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Title

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Abstract

Feather pecking (FP) is a major welfare and economic issue in the egg production industry. Behavioural characteristics, such as fearfulness, have been related to FP. However, it is unknown how divergent selection on FP affects fearfulness in comparison to no selection on FP. Therefore, we compared responses of birds selected on low (LFP) and high feather pecking (HFP) with birds from an unselected control line (CON) to several behavioural tests (i.e. novel object (NO), novel environment (NE), open field (OF) and tonic immobility (TI)) at young and adult ages. Furthermore, the relation between actual FP behaviour (i.e. FP phenotypes) and fearfulness is not well understood. Therefore, we compared responses of birds with differing FP phenotypes. Feather pecking phenotypes of individual birds were identified via FP observations at several ages. The number of severe feather pecks given and received was used to categorize birds as feather peckers, feather pecker-victims, victims or neutrals. Here we show that HFP birds repeatedly had more active responses (i.e. they approached a NO sooner, vocalized sooner and more, showed more flight attempts and had shorter TI durations), which could indicate lower fearfulness, compared to CON and LFP birds at both young and adult ages. Within the HFP line, feather peckers had more active responses (i.e. they tended to show more flight attempts compared to victims and tended to walk more compared to neutrals), suggesting lower fearfulness, compared to victims and neutrals. Thus, in this study high FP seems to be related to low fearfulness, which is opposite to what previously has been found in other experimental and commercial lines. This stresses the need for further research into the genetic and phenotypic correlations between FP and fearfulness in various populations of chickens, especially in commercial lines. Findings from experimental lines should be used with caution when developing control and/or prevention methods that are to be applied in commercial settings. Furthermore, activity and/or coping style might overrule fearfulness within the HFP line, as HFP birds and feather peckers within the HFP line had more active responses. This might indicate a complex interplay between fearfulness, activity and coping style that could play a role in the development of FP.

Keywords Feather pecking; phenotype; genotype; fearfulness; activity; coping style.

1. Introduction

Feather pecking (FP) is a major behavioural problem in the egg production industry and involves laying hens pecking and pulling at feathers of conspecifics. Different types of FP have been defined: gentle feather pecking (GFP) consists of nibbling or gentle pecks at the feathers and causes little or no damage; and severe feather pecking (SFP) consists of forceful pecks and pulls of feathers and can thus cause serious damage to the recipient and can even develop into cannibalistic pecking (Savory, 1995). Preventing or controlling FP is difficult as it is influenced by many factors, both environmental and genetic (Rodenburg et al., 2013).

Certain behavioural characteristics, such as fearfulness, have been related to FP. Fearfulness can be defined as the tendency of an animal to be easily frightened in response to potentially dangerous stimuli (Boissy, 1995; Jones, 1996). Selection on egg production traits resulted in a high (HP) and low (LP) FP line (Korte et al., 1997). HP chicks showed a longer duration of freezing, and vocalized and walked later in an open field (OF) test than LP chicks, but no difference was found in tonic immobility (TI) duration (Jones et al., 1995). In a commercial line comparison, fewer Rhode Island Red (RIR) birds moved away from a novel object (NO) than White Leghorn (WL) birds at adult age and WL birds had more feather damage, indicating that WL birds were more fearful and showed more FP than RIR birds (Uitdehaag et al., 2008). On an individual level Rodenburg et al. (2004) found a strong negative correlation between OF activity at a young age and high levels of FP at adult age, indicating that fearful chicks are more likely to develop FP as adult birds. This is supported by de Haas et al. (2014) on farm level who showed that fear of humans during the rearing period is a predictor for feather damage at adult ages. These findings indicate that FP is related to high fearfulness in young and adult birds.

In lines divergently selected on FP, resulting in a high (HFP) and a low (LFP) FP line (Kjaer et al., 2001), first indications were found that they differ in fearfulness. However, the relationship between fearfulness and FP seems to be the opposite to that described above. Kops et al. (2017) found that HFP chicks vocalized and walked sooner in an isolation test, approached a NO faster and more chicks approached the NO compared to LFP chicks and similar results were found in a human approach

(HA) test, suggesting HFP chicks were less fearful compared to LFP chicks. Lines did not differ in the number of steps or vocalizations, or in the latency to vocalize in an OF test at adolescent age (Kops et al., 2017). In a novel maze, HFP birds walked a longer distance, spent a larger proportion of time walking and vocalized sooner compared to LFP birds at adult age (de Haas et al., 2010). Bögelein et al. (2014) found that adult HFP birds had a shorter TI duration, shorter latency to step and vocalize in an OF test and shorter latency to emerge in an emerge test compared to LFP birds. The findings from these studies suggest that HFP birds are less fearful compared to LFP birds at an adult age. Another study, however, found no differences between the HFP and LFP line in TI, HA or NO test at an adult age (Rodenburg et al., 2010). Taken together, there is inconsistency on whether the FP selection lines differ with regard to fearfulness, especially at an adult age. At a young age HFP chicks seem to be less fearful and show more active responses compared to LFP chicks. Thus, the FP selection lines show a different relation between FP and fearfulness compared to commercial lines and other experimental lines (i.e. HP and LP lines). Yet, other factors such as coping style and/or activity could play a role in the behavioural responses of the FP selection lines as suggested by previous studies (de Haas et al., 2010; Kjaer, 2009; Kops et al., 2017).

In order to better understand the development of FP it is crucial to identify how actual FP behaviour is related to behavioural characteristics, since animals can become feather peckers, feather pecker-victims, victims or neutrals (i.e. FP phenotypes). Only a few studies to date have related actual FP behaviour to fearfulness. Vestergaard et al. (1993) found a positive correlation between TI duration and rate of SFP given, indicating that feather peckers are more fearful. Jensen et al. (2005) showed that adult feather peckers were faster at approaching both novel food and a NO compared to non-feather peckers, but feather peckers and non-feather peckers did not differ in TI duration. In the FP selection lines, Bögelein et al. (2014) found low correlations between FP and several fear criteria, suggesting that fear might not be related to FP. Thus, FP phenotypes seem to differ in fearfulness, but the direction of the relation remains unclear and may depend on the genotype used.

