production by monocytes, SpAb and N(A)Ab titers, but did not affect immune cell subsets. The HFP line showed higher NO production by monocytes and higher IgG N(A)Ab titers compared to the LFP line. Furthermore the HFP line tended to have lower IgM NAAb titers, but higher IgM and IgG SpAb titers compared to the LFP line. Thus, divergent selection on FP affects the innate and adaptive immune system, where the HFP line seems to have a more responsive immune system compared to the LFP line. Although causation cannot be established in the present study, it is clear that relationships between the immune system and FP exist. Therefore, it is important to take these relationships into account when selecting on behavioral or immunological traits.

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1. Introduction

In vertebrates the immune system and behavior are deeply connected. Individual differences in behavioral patterns are associated with variation in pathogen and parasite exposure [[1],[2]], which could lead to differences in immune characteristics. In turn, the immune system has been shown to be involved in brain development and regulation of behaviors [[3–5]], and could thereby influence behavioral characteristics. Behavioral and immune characteristics may also be linked through factors that simultaneously affect these characteristics, such as genetics, early-life experiences or the gut microbiota [[6]].

It is crucial to identify whether these relations between behavioral and immune characteristics also exist in production animals, as selection on behavioral characteristics could influence animal health and selection on immune characteristics could influence animal behavior. Especially of interest are damaging behaviors as these behaviors reduce animal welfare and productivity [[7]]. Feather pecking (FP) is such a damaging behavior in chickens, which involves hens pecking and pulling at feathers of conspecifics. Chickens have a high motivation to explore and forage which could lead to the expression of FP in animal husbandry [[8],[9]]. In addition, chickens with a higher motivation to explore and forage are more likely to be exposed to pathogens, as they explore more of their environment, food sources and interact more with conspecifics [[1]], but they are also more likely to develop FP. This points to a potential relationship between the immune system and FP.

Indeed, multiple studies have found relationships between the immune system and FP. Genetic mutations in cytokine Interleukin 4 (IL4), IL9, nuclear factor-kappa-B (NFKB) and chemokine (CCL4) genes of cage mates were associated with feather damage of individuals [[10]], where feather damage is an indicator of severe FP [[11]]. Genetic mutations in the IL4 and IL9 genes were further associated with IgM and IgG natural antibodies (NAb) titers [[12]], where NAb are antibodies that can bind antigen without prior intentional exposure to that antigen [[13],[14]]. These associations were all associative genetic effects on feather damage (the genetic effect of the genotype of cage mates on an individual's feather damage), suggesting that IL4, IL9, NFKB and CCL4 genes and NAb titers may be related to the propensity to perform FP. This is supported by the finding that when cage mates had higher IgG NAb, the individual had more feather damage [[15]]. Furthermore, a strong genetic correlation was found between FP and the specific antibody (SpAb) response [[16]]. These findings suggest a (genetic) relation between the immune system and FP.

Further evidence for a relationship between the immune system and FP comes from lines divergently selected on FP. The high FP (HFP) line had a higher specific antibody (SpAb) response to vaccination, while the low FP (LFP) line had a higher relative abundance of white blood cells, T helper cells, double positive T cells and higher expression of MHC class I molecules on T and B cells [[17]]. The HFP line further had lower IgM NAb, but higher IgG NAb compared to the LFP line [[18]]. These findings suggest that the FP selection lines differ in immune responsiveness and give further support for a relationship between the immune system and FP.

To further identify relationships between the immune system and FP we characterized the FP selection lines with regard to immune characteristics. We identified nitric oxide (NO) production by monocytes, specific antibody (SpAb) titers, natural (auto)antibody (N(A)Ab) titers and immune cell subsets of the HFP and LFP lines. NO production by blood derived monocytes was measured *ex vivo*, as indicator for innate pro-inflammatory immune functioning [[19],[20]]. SpAb titers to human serum albumin (HuSA) were measured as part of the adaptive immune system. N(A)Ab titers were measured as they play an essential role in both innate and adaptive immunity, for example by maintaining homeostasis, increasing disease resistance and linking the two types of immunity [[21–24]], where NAb bind to non-self-antigen and NAAb bind to self-antigen. We further included immune cell subsets to identify whether differences in immune characteristics were reflected by differences in the relative abundance of immune cell subsets. We hypothesized that the HFP line would have a more responsive immune system (i.e. higher NO production, SpAb and IgG N(A)Ab titers), as it previously had a higher specific immune response and IgG NAb compared to the LFP line [[17],[18]]. We further expected higher NO production in the HFP line, as macrophages from a line selected on high antibody response produced more NO compared to a line selected on low antibody response in chickens [[25]].

2. Material and methods

2.1. Animals and housing

Offspring from White Leghorn birds from the 18th generation of lines selected on high (HFP) respectively low feather pecking (LFP) were used (see Kjaer et al. [[26]] for the selection procedure). The HFP and LFP line were divergently selected on feather pecking (FP) for seven generations and were maintained in subsequent generations. The parent stock was between 50 and 54 weeks of age at the time of egg collection. A total of 120 birds were produced in one batch of eggs that were incubated at an average egg shell temperature of 37.8 °C and average relative humidity of 54.9%. Non-beak-trimmed female birds were used that had a neck tag (Roxan) with a unique number. At 3 and 4 weeks of age, birds were color marked on the neck and/or back for individual identification (colors: black, purple, green and orange). From 7 weeks of age onwards, birds were equipped with a light weight backpack with a number for individual identification. Birds were housed per line in 10 pens and in groups of 12. At 8 weeks of age group size was reduced by 2 birds (n = 9–10 birds per pen).

