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1 **Title**

2 Feather pecking genotype and phenotype affect behavioural responses of laying hens

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14   **Abstract**

15   Feather pecking (FP) is a major welfare and economic issue in the egg production industry.

16   Behavioural characteristics, such as fearfulness, have been related to FP. However, it is unknown how

17   divergent selection on FP affects fearfulness in comparison to no selection on FP. Therefore, we

18   compared responses of birds selected on low (LFP) and high feather pecking (HFP) with birds from

19   an unselected control line (CON) to several behavioural tests (i.e. novel object (NO), novel

20   environment (NE), open field (OF) and tonic immobility (TI)) at young and adult ages. Furthermore,

21   the relation between actual FP behaviour (i.e. FP phenotypes) and fearfulness is not well understood.

22   Therefore, we compared responses of birds with differing FP phenotypes. Feather pecking phenotypes

23   of individual birds were identified via FP observations at several ages. The number of severe feather

24   pecks given and received was used to categorize birds as feather peckers, feather pecker-victims,

25   victims or neutrals. Here we show that HFP birds repeatedly had more active responses (i.e. they

26   approached a NO sooner, vocalized sooner and more, showed more flight attempts and had shorter TI

27   durations), which could indicate lower fearfulness, compared to CON and LFP birds at both young

28   and adult ages. Within the HFP line, feather peckers had more active responses (i.e. they tended to

29   show more flight attempts compared to victims and tended to walk more compared to neutrals),

30   suggesting lower fearfulness, compared to victims and neutrals. Thus, in this study high FP seems to

31   be related to low fearfulness, which is opposite to what previously has been found in other

32   experimental and commercial lines. This stresses the need for further research into the genetic and

33   phenotypic correlations between FP and fearfulness in various populations of chickens, especially in

34   commercial lines. Findings from experimental lines should be used with caution when developing

35   control and/or prevention methods that are to be applied in commercial settings. Furthermore, activity

36   and/or coping style might overrule fearfulness within the HFP line, as HFP birds and feather peckers

37   within the HFP line had more active responses. This might indicate a complex interplay between

38   fearfulness, activity and coping style that could play a role in the development of FP.

39   **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

67 (HA) test, suggesting HFP chicks were less fearful compared to LFP chicks. Lines did not differ in the

68 number of steps or vocalizations, or in the latency to vocalize in an OF test at adolescent age (Kops et

69 al., 2017). In a novel maze, HFP birds walked a longer distance, spent a larger proportion of time

70 walking and vocalized sooner compared to LFP birds at adult age (de Haas et al., 2010). Bögelein et

71 al. (2014) found that adult HFP birds had a shorter TI duration, shorter latency to step and vocalize in

72 an OF test and shorter latency to emerge in an emerge test compared to LFP birds. The findings from

73 these studies suggest that HFP birds are less fearful compared to LFP birds at an adult age. Another

74 study, however, found no differences between the HFP and LFP line in TI, HA or NO test at an adult

75 age (Rodenburg et al., 2010). Taken together, there is inconsistency on whether the FP selection lines

76 differ with regard to fearfulness, especially at an adult age. At a young age HFP chicks seem to be less

77 fearful and show more active responses compared to LFP chicks. Thus, the FP selection lines show a

78 different relation between FP and fearfulness compared to commercial lines and other experimental

79 lines (i.e. HP and LP lines). Yet, other factors such as coping style and/or activity could play a role in

80 the behavioural responses of the FP selection lines as suggested by previous studies (de Haas et al.,

81 2010; Kjaer, 2009; Kops et al., 2017).

82 In order to better understand the development of FP it is crucial to identify how actual FP behaviour is

83 related to behavioural characteristics, since animals can become feather peckers, feather pecker-

84 victims, victims or neutrals (i.e. FP phenotypes). Only a few studies to date have related actual FP

85 behaviour to fearfulness. Vestergaard et al. (1993) found a positive correlation between TI duration

86 and rate of SFP given, indicating that feather peckers are more fearful. Jensen et al. (2005) showed

87 that adult feather peckers were faster at approaching both novel food and a NO compared to non-

88 feather peckers, but feather peckers and non-feather peckers did not differ in TI duration. In the FP

89 selection lines, Bögelein et al. (2014) found low correlations between FP and several fear criteria,

90 suggesting that fear might not be related to FP. Thus, FP phenotypes seem to differ in fearfulness, but

91 the direction of the relation remains unclear and may depend on the genotype used.