As it is unknown how divergent selection on FP affects fearfulness in comparison to no selection on FP, we compared responses of HFP and LFP birds with those of birds from an unselected control line

(CON) to several behavioural tests at young and adult ages. Furthermore, as the relation between actual FP behaviour (i.e. FP phenotypes) and fearfulness is not well understood, we compared the responses of birds with differing phenotypes. Therefore, the aim of this study was to investigate fearfulness in relation to FP genotype (divergent selection on FP and no selection on FP) and FP phenotype (actual FP behaviour). We hypothesized that HFP birds would be less fearful than LFP and CON birds at both young and adult ages. Based on previous findings the relation between fearfulness and FP phenotypes remains unclear, so we had no a priori hypothesis for differences in fearfulness between FP 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., 2001) for a detailed description of the selection procedure). The HFP and LFP line were divergently selected on FP for seven generations and were maintained in subsequent generations. The parent stock was between 38 and 43 weeks of age at the time of egg collection. 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. Only non-beak-trimmed female birds were used for the experiment. Birds were housed per line in 24 floor pens (height 2 m, length 2 m, width 1 m) in groups of 19 birds. At 1 day, 5 weeks and 10 weeks of age group size was reduced (n = 16-17 birds per pen, n = 10-15 birds per pen and n = 8-12 birds per pen, respectively). At 20 weeks of age, group size was levelled out at 8-9 birds per pen, with a total of 63 LFP, 72 HFP and 71 CON birds. All birds were individually marked with a small neck tag with a colour/number combination (Roxan, Selkirk, Scotland) for individual identification. At 3 and 4 weeks of age, birds were colour marked on the neck and/or back for individual identification (colours: black, purple, green, blue and orange). The same colours were used in a previous study where no effect on FP was found

(Rodenburg et al., 2003). At 7 weeks of age, the birds were equipped with a light weight backpack with a number for individual identification.

At all times, water and feed were provided *ad libitum.* Birds received a standard rearing diet 1 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. Each pen was provided with wood shavings on the floor, a perch installed 5 cm above the floor from 3 to 5 weeks of age and a perch installed 45 cm above the floor from 6 weeks of age onwards. Post hatch, temperature was kept around 33°C and gradually lowered to 24°C at 4 weeks of age. From 19 weeks of age onwards, temperature was kept around 21°C. 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 13L:11D at 20 weeks of age. At 22 weeks of age, the light regime was increased to 16L:8D. Light intensity for each pen was measured with a Voltcraft MS-1300 light meter (Conrad Electric Benelux, Oldenzaal, the Netherlands) and ranged between 34.8-68.2 LUX (average 48.1 LUX) during the first 3 weeks of life. At 3 weeks of age the light intensity was lowered, ranging between 2.74-7.09 LUX (average 4.68 LUX) to reduce the risk of cannibalism. Straw was provided in racks from 3 to 20 weeks of age to enrich the environment and reduce the risk of cannibalism. At 20 weeks of age straw racks were removed. A wooden nest box was placed in front of the pen at 15 weeks of age. Visual barriers of approximately 1.5 m high were placed between pens at the start of the experiment to prevent birds in adjacent pens of seeing each other. Birds received vaccinations against Marek's disease (day 0, intramuscular (i.m.)), Infectious Bronchitis (day 0, 14, 56 and 108, via spray), Newcastle Disease (day 7, 28, 70 via spray and day 84 i.m.), Infectious Bursal Disease/Gumboro (day 25, via drinking water), Avian Encephalomyelitis and Pox Diphteria (day 84, via wing web injection) and Infectious Laryngo Tracheitis (day 84, via eye drops). The experiment was approved by the Central Authority for Scientific Procedures on Animals according to Dutch law (no: AVD104002015150).

2.2 Behavioural Observations and Tests

Feather pecking behaviour was observed between 3 and 29 weeks of age. Birds were subjected to four behavioural tests that are related to fearfulness: novel object test, novel environment test, open field test and tonic immobility test. The novel object test and tonic immobility test were performed twice. A timeline of the feather pecking observations and behavioural tests performed at specific ages is provided in Figure 1. The order for testing and observations was always randomized on pen level. Order for testing during the open field test and tonic immobility test were randomized on individual level. The experimenters were blinded to the lines.

2.2.1. Feather Pecking Observations

Feather pecking behaviour was observed on an individual level from week 3-4, 8-9, 12-13, 15-16 and 28-29. In week 3-4 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 5 min habituation time. In week 8-9, 12-13, 15-16 and 28-29 behaviour 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 programme (Noldus Information Technology B.V., Wageningen, the Netherlands) was used for video analysis of FP, categorized according to Table 1 (derived from Newberry et al., 2007) in gentle feather pecks (subdivided into exploratory gentle feather pecks (EFP) and bouts of stereotyped gentle feather pecking (StFP)) and severe feather pecks (SFP). Feather pecking behaviours were summed over two subsequent weeks, thus including one morning and one afternoon observation with a total observation period of 60 min for week 3-4 and a total observation period of 30 min for all other time points. The summed number of SFP, either given or received, was used to identify FP phenotypes. Classification of phenotypes was adapted from Daigle et al. (2015). When a bird gave more than one SFP it was defined as a feather pecker (P). When a bird received more than one SFP it was defined as a victim (V). When a bird gave and received more than one SFP it was defined as a feather pecker-victim (P-V). When a bird gave and received zero or one SFP it was defined as a neutral (N).

2.2.2. Novel Object Test

At 4 days and 10 weeks of age, the response to a novel object (NO) was tested. At 4 days of age, the NO was a wooden block (height 8 cm, length 5 cm, width 2.5 cm) wrapped with coloured tape (green, white, black, yellow, and red) (n = 24, see de Haas et al., 2014 for a detailed description of the test method). The test started 10 sec. after the experimenter had placed the NO on the floor in the centre of the home pen (n = 24). The latency for three different birds to approach the NO at a distance of < 25 cm and the number of birds that were within < 25 cm of the NO was recorded every 10 sec for the 2 min test duration. At 10 weeks of age, the NO test was repeated (n = 24). The NO was a plastic stick (length 50 cm, diameter 3.5 cm) wrapped with coloured tape (red, white, green, black, and yellow)(based on Welfare Quality®, 2009). The same experimenter tested all pens at 4 days and 10 weeks of age.

2.2.3. Novel Environment Test

At 4 weeks of age, the response to a novel environment (NE) was tested for a duration of 1 min (n = 387, see de Haas et al., 2014 for a detailed description of the test method). All birds from a pen were taken and transported in a cardboard box to a room near the testing rooms. The average time difference between the first and last bird to be tested was 25 min. Birds were taken out of the cardboard box to one of two test locations, where birds were placed inside a white bucket (height 57 cm, length 32 cm, width 22 cm). The bucket was covered with a wire mesh to prevent birds from escaping. The experimenter was out of sight of the bird while testing, but was able to record latency to vocalize, number of vocalizations and number of flight attempts. After testing, birds were returned to a second cardboard box and when all birds from a pen were tested they were returned to their home pen. Together, two experimenters tested all birds where each experimenter tested approximately half of the birds alone.

2.2.4. Open Field Test

At 15 weeks of age, birds were individually subjected to an open field (OF) test for a duration of 5 min (n = 244, see Rodenburg et al. (2009) for a detailed description of the test method). Birds were individually transported to and from the test room in a cardboard box. A square wooden enclosure (height 1.22 m, length 1.15 m, width 1 m) was used. Wire mesh prevented birds from escaping. The front of the enclosure consisted of Plexiglas. A video-camera was placed approximately 1.0 m in front of the Plexiglas. A bird was placed in the middle of the OF at the start of the test. The experimenter was out of sight of the bird while testing, but was able to record latency to step and number of steps from a monitor and latency to vocalize and number of vocalizations. One experimenter tested all birds.