At all times, water and feed were provided ad libitum. Birds received a standard rearing diet 1 from hatch until 8 weeks of age, a standard rearing diet 2 from 8 until 16 weeks of age and a standard laying diet from 16 weeks of age onwards until the end of the experiment. Each floor pen (h: 2 m, l: 2 m, w: 1 m) had wood shavings on the floor, a platform installed 45 cm above the floor and visual barriers of 1.5 m high to prevent birds in adjacent pens of seeing each other. Post hatch, temperature was kept around 33 °C and gradually lowered to 21 °C at 5 weeks of age. The light regime was 23L:1D post hatch, and was weekly, gradually reduced to 8L:16D at 4 weeks of age. From 15 weeks of age, the light regime was weekly extended with 1 h until 16L:8D at 22 weeks of age. Light intensity in pens ranged between 25.8 and 68.2 LUX (average 49.8 LUX) during the first 6 weeks of life, thereafter light intensity was reduced and ranged between 3.5 and 5.8 LUX (average 4.8 LUX) as measured with a Voltcraft MS-1300 light meter (Conrad Electric Benelux). A wooden nest box was placed in front of the pen at 15 weeks of age. The experimental set-up (housing conditions, vaccinations, etc.) was designed to reflect commercial conditions as FP is an issue in the egg laying industry. The experiment was approved by the Central Authority for Scientific Procedures on Animals according to Dutch law (no: AVD104002015150).

2.2. Feather pecking observations

FP behavior was observed on individual level in week 4–5, 9–10, 14–15, 19 + 21, 24–25 and 28–29. In week 4–5 birds were observed by direct observation. Each observation lasted 30 min, either in the morning (8:30 h-12:00 h) or in the afternoon (12:30 h-16:00 h), after a 2 min habituation period. For all other weeks FP was observed from video recordings. Each observation lasted 15 min, either in the morning (10:40 h-10:55 h) or in the afternoon (14:40 h-14:55 h). The Observer XT 10 program (Noldus Information Technology) was used for video analysis of FP, categorized into gentle FP (subdivided into exploratory FP and bouts of stereotyped FP) and severe FP (Table 1). Inter-observer reliability for video analysis was high for all FP behaviors (Pearson correlations: exploratory FP = 0.99, stereotyped FP = 0.96 and severe FP = 0.98). The order for observations was randomized at pen level and

Table 1
Ethogram of the feather pecking (FP) observations (after Newberry et al. [[27]]).

Behavior	Description
Exploratory FP	Bird makes gentle beak contact with the feathers of another bird without visibly altering the position of the feathers. The recipient makes no apparent response. Each peck is recorded.
Stereotyped FP Bout	Bird makes ≥3 gentle pecks at a single body region at intervals of ≤1 s. Each series of pecks (bout) is recorded. Bout ends when birds separate, or when pecking is directed to another target on the same, or another, bird.
Severe FP	Bird grips and pulls or tears vigorously at a feather of another bird with her beak, causing the feather to lift up, break or be pulled out. The recipient reacts to the peck by vocalizing, moving away or turning towards the pecking bird. Each peck is recorded.

observers were blinded to the lines. FP behaviors were summed over two subsequent weeks, thus including one morning and one afternoon observation with a total observation period of 60 min for week 4–5 and a total observation period of 30 min for all other weeks.

2.3. Vaccinations, immune challenge, blood collection and analysis

All birds received vaccinations against Marek's disease (day 0 intramuscular), Infectious Bronchitis (day 0 and week 2 eye drops, week 8 and 15 spray), Newcastle Disease (week 1, 4, 10 spray and week 12 injection), Infectious Bursal Disease (day 25 drinking water), Avian Encephalomyelitis and Pox Diphtheria (week 12 wingweb) and Infectious Laryngo Tracheitis (week 12 eye drops). At 8 weeks of age, 15 birds per line (3 per pen) were intra-tracheally immunized with Human Serum Albumin (HuSA) (0.5 mg/kg, Sigma-Aldrich A3782) using a blunted needle [[28]].

Blood was collected from all birds at 5, 10, 15, 20, 25 and 30 weeks of age. Blood was additionally collected from immunized birds at day 0, prior to HuSA immunization (8 weeks of age), day 4, day 7 (9 weeks of age) and day 14 (10 weeks of age) post HuSA immunization (see Fig. 1 for overview). Blood was taken from the wing vein using a heparinized syringe and kept on ice. In the laboratory, blood samples for natural (auto)antibody (N(A)Ab) titers and specific antibody (SpAb) titers were centrifuged at 5250 x *g* for 10 min at room temperature and plasma was stored at −20 °C until further analysis. Blood samples for leukocyte isolation were not kept on ice and analyzed directly in the laboratory.

2.3.1. Plasma IgM and IgG natural (auto)antibody titers

Samples from all birds at 5, 10, 15, 20, 25 and 30 weeks of age were used for determination of IgM and IgG NAb titers against keyhole limpet hemocyanin (KLH) and for determination of IgM and IgG NAAb titers against phosphorylcholine conjugated to bovine serum albumin (PC-BSA) and Herring sperm DNA. N(A)Ab titers against KLH (2 µg/mL, Sigma-Aldrich H8283), PC-BSA (1 µg/mL, LGC Biosearch Technologies PC-1011-10) and Herring sperm DNA (5 µg/mL, Sigma-Aldrich D6898) were determined by an indirect enzyme-linked immunosorbent assay (ELISA) as described previously [[29]] with the following modifications. Plasma samples were added on plates in a four step dilution starting at dilution 1:40 in phosphate buffer saline (PBS) containing 0.05% Tween 20 and 1% horse plasma (100 µL in each well). Peroxidase conjugated goat-anti-chicken IgM (Bethyl Laboratories A30-102P, dilution 1:20,000) or goat-anti-chicken IgG (Bethyl Laboratories A30-104P, dilution 1:20,000) was used as secondary antibody (100 µL in each well). Substrate buffer (tetramethylbenzidine and 0.05% H_2O_2) was added (100 µL in each well) and after 20 min the reaction was stopped with 50 µL of 1.25 M H_2SO_4. Extinctions were measured with a Thermo Scientific Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific) at 450 nm. Titers were expressed as log2 values of the dilutions that gave an extinction closest to 50% of Emax where Emax represents the highest mean extinction of a standard positive (pooled) plasma present on every plate.

