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PHYSIOLOGY & BEHAVIOR

Title

Stress response, peripheral serotonin and natural antibodies in feather pecking genotypes and phenotypes and their relation with coping style

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Keywords: Feather pecking; genotype; phenotype; stress response; natural antibody; serotonin

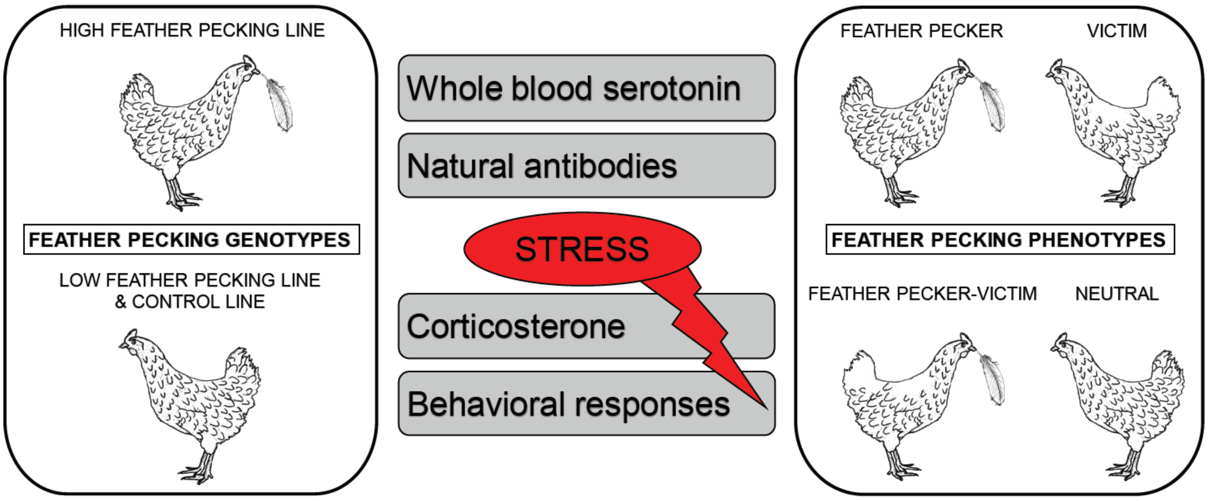
Highlights

- Physiological & behavioral measures were studied in relation to feather pecking (FP)
- Stress response, natural antibody titers, corticosterone & 5-HT level were identified
- FP genotypes differed in behavioral responses, 5-HT level & natural antibody titers
- FP phenotypes differed in behavioral responses & 5-HT level
- FP genotypes and FP phenotypes could not be categorized into coping styles

Abstract

Feather pecking (FP), a serious welfare and economic issue in the egg production industry, has been related to coping style. Proactive and reactive coping styles differ in, among others, the stress response, serotonergic activity and immune activity. Yet, it is unknown whether genetic lines divergently selected on FP (i.e. FP genotypes) or individuals differing in FP (i.e. FP phenotypes) can be categorized into coping styles. Therefore, we determined peripheral serotonin (5-HT) levels, natural antibody (NAb) titers, behavioral and corticosterone (CORT) responses to manual restraint (MR) in FP genotypes (high FP (HFP), low FP (LFP) and unselected control (CON) line) and FP phenotypes (feather pecker, feather pecker-victim, victim and neutral). We further examined the consistency of and relationships between behavioral and physiological measures. FP genotypes differed in behavioral responses to MR, 5-HT levels and NAb titers, but not in CORT levels after MR. HFP birds had less active responses at adolescent age, but more active responses at adult age compared to LFP and CON birds. The CON line had higher 5-HT levels at adolescent age, while the HFP line had lower 5-HT levels than the other lines at adult age. Overall, the HFP line had lower IgM NAb titers, while the LFP line had lower IgG NAb titers compared to the other lines. FP phenotypes differed in behavioral responses to MR and 5-HT levels, but not in CORT levels after MR or NAb titers. Within the HFP line, feather peckers tended to have less active responses compared

to neutrals at adolescent age, while victims had more active responses compared to the other phenotypes at adult age. Feather peckers had higher 5-HT levels than neutrals at adult age. Behavioral and CORT responses to MR were not consistent over time, suggesting that responses to MR might not reflect coping style in this study. Furthermore, proactive behavioral responses were correlated with reactive physiological measures and vice versa. Thus, it was not possible to categorize FP genotypes or FP phenotypes into specific coping styles.



1. Introduction

Feather pecking (FP) is a serious welfare and economic issue in the egg production industry. It involves hens pecking and pulling at feathers of conspecifics, thereby negatively affecting welfare and production. Previous studies have indicated that FP might be related to coping style [1,2]. Coping style can be defined as a coherent set of behavioral and physiological stress responses which is consistent over time and situations (proactive vs. reactive, [3]). In several animal species coping styles are shown to differ in behavioral and physiological responses, where a proactive coping style is, among others, associated with active behavioral responses, low baseline activity and stress reactivity of the hypothalamic-pituitary-adrenal (HPA)-axis, low central serotonergic activity, low humoral immunity, high cellular immunity and innate immune activity compared to a reactive coping style [4–7].

We here focus on the stress response and serotonergic system, as these have been indicated to be involved in FP [8–10]. We further focus on the immune system as it has been related to FP [11,12], specifically on natural antibodies (NAb), antibodies that can bind antigens without prior exposure to that antigen [13]. Natural antibodies 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 [14–17]. Some indications have been found that NAb, specifically NAb binding keyhole limpet hemocyanin (KLH), might be related to FP. Certain genetic mutations were associated with both NAb titers and feather damage (as indicator of FP, [18]) [19,20], and an associative effect of NAb titers on feather damage was detected [21]. These findings reveal a genetic basis for a relation between NAb and FP.

Laying hens divergently selected on FP, resulting in high (HFP) and low FP (LFP) lines [22], differ in their responses to behavioral tests. HFP birds respond more actively compared to LFP birds [23–26] and compared to unselected control (CON) birds [26], suggesting that HFP birds have a more proactive coping style. These FP selection lines further differ in their stress response and serotonergic activity. HFP birds had higher corticosterone (CORT) levels after manual restraint (MR) [27] and vocalized sooner and more, but struggled later and less compared to LFP birds during MR [25]. Furthermore, HFP birds had lower central serotonergic activity at young age, but higher central serotonergic activity compared to LFP birds at adult age [25]. To date, no studies have identified NAb titers in these FP selection lines, but a previous study gave first indications that HFP birds differ from LFP birds in immune reactivity and competence [28]. These findings indicate that divergent selection on FP affects stress responses, serotonergic activity and immune competence. However, results remain inconsistent with regard to lines being categorized as proactive or reactive. This might be explained by the fact that these studies identified differences between genetic lines, but individuals within a genetic line could be proactive or reactive copers.