92 As it is unknown how divergent selection on FP affects fearfulness in comparison to no selection on

93 FP, we compared responses of HFP and LFP birds with those of birds from an unselected control line

94   (CON) to several behavioural tests at young and adult ages. Furthermore, as the relation between

95   actual FP behaviour (i.e. FP phenotypes) and fearfulness is not well understood, we compared the

96   responses of birds with differing phenotypes. Therefore, the aim of this study was to investigate

97   fearfulness in relation to FP genotype (divergent selection on FP and no selection on FP) and FP

98   phenotype (actual FP behaviour). We hypothesized that HFP birds would be less fearful than LFP and

99   CON birds at both young and adult ages. Based on previous findings the relation between fearfulness

100   and FP phenotypes remains unclear, so we had no a priori hypothesis for differences in fearfulness

101   between FP phenotypes.

102   **2.   Material and Methods**

103   2.1. Animals and Housing

104   White Leghorn birds from the 18th generation of an unselected control (CON) line and lines selected

105   on high (HFP) respectively low feather pecking (LFP) were used (see (Kjaer et al., 2001) for a

106   detailed description of the selection procedure). The HFP and LFP line were divergently selected on

107   FP for seven generations and were maintained in subsequent generations. The parent stock was

108   between 38 and 43 weeks of age at the time of egg collection. A total of 456 birds were produced in

109   two batches of eggs that were incubated at an average egg shell temperature of 37.3 ºC and average

110   relative humidity of 55.6 %. The two batches had the same housing conditions and experimental set-

111   up with 4 pens per line, but with two weeks between batches. Only non-beak-trimmed female birds

112   were used for the experiment. Birds were housed per line in 24 floor pens (height 2 m, length 2 m,

113   width 1 m) in groups of 19 birds. At 1 day, 5 weeks and 10 weeks of age group size was reduced (n =

114   16-17 birds per pen, n = 10-15 birds per pen and n = 8-12 birds per pen, respectively). At 20 weeks of

115   age, group size was levelled out at 8-9 birds per pen, with a total of 63 LFP, 72 HFP and 71 CON

116   birds. All birds were individually marked with a small neck tag with a colour/number combination

117   (Roxan, Selkirk, Scotland) for individual identification. At 3 and 4 weeks of age, birds were colour

118   marked on the neck and/or back for individual identification (colours: black, purple, green, blue and

119   orange). The same colours were used in a previous study where no effect on FP was found

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

121   with a number for individual identification.

122   At all times, water and feed were provided *ad libitum.* Birds received a standard rearing diet 1 until 8

123   weeks of age, a standard rearing diet 2 from 8 until 16 weeks of age and a standard laying diet from

124   16 weeks of age onwards. Each pen was provided with wood shavings on the floor, a perch installed 5

125   cm above the floor from 3 to 5 weeks of age and a perch installed 45 cm above the floor from 6 weeks

126   of age onwards. Post hatch, temperature was kept around 33°C and gradually lowered to 24°C at 4

127   weeks of age. From 19 weeks of age onwards, temperature was kept around 21°C. The light regime

128   was 23L:1D post hatch, and was weekly, gradually reduced to 8L:16D at 4 weeks of age. From 15

129   weeks of age, the light regime was weekly extended with 1 h until 13L:11D at 20 weeks of age. At 22

130   weeks of age, the light regime was increased to 16L:8D. Light intensity for each pen was measured

131   with a Voltcraft MS-1300 light meter (Conrad Electric Benelux, Oldenzaal, the Netherlands) and

132   ranged between 34.8-68.2 LUX (average 48.1 LUX) during the first 3 weeks of life. At 3 weeks of

133   age the light intensity was lowered, ranging between 2.74-7.09 LUX (average 4.68 LUX) to reduce

134   the risk of cannibalism. Straw was provided in racks from 3 to 20 weeks of age to enrich the

135   environment and reduce the risk of cannibalism. At 20 weeks of age straw racks were removed. A