2.2.5. Tonic Immobility Test

At 13 weeks of age, birds were individually subjected to a tonic immobility (TI) test for a maximum duration of 5 min (n = 248, see Jones and Faure (1981) for a more detailed description of the test method). The TI test was performed on two consecutive days in the afternoon and birds were randomly assigned to a test day with half of a pen being tested on the first day and the other half on the second day. Half of the birds in a pen were taken and transported in a cardboard box to a room near the testing rooms. The average time difference between the first and last bird to be tested was 15 min. Birds were taken out of the cardboard box to one of two test locations, where they were placed in supine position in a metal cradle with their head suspended from the side of the cradle. The right hand of the experimenter was placed on the breast of the bird, while the left hand gently forced the bird's head down lightly while cupping its head. Each bird was restrained in this position for 10 sec. When after releasing, the bird remained in this position, TI duration was recorded until the bird returned to upright position. If this happened within 10 sec after release, TI was induced again, with a maximum of three attempts at inducing TI. Eye contact with the bird was avoided, but the experimenter was visible for the bird during the test. The experimenter recorded the number of induction attempts needed and the duration of TI (latency to self-righting). After testing, birds were returned to a second cardboard box and when all birds from a cardboard box had been tested they were returned to their home pen. Together, three experimenters tested all birds where each experimenter tested approximately a third of the birds alone.

At 28 weeks of age, the tonic immobility test was repeated (n = 205). The average time difference between the first and last bird to be tested was 12 min. Together, two experimenters tested all birds where each experimenter tested approximately half of the birds alone.

2.3 Statistical Analysis

SAS Software version 9.3 was used for statistical analysis (SAS Institute Inc., Cary, USA). Linear mixed models for line effects were tested for each age separately and consisted of fixed effects of line and batch and the random effect of pen within line, except for the NO test, which was tested at pen level. Phenotype effects were tested only in the HFP line as on average less than 10% of birds was categorized as P, P-V or V within the LFP and CON lines (See Table 3). Linear mixed models for phenotype effects in the HFP line consisted of fixed effects of FP phenotype and batch and the random effect of pen. Phenotype effects were tested for each behavioural test separately using the most recent FP phenotype categorization (for example, FP phenotypes based on FP observations from week 3 and 4 were used to identify phenotype effects in the NE test). Phenotype effects in the NO test at 4 days of age were not tested as we could not identify FP phenotypes at that age. Test time (morning 8:00 h-12:30 h or afternoon 12:30 h-18:00 h) was added as fixed effect for the NE test and the OF test. Experimenter was added as fixed effect for the NE test and the TI test. Testing order was included as fixed effect for the TI test. The model residuals were visually examined for normality. Variables were square root transformed (i.e. percentage of birds that approached the NO; latency to vocalize and frequency of vocalizations in the NE test; latency to vocalize and step, frequency of steps and vocalizations in the OF test; and TI duration) to obtain normality of model residuals. A Kruskal Wallis test was used to analyse line effects for latency to approach the NO and post hoc comparisons were made with Dunn's test. A generalized linear mixed model with a Binary distribution was used to test line and phenotype effects in the HFP line for flight attempts in the NE test. A generalized linear mixed model with a Poisson distribution was used to test line effects for all FP behaviours. A backward regression procedure was used when fixed effects (i.e. test time, experimenter or testing order) had $P > 0.1$. Post hoc pairwise comparisons were corrected by Tukey–Kramer adjustment. 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. Feather Pecking Observations

An overview of the line effects on feather pecking behaviour at different ages is given in Table 2. Line effects were found for exploratory feather pecks (EFP) given at 8-9 ($F_{2,20}$ = 5.36, P < 0.05), 12-13 ($F_{2,20}$ = 3.62, P < 0.05) and line tended to affect EFP given at 15-16 weeks of age ($F_{2,20}$ = 3.35, P < 0.1). LFP birds showed less EFP at 8-9 weeks of age compared to HFP and CON birds (P < 0.05), but HFP and CON birds did not differ in EFP at this age. HFP birds showed more EFP at 12-13 and tended to show more EFP at 15-16 weeks of age compared to CON birds (P < 0.05 and P < 0.1, respectively), but LFP birds did not differ in EFP compared to HFP and CON birds at both ages.

Line effects were also found for stereotyped feather pecking bouts (StFP) given at 3-4 ($F_{2,20}$ = 6.18, P < 0.01), 8-9 ($F_{2,20}$ = 10.09, P < 0.01) and 12-13 weeks of age ($F_{2,20}$ = 4.96, P < 0.05). HFP birds tended to show more StFP at 3-4 (P < 0.1) and showed more StFP at 8-9 weeks of age (P < 0.01) compared to LFP birds. Furthermore, HFP birds showed more StFP at 3-4 (P < 0.01) and 8-9 weeks of age (P < 0.05) compared to CON birds, but LFP and CON birds did not differ in StFP at these ages. CON birds showed less StFP at 12-13 weeks of age compared to HFP and LFP birds (P < 0.05), but HFP and LFP birds did not differ in StFP at this age.

Finally, line effects were found for severe feather pecks (SFP) given at 3-4 ($F_{2,20}$ = 4.25, P < 0.05), 8-9 ($F_{2,20}$ = 7.38, P < 0.01), 15-16 ($F_{2,20}$ = 7.31, P < 0.01) and 28-29 weeks of age ($F_{2,19}$ = 14.09, P < 0.01). HFP birds showed more SFP at 3-4 (P < 0.05), 8-9 (P < 0.05), 15-16 (P < 0.01) and 28-29 weeks of age (P < 0.01) compared to LFP birds. HFP birds showed more SFP at 8-9 and 28-29 weeks of age (P < 0.01) and tended to show more SFP at 15-16 weeks of age compared to CON birds (P < 0.1). LFP and CON birds did not differ in SFP at all ages.

3.1.2. Feather Pecking Phenotypes

Birds were categorized as feather pecker (P), feather pecker – victim (P-V), victim (V) or neutral (N). The number (and percentage) of hens within each category at different ages is given in Table 3. On average the largest percentage of hens was categorized as N across all ages in all three lines (HFP 51.7%; CON 80.8%; LFP 85.2%). The smallest percentage of hens was categorized as P-V in all three lines (HFP 10.5%; CON 2.7%; LFP 2.1%). The remainder of hens was categorized as P (HFP 14.9%; CON 8.1%; LFP 7.7%) and V (HFP 23.0%; CON 8.4%; LFP 5.1%).