To get a better understanding of the relation between FP and coping style it is important to identify the coping style of individual birds and relate this to their FP behavior, since birds can become feather peckers, feather pecker-victims, victims or neutrals (i.e. FP phenotypes). Feather peckers and victims within the HFP line seemed to respond more actively to behavioral tests [26], indicating that these birds might have a proactive coping style. Only a few studies to date have related actual FP behavior to the stress response, serotonergic- and immune-systems. FP phenotypes have been shown to differ in serotonergic activity, but the direction of the relation is dependent on brain area studied [29]. FP phenotypes further differed in whole blood serotonin (5-HT) levels, but not in CORT levels after MR [30]. This is supported by a study where FP phenotypes did not differ in CORT levels after MR, but they did differ in behavioral responses to MR [31]. To date, no studies have identified NAb titers in FP phenotypes, but genes associated with the immune system were either upregulated or downregulated in the brain when comparing FP phenotypes [32]. These findings indicate that

FP phenotypes might differ in immune competence, serotonergic activity and behavioral stress responses, yet no findings indicate that FP phenotypes differ in physiological stress responses. Similar to the findings from the FP selection lines, results remain inconsistent with regard to FP phenotypes being categorized as proactive or reactive.

Although differences in FP have been analyzed in relation to the stress response, serotonergic- and immune-systems, no studies to our knowledge have examined these variables in conjunction. Furthermore, most studies to date have compared genetic lines differing in FP, but only a few have compared individuals differing in FP with regard to these variables. Therefore, the aim of this study was to investigate behavioral responses and physiological measures, with a focus on the stress response, serotonergic- and immune-systems, in relation to FP genotype (HFP, LFP and CON lines) and FP phenotype (feather pecker, feather pecker-victim, victim and neutral). Whole blood 5-HT level was used as indicator for central 5-HT [33], CORT level after MR was used as indicator for HPA-axis activity [27] and KLH-binding NAb titer was used as a general indicator for immune competence [34]. The MR test was performed twice, at an adolescent and adult age, to examine consistency in individual differences. We further examined the relation between behavioral responses and physiological measures within FP genotypes and FP phenotypes. Based on previous findings where HFP and feather peckers within the HFP line responded more actively to several behavioral tests, we hypothesized that HFP birds would have a more proactive coping style compared to LFP and CON birds. Furthermore, we hypothesized that feather peckers within the HFP line would have a more proactive coping style compared to other phenotypes.

2. Material and Methods

2.1. Animals and Housing

White Leghorn birds from the 18th generation of an unselected control (CON) line and lines selected on high (HFP) respectively low feather pecking (LFP) were used (see Kjaer et al. [22] 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. A total of 456 birds were produced in two batches of eggs that were incubated at an average egg shell temperature of 37.3 ºC and average relative humidity of 55.6 %. The two batches had the same housing conditions and experimental set-up with 4 pens per line, but with two weeks between batches (see van der Eijk et al. [26] for more details). The experiment was approved by the Central Authority for Scientific Procedures on Animals according to Dutch law (no: AVD104002015150).

2.2 Behavioral Observations and Tests

Feather pecking was observed between 3 and 29 weeks of age. Birds were subjected to a manual restraint test at 14 and 24 weeks of age. The order for observations was always randomized on pen level. The order for testing was randomized on individual level. The experimenters were blinded to the lines and phenotypes.

2.2.1. Feather Pecking Observations

Feather pecking was observed on an individual level from week 3-4, 8-9, 15-16, 18-19, 24-25 and 28-29. In week 3-4, birds were observed by direct observation. Each week birds were observed for 30 min, either in the morning (8:30 h-12:00 h) or in the afternoon (12:30 h-16:00 h), after a 5 min habituation time. Thus, in week 3-4 the total observation time was 60 min. In week 8-9, 15-16, 18-19, 24-25 and 28-29, behavior was observed from video recordings. Each week birds were observed for 15 min, either in the morning (10:40 h-10:55 h) or in the afternoon (14:40 h-14:55 h), with a total observation time of 30 min over two weeks. Feather pecking was categorized according to Table 1 in exploratory FP, bouts of stereotyped FP and severe FP (derived from Newberry et al. [35]). Feather pecking behaviors were summed over two subsequent weeks and the summed number of severe FP, either given or received, was used to identify FP phenotypes. Classification of FP phenotypes was adapted from Daigle et al. [30]. When a bird gave more than one, but received zero or one severe FP it was defined as a feather pecker (P). When a bird gave zero or one, but received more than one severe FP it was defined as a victim (V). When a bird gave and received more than one severe FP it was defined as a feather pecker-victim (P-V). When a bird gave and received zero or one severe FP it was defined as a neutral (N) (see Appendix I and van der Eijk et al. [26] for feather pecking results).

Table 1. Ethogram of the feather pecking observations (after Newberry et al. [35]).

Behavior	**Description**
Exploratory Feather Pecking	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 Feather Pecking Bout	Bird makes ≥ 3 gentle pecks at intervals ≤ 1 s at a single body region. 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 Feather Pecking	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.

2.2.2. Manual Restraint Test

At 14 weeks of age, birds were individually subjected to a manual restraint (MR) test in the same room as their home pens (n = 247) (see Bolhuis et al. [36] for test method). For both batches, the MR test was performed on two days. Birds were caught individually from their pens and placed on their right side on a table covered with cardboard, with the right hand of the experimenter covering the bird's back and the left hand gently stretching the bird's legs. Birds were restrained in this position for 5 min. The latencies to vocalize and to struggle and the number of vocalizations and struggles were recorded. Together, five experimenters tested the birds, where each experimenter tested approximately one fifth of the birds alone. Distribution of birds over experimenters and time of day was random for pens and lines. Fifteen min after the start of the MR test, blood samples were drawn from the wing vein for assessment of the peak in plasma corticosterone (CORT) level [37], whole blood serotonin (5-HT) level and plasma natural antibody (NAb) titers.

At 24 weeks of age, the MR test was repeated using the same method as described above (n = 206), with the following modifications. Birds were caught individually from their pens and placed in a cardboard box. Birds were then moved to one of two testing rooms. Together, three experimenters tested the birds, where each experimenter tested approximately one third of the birds alone.

2.3 Blood Collection and Analyses

Blood was collected from all birds at 4, 9, 14, 19, 24 and 29 weeks of age. Blood was taken from the wing vein using a heparinized syringe and kept on ice after blood sampling. In the laboratory, whole blood samples (1 ml) for determination of 5-HT were stored at -20 °C until further analysis. Blood samples for CORT and NAb were centrifuged at 5250 x g for 10 min at room temperature and the obtained plasma was stored at -20 °C until further analysis.