136   wooden nest box was placed in front of the pen at 15 weeks of age. Visual barriers of approximately

137   1.5 m high were placed between pens at the start of the experiment to prevent birds in adjacent pens

138   of seeing each other. Birds received vaccinations against Marek's disease (day 0, intramuscular

139   (i.m.)), Infectious Bronchitis (day 0, 14, 56 and 108, via spray), Newcastle Disease (day 7, 28, 70 via

140   spray and day 84 i.m.), Infectious Bursal Disease/Gumboro (day 25, via drinking water), Avian

141   Encephalomyelitis and Pox Diphteria (day 84, via wing web injection) and Infectious Laryngo

142   Tracheitis (day 84, via eye drops). The experiment was approved by the Central Authority for

143   Scientific Procedures on Animals according to Dutch law (no: AVD104002015150).

144   2.2 Behavioural Observations and Tests

145   Feather pecking behaviour was observed between 3 and 29 weeks of age. Birds were subjected to four

146   behavioural tests that are related to fearfulness: novel object test, novel environment test, open field

6

147   test and tonic immobility test. The novel object test and tonic immobility test were performed twice.

148   A timeline of the feather pecking observations and behavioural tests performed at specific ages is

149   provided in Figure 1. The order for testing and observations was always randomized on pen level.

150   Order for testing during the open field test and tonic immobility test were randomized on individual

151   level. The experimenters were blinded to the lines.

152   2.2.1.   Feather Pecking Observations

153   Feather pecking behaviour was observed on an individual level from week 3-4, 8-9, 12-13, 15-16 and

154   28-29. In week 3-4 birds were observed by direct observation. Each observation lasted 30 min, either

155   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.

156   In week 8-9, 12-13, 15-16 and 28-29 behaviour was observed from video recordings. Each

157   observation lasted 15 min, either in the morning (10:40 h-10:55 h) or in the afternoon (14:40 h-14:55

158   h). The Observer XT 10 programme (Noldus Information Technology B.V., Wageningen, the

159   Netherlands) was used for video analysis of FP, categorized according to Table 1 (derived from

160   Newberry et al., 2007) in gentle feather pecks (subdivided into exploratory gentle feather pecks (EFP)

161   and bouts of stereotyped gentle feather pecking (StFP)) and severe feather pecks (SFP). Feather

162   pecking behaviours were summed over two subsequent weeks, thus including one morning and one

163   afternoon observation with a total observation period of 60 min for week 3-4 and a total observation

164   period of 30 min for all other time points. The summed number of SFP, either given or received, was

165   used to identify FP phenotypes. Classification of phenotypes was adapted from Daigle et al. (2015).

166   When a bird gave more than one SFP it was defined as a feather pecker (P). When a bird received

167   more than one SFP it was defined as a victim (V). When a bird gave and received more than one SFP

168   it was defined as a feather pecker-victim (P-V). When a bird gave and received zero or one SFP it was

169   defined as a neutral (N).

170   2.2.2.   Novel Object Test

171   At 4 days and 10 weeks of age, the response to a novel object (NO) was tested. At 4 days of age, the

172   NO was a wooden block (height 8 cm, length 5 cm, width 2.5 cm) wrapped with coloured tape (green,

173   white, black, yellow, and red) (n = 24, see de Haas et al., 2014 for a detailed description of the test

174   method). The test started 10 sec. after the experimenter had placed the NO on the floor in the centre of

175   the home pen (n = 24). The latency for three different birds to approach the NO at a distance of < 25

176   cm and the number of birds that were within < 25 cm of the NO was recorded every 10 sec for the 2

177   min test duration. At 10 weeks of age, the NO test was repeated (n = 24). The NO was a plastic stick

178   (length 50 cm, diameter 3.5 cm) wrapped with coloured tape (red, white, green, black, and

179   yellow)(based on Welfare Quality®, 2009). The same experimenter tested all pens at 4 days and 10

180   weeks of age.