2.3.2. Plasma IgM and IgG specific antibody titers

Samples from immunized birds at day 0, prior to HuSA immunization, day 4, day 7 and day 14 post HuSA immunization were used for determination of IgM and IgG SpAb titers against HuSA (4 µg/mL, Sigma-Aldrich A3782). SpAb titers against HuSA were determined by an indirect ELISA as described above, with the following modifications. Peroxidase conjugated goat-anti-chicken IgM (Bethyl Laboratories A30-102P, dilution 1:20,000) or goat-anti-chicken IgG (Bethyl Laboratories A30-104P, dilution 1:40,000) was used as secondary antibody (100 µL in each well).

2.3.3. Leukocyte isolation and stimulation experiment

Samples from immunized birds at 8 and 10 weeks of age were used for leukocyte isolation and *ex vivo* stimulation. Samples were diluted 1:1 in RPMI 1640 (Gibco). Diluted blood was loaded onto a Histopaque-1119 gradient (Sigma-Aldrich Histopaque-1119) and centrifuged at 700 x *g* for 40 min at room temperature. The interphase containing the leukocytes was collected, washed 2 times and re-suspended in complete culture medium RPMI 1640 (Gibco) supplemented with HEPES, Glutamax, 10% heat-inactivated chicken serum (Gibco) and 0.5%

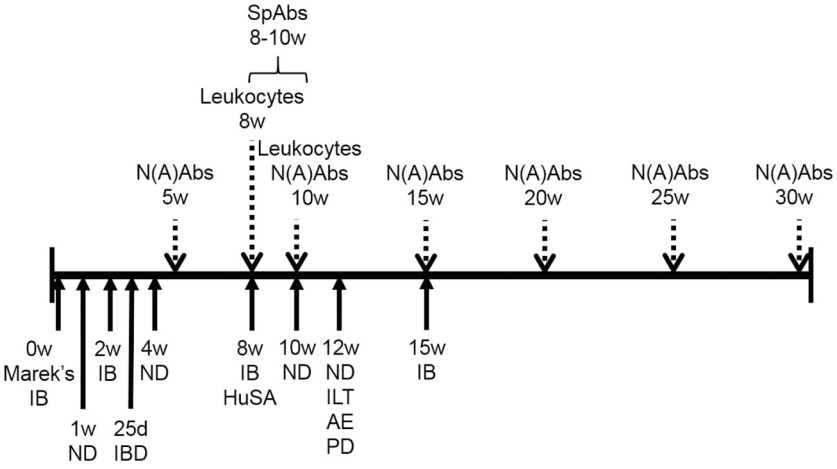

Fig. 1. Timeline of blood sampling (above line), vaccinations and immune challenge (below line) performed at specific ages in days (d) or weeks (w). N(A)Abs = natural (auto)antibodies, Leukocytes = leukocyte isolation, SpAbs = specific antibodies, Marek's = Marek's disease, IB = Infectious Bronchitis, ND = Newcastle Disease, IBD = Infectious Bursal Disease, HuSA = Human Serum Albumin immunization, ILT = Infectious Laryngo Tracheitis, AE = Avian Encephalomyelitis, PD = Pox Diphtheria.

antibiotics (final concentration of 50 U/mL penicillin and 50 μg/mL streptomycin, Gibco). Leukocytes were seeded at a concentration of $1*10^6$ cells per well to a 96-wells flat-bottom plate (Greiner CELLSTAR) with a total volume of 100 μL per well. The cells were incubated overnight at 41 °C in 5% CO_2 and 95% humidity. The next day, non-adherent cells were washed away with pre-warmed culture medium. Adherent cells were stimulated with 200 μL per well culture medium (control), lipopolysaccharides (LPS) from *E. coli* serotype O55:B5 (10 μg/mL, Sigma-Aldrich L6529), recombinant chicken interferon gamma (IFN-γ) (100 ng/mL, Kingfisher Biotech) or a combination of LPS + IFNγ with the same concentrations for 48 h for the nitric oxide production assay.

2.3.4. Flow cytometry

Subsets of innate and adaptive immune cells in the blood were analyzed by flow cytometry. All antibodies were obtained from Southern biotech, except for the mouse-anti-chicken CD40 and the secondary antibodies goat-anti-mouse IgG2a -APC and IgG3-PE which were obtained from Biorad and BD biosciences, respectively. The chicken natural killer (NK) cell markers were kindly provided by professor T. Göbel, LMU Munich.

500,000 isolated leukocytes were washed with FACS buffer (PBS w/o CaMg (Gibco) supplemented with 0.5% BSA and 0.005% Sodium azide (Sigma-Aldrich)). The cells were transferred to a 96-wells round-bottom plate and stained with monoclonal antibodies specific for chicken immune cells. Mix 1 includes a combination of mouse-anti-chicken β2M FITC (clone F21-F21, IgG1), mouse-anti-chicken CD3-PE (clone CT3; IgG1), mouse-anti-chicken CD4-biotin (clone CT4, IgG1) and mouse-anti-chicken CD8α-APC (CT8, IgG1). In mix 2 a combination of mouse-anti-chicken TCR1-FITC (TCRγδ, IgG1), mouse-anti-chicken MHC II-PE (CIa, IgM) and mouse-anti-chicken CD40 (AV79, IgG2a) was used. In mix 3 mouse-anti-chicken-Bu-1-FITC (AV20, IgG1) was used together with mouse-anti-chicken CD3 APC (CT3, IgG1) and the mouse-anti-chicken NK markers 28–4 (IgG3) [[30]] and 20E5-biotin (IgG1) [[31]]. Cells were incubated with antibodies for 20 min at 4 °C. Next, cells were washed 2 times in FACS buffer. Afterwards, streptavidin-Percp (BD biosciences) was added to the samples stained with mix 1, goat-anti-mouse IgG2a-APC to samples stained with mix 2, a combination of streptavidin-Percp and goat-anti-mouse IgG3-PE to cells stained with mix 3, or a combination of all secondary antibodies was added to samples without a primary antibody mix as negative control (Sup. Table 1). Cells were stained for 20 min at 4 °C and washed in PBS. Next, cells were re-suspended in PBS and an equal volume of 4% paraformaldehyde (Merck) was added to fixate the cells. Cells were incubated for 10 min at room temperature, washed 1 time using FACS buffer and re-suspended in FACS buffer. Flow cytometry was performed using a FACS Canto flow cytometer (BD Biosciences) and at least 100,000 lymphocytes were collected. Data were analyzed using FlowJo software (Threestar).