2.3.1 Plasma Corticosterone

Samples from week 14 and 24 were used for determination of plasma CORT concentrations via a radioimmunoassay kit (MP Biomedicals, LLC, Orangeburg, USA) as described previously [38].

2.3.2 Whole Blood Serotonin

Samples from week 14 and 24 were used for determination of whole blood 5-HT concentration (nmol/mL) via a fluorescence assay as described previously [36]. The centrifugation steps were performed at 931 x g and fluorescence was determined in a Perkin-Elmer 2000 Fluorescence spectrophotometer (PerkinElmer Inc., Waltham, USA) at 295 and 540 nm.

2.3.3 Plasma IgM and IgG Natural Antibody Titers

Samples from all weeks were used for determination of IgM and IgG NAb titers against keyhole limpet hemocyanin (KLH). Strictly, birds produce IgY and not IgG. However, since bird IgY shares homology in function with mammal IgG we refer to IgY as IgG in this study [39]. NAb titers against KLH were determined by an indirect enzyme-linked immunosorbent assay (ELISA) as described previously [40], with the following modifications. Serial dilutions of plasma were made in four steps starting at dilution 1:40,000 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 (catalog A30-102P, Bethyl Laboratories Inc., Montgomery, USA; dilution 1:20,000) or goat-anti-chicken IgG (catalog A30-104P, Bethyl Laboratories Inc., Montgomery, USA; dilution 1:20,000) was used as secondary antibody (100 µl in each well). Substrate buffer was added (100 µl in each well) and after 20 min the reaction was stopped with 50 µL of 1.25M H_2SO_4. Extinctions were measured with a Thermo Scientific Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) 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.

2.4 Statistical Analysis

SAS Software version 9.3 was used for statistical analysis (SAS Inst. Inc., Cary, NC, USA). Linear mixed models for line effects tested per age consisted of fixed effects line and batch and the random effect pen within line. Linear mixed models for line effects on NAb titers (IgM and IgG) consisted of fixed effects line * age, line, age and batch. The random effect consisted of pen within line with a repeated statement for age with chicken ID as subject and an unstructured covariance structure. The unstructured covariance structure gave the best fitting model. Phenotype effects were tested only in the HFP line as on average less than 10% of birds was categorized as feather pecker, feather pecker-victim or victim within the LFP and CON lines (see Table 2 in Appendix I and Table 3 in van der Eijk et al. [26]). Linear mixed models for phenotype effects tested per age consisted of fixed effects phenotype and batch and the random effect pen. Test time (morning 8:00 h-12:30 h or afternoon 12:30 h-18:00 h) and experimenter were added as fixed effects for the MR test (including behavioral responses, CORT and 5-HT levels). The model residuals were visually examined for normality. Variables were square root transformed (i.e. latency to struggle and vocalize, vocalization and struggle frequency, 5-HT level) or log transformed (i.e. CORT level) to obtain normality of model residuals. A generalized linear mixed model with a Poisson distribution was used to test line effects per age for all FP behaviors. A backward regression procedure was used when fixed effects (i.e. test time or experimenter) had a P-value > 0.1. Post hoc pairwise comparisons were corrected by Tukey–Kramer adjustment. Principal component analysis (PCA) was used to establish data reduction for each age separately (14 and 24 weeks of age). The four behavioral measures during MR were included in the PCA for both ages: square root transformed latencies and frequencies of struggles and vocalizations. Only principal components with eigenvalues equal to or larger than 1 were considered for further analyses. PCA loadings were considered significant when loadings were > 0.4 or < -0.4. Pearson correlations were calculated to determine the relationships between behavioral and physiological measures and to establish whether individual differences were consistent over time. P-values < 0.05 were considered to be significant. P-values between 0.05 and 0.1 were considered to indicate a tendency. All data is presented as (untransformed) mean ± standard error of the mean (SEM).

3. Results

3.1. Line Effects

3.1.1. Manual Restraint Test

At 14 weeks of age, line effects were found for latency to struggle ($F_{2,20} = 5.91$, $P < 0.01$) and struggle frequency ($F_{2,20} = 4.26$, $P < 0.05$) during manual restraint (MR). High feather pecking (HFP) birds struggled later and less compared to unselected control (CON) birds ($P < 0.05$). HFP birds struggled later ($P < 0.05$) and tended to struggle more compared to low feather pecking (LFP) birds ($P < 0.1$), while LFP and CON birds did not differ in latency to struggle or struggle frequency (Figure 1A & C). We found no line effects on latency to vocalize or vocalization frequency.

At 24 weeks of age, line effects were found for latency to vocalize ($F_{2,19} = 8.60$, $P < 0.01$) and vocalization frequency ($F_{2,19} = 9.28$, $P < 0.01$). HFP birds vocalized sooner and more compared to LFP ($P < 0.05$ and $P < 0.01$, respectively) and CON birds ($P < 0.01$ and $P < 0.05$, respectively) (Figure 1B & D). LFP and CON birds did not differ in latency to vocalize or vocalization frequency. No line effects were found on latency to struggle or struggle frequency.