181   2.2.3.   Novel Environment Test

182   At 4 weeks of age, the response to a novel environment (NE) was tested for a duration of 1 min (n =

183   387, see de Haas et al., 2014 for a detailed description of the test method). All birds from a pen were

184   taken and transported in a cardboard box to a room near the testing rooms. The average time

185   difference between the first and last bird to be tested was 25 min. Birds were taken out of the

186   cardboard box to one of two test locations, where birds were placed inside a white bucket (height 57

187   cm, length 32 cm, width 22 cm). The bucket was covered with a wire mesh to prevent birds from

188   escaping. The experimenter was out of sight of the bird while testing, but was able to record latency to

189   vocalize, number of vocalizations and number of flight attempts. After testing, birds were returned to

190   a second cardboard box and when all birds from a pen were tested they were returned to their home

191   pen. Together, two experimenters tested all birds where each experimenter tested approximately half

192   of the birds alone.

193   2.2.4.   Open Field Test

194   At 15 weeks of age, birds were individually subjected to an open field (OF) test for a duration of 5

195   min (n = 244, see Rodenburg et al. (2009) for a detailed description of the test method). Birds were

196   individually transported to and from the test room in a cardboard box. A square wooden enclosure

197   (height 1.22 m, length 1.15 m, width 1 m) was used. Wire mesh prevented birds from escaping. The

198   front of the enclosure consisted of Plexiglas. A video-camera was placed approximately 1.0 m in front

199 of the Plexiglas. A bird was placed in the middle of the OF at the start of the test. The experimenter

200 was out of sight of the bird while testing, but was able to record latency to step and number of steps

201 from a monitor and latency to vocalize and number of vocalizations. One experimenter tested all

202 birds.

203 2.2.5.   Tonic Immobility Test

204 At 13 weeks of age, birds were individually subjected to a tonic immobility (TI) test for a maximum

205 duration of 5 min (n = 248, see Jones and Faure (1981) for a more detailed description of the test

206 method). The TI test was performed on two consecutive days in the afternoon and birds were

207 randomly assigned to a test day with half of a pen being tested on the first day and the other half on

208 the second day. Half of the birds in a pen were taken and transported in a cardboard box to a room

209 near the testing rooms. The average time difference between the first and last bird to be tested was 15

210 min. Birds were taken out of the cardboard box to one of two test locations, where they were placed in

211 supine position in a metal cradle with their head suspended from the side of the cradle. The right hand

212 of the experimenter was placed on the breast of the bird, while the left hand gently forced the bird's

213 head down lightly while cupping its head. Each bird was restrained in this position for 10 sec. When

214 after releasing, the bird remained in this position, TI duration was recorded until the bird returned to

215 upright position. If this happened within 10 sec after release, TI was induced again, with a maximum

216 of three attempts at inducing TI. Eye contact with the bird was avoided, but the experimenter was

217 visible for the bird during the test. The experimenter recorded the number of induction attempts

218 needed and the duration of TI (latency to self-righting). After testing, birds were returned to a second

219 cardboard box and when all birds from a cardboard box had been tested they were returned to their

220 home pen. Together, three experimenters tested all birds where each experimenter tested

221 approximately a third of the birds alone.

222 At 28 weeks of age, the tonic immobility test was repeated (n = 205). The average time difference

223 between the first and last bird to be tested was 12 min. Together, two experimenters tested all birds

224 where each experimenter tested approximately half of the birds alone.

2.3 Statistical Analysis

226 SAS Software version 9.3 was used for statistical analysis (SAS Institute Inc., Cary, USA). Linear

227 mixed models for line effects were tested for each age separately and consisted of fixed effects of line

228 and batch and the random effect of pen within line, except for the NO test, which was tested at pen

229 level. Phenotype effects were tested only in the HFP line as on average less than 10% of birds was

230 categorized as P, P-V or V within the LFP and CON lines (See Table 3). Linear mixed models for

231 phenotype effects in the HFP line consisted of fixed effects of FP phenotype and batch and the

232 random effect of pen. Phenotype effects were tested for each behavioural test separately using the

233 most recent FP phenotype categorization (for example, FP phenotypes based on FP observations from

234 week 3 and 4 were used to identify phenotype effects in the NE test). Phenotype effects in the NO test

235 at 4 days of age were not tested as we could not identify FP phenotypes at that age. Test time

236 (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

237 the OF test. Experimenter was added as fixed effect for the NE test and the TI test. Testing order was

238 included as fixed effect for the TI test. The model residuals were visually examined for normality.