2.3.5. Nitric oxide production assay

The nitric oxide (NO) production assay was performed 48 h after the *ex vivo* stimulation. The reactive nitrogen oxide intermediate NO was indirectly measured by quantifying the production of the more stable nitrite (NO_2-), using Griess reagents [[32]]. The assay is a colorimetric assay and quantifies the accumulation of NO in the culture medium. A volume of 50 μL culture supernatant was transferred to a 96-wells flat-bottom plate (Greiner CELLSTAR) and combined with 50 μL of the Griess reagent. The Griess reagent consists of a 1:1 mixture: Griess reagents solution A (2% Sulphanilamide in 5% H_3PO_4) and Griess reagents solution B (0.2% N-(1-naphthyl)ethylenediamine dihydrochloride in H_2O). The plate was incubated for 10 min at room temperature. NO concentration was determined by measuring the optical density (OD_{540}) with a Thermo Scientific Multiskan GO microplate spectrophotometer. The amount is determined by a calibration line using two fold dilutions of a sodium nitrite solution ($NaNO_2$) in the range of 100 μM – 0 μM.

2.4. Statistical analysis

SAS Software version 9.4 was used for statistical analysis (SAS Inst.). Linear mixed models for line effects on N(A)Ab titers consisted of fixed effects line * age, line and age. The random effect consisted of pen within line, a repeated statement for age with chicken ID as subject and an unstructured covariance structure. The unstructured covariance structure gave the best fitting model (model 1). Linear mixed models for line effects on SpAb titers consisted of fixed effects line * age, line and age. The random effect consisted of pen within line (model 2). Linear mixed models for line effects on NO production and SpAb titers tested per age consisted of fixed effects line and the random effect pen within line (model 3). The model assumptions were visually examined. NO production at 10 weeks of age was log transformed to obtain normality of model residuals. Post hoc pairwise comparisons were corrected by Tukey–Kramer adjustment. A Kruskal Wallis test was used to identify line effects on FP behavior, the relative abundance of immune cell subsets and NO production at 8 weeks of age. All data is presented as (untransformed) mean ± standard error (SE).

Model 1:

$$\gamma_{ijkl} = \mu + LINE_i + AGE_j + LINE_i*AGE_j + (pen_k/line_i) + id_l + e_{ijkl}$$

γ = N(A)Ab titers. μ = overall mean. line = fixed effect of line (i = HFP or LFP). age = fixed effect of age, week at which blood was collected for measurement (j = 5, 10, 15, 20, 25 or 30). pen/line = random effect of line within pen (k = 1–10). id = random effect of chicken id (l = 1–120) with repeated observations assumed to be distributed as ~N(0,Tσ2id) in which T is the unstructured covariance matrix with the chicken id as the subject and age as the repeated effect, σ2id is the variance between chickens. e = residual effect.

Model 2:

$$\gamma_{ijk} = \mu + LINE_i + AGE_j + LINE_i*AGE_j + (pen_k/line_i) + e_{ijk}$$

γ = SpAb titers. μ = overall mean. line = fixed effect of line (i = HFP or LFP). age = fixed effect of age, days post immunization at which blood was collected for measurement (j = 0, 4, 7 or 14). pen/line = random effect of line within pen (k = 1–10). e = residual effect.

Model 3:

$$\gamma_{ij} = \mu + LINE_i + (pen_j/line_i) + e_{ij}$$

γ = NO production or SpAb titers. μ = overall mean. line = fixed effect of line (i = HFP or LFP). pen/line = random effect of line within pen (j = 1–10). e = residual effect.

3. Results

3.1. Divergent selection on feather pecking affects feather pecking behavior

Feather pecking (FP) behavior was observed at an individual level throughout the experiment to identify whether divergent selection on FP actually resulted in differences in FP. FP was categorized into gentle FP (subdivided into exploratory and stereotyped FP) and severe FP, where gentle FP usually does not result in damage and severe FP is the problematic behavior in terms of damage to the recipient [[8]]. An overview of line effects on the different types of FP is given in Table 2. HFP birds showed more exploratory FP at all ages and showed more stereotyped FP at 4–5, 14–15, 24–25 and 28–29 weeks of age compared to LFP birds ($P < 0.05$). Furthermore, HFP birds tended to show more severe FP at 4–5 and 19 + 21 weeks of age ($P < 0.1$) and showed more severe FP at 14–15, 24–25 and 28–29 weeks of age compared to LFP birds ($P < 0.05$). These results indicate that divergent selection on FP indeed altered FP behavior, with the HFP line showing more FP compared to the LFP line.

Table 2
Feather pecking (FP) behavior (exploratory FP, stereotyped FP (bouts) and severe FP) of the high (HFP) and low feather pecking (LFP) lines at different ages.