3.1.2. Behavioural Tests

3.1.2.1. Novel Object Test

Line effects were found for the average percentage of birds that approached the novel object (NO) and the latency for three birds to approach the NO at 4 days ($F_{2,20} = 17.73$, $P < 0.01$ and $X^2 = 15.55$, $P < 0.01$, respectively) and 10 weeks of age ($F_{2,20} = 7.03$, $P < 0.01$ and $X^2 = 11.39$, $P < 0.01$, respectively). More HFP birds approached the NO and they approached it faster at 4 days of age compared to LFP and CON birds ($P < 0.01$). At 10 weeks of age, more HFP birds approached the NO and they approached it faster compared to LFP birds ($P < 0.01$) and more HFP birds tended to approach the NO and they tended to approach it faster compared to CON birds ($P < 0.1$) (Figure 2A & B). LFP and CON birds did not differ in their response to the NO at both ages.

3.1.2.2. Novel Environment Test

Line effects were found for latency to vocalize ($F_{2,20} = 13.21$, $P < 0.01$), vocalization frequency ($F_{2,20} = 24.69$, $P < 0.01$) and number of flight attempts ($F_{2,20} = 11.48$, $P < 0.01$) in the novel environment (NE) test at 4 weeks of age. HFP birds vocalized sooner and more compared to LFP and CON birds ($P < 0.01$) (Figure 3A & B). HFP birds showed more flight attempts compared to LFP ($P < 0.01$) and CON birds ($P < 0.05$) (Figure 3C). LFP and CON birds did not differ in their latency to vocalize, vocalization frequency or number of flight attempts.

3.1.2.3. Open Field Test

Line tended to affect the latency to first step ($F_{2,20}$ = 3.21, P < 0.1) and line affected latency to vocalize ($F_{2,20}$ = 4.95, P < 0.05) in the open field (OF) test at 15 weeks of age. HFP birds walked sooner compared to CON birds (P < 0.05) and vocalized sooner compared to LFP birds (P < 0.05) (Figure 4A). LFP birds did not differ in latency to first step compared to HFP and CON birds. CON birds did not differ in latency to vocalize compared to HFP and LFP birds. Line tended to affect step frequency ($F_{2,20}$ = 3.30, P < 0.1) and vocalization frequency ($F_{2,20}$ = 3.34, P < 0.1). HFP birds tended to show more steps compared to CON birds (P < 0.1), while LFP birds did not differ in step frequency compared to HFP and CON birds. CON birds vocalized more compared to LFP birds (P < 0.05), while HFP birds did not differ in vocalization frequency compared to LFP and CON birds (Figure 4B).

3.1.2.4. Tonic Immobility Test

Line affected tonic immobility (TI) duration at 13 ($F_{2,20}$ = 12.89, P < 0.01) and 28 weeks of age ($F_{2,19}$ = 6.35, P < 0.01). HFP birds had a shorter TI duration compared to LFP and CON birds at 13 weeks of age (P < 0.01), while LFP and CON birds did not differ. LFP birds had a longer TI duration than HFP birds (P < 0.01) and tended to have a longer TI duration than CON birds (P < 0.1) at 28 weeks of age, while HFP and CON birds did not differ (Figure 5).

3.2. Phenotype Effects in the HFP Line

Phenotype affected the number of flight attempts ($F_{3,119}$ = 3.18, P < 0.05) during the NE test. Victims (V) showed more flight attempts compared to neutrals (N) (P < 0.05) and tended to show fewer flight attempts compared to feather peckers (P) (P < 0.1). Feather pecker-victims (P-V) did not differ from P, V or N (Figure 6A). Phenotype tended to affect step frequency ($F_{3,75}$ = 2.64, P < 0.1) during the OF test. P tended to walk more compared to N (P < 0.1), while all other phenotype combinations did not differ (Figure 6B). We found no phenotype effects in the NO or TI test.

4. Discussion

The aim of this study was to investigate fearfulness in relation to feather pecking (FP) genotype (divergent selection on FP and no selection on FP) and FP phenotype (actual FP behaviour). Our results show that FP genotypes differ in their responses to several behavioural tests at young and adult ages. The high FP (HFP) line showed more active responses (i.e. approached a novel object sooner, vocalized sooner and more, showed more flight attempts and had shorter tonic immobility durations), which could suggest lower fearfulness, compared to the unselected control (CON) and low FP (LFP) line. Our results give first indications that FP phenotypes within the same genetic line (HFP line) differ in their responses. Feather peckers tended to show more active responses (i.e. they tended to show more flight attempts compared to victims and tended to walk more compared to neutrals), which could suggest lower fearfulness, compared to victims at a young age and compared to neutrals at an adolescent age. Neutrals showed more passive responses (i.e. less flight attempts), which could suggest higher fearfulness, compared to victims at a young age.

4.1. Line Effects

4.1.1. Feather Pecking Observations

Our findings indicate that selection for FP results in altered FP behaviour compared to no selection or selection against FP. LFP birds showed less exploratory feather pecking (EFP) compared to CON and HFP birds at a young age, whereas HFP birds showed more EFP compared to CON birds at adolescent ages. Furthermore, HFP birds showed more stereotyped feather pecking bouts (StFP) compared to CON and LFP birds at young ages, whereas CON birds showed less StFP compared to HFP and LFP birds at an adolescent age. We found no differences between the lines in EFP or StFP at adult ages. At both young and adult ages, HFP birds showed more severe feather pecking (SFP) compared to LFP and CON birds.

The HFP and LFP lines were divergently selected on a combination of severe and gentle feather pecking. However, selection did not favour gentle feather pecking, because gentle pecks in series were counted as a single bout (like for StFP in the present study). This could have resulted in a higher selection pressure on SFP than on gentle feather pecking (identified as EFP and StFP in the present study)(Kjaer et al., 2001) and this might explain why we see more consistent differences in SFP and less consistent or no differences in EFP and StFP. Furthermore, gentle and severe feather pecking are regarded as behaviours with a different motivational background (Kjaer and Vestergaard, 1999). Gentle feather pecking typically decreases with age (Rodenburg et al., 2004) which could explain why we see no differences in EFP and StFP at adult ages. Previous studies showed similar differences in FP between the HFP and LFP line (Bessei et al., 2013; Bögelein et al., 2015, 2014; Kjaer, 2009; Kjaer et al., 2001; Kjaer and Guémené, 2009; Kops et al., 2017; Piepho et al., 2017). For the first time we show that the LFP and CON line did not differ greatly in FP, especially not in SFP. The LFP and CON line also had similar percentages of birds categorized as feather peckers. Thus, selection for FP is more effective in increasing FP than selection against FP is in reducing FP. This is supported by Piepho et al. (2017) who showed that there are still some extreme feather peckers present in the LFP line. This can be explained by the change in phenotypic variability seen after some generations of selection when the mean level of FP becomes low (Kjaer et al., 2001). Feather pecking is a threshold trait (Kjaer and Jørgensen, 2011) and when the general level of FP is low, most birds will not show any FP even if they differ in their genetic propensity to perform FP. This makes it impossible to distinguish feather peckers from neutrals for selection and the selection for less FP is no longer effective.