239 Variables were square root transformed (i.e. percentage of birds that approached the NO; latency to

240 vocalize and frequency of vocalizations in the NE test; latency to vocalize and step, frequency of steps

241 and vocalizations in the OF test; and TI duration) to obtain normality of model residuals. A Kruskal

242 Wallis test was used to analyse line effects for latency to approach the NO and post hoc comparisons

243 were made with Dunn's test. A generalized linear mixed model with a Binary distribution was used to

244 test line and phenotype effects in the HFP line for flight attempts in the NE test. A generalized linear

245 mixed model with a Poisson distribution was used to test line effects for all FP behaviours. A

246 backward regression procedure was used when fixed effects (i.e. test time, experimenter or testing

247 order) had $P > 0.1$. Post hoc pairwise comparisons were corrected by Tukey–Kramer adjustment. P-

248 values < 0.05 were considered to be significant. P-values between 0.05 and 0.1 were considered to

249 indicate a tendency. All data is presented as (untransformed) mean $\pm$ standard error of the mean

250 (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.

11

274 3.1.2. Feather Pecking Phenotypes

275 Birds were categorized as feather pecker (P), feather pecker – victim (P-V), victim (V) or neutral (N).

276 The number (and percentage) of hens within each category at different ages is given in Table 3. On

277 average the largest percentage of hens was categorized as N across all ages in all three lines (HFP

278 51.7%; CON 80.8%; LFP 85.2%). The smallest percentage of hens was categorized as P-V in all three

279 lines (HFP 10.5%; CON 2.7%; LFP 2.1%). The remainder of hens was categorized as P (HFP 14.9%;

280 CON 8.1%; LFP 7.7%) and V (HFP 23.0%; CON 8.4%; LFP 5.1%).

281 3.1.2. Behavioural Tests

282 3.1.2.1. Novel Object Test

283 Line effects were found for the average percentage of birds that approached the novel object (NO) and

284 the latency for three birds to approach the NO at 4 days ($F_{2,20} = 17.73$, $P < 0.01$ and $X^2 = 15.55$, $P <$

285 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).

286 More HFP birds approached the NO and they approached it faster at 4 days of age compared to LFP

287 and CON birds ($P < 0.01$). At 10 weeks of age, more HFP birds approached the NO and they

288 approached it faster compared to LFP birds ($P < 0.01$) and more HFP birds tended to approach the NO

289 and they tended to approach it faster compared to CON birds ($P < 0.1$) (Figure 2A & B). LFP and

290 CON birds did not differ in their response to the NO at both ages.

291 3.1.2.2. Novel Environment Test

292 Line effects were found for latency to vocalize ($F_{2,20} = 13.21$, $P < 0.01$), vocalization frequency ($F_{2,20}$

293 $= 24.69$, $P < 0.01$) and number of flight attempts ($F_{2,20} = 11.48$, $P < 0.01$) in the novel environment

294 (NE) test at 4 weeks of age. HFP birds vocalized sooner and more compared to LFP and CON birds

295 ($P < 0.01$) (Figure 3A & B). HFP birds showed more flight attempts compared to LFP ($P < 0.01$) and

296 CON birds ($P < 0.05$) (Figure 3C). LFP and CON birds did not differ in their latency to vocalize,

297 vocalization frequency or number of flight attempts.

298 3.1.2.3. Open Field Test

299   Line tended to affect the latency to first step ($F_{2,20} = 3.21$, $P < 0.1$) and line affected latency to

300   vocalize ($F_{2,20} = 4.95$, $P < 0.05$) in the open field (OF) test at 15 weeks of age. HFP birds walked

301   sooner compared to CON birds ($P < 0.05$) and vocalized sooner compared to LFP birds ($P < 0.05$)

302   (Figure 4A). LFP birds did not differ in latency to first step compared to HFP and CON birds. CON

303   birds did not differ in latency to vocalize compared to HFP and LFP birds. Line tended to affect step

304   frequency ($F_{2,20} = 3.30$, $P < 0.1$) and vocalization frequency ($F_{2,20} = 3.34$, $P < 0.1$). HFP birds tended

305   to show more steps compared to CON birds ($P < 0.1$), while LFP birds did not differ in step frequency

306   compared to HFP and CON birds. CON birds vocalized more compared to LFP birds ($P < 0.05$),

307   while HFP birds did not differ in vocalization frequency compared to LFP and CON birds (Figure

308   4B).