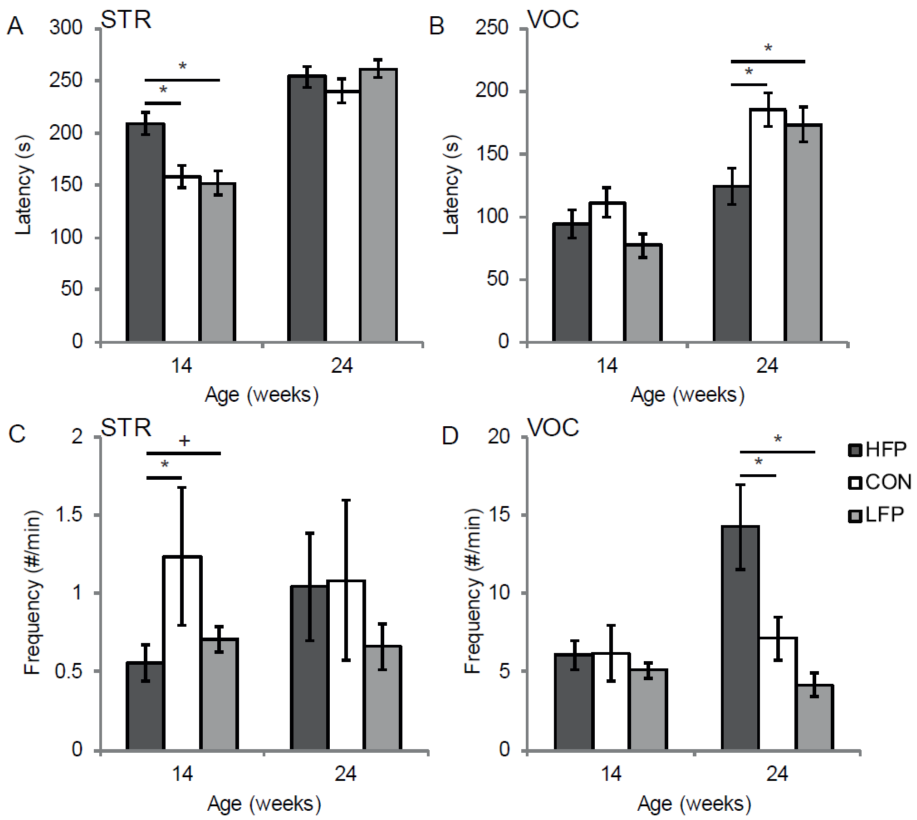


Figure 1. A) Mean latency (± SEM) to struggle (STR), B) mean latency (± SEM) to vocalize (VOC), C) mean struggle frequency (± SEM) and D) mean vocalization frequency (± SEM) during manual restraint at 14 and 24 weeks of age for the high (HFP, n = 87 (14 weeks) and n = 72 (24 weeks)), control (CON, n = 81 (14 weeks) and n = 70 (24 weeks)) and low feather pecking (LFP, n = 79 (14 weeks) and n = 63 (24 weeks)) lines. $^{+}$ show tendencies ($P < 0.1$) and * show significant differences ($P < 0.05$) between lines.

3.1.2. Corticosterone

No line effects were found for corticosterone (CORT) levels after MR at 14 (HFP = 5.35 ng/mL, CON = 4.54 ng/mL and LFP = 5.29 ng/mL) or 24 weeks of age (HFP = 4.22 ng/mL, CON = 5.45 ng/mL and LFP = 4.02 ng/mL).

3.1.3. Serotonin

Line effects were found for whole blood serotonin (5-HT) levels at 14 ($F_{2,20} = 18.24$, $P < 0.01$) and 24 weeks of age ($F_{2,19} = 8.26$, $P < 0.01$). CON birds had higher 5-HT levels compared to LFP and HFP birds (P < 0.01), while HFP and LFP birds did not differ in 5-HT levels at 14 weeks of age. At 24 weeks of age, HFP birds had lower 5-HT levels compared to LFP (P < 0.05) and CON birds (P < 0.01), while LFP and CON birds did not differ in 5-HT levels (Figure 2).

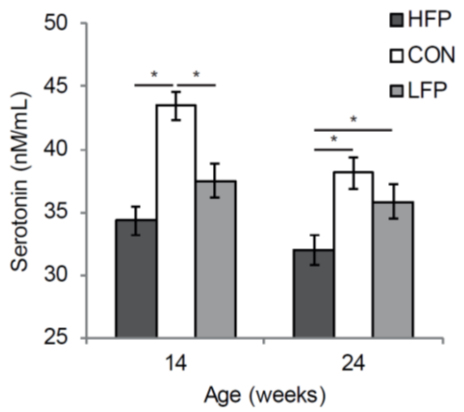


Figure 2. Mean whole blood serotonin level (± SEM) at 14 and 24 weeks of age for the high (HFP, n = 84 (14 weeks) and n = 68 (24 weeks)), control (CON, n = 81 (14 weeks) and n = 68 (24 weeks)) and low feather pecking (LFP, n = 74 (14 weeks) and n = 57 (24 weeks)) lines. * show significant differences (P < 0.05) between lines.

3.1.4. IgM and IgG Natural Antibody Titers

A line * age interaction effect was found for both IgM natural antibody (NAb) titers ($F_{10,1537}$ = 9.47, P < 0.01) and IgG NAb titers ($F_{10,1535}$ = 3.70, P < 0.01) against keyhole limpet hemocyanin (KLH). Overall, HFP birds had lower IgM titers compared to CON and LFP birds (HFP = 5.76, CON = 6.32 and LFP = 6.38, P < 0.01), but CON and LFP birds did not differ significantly. Furthermore, all lines differed significantly from each other for IgG titers, with HFP birds having intermediate, CON birds having the highest and LFP birds having the lowest IgG titers (HFP = 6.08, CON = 6.60 and LFP = 5.46, P < 0.01). For specific comparisons of IgM and IgG titers between lines per age see Figure 3A & 3B, respectively.

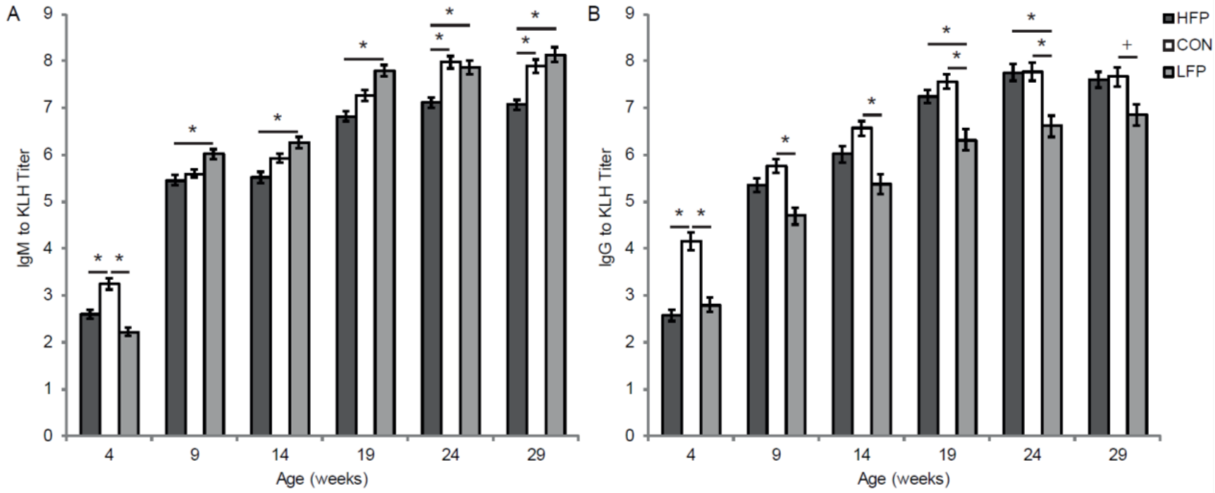


Figure 3. A) Mean natural antibody titers of IgM (± SEM) and B) mean titers of IgG (± SEM) against keyhole limpet hemocyanin (KLH) at 4, 9, 14, 19, 24 and 29 weeks of age for the high (HFP), control (CON) and low feather pecking (LFP) lines. $^{+}$ show tendencies ($P < 0.1$) and * show significant differences ($P < 0.05$) between lines.