Variables	HFP	LFP	*P*-value	χ2
Age (4–5 weeks)	**n = 59**	**n = 59**		
Exploratory FP	3.91 ± 0.54	1.36 ± 0.22	< 0.01	21.44
Stereotyped FP (bouts)	1.01 ± 0.19	0.35 ± 0.07	< 0.01	7.91
Severe FP	0.57 ± 0.13	0.30 ± 0.07	< 0.1	3.72
Age (9–10 weeks)	**n = 48**	**n = 49**		
Exploratory FP	4.08 ± 0.89	2.00 ± 0.58	< 0.05	6.02
Stereotyped FP (bouts)	0.50 ± 0.16	0.24 ± 0.13	ns	2.20
Severe FP	0.30 ± 0.30	0.08 ± 0.08	ns	0.00
Age (14–15 weeks)	**n = 46**	**n = 47**		
Exploratory FP	3.52 ± 0.66	1.19 ± 0.39	< 0.01	12.76
Stereotyped FP (bouts)	0.17 ± 0.08	–	< 0.05	4.13
Severe FP	0.43 ± 0.15	0.09 ± 0.06	< 0.05	4.09
Age (19 + 21 weeks)	**n = 45**	**n = 42**		
Exploratory FP	3.60 ± 1.44	0.74 ± 0.23	< 0.05	4.71
Stereotyped FP (bouts)	0.20 ± 0.10	0.07 ± 0.04	ns	0.93
Severe FP	0.38 ± 0.19	0.05 ± 0.05	< 0.1	3.44
Age (24–25 weeks)	**n = 43**	**n = 39**		
Exploratory FP	6.47 ± 1.80	0.87 ± 0.31	< 0.01	14.42
Stereotyped FP (bouts)	0.56 ± 0.23	0.10 ± 0.10	< 0.05	4.19
Severe FP	1.07 ± 0.38	0.05 ± 0.05	< 0.05	5.58
Age (28–29 weeks)	**n = 43**	**n = 36**		
Exploratory FP	2.98 ± 0.59	0.72 ± 0.33	< 0.01	10.80
Stereotyped FP (bouts)	0.42 ± 0.14	0.06 ± 0.06	< 0.05	4.83
Severe FP	1.07 ± 0.47	0.11 ± 0.08	< 0.05	4.95

Average number of pecks or bouts per bird per hour (age 4–5 weeks: 60 min total observation time per bird; age 9–10, 14–15, 19 + 21, 24–25 and 28–29 weeks: 30 min total observation time per bird).

3.2. Divergent selection on feather pecking does not affect immune cell subsets

Leukocytes were analyzed by flow cytometry to identify the relative abundance of immune cell subsets in the FP selection lines. An example of the gating strategy is shown in Sup. Fig. 1 and an overview of the results is shown in Sup. Table 2. We found no differences between lines for any of the immunological cell types (relative abundance or expression), except for the expression (mean fluorescent intensity) of MHC class I on T helper cells (CD4) ($\chi^2 = 4.99$, $P < 0.05$) and on CD4+ CD8α + ($\chi^2 = 4.79$, $P < 0.05$), which were lower in the HFP line compared to the LFP line. These results suggest that the FP selection lines did not differ in relative abundance of immune cell subsets.

3.3. Divergent selection on feather pecking affects nitric oxide production by blood derived monocytes

Nitric oxide (NO) production by blood derived monocytes was measured *ex vivo* as indicator for innate pro-inflammatory immune functioning [[19],[20]]. At 8 and 10 weeks of age, the HFP line had higher NO production by blood derived monocytes stimulated *ex vivo* with lipopolysaccharides (LPS) ($\chi^2 = 5.04$, $P < 0.05$ and $F_{1,8} = 23.81$, $P < 0.01$, respectively) or LPS + interferon gamma (IFNγ) ($\chi^2 = 7.68$, $P < 0.01$ and $F_{1,8} = 11.30$, $P < 0.01$, respectively) compared to the LFP line, but no differences were found for the control (medium) or stimulation with IFNγ (Fig. 2). When comparing the control to stimulation with LPS, IFNγ or LPS + IFNγ, we found that in the HFP line NO production increased by addition of LPS ($P < 0.05$) or LPS + IFNγ ($P < 0.01$) at 8 weeks of age ($F_{3,44} = 88.36$, $P < 0.01$). In the LFP line, NO production only increased by addition of LPS + IFNγ ($P < 0.01$) compared to the control ($F_{3,33} = 11.16$, $P < 0.01$). At 10 weeks of age, NO production increased for both lines by addition of LPS or LPS + IFNγ ($P < 0.01$), but not by addition of IFNγ compared to the control (HFP: $F_{3,55} = 105.53$, $P < 0.01$ and LFP: $F_{3,51} = 21.02$, $P < 0.01$). These results suggest that divergent selection on FP affects monocyte activity, as we found no differences in the relative abundance of CD40+ MHC II+ cells, which includes monocytes, with the HFP line having higher monocyte activity compared to the LFP line.

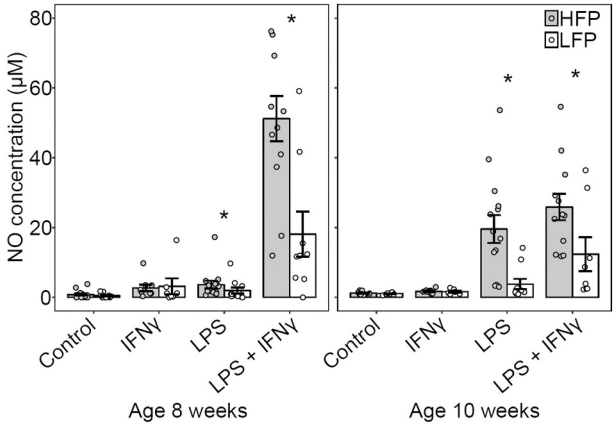

Fig. 2. Mean nitric oxide (NO) concentration (± SE) by blood derived monocytes after stimulation with medium (Control), interferon gamma (IFNγ), lipopolysaccharides (LPS) or LPS + IFNγ for immunized high (HFP, n = 9–15) and low feather pecking (LFP, n = 7–14) birds at 8 and 10 weeks of age. * indicates significant differences ($P < 0.05$) between lines.