4.1.2. Behavioural Tests

The present findings indicate that birds selected for FP show consistent responses in a set of behavioural tests at both young and adult ages and differ from birds that were unselected or selected against FP. Responses to the novel object (NO) (i.e. more birds approached a NO and they approached it sooner) indicate reduced fearfulness (Forkman et al., 2007) in HFP birds compared to CON and LFP birds. In the novel environment (NE) test, HFP birds seem to be less fearful (i.e. vocalized sooner and more and showed more flight attempts) compared to CON and LFP birds as silence and inactivity have been related to high fearfulness (Forkman et al., 2007; Jones, 1996; Suarez and Gallup, 1983). HFP birds seem to be less fearful (i.e. walked sooner and tended to walk more) compared to CON birds in the open field (OF) test, while LFP birds seem to be more fearful (i.e. vocalized less) compared to CON and more fearful (i.e. vocalized later) compared to HFP birds. In the tonic immobility (TI) test at adolescent age, HFP birds were less fearful (i.e. shorter TI duration) compared to CON and LFP birds as long TI durations have been related to high fearfulness (Forkman et al., 2007; Jones, 1996). Further, LFP birds were more fearful (i.e. longer TI duration) compared to HFP birds and seem to be more fearful (i.e. tended to have longer TI duration) compared to CON birds at adult age. In general, HFP birds appeared less fearful compared to CON and LFP birds in all behavioural tests, especially at young ages. For the first time, we show that CON and LFP birds did not differ in fearfulness at young ages, but LFP birds seem to be more fearful compared to CON birds at adult ages. Overall, selection for FP can alter behavioural characteristics other than FP (i.e. fearfulness) at young and adult ages. Selection against FP seems to alter fearfulness at an adult age.

These results are consistent with previous findings where young (< 16 weeks) HFP chicks were indicated as being less fearful compared to LFP chicks (Kops et al., 2017) and where responses of adult (> 33 weeks) HFP birds suggest that they were less fearful compared to LFP birds (Bögelein et al., 2014; de Haas et al., 2010). However, Rodenburg et al. (2010) found no differences in fearfulness between the HFP and LFP line at an adult age (> 25 weeks) when housed in conventional cages. In other experimental and commercial lines, high FP (indicated by actual FP behaviour or feather damage) has been related to high fearfulness (high vs. low FP line: Jones et al., 1995 (< 5 weeks); Rodenburg et al., 2004; White Leghorn vs. Rhode Island Red: Uitdehaag et al., 2008 (> 23 weeks)) and de Haas et al. (2014) found the same relation in commercial flocks (ISA Brown and Dekalb White). Even though cause and effect can be discussed in some of these studies, it indicates that genetic correlations between FP and fearfulness might have opposite directions in different genotypes. Thus, findings from the FP selection lines should be used with caution when developing control and/or prevention methods that are to be applied in commercial settings. Furthermore, the responses seen in the behavioural tests in the present study might not only be affected by fear. Fear-related responses are complex and it is unlikely that a particular behaviour is only related to fear (Forkman et al., 2007). Several other factors could have influenced birds’ responses, such as coping style, activity, exploration and social motivation (Forkman et al., 2007; Jones, 1996; Koolhaas et al., 1999). For example, in the NE and OF test social isolation can also induce vocal responses, especially in isolated young chicks that seek safety by calling for conspecifics (Gallup and Suarez, 1980; Jones et al., 1995).

Previous studies have indicated that FP might be related to coping style (de Haas et al., 2010; Jensen et al., 2005; Kops et al., 2017; Korte et al., 1997; van Hierden et al., 2002). Coping style is defined as a coherent set of behavioural and physiological stress responses which is consistent over time and situations (proactive vs. reactive, Koolhaas et al., 1999). Although we did find a consistent difference in behavioural responses between lines over time, with HFP birds showing a more proactive coping style than LFP and CON birds, physiological responses should be considered as well. Kjaer and Guémené (2009) showed that HFP birds had higher corticosterone levels after manual restraint compared to LFP birds, while CON birds had intermediate corticosterone levels, suggesting that HFP birds are more reactive and LFP birds are more proactive. However, preliminary results showed no difference in corticosterone levels between the HFP and LFP lines after manual restraint (van der Eijk et al., 2017). Furthermore, HFP birds had a higher heart rate and lower heart rate variability compared to LFP birds (Kjaer and Jørgensen, 2011), suggesting that HFP birds are more proactive and LFP birds are more reactive. Thus, there is inconsistency between behavioural and physiological findings with regard to coping style in the FP selection lines and further research is needed to indicate whether HFP and LFP birds can be classified into different coping styles. Studies should include behavioural, physiological and neuroendocrine characteristics as coping styles differ in these aspects (Koolhaas et al., 1999).

The present and previous studies show that HFP birds had more active responses to several behavioural tests compared to LFP birds (Bögelein et al., 2014; de Haas et al., 2010; Kops et al., 2017). For the first time, we show that HFP birds had more active responses to several behavioural tests compared to CON birds. Kjaer (2009) showed that HFP birds had higher home-pen locomotor activity compared to LFP and CON birds. Similar results were found in an individual NE test where HFP birds walked a longer distance than LFP birds (de Haas et al., 2017a). Kjaer (2009) suggested that FP in the HFP line might be linked to changes in intrinsic motivation, which either directly or indirectly leads to higher locomotor activity and could thus be a result of a genetically based hyperactivity disorder. When HFP birds are indeed more active in general because of changes in their intrinsic motivation this might result in a more active response to any type of behavioural test. A higher general level of activity in the behavioural tests may suggest that HFP birds are less fearful while this might not be the case. Even responses to the TI test, which is considered a validated test for fearfulness (Forkman et al., 2007), might be affected by activity and/or coping style. Especially when birds have their eyes open but remain lying down during a TI test, latency to self-righting might be more related to activity and/or coping style than to fearfulness as was suggested in pigs by Erhard and Mendl (1999). The comparable responses of LFP and CON birds indicate that selection against FP might not alter fearfulness or intrinsic motivation. Based on the present findings we suggest that activity and/or coping style might overrule fearfulness within the HFP line, suggesting a complex interplay between fearfulness, activity and coping style that might play a role in the development of FP. Such an interplay between fearfulness, activity and coping style has been suggested before to affect behavioural responses of calves to several behavioural tests (van Reenen et al., 2005, 2004).