3.2. Phenotype Effects in the HFP Line

3.2.1. Manual Restraint Test

Phenotype effects were found for vocalization frequency ($F_{3,75} = 2.81$, $P < 0.05$) during MR at 14 weeks of age. Neutrals tended to vocalize more compared to feather peckers ($P < 0.1$) (Figure 4D). We found no phenotype effects for latency to struggle, latency to vocalize or struggle frequency (Figure 4A, 4B & 4C).

At 24 weeks of age, phenotype effects were found for latency to struggle ($F_{3,58} = 3.67$, $P < 0.05$), latency to vocalize ($F_{3,59} = 3.27$, $P < 0.05$) and vocalization frequency ($F_{3,61} = 4.61$, $P < 0.01$). Victims struggled sooner compared to feather peckers ($P < 0.05$) and tended to struggle sooner compared to feather pecker-victims and neutrals ($P < 0.1$) (Figure 4A). Victims vocalized sooner compared to feather pecker-victims ($P < 0.05$) and tended to vocalize sooner compared to feather peckers ($P < 0.1$) (Figure 4B). Victims vocalized more compared to all other phenotypes (P < 0.05) (Figure 4D). We found no phenotype effects for struggle frequency (Figure 4C).

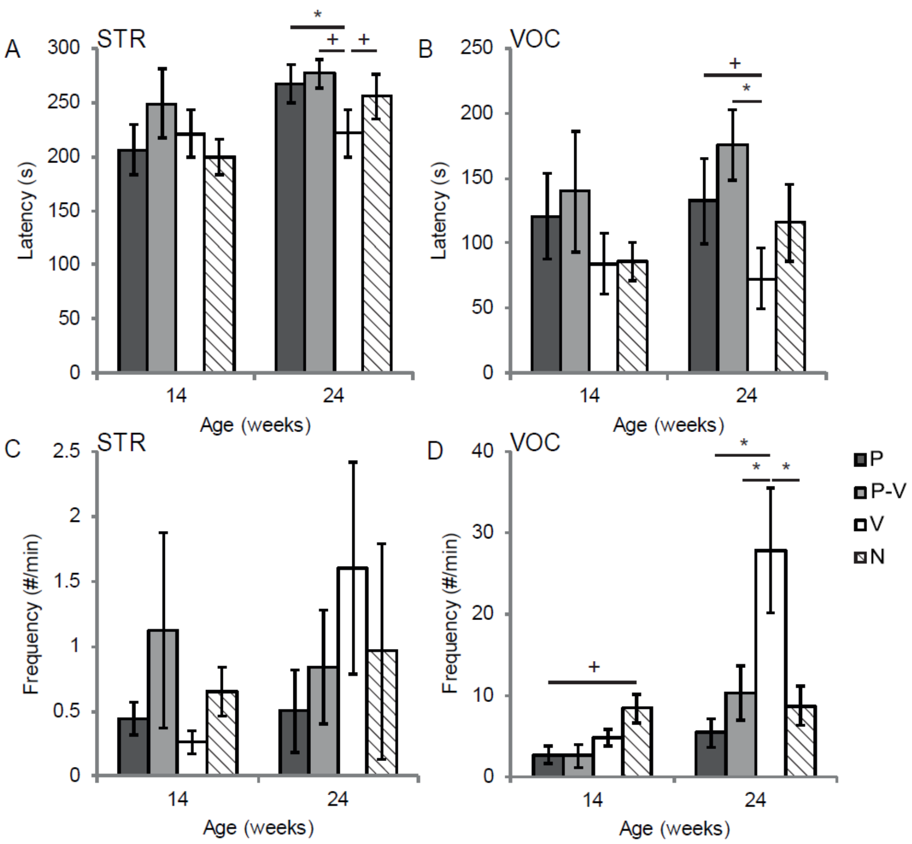


Figure 4. A) Mean latency (± SEM) to struggle (STR), B) mean latency (± SEM) to vocalize (VOC), C) mean struggle frequency (± SEM) and D) mean vocalization frequency (± SEM) during manual restraint at 14 and 24 weeks of age for feather peckers (P, n = 13 (14 weeks) and n = 11 (24 weeks)), feather pecker-victims (P-V, n = 7 (14 weeks) and n = 22 (24 weeks)), victims (V, n = 23 (14 weeks) and n = 21 (24 weeks)) and neutrals (N, n = 43 (14 weeks) and n = 18 (24 weeks)). $^{+}$ show tendencies (P < 0.1) and * show significant differences (P < 0.05) between phenotypes.

3.2.2. Corticosterone

No phenotype effects were found for CORT levels after MR at 14 (feather peckers = 4.85 ng/mL, feather pecker-victims = 4.59 ng/mL, victims = 5.41 ng/mL and neutrals = 5.64 ng/mL) or 24 weeks of age (feather peckers = 6.79 ng/mL, feather pecker-victims = 3.45 ng/mL, victims = 4.49 ng/mL and neutrals = 3.26 ng/mL).

3.2.3. Serotonin

No phenotype effects were found for whole blood 5-HT levels at 14 weeks of age. Phenotype effects were found for 5-HT levels at 24 weeks of age ($F_{3,56} = 3.48$, $P < 0.05$), where feather peckers had higher 5-HT levels compared to neutrals ($P < 0.05$) (Figure 5).

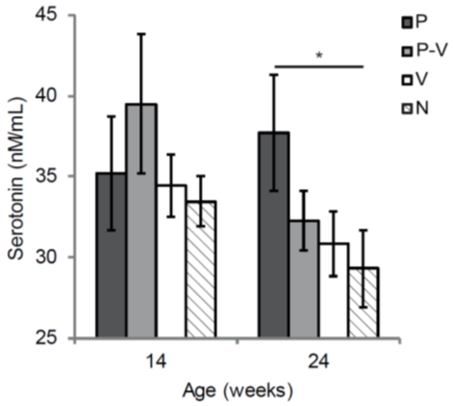


Figure 5. Mean whole blood serotonin level (± SEM) at 14 and 24 weeks of age for feather peckers (P, n = 13 (14 weeks) and n = 11 (24 weeks)), feather pecker-victims (P-V, n = 7 (14 weeks) and n = 20 (24 weeks)), victims (V, n = 22 (14 weeks) and n = 20 (24 weeks)) and neutrals (N, n = 41 (14 weeks) and n = 17 (24 weeks)). * show significant differences ($P < 0.05$) between phenotypes.

3.2.4. IgM and IgG Natural Antibody Titers

Unfortunately, we could not test for phenotype * age interaction effects on IgM or IgG NAb titers as birds switched between phenotypes. No phenotype effects were found for IgM or IgG NAb titers against KLH at 4, 9, 14, 19, 24 or 29 weeks of age.