3.4. Divergent selection on feather pecking affects IgM and IgG specific antibody titers

We measured specific antibody (SpAb) titers to human serum albumin (HuSA) in the FP selection lines to identify differences in the specific humoral immune response as part of the adaptive immune system. No significant line * age interactions were found on IgM or IgG SpAb titers to HuSA. When identifying differences between lines for ages separately, we found that the HFP line tended to have higher IgM ($F_{1,8} = 5.14$, $P < 0.1$) (Fig. 3A) and IgG SpAb titers ($F_{1,8} = 3.50$, $P < 0.1$) (Fig. 3B) compared to the LFP line at day 14 post immunization. These findings suggest that divergent selection on FP affects the specific immune response, where the HFP line seems to have a higher specific immune response compared to the LFP line.

3.5. Divergent selection on feather pecking affects IgG natural (auto) antibody titers, but not IgM natural (auto)antibody titers

We measured natural (auto)antibody (N(A)Ab) titers in the FP selection lines as they play an essential role in both innate and adaptive immunity [[21],[33]], with NAb binding to non-self-antigen and NAAb

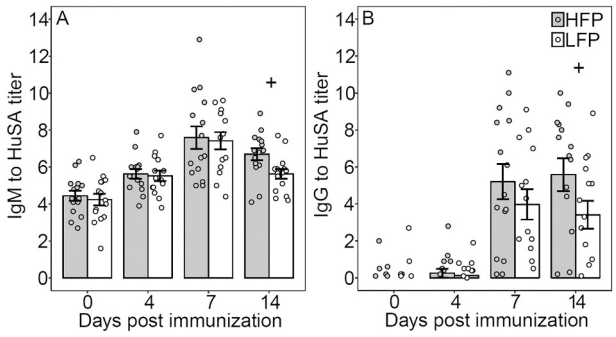

Fig. 3. A) Mean specific antibody titers of IgM (± SE) and B) IgG to human serum albumin (HuSA) of immunized high (HFP, n = 15) and low feather pecking (LFP, n = 14–15) birds at day 0 (prior to immunization), 4, 7 and 14 post immunization. + indicates tendencies ($P < 0.1$) between lines.

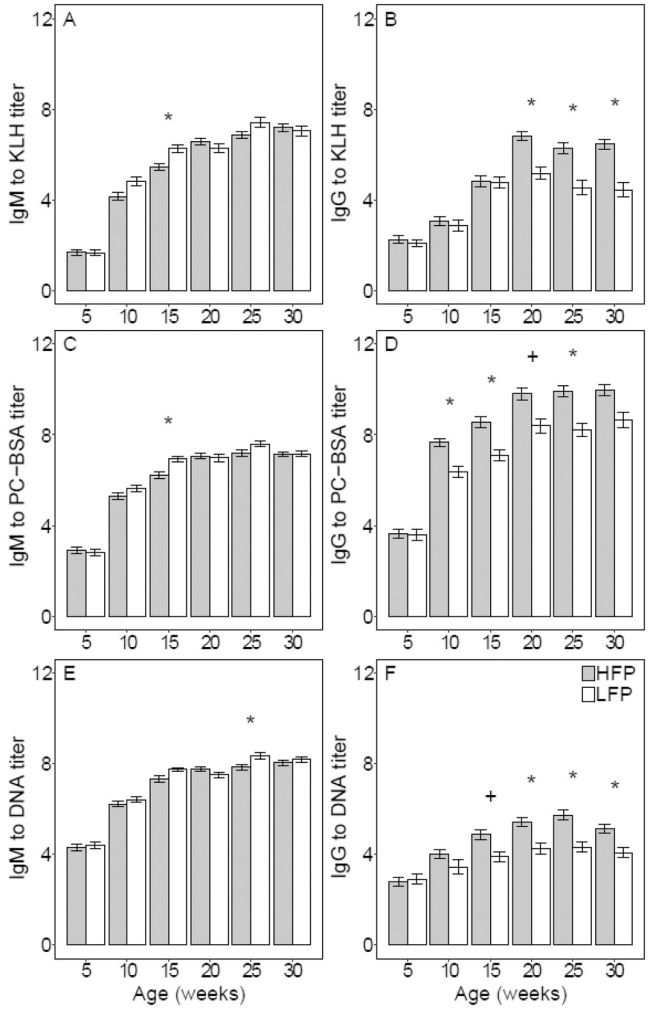

Fig. 4. Mean natural (auto)antibody titers (± SE) of IgM (A, C and E) and IgG (B, D and F) to keyhole limpet hemocyanin (KLH) (A and B); phosphorylcholine conjugated to bovine serum albumin (PC-BSA) (C and D) and Herring DNA (E and F) of the high (HFP) and low feather pecking (LFP) lines at 5, 10, 15, 20, 25 and 30 weeks of age. + indicates tendencies ($P < 0.1$) and * indicates significant differences ($P < 0.05$) between lines.

binding to self-antigen.

Significant line * age interactions were seen on IgM and IgG NAb titers to keyhole limpet hemocyanin (KLH) (IgM: $F_{5,498} = 10.78$, $P < 0.01$; IgG: $F_{5,497} = 7.94$, $P < 0.01$; Fig. 4A-B). Overall, no differences between lines were found for IgM to KLH, but the HFP line did have higher IgG to KLH ($P < 0.01$) compared to the LFP line.

Significant line * age interactions were also seen on IgM and IgG NAAb titers to phosphorylcholine conjugated to bovine serum albumin (PC-BSA)(IgM: $F_{5,495} = 5.62$, $P < 0.01$; IgG: $F_{5,494} = 3.37$, $P < 0.01$; Fig. 4C-D) and to Herring DNA (IgM: $F_{5,495} = 5.35$, $P < 0.01$; IgG: $F_{5,495} = 3.02$, $P < 0.05$; Fig. 4E-F). Overall, the HFP line tended to have lower IgM to PC-BSA and Herring DNA ($P < 0.1$), but had higher IgG to PC-BSA and Herring DNA ($P < 0.01$) compared to the LFP line. For specific comparisons of IgM and IgG N(A)Ab titers between lines per age see Fig. 4.