4.2. Phenotype Effects in the HFP Line

The present findings give first indications that birds which differ in actual FP behaviour (i.e. FP phenotypes) within the same genetic line (HFP line) seem to differ in fearfulness. Previous studies either found a positive (Vestergaard et al., 1993), negative (Jensen et al., 2005) or no relation (Bögelein et al., 2014) between fearfulness and actual FP behaviour. Here we show that feather peckers tended to show more flight attempts compared to victims, while victims showed more flight attempts compared to neutrals in the NE test. In the OF test, feather peckers tended to walk more compared to neutrals. These findings suggest that feather peckers were less fearful (i.e. tended to show more flight attempts) compared to victims at young age and less fearful (i.e. tended to walk more) compared to neutrals at adolescent age. Neutrals seem to be more fearful (i.e. less flight attempts) compared to victims at young age and compared to feather peckers (i.e. tended to walk less) at adolescent age. These findings suggest that victims were more fearful compared to feather peckers and neutrals more fearful compared to feather peckers and victims. The higher fearfulness in victims might be a consequence of being feather pecked as also indicated by earlier studies (Hughes and Duncan, 1972; Rodenburg et al., 2010). It should be noted, that we found no phenotype effects in the TI test, which is considered a validated test for fearfulness (Forkman et al., 2007). Yet, we did find phenotype effects in the NE and OF test, where behavioural responses could also be related to coping style, activity, etc. (Forkman et al., 2007; Jones, 1996; Koolhaas et al., 1999). A similar line of reasoning, as for the differences seen between the FP selection lines, might be true for the differences seen between feather peckers and other FP phenotypes. Feather peckers might be more active in general and have a more proactive coping style compared to other FP phenotypes. In order to classify FP phenotypes into a certain coping style physiological responses should be considered as well. First indications have been found that phenotypes can differ with regard to their physiology. Brunberg et al. (2011) identified differences in brain gene expression when comparing feather peckers to victims and control birds. Furthermore, phenotypes were shown to differ in serotonergic neurotransmission parameters in several brain areas, although no or small differences were found in dopaminergic neurotransmission parameters (Kops et al., 2013). However, Daigle et al. (2015) found no differences in corticosterone or whole blood serotonin levels after manual restraint between phenotypes. First indications have been found that phenotypes can differ in activity. Feather peckers walked a longer distance than victims in a NE test (de Haas et al., 2017b), suggesting that feather peckers are more active. Furthermore, Newberry et al. (2007) found that birds that performed more foraging behaviour when young were more likely to become feather peckers as adults, indicating that feather peckers might be more active. To shed more light on whether FP phenotypes differ in activity levels and whether they can be classified into different coping styles, further research is needed.

A limitation in our study is that we observed FP behaviour for a limited amount of time which might have led to FP behaviour not being observed. However, continuous observation is impractical. Daigle et al. (2015) showed that around half of the birds were classified with the same phenotype at three out of five ages, suggesting that birds are able to switch phenotypes and are not consistent over time. Unfortunately, we could not identify phenotype consistency as several birds (specifically feather peckers and neutrals) were sacrificed during the experiment for other purposes. However, 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 a particular behavioural test. We emphasize the importance of identifying FP phenotypes as they seem to differ in their responses to several behavioural tests.

5. Conclusion

Feather pecking genotypes and feather pecking phenotypes within the same genetic line differ in their responses to several behavioural tests at both young and adult ages. The high FP line and feather peckers within the high FP line showed more active responses, suggesting lower fearfulness.

Selection for FP has been effective in increasing FP behaviour and altering other behavioural characteristics (i.e. activity, fearfulness), whereas selection against FP has been less effective in reducing FP and altering other behavioural characteristics.

High FP seems to be related to low fearfulness, which is opposite to what has been found in other experimental and commercial lines. This stresses the need for further research into the genetic and phenotypic correlations between FP and fearfulness in various populations of chickens.

Activity and/or coping style might overrule fearfulness within the high FP line, suggesting a complex interplay between fearfulness, activity and coping style that might play a role in the development of FP.

Acknowledgements

Dr. Elske de Haas is gratefully acknowledged for commenting on previous versions of the manuscript. We thank Camille Buquet and Tessa van der Helm for helping with the behavioural tests and feather pecking observations. We thank the staff of experimental farm “CARUS” for their excellent animal care. This study is in part funded by the project “WIAS Graduate Programme” (no: 022.004.005) which is financially supported by the Netherlands Organisation for Scientific Research (NWO).

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Table 1. Ethogram of the feather pecking observations (after Newberry et al., 2007).

Behaviour	**Description**
Exploratory Feather Pecking (EFP)	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 (StFP)	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 (SFP)	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.

Table 2. Feather pecking behaviour (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 (3-4 weeks)	**n = 131**	**n = 126**	**n = 125**	
EFP	2.89 ± 0.26	2.51 ± 0.26	2.35 ± 0.57	ns
StFP (bouts)	4.45 ± 1.00^{a}	0.99 ± 0.17^{b}	1.59 ± 0.46^{ab}	< 0.01
SFP	2.37 ± 1.27^{a}	0.44 ± 0.14^{ab}	0.30 ± 0.07^{b}	< 0.05
Age (8-9 weeks)	**n = 110**	**n = 103**	**n = 101**	
EFP	2.82 ± 0.32^{a}	3.03 ± 0.36^{a}	1.76 ± 0.29^{b}	< 0.05
StFP (bouts)	3.02 ± 0.47^{a}	1.42 ± 0.26^{b}	1.05 ± 0.19^{b}	< 0.01
SFP	2.40 ± 0.51^{a}	0.50 ± 0.13^{b}	0.55 ± 0.19^{b}	< 0.01
Age (12-13 weeks)	**n = 88**	**n = 81**	**n = 79**	
EFP	7.45 ± 0.99^{a}	4.64 ± 0.71^{b}	5.27 ± 0.70^{ab}	< 0.05
StFP (bouts)	0.98 ± 0.27^{a}	0.20 ± 0.07^{b}	0.76 ± 0.18^{a}	< 0.05
SFP	2.55 ± 0.33	1.98 ± 0.39	1.34 ± 0.24	ns
Age (15-16 weeks)	**n = 86**	**n = 81**	**n = 77**	
EFP	6.70 ± 0.71	4.37 ± 0.51	4.83 ± 0.48	ns
StFP (bouts)	0.53 ± 0.16	0.47 ± 0.14	0.52 ± 0.14	ns
SFP	2.74 ± 0.78^{a}	0.99 ± 0.23^{ab}	0.49 ± 0.17^{b}	< 0.01
Age (28-29 weeks)	**n = 71**	**n = 70**	**n = 63**	
EFP	4.62 ± 0.66	3.89 ± 0.46	3.43 ± 0.70	ns
StFP (bouts)	0.70 ± 0.25	0.54 ± 0.16	0.60 ± 0.23	ns
SFP	6.25 ± 1.87^{a}	0.63 ± 0.14^{b}	0.48 ± 0.14^{b}	< 0.01

Average number of pecks or bouts per bird per hour (age 3-4 weeks: 60 min total observation time per bird; age 8-9, 12-13, 15-16 and 28-28 weeks: 30 min total observation time per bird). Differing superscript letters (a,b) indicate significant differences ($P < 0.05$) between lines.