3.3. Principal Component Analysis

At 14 and 24 weeks of age PCA produced one principal component with eigenvalue larger than 1 (2.00 and 1.94, respectively). All behavioral responses to MR loaded highly on the first principal component at both ages (the percentage of variance explained was 50% and 48%, respectively). We used this behavioral component to identify consistency in behavioral responses to MR over time and to identify relations with physiological measures. At both ages, the behavioral component had high negative loadings for latencies to struggle and vocalize, and high positive loadings for struggle and vocalization frequencies. Thus, chickens with high component scores struggled and vocalized sooner and more and vice versa.

3.4. Consistency of Measures over Time

We will focus on presenting Pearson correlation coefficients that were significant ($P < 0.05$) and above 0.2, as correlation coefficients below 0.2 are thought to show almost negligible relationships [41]. We identified consistency of measures over time within FP genotypes (HFP, CON and LFP). Unfortunately, we were unable to identify consistency over time within FP phenotypes as birds switched between phenotypes. Between 14 and 24 weeks of age, individual differences in 5-HT level, IgM and IgG NAb titers were consistent over time for the HFP line (correlations 0.52, 0.25 and 0.47, respectively). Furthermore, IgM and IgG NAb titers were consistent over time for the LFP line (correlations 0.46 and 0.44, respectively) and CON line (correlations 0.27 and 0.32, respectively). However, scores of the behavioral component and CORT levels were not consistent between 14 and 24 weeks of age for any of the lines (Table 2).

Table 2. Consistency[a] over time of individual differences in behavioral component score and physiological measures as identified in high (HFP), control (CON) and low feather pecking (LFP) lines at 14 and 24 weeks of age.

Measures	Correlations between 14 & 24 weeks of age		
	HFP	CON	LFP
Behavioral component[b]	0.22	0.03	0.07
Corticosterone	0.07	0.06	0.01
Serotonin	0.52**	0.16	0.24
Natural antibody IgM	0.25*	0.27*	0.46**
Natural antibody IgG	0.47**	0.32**	0.44**

[a] Pearson correlations across measures at 14 and 24 weeks of age.

[b] Behavioral component was extracted by principal component analysis of four behavioral responses to manual restraint at both 14 and 24 weeks of age.

*P < 0.05

**P < 0.01

3.5. Relations between Behavioral and Physiological Measures

3.5.1. Line Effects

At 14 weeks of age, the behavioral component was correlated with 5-HT level in CON birds (-0.23), indicating that CON birds which struggled and vocalized sooner and more during MR had low 5-HT levels. At 24 weeks of age, the behavioral component was correlated with 5-HT level, IgM and IgG NAb titers in CON birds (0.26, -0.29 and -0.34, respectively), indicating that CON birds which struggled and vocalized sooner and more during MR had high 5-HT levels, but low IgM and low IgG NAb titers. Behavioral component scores were not correlated with any of the physiological measures for the HFP or LFP lines at both ages.

3.5.2. Phenotype Effects in the HFP line

At 14 weeks of age, we found no significant correlations between the behavioral component and physiological measures for FP phenotypes. At 24 weeks of age, the behavioral component was correlated with CORT level in feather peckers (0.81), suggesting feather peckers that struggled and vocalized sooner and more had high CORT levels. We found no further significant correlations between the behavioral component and physiological measures for FP phenotypes.

4. Discussion

In this study, we investigated behavioral responses and physiological measures, with a focus on the stress response, serotonergic- and immune-systems, in relation to feather pecking (FP) genotype (high FP (HFP), low FP (LFP) and unselected control (CON) line) and FP phenotype (feather pecker, feather pecker-victim, victim and neutral). Tests were performed at adolescent and adult age to examine consistency of individual differences within FP genotypes. We further examined relationships between behavioral responses and physiological measures within FP genotypes and within FP phenotypes of the HFP line.

4.1. Feather Pecking Genotype and Phenotype

4.1.1. Stress Response

HFP birds responded passively (i.e. struggled later and less) at adolescent age and actively (i.e. vocalized sooner and more) at adult age during manual restraint (MR). This is consistent with previous findings where HFP birds struggled later and less, but vocalized sooner and more compared to LFP birds at adolescent age [25] and where HFP birds responded more actively to several behavioral tests at various ages [23,25,26]. Within the HFP line, feather peckers tended to respond passively (i.e. vocalized less) compared to neutrals at adolescent age and victims responded actively (i.e. struggled sooner, vocalized sooner and more) compared to the other phenotypes at adult age during MR. In a previous study, feather peckers were more active during a MR test compared to non-peckers at adult age [31], which is opposite to what we find here. Previously, we also found that feather peckers tended to respond more actively compared to victims and neutrals, and victims responded more actively compared to neutrals in other behavioral tests [26]. Yet, FP genotypes and FP phenotypes did not differ in corticosterone (CORT) levels after MR, thus providing no physiological support for our behavioral findings. Furthermore, this suggests that divergent selection on FP does not affect HPA-axis activity and that FP phenotypes do not differ in HPA-axis activity, indicating that FP genotypes and FP phenotypes might not differ in stress sensitivity. Previously, HFP birds were found to have higher CORT levels after MR compared to LFP birds with CON birds having intermediate levels at adult age [27], suggesting that HFP birds are more reactive. This discrepancy between studies might be explained by the fact that we used birds from the 18th generation, while the previous study used birds from the 6th generation. These birds were selected as parents of the 7th generation, thus containing extreme individuals with regard to FP [27]. Furthermore, the FP selection lines were maintained for subsequent generations which could have caused physiological effects to become less pronounced. In addition, HFP birds had increased heart rate and reduced heart rate variability compared to LFP birds [42], suggesting that HFP birds are more proactive. When comparing other lines, selected on egg production traits but also differing in FP, the opposite was found with high FP being related to low CORT levels after MR [1,12,43]. Furthermore, no differences in CORT levels were found between FP phenotypes in previous studies [30,31]. Thus, there is inconsistency in findings with regard to the relation between high FP and CORT levels within FP genotypes, whereas FP phenotypes do not seem to differ in CORT levels after MR.

It should be noted that behavioral and physiological responses to MR in this study might not be indicative of a stress response, as CORT levels after MR were generally low (average 4.8 ng/mL). Previous studies found peaks above 6.5 ng/mL [27,36,37]. Low CORT levels might be explained by the fact that we performed multiple behavioral tests (see van der Eijk et al. [26]), causing birds to become habituated to handling. In repeatedly handled birds CORT levels reduced faster after handling compared to unhandled birds [44]. Thus, our MR test possibly did not induce a strong stress response, making behavioral and physiological findings difficult to interpret in relation to the stress response. Based on our findings we suggest that divergent selection on FP affects behaviors other than FP (i.e. activity) and that FP phenotypes differ in their behavioral responses.