These results suggest that divergent selection on FP affects N(A)Ab titers of the IgG isotype, where the HFP line had higher IgG N(A)Ab titers compared to the LFP line with even almost four fold higher titers at adult ages. Yet, divergent selection on FP does not seem to affect N(A)Ab titers of the IgM isotype, although the HFP line had lower IgM N(A)Ab titers compared to the LFP line at specific ages.

4. Discussion

To further identify relationships between the immune system and feather pecking (FP) we characterized lines divergently selected on FP with regard to immune characteristics. We identified nitric oxide (NO) production by monocytes, specific antibody (SpAb) titers, natural (auto)antibody (N(A)Ab) titers and immune cell subsets of the high FP (HFP) and low FP (LFP) lines. NO production by monocytes was measured as indicator for innate pro-inflammatory immune functioning, SpAb titers were measured as part of the adaptive immune system and N(A)Ab titers were measured as they play an essential role in both innate and adaptive immunity. We further included immune cell subsets to identify whether differences in immune characteristics were reflected in the relative abundance of immune cell subsets. We hypothesized that the HFP line would have a more responsive immune system (i.e. higher NO production, SpAb and IgG N(A)Ab titers) compared to the LFP line.

Divergent selection on FP affected FP behavior, where the HFP line showed more FP compared to the LFP line. Divergent selection on FP further affected NO production by monocytes, SpAb and N(A)Ab titers, but not the relative abundance of immune cell subsets. The HFP line had higher NO production by blood derived monocytes compared to the LFP line. We assumed NO was mainly produced by viable monocytes, as the majority of the cell population would consist of monocytes since previous studies showed that the number of viable thrombocytes decreases rapidly within the first 24 h and decreases even further in the next 48 h [[34–36]]. Furthermore, the HFP line tended to have higher IgM and IgG SpAb titers, lower IgM NAAb titers and had higher IgG N(A)Ab titers compared to the LFP line. Previously, it was suggested that IgG NAb are dependent upon exogenous antigen stimulation, while IgM NAb are not [[37]]. This indicates that differences in IgM NAb titers might be explained by genetic alterations, while differences in IgG NAb titers might be explained by a difference in environmental influences or immune responsiveness to environmental influences. As both lines were exposed to similar environmental conditions, we suggest that the HFP line had an increased immune responsiveness to environmental influences compared to the LFP line. This is further supported by the higher SpAb titers in the HFP line. Overall, these findings suggest that the HFP line had a more responsive immune system (both innate and adaptive immune system) compared to the LFP line.

Our findings are supported by previous studies in the FP selection lines, where the HFP line had lower IgM NAb, but higher IgG NAb and SpAb compared to the LFP line [[17],[18]], suggesting a more responsive immune system in the HFP line. Yet, Buitenhuis et al. [[17]] did find differences in immune cell subsets, where the HFP line had a lower relative abundance of T helper cells (CD4) and double positive T cells (CD4 + CD8 +) compared to the LFP line. Further support for our findings comes from previous studies in chickens, where a genetic correlation was found between FP and SpAb response [[16]] and high NAb titers were suggested to be related to the propensity to perform FP [[12],[15]]. Thus, overall high FP seems to be related to increased immune responsiveness.

It should be noted that we did not include the unselected control line in the present study. Therefore we cannot compare the effects of selection for or against FP with the effects of no selection on FP. Previously, we found that the LFP line had the lowest IgG NAb titers, the HFP line had intermediate IgG NAb titers and the unselected control line had the highest IgG NAb titers [[18]]. This suggests that selection against FP reduces immune responsiveness compared to no selection, rather than that selection for FP increases immune responsiveness compared to no selection. Yet, the HFP line also had higher SpAb responses compared to the unselected control and LFP lines one week post vaccination [[17]]. Overall, our and previous findings suggest that divergent selection on FP affects the responsiveness of the innate and adaptive immune system.

Still, these findings in the FP selection lines could be caused by differences in behavior, differences in immune system functioning or

factors simultaneously affecting behavior and immune characteristics, such as genetics, gut microbiota, serotonin or stress. We will now briefly discuss each of these options.

4.1. Differences in behavior lead to differences in immune characteristics

Chickens have a high motivation to explore and forage [[8],[38]] and FP is considered to be a redirected foraging behavior [[9],[39]]. Chickens with a higher motivation to explore and forage are more likely to be exposed to pathogens, as they explore more of their environment, food sources and interact more with conspecifics [[1],[40]].

Previously, HFP birds showed more explorative pecking [[41]], pecked more in an operant conditioning test to obtain mealworms [[42]] and showed a higher number of responses in a Skinner box to gain access to feathers and wood shavings compared to LFP birds [[43]]. These findings indicate a higher motivation to explore and forage in the HFP line which could lead to increased immune responsiveness. Indeed, more explorative birds invested more in innate immune function, such as complement activity and NAbs [[44]]. However, the opposite has also been found, where more explorative birds had lower NAb [[45]]. High locomotor activity can further lead to increased exposure to pathogens, for example more active fish had a higher parasite load compared to non-active fish [[46]]. HFP birds showed higher locomotor activity [[47]] and more active responses to various behavioral tests [[48],[49]]. Thus, the differences in immune responsiveness between the FP selection lines could be caused by increased locomotor activity, exploration and foraging, which leads to increased exposure to pathogens, thereby potentially altering immune system functioning.