Table 3. 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 (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%)

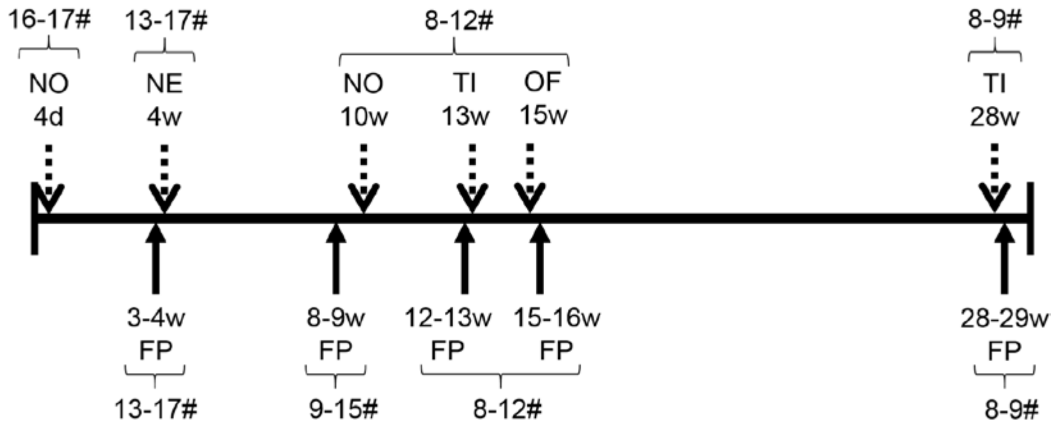

Figure 1. Timeline of feather pecking observations (below line) and behavioural tests (above line) performed at specific ages in days (d) or weeks (w) and the range of group sizes in pens (#). FP = feather pecking observations, NO = novel object test, NE = novel environment test, TI = tonic immobility test and OF = open field test.

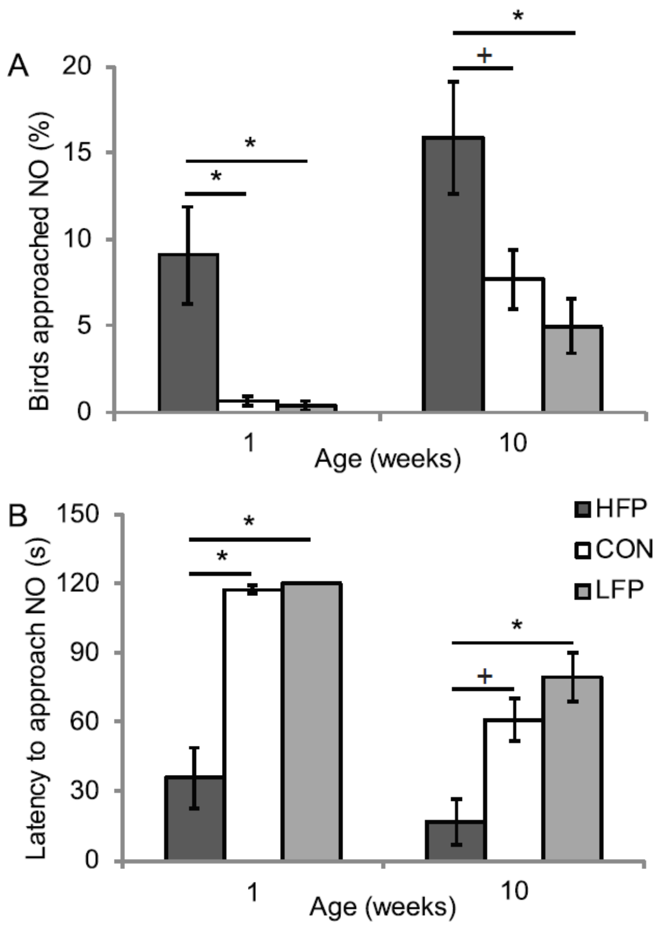

Figure 2. A) Mean percentage (± SEM) of birds approaching the novel object (NO) and B) mean latency (± SEM) for three birds to approach the NO in the NO test at 4 days (indicated as 1 week of age) and 10 weeks of age for the high (HFP, n = 8), control (CON, n = 8) and low feather pecking (LFP, n = 8) lines. $^{+}$ show tendencies ($P < 0.1$) and * show significant differences ($P < 0.05$) between lines.

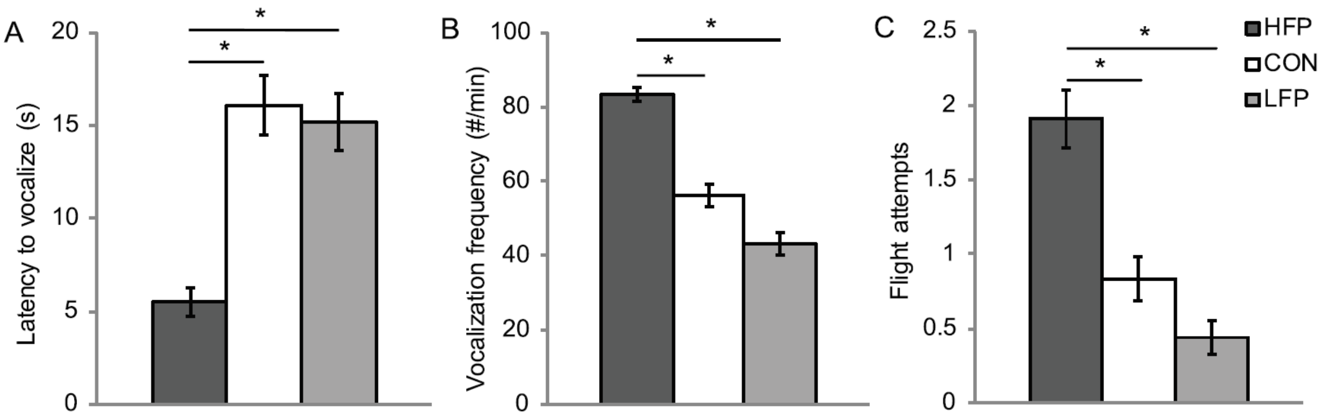

Figure 3. A) Mean latency (± SEM) to vocalize, B) mean vocalization frequency (± SEM) and C) mean number of flight attempts (± SEM) in the novel environment test at 4 weeks of age for the high (HFP, n = 132), control (CON, n = 128) and low feather pecking (LFP, n = 128) lines. * show significant differences ($P < 0.05$) between lines.