4.1.2. Serotonergic System

CON birds had higher whole blood serotonin (5-HT) levels compared to HFP and LFP birds at adolescent age, while HFP birds had lower whole blood 5-HT levels compared to CON and LFP birds at adult age. A previous study found the opposite relationship, with HFP birds having higher plasma 5-HT levels than LFP birds [28]. This discrepancy with our study might be explained by the methods used (plasma vs. whole blood), as whole blood 5-HT more likely reflects storage concentration of 5-HT, while plasma 5-HT reflects unbound 5-HT [45]. Previous studies support our findings, where lines with a high FP tendency had lower whole blood 5-HT levels at adult ages (> 40 weeks) [33,46,47], suggesting that high FP is related to low peripheral 5-HT levels. Although FP phenotypes did not differ in whole blood 5-HT at adolescent age, feather peckers within the HFP line had higher whole blood 5-HT levels compared to neutrals at adult age. Previously the opposite was found where neutrals had higher whole blood 5-HT compared to victims and feather pecker-victims at adult age [30]. The peripheral and central serotonergic system show similar characteristics in their transporters and receptors [48] and whole blood 5-HT was correlated with central 5-HT, 5-HIAA (5-HT metabolite) and 5-HT turnover (5-HIAA/5-HT) in chickens [33]. However, caution is needed when extrapolating whole blood 5-HT levels to central 5-HT levels as 5-HT cannot cross the blood-brain barrier [49]. Yet, in a previous study the FP selection lines were shown to differ in central serotonergic activity, where HFP chicks had lower central serotonergic activity compared to LFP chicks in several brain areas. At adult age the differences had disappeared or were opposite to what was found at young age [25]. Low central serotonergic activity might thus predispose chickens to develop FP, while at an adult age high FP seems to be related to high central serotonergic activity (see de Haas and van der

Eijk, [10] for a review). This shift in activity might be linked to performing or receiving FP as FP phenotypes were shown to differ in central serotonergic activity, where feather peckers had higher central serotonergic activity compared to neutrals [29].

It is interesting to note that we found a similar opposite relation between FP and whole blood 5-HT level, with HFP birds having lowest 5-HT but feather peckers within the HFP line having highest 5-HT. The actual performance of FP might increase peripheral 5-HT levels, possibly due to feather eating. HFP birds are more prone to eat feathers compared to LFP birds [50,51] and feather peckers showed more feather eating compared to non-peckers [52]. Ingestion of feathers may increase peripheral 5-HT by providing structural components as the gut releases 5-HT in reaction to sensory perception of the mucosal layer [53]. However, this relation between feather eating and increased peripheral 5-HT remains speculative and further research is needed. Based on our findings we suggest that divergent selection on FP affects whole blood 5-HT, potentially via mutations and/or alterations in expression of genes involved in the serotonergic system as previously found in relation to feather damage [20] and in the FP selection lines [54,55]. This is supported by the finding that whole blood 5-HT level was consistent between ages in the HFP line, but not in the CON and LFP lines. We further show that FP phenotypes differ in whole blood 5-HT. Since birds in our study already started to feather peck at a young age, we cannot distinguish between cause or consequence of FP in relation to whole blood 5-HT. Therefore, it would be interesting to identify whole blood 5-HT levels in birds prior to and after the development of FP.

4.1.3. Immune System

Overall, HFP birds had lower IgM NAb titers compared to CON and LFP birds, while LFP birds had lower IgG NAb titers compared to CON birds with HFP birds having intermediate titers. FP phenotypes did not differ in IgM or IgG NAb titers. Thus, we only found differences between FP genotypes but not between FP phenotypes. This could suggest that there are genes simultaneously involved in FP and the immune system as indicated by previous studies [20,56] even in the FP selection lines [28,57]. Indeed, both NAb titers and the performance of FP have been shown to be heritable traits [40,58]. This is further supported by our finding that both IgM and IgG NAb titers are consistent over time. Findings from a previous study in the FP selection lines, suggest that HFP birds differ from LFP birds in immune reactivity and competence [28]. Furthermore, when conspecifics within a cage had higher IgG NAb, the individual might have more feather damage [21]. This is consistent with our study where HFP birds had higher IgG NAb titers compared to LFP birds, although CON birds did not differ from HFP birds in IgG NAb titers. Interestingly, the HFP line had lower IgM NAb titers, while the LFP line had lower IgG NAb titers compared to the other lines. Previously, it was suggested that IgG NAb are dependent upon exogenous antigen stimulation, while IgM NAb are not [59]. Thus, IgM NAb may be more under genetic influence, while IgG NAb may reflect immunomodulating environmental influences. This is further supported by a study that found high genetic correlations, but low phenotypic correlations between IgM and IgG NAb [40]. In the FP selection lines, this could mean that lower IgM NAb titers in the HFP line might be explained by alterations in their genetic make-up, while the lower IgG NAb titers in the LFP line might be explained by a difference in environmental influences or immune responsiveness to environmental influences. As lines were exposed to similar environmental conditions, we suggest that the LFP line had reduced immune responsiveness to environmental influences compared to the HFP and CON lines. Previously, the HFP line had higher responses to infectious bursal disease virus compared to the LFP and CON lines [28], suggesting that the HFP line had increased specific antibody responsiveness. Together with our findings this might indicate that HFP birds show increased immune responsiveness (i.e. they are more responsive to the environment) than LFP birds. In this study we focused on

NAb titers, yet further research is needed to identify whether the FP selection lines differ in immune responsiveness by for example, measuring innate and cellular responses to environmental challenges. Furthermore, high NAb titers (both IgM and IgG) have been related to increased survival in laying hens and NAb titer has been suggested as an indicator for general disease resistance [34,60,61]. Therefore, divergent selection on FP could potentially affect survival and health via altering NAb titers.

4.2. Coping style

Although previous studies have found differences in coping styles between lines which differ in FP tendency [1,62], we did not find such a clear relation here for FP genotypes or FP phenotypes. Behavioral responses to MR (as indicated by the behavioral component) and CORT levels were inconsistent between ages, suggesting that behavioral and physiological responses to MR in this study might not reflect coping style. Furthermore, for both FP genotypes and FP phenotypes proactive behavioral responses were correlated with reactive physiological measures (either NAb titers, CORT or 5-HT levels) and vice versa. Thus, we cannot categorize FP genotypes or FP phenotypes into specific coping styles.