4.2. Differences in immune responsiveness lead to differences in behavioral characteristics

Having a more responsive immune system might make HFP birds more at risk of developing FP compared to LFP birds. The increased immune responsiveness might result in HFP birds responding stronger to immune challenges or vaccinations with the synthesis and release of pro-inflammatory cytokines. Increased monocyte activity in the HFP line, as indicated by higher NO production, suggests increased production of pro-inflammatory cytokines [[50],[51]]. Although we measured NO production *ex vivo*, our *in vitro* findings suggest higher potential of macrophages (i.e. differentiated monocytes) to produce NO and pro-inflammatory cytokines in the HFP line. HFP birds had higher NAAb, indicating increased cell damage [[52]] potentially caused by higher NO production and pro-inflammatory activity of macrophages [[53]]. HFP birds also had higher IgG N(A)Ab and SpAb, suggesting increased inflammation as IgG has pro-inflammatory activity [[54]] and this increased inflammation might be caused by pro-inflammatory cytokines [[55]]. Furthermore, nuclear-factor-kappa-B (NFKB) and chemokine (CCL4) genes were suggested to be related to the propensity to perform FP [[10]]. Interestingly, NFKB can induce transcription of inducible nitric oxide synthase (iNOS), the enzyme responsible for NO production [[56]], and plays a role in pro-inflammatory cytokine production and release [[57]]. CCL4 (also known as macrophage inflammatory protein-1β [[58]]) has been suggested as marker for macrophages that produce pro-inflammatory cytokines [[59]]. These findings point to a potential relation between pro-inflammatory cytokines and FP.

Peripherally produced pro-inflammatory cytokines can act on the brain via various routes [[60]] where they reduce serotonergic and dopaminergic neurotransmission [[61]]. A deficient serotonergic system was suggested to predispose chickens to develop FP [[62]] and young HFP birds had lower central serotonergic and dopaminergic activity compared to young LFP birds [[48]]. Pro-inflammatory cytokines can further alter hypothalamus-pituitary-adrenal axis sensitization, thereby increasing susceptibility to stressors [[63],[64]]. Stress sensitivity has been suggested to play a role in the development of FP [[8]] and the HFP line had higher stress sensitivity compared to the LFP line [[65],[66]]. Thus, increased immune responsiveness might result in the development of FP via the production of pro-inflammatory cytokines which act on the brain and alter neurotransmission. However, we did not measure pro-inflammatory cytokine levels in this study, as detection of avian cytokines is still limited by the lack of specific antibodies and reliable tests for cytokine production [[67],[68]].

A first indication for a role of the immune system in FP was found, where activation of the specific immune response at a young age increased feather damage at adult age, suggesting that stimulation of the specific immune response predisposes chickens for FP [[69]]. Whether differences in immune responsiveness could be causal to FP or are a consequence of increased locomotor activity, foraging or exploration remains to be elucidated.

4.3. Factors simultaneously affecting behavioral and immune characteristics, such as genetics, gut microbiota, serotonin and stress

The differences seen in immune responsiveness between the FP selection lines could be caused by genes simultaneously involved in FP and the immune system, as indicated by previous studies [[10],[17],[70],[71]]. Apart from the findings in the FP selection lines, other genetic associations have been found between the immune system and feather damage or FP [[10],[15],[16]]. Furthermore, divergent selection on NAb resulted in more feather damage in the high NAb line compared to the low NAb line [[72]], suggesting selection for high NAb results in more FP. A chicken line with less feather damage showed downregulation of genes related to immune system processes in the cerebrum compared to a chicken line with more feather damage [[73],[74]], suggesting low FP is related to downregulation of immune functioning. Feather peckers showed upregulation or downregulation of hypothalamic gene expression involved in immunomodulation compared to neutrals [[75]]. These studies provide evidence for a genetic link between the immune system and FP.

Another factor that has been shown to influence both behavior and the immune system is the gut microbiota [[76–78]]. Interestingly, the FP selection lines were shown to differ in microbiota composition determined from cecal droppings and luminal gut microbiota composition, where the HFP line had a higher relative abundance of Clostridiales but lower relative abundance of *Lactobacillus* compared to the LFP line [[79],[80]]. Administration of *Lactobacillus* species altered SpAb responses [[81]] and *Lactobacillus* species were found to alter NO production by macrophages *in vitro* [[82]]. However, we did not identify microbiota composition in the present study and it should be noted that the relative abundance of *Lactobacillus* was not determined at the species level, but at genus level in previous studies. Yet, the orders of Clostridiales and Lactobacillales were associated with the development of NAb repertoire in mice [[83]], suggesting these orders may regulate production of NAb. Whether the line differences in FP and immune responsiveness are due to differences in microbiota composition remains to be determined.

The serotonergic system, which is involved in regulating many types of behavior, and the hypothalamic-pituitary-adrenal axis, which is the major stress regulatory system, have been indicated to be involved in the development of FP [[8],[84]] and can further influence the immune system. For example, serotonin and glucocorticoid receptors are present on various immune cells, such as lymphocytes, monocytes, macrophages and dendritic cells [[85],[86]]. Furthermore, serotonin seems to play a role in functioning of monocytes and macrophages, gut inflammation and autoimmunity [[85]] and it is well established that stress can alter immune function as mediated by glucocorticoids (i.e. corticosterone) [[86]]. However, stress-induced corticosterone levels and whole blood serotonin levels did not differ between the FP selection lines in the present study (data not shown). Therefore, these systems might not explain the line differences in immune responsiveness seen in the present study.

5. Conclusion

In conclusion, divergent selection on FP affects nitric oxide production by blood derived monocytes, specific antibody titers and natural (auto)antibody titers, but not the relative abundance of immune cell subsets. Thus, divergent selection on FP affects different arms of the immune system, where the HFP line had a more responsive immune system (i.e. higher nitric oxide production, higher IgM and IgG specific antibody titers and higher IgG natural (auto)antibody titers) compared to the LFP line. Although causation cannot be established in the present study, it provides further evidence that relationships between the immune system and FP exist either through behavioral differences, immunological differences, genetics, gut microbiota, serotonin or stress. Based on our and previous findings in the FP selection lines, genetics and immunological differences seem to be the most likely explanations for relationships found. Thus, it is important to take these relationships into account when selecting on certain behavioral or immunological traits.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.physbeh.2019.112680.

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