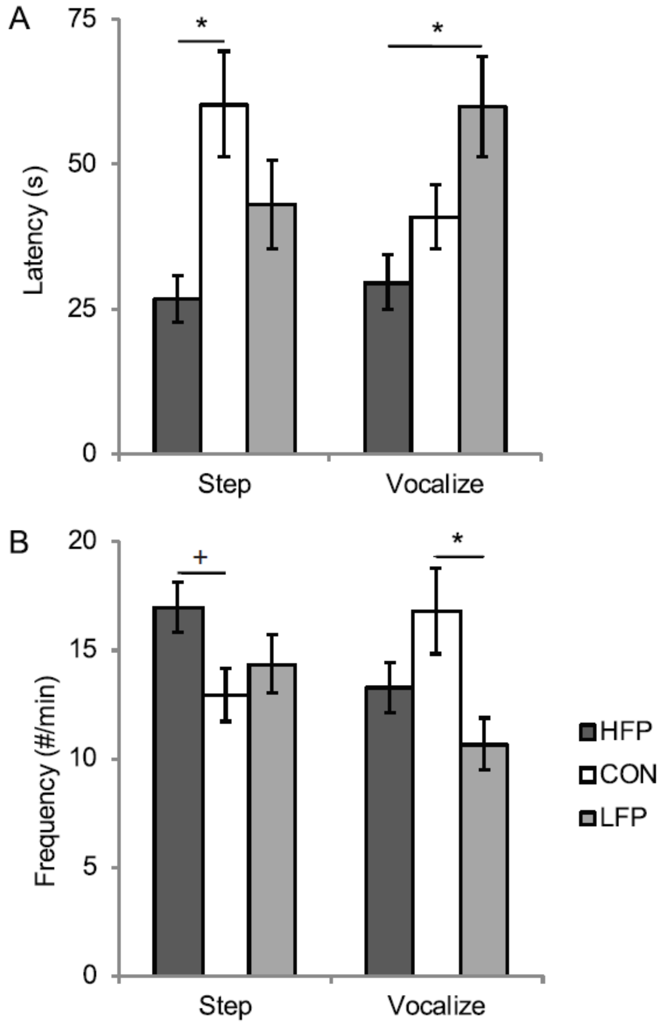

Figure 4. A) Mean latencies (± SEM) to first step and to vocalize and B) mean step and vocalization frequencies (± SEM) in the open field test at 15 weeks of age for the high (HFP, n = 86), control (CON, n = 81) and low feather pecking (LFP, n = 77) lines. $^{+}$ show tendencies ($P < 0.1$) and * show significant differences ($P < 0.05$) between lines.

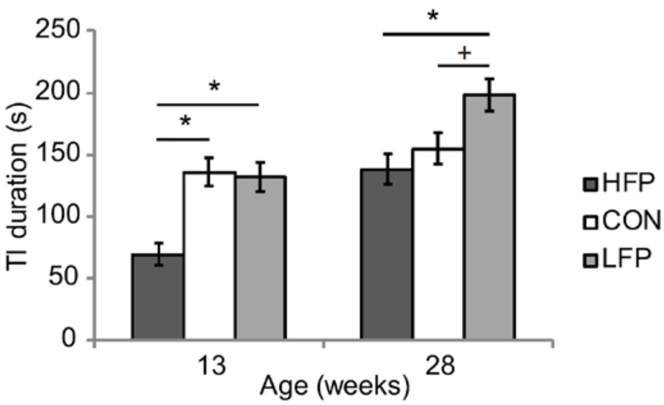

Figure 5. Mean tonic immobility (TI) durations (± SEM) in the TI test at 13 and 28 weeks of age for the high (HFP, n = 88 (13 weeks) and n = 72 (28 weeks)), control (CON, n = 81 (13 weeks) and n = 70 (28 weeks)) and low feather pecking (LFP, n = 79 (13 weeks) and n = 63 (28 weeks)) lines. $^{+}$ show tendencies ($P < 0.1$) and * show significant differences ($P < 0.05$) between lines.

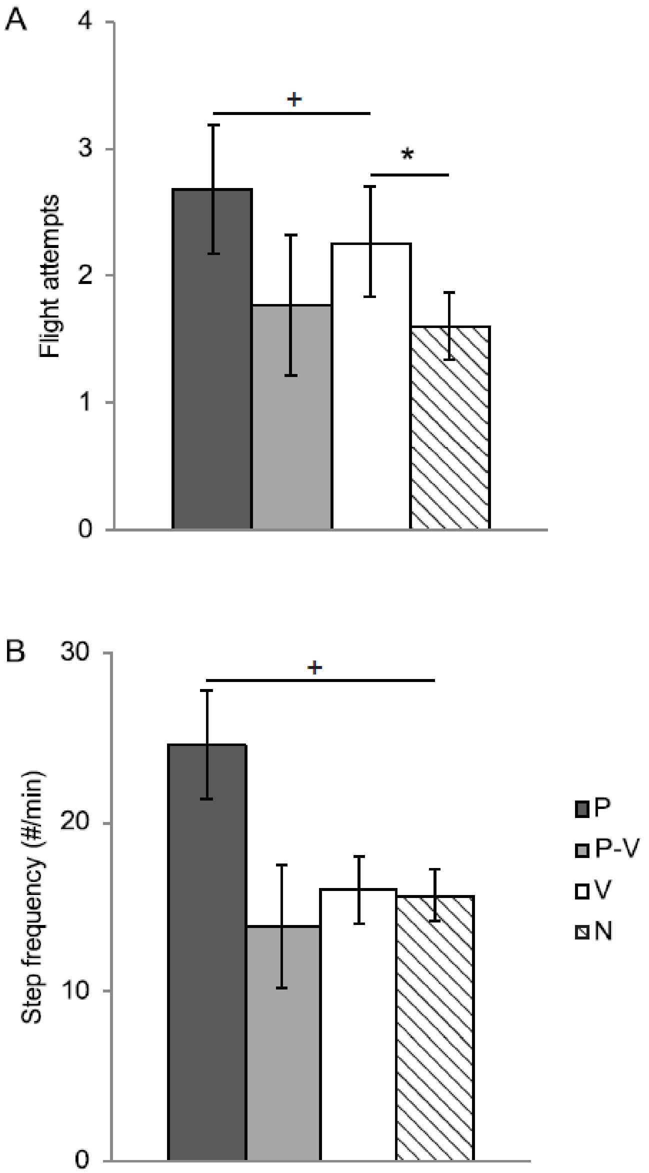

Figure 6. A) Mean number of flight attempts (± SEM) of feather peckers (P, n = 16), feather pecker-victims (P-V, n = 13), victims (V, n = 34) and neutrals (N, n = 68) of the high feather pecking line in the novel environment (NE) test at 4 weeks of age and B) mean step frequency (± SEM) of feather peckers (P, n = 13), feather pecker-victims (P-V, n = 7), victims (V, n = 23) and neutrals (N, n = 43) of the high feather pecking line in the open field test at 15 weeks of age. $^{+}$ show tendencies ($P < 0.1$) and * show significant differences ($P < 0.05$) between phenotypes.