A limitation in our study is that we observed FP behavior for a limited amount of time which might have led to FP behavior not being observed. However, continuous observation is impractical and the strength of this study was that we identified phenotype effects using the most recent FP phenotype categorization that was based on FP observations closest to the MR test at 14 or 24 weeks of age and to blood sampling at 4, 9, 19 and 29 weeks of age. We emphasize the importance of identifying FP phenotypes as they seem to differ in their behavioral responses and in whole blood 5-HT levels.

5. Conclusion

Divergent selection on feather pecking (FP) affects behavioral characteristics other than FP (i.e. activity), serotonergic- (i.e. peripheral serotonin) and immune-systems (i.e. natural antibodies), but FP genotypes did not differ in HPA-axis activity (i.e. corticosterone) in the present study.

Feather pecking phenotypes seem to differ in behavioral responses and the serotonergic system (i.e. peripheral serotonin), but differences in HPA-axis activity (i.e. corticosterone) or immune system (i.e. natural antibodies) were not found.

The present study could not support the categorization of FP genotypes or FP phenotypes into specific coping styles.

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Supplementary data

Feather Pecking Observations

An overview of the line effects on feather pecking behavior at different ages is given in Table 1. Lines did not differ in exploratory feather pecks (EFP) or stereotyped feather pecking bouts (StFP) at 18-19 and 24-25 weeks of age. Line effects were found for severe feather pecks (SFP) given at 18-19 ($F_{2,20}$ = 11.90, P < 0.01) and 24-25 weeks of age ($F_{2,19}$ = 10.16, P < 0.01). HFP birds showed more SFP at 18-19 (P <0.01) and 24-25 weeks of age (P < 0.01) compared to LFP birds. HFP birds showed more SFP at 24-25 weeks of age (P < 0.05) and tended to show more SFP at 18-19 weeks of age compared to CON birds (P < 0.1). LFP and CON birds only differed in SFP at 18-19 weeks of age, with CON birds showing more SFP compared to LFP birds (P < 0.05).

Table 1. Feather pecking behavior (exploratory feather pecking (EFP), stereotyped feather pecking (StFP) (bouts) and severe feather pecking(SFP)) of the high (HFP), control (CON) and low feather pecking (LFP) lines at different ages.

Variables	**HFP**	**CON**	**LFP**	**P-value**
Age (18-19 weeks)	**n = 86**	**n = 81**	**n = 77**	
EFP	8.81 ± 1.50	6.96 ± 0.84	5.51 ± 0.64	ns
StFP (bouts)	1.63 ± 0.32	1.28 ± 0.35	1.27 ± 0.23	ns
SFP	5.88 ± 2.34^{a}	1.19 ± 0.30^{a}	0.16 ± 0.07^{b}	< 0.01
Age (24-25 weeks)	**n = 72**	**n = 70**	**n = 63**	
EFP	4.14 ± 0.61	3.86 ± 0.57	2.99 ± 0.44	ns
StFP (bouts)	0.67 ± 0.19	0.26 ± 0.11	0.22 ± 0.08	ns
SFP	7.67 ± 2.03^{a}	2.09 ± 0.44^{b}	1.08 ± 0.27^{b}	< 0.01

Average number of pecks or bouts per bird per hour (30 min total observation time per bird). Differing lowercase letters (a,b) show significant differences (P < 0.05) between lines.

Feather Pecking Phenotypes

Birds were categorized as feather pecker, feather pecker – victim, victim and neutral. The number (and percentage) of hens within each category at different ages is given in Table 2. On average the largest percentage of hens was categorized as neutrals across all ages in all three lines (HFP 47.8%; CON 77.6%; LFP 85.2%). The remainder of hens was categorized as feather pecker (HFP 14.5%; CON 9.4%; LFP 7.5%), feather pecker - victim (HFP 13.7%; CON 3.3%; LFP 1.9%) and victim (HFP 24.1%; CON 9.6%; LFP 5.4%).

Table 2. The number (and percentage) of hens per phenotype category (feather pecker (P), feather pecker-victim (P-V), victim (V) and neutral (N)) within the high (HFP), control (CON) and low feather pecking (LFP) lines based on the number of severe feather pecks (SFP) given or received at different ages.

	P	P-V	V	N
Criteria	Give > 1 SFP Receive 0 or 1 SFP	Give > 1 SFP Receive > 1 SFP	Give 0 or 1 SFP Receive > 1 SFP	Give 0 or 1 SFP Receive 0 or 1 SFP
		Age (3-4 weeks)		
HFP	16 (12.2%)	13 (9.9%)	34 (26.0%)	68 (51.9%)
CON	7 (5.6%)	2 (1.6%)	10 (7.9%)	107 (84.9%)
LFP	7 (5.6%)	5 (4.0%)	4 (3.2%)	109 (87.2%)
		Age (8-9 weeks)		
HFP	19 (17.3%)	3 (2.7%)	16 (14.6%)	72 (65.5%)
CON	6 (5.8%)	1 (1.0%)	5 (4.9%)	91 (88.4%)
LFP	5 (5.0%)	0 (0.0%)	4 (4.0%)	92 (91.1%)
		Age (12-13 weeks)		
HFP	19 (21.6%)	8 (9.1%)	17 (19.3%)	44 (50.0%)
CON	12 (14.8%)	8 (9.9%)	11 (13.6%)	50 (61.7%)
LFP	13 (16.5%)	4 (5.1%)	9 (11.4%)	53 (67.1%)
		Age (15-16 weeks)		
HFP	13 (15.1%)	7 (8.1%)	23 (26.7%)	43 (50.0%)
CON	7 (8.6%)	1 (1.2%)	9 (11.1%)	64 (79.0%)
LFP	4 (5.2%)	1 (1.3%)	4 (5.2%)	68 (88.3%)
		Age (18-19 weeks)		
HFP	10 (11.6%)	11 (12.8%)	21 (24.4%)	44 (51.2%)
CON	9 (11.1%)	2 (2.5%)	8 (9.9%)	62 (76.5%)
LFP	1 (1.3%)	0 (0.0%)	1 (1.3%)	75 (97.4%)
		Age (24-25 weeks)		
HFP	11 (15.3%)	22 (30.6%)	21 (29.2%)	18 (25.0%)
CON	10 (14.3%)	5 (7.1%)	11 (15.7%)	44 (62.9%)
LFP	8 (12.7%)	2 (3.2%)	7 (11.1%)	46 (73.0%)
		Age (28-29 weeks)		
HFP	6 (8.5%)	16 (22.5%)	20 (28.2%)	29 (40.9%)
CON	4 (5.7%)	0 (0.0%)	3 (4.3%)	63 (90.0%)
LFP	4 (6.3%)	0 (0.0%)	1 (1.6%)	58 (92.1%)

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