309   3.1.2.4. Tonic Immobility Test

310   Line affected tonic immobility (TI) duration at 13 ($F_{2,20} = 12.89$, $P < 0.01$) and 28 weeks of age ($F_{2,19}$

311   $= 6.35$, $P < 0.01$). HFP birds had a shorter TI duration compared to LFP and CON birds at 13 weeks

312   of age ($P < 0.01$), while LFP and CON birds did not differ. LFP birds had a longer TI duration than

313   HFP birds ($P < 0.01$) and tended to have a longer TI duration than CON birds ($P < 0.1$) at 28 weeks of

314   age, while HFP and CON birds did not differ (Figure 5).

315   3.2. Phenotype Effects in the HFP Line

316   Phenotype affected the number of flight attempts ($F_{3,119} = 3.18$, $P < 0.05$) during the NE test. Victims

317   (V) showed more flight attempts compared to neutrals (N) ($P < 0.05$) and tended to show fewer flight

318   attempts compared to feather peckers (P) ($P < 0.1$). Feather pecker-victims (P-V) did not differ from

319   P, V or N (Figure 6A). Phenotype tended to affect step frequency ($F_{3,75} = 2.64$, $P < 0.1$) during the OF

320   test. P tended to walk more compared to N ($P < 0.1$), while all other phenotype combinations did not

321   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

14

348 selection pressure on SFP than on gentle feather pecking (identified as EFP and StFP in the present

349 study)(Kjaer et al., 2001) and this might explain why we see more consistent differences in SFP and

350 less consistent or no differences in EFP and StFP. Furthermore, gentle and severe feather pecking are

351 regarded as behaviours with a different motivational background (Kjaer and Vestergaard, 1999).

352 Gentle feather pecking typically decreases with age (Rodenburg et al., 2004) which could explain why

353 we see no differences in EFP and StFP at adult ages. Previous studies showed similar differences in

354 FP between the HFP and LFP line (Bessei et al., 2013; Bögelein et al., 2015, 2014; Kjaer, 2009; Kjaer

355 et al., 2001; Kjaer and Guémené, 2009; Kops et al., 2017; Piepho et al., 2017). For the first time we

356 show that the LFP and CON line did not differ greatly in FP, especially not in SFP. The LFP and

357 CON line also had similar percentages of birds categorized as feather peckers. Thus, selection for FP

358 is more effective in increasing FP than selection against FP is in reducing FP. This is supported by

359 Piepho et al. (2017) who showed that there are still some extreme feather peckers present in the LFP

360 line. This can be explained by the change in phenotypic variability seen after some generations of

361 selection when the mean level of FP becomes low (Kjaer et al., 2001). Feather pecking is a threshold

362 trait (Kjaer and Jørgensen, 2011) and when the general level of FP is low, most birds will not show

363 any FP even if they differ in their genetic propensity to perform FP. This makes it impossible to

364 distinguish feather peckers from neutrals for selection and the selection for less FP is no longer

365 effective.

366 4.1.2. Behavioural Tests

367 The present findings indicate that birds selected for FP show consistent responses in a set of

368 behavioural tests at both young and adult ages and differ from birds that were unselected or selected

369 against FP. Responses to the novel object (NO) (i.e. more birds approached a NO and they

370 approached it sooner) indicate reduced fearfulness (Forkman et al., 2007) in HFP birds compared to

371 CON and LFP birds. In the novel environment (NE) test, HFP birds seem to be less fearful (i.e.

372 vocalized sooner and more and showed more flight attempts) compared to CON and LFP birds as

373 silence and inactivity have been related to high fearfulness (Forkman et al., 2007; Jones, 1996; Suarez

374 and Gallup, 1983). HFP birds seem to be less fearful (i.e. walked sooner and tended to walk more)

375 compared to CON birds in the open field (OF) test, while LFP birds seem to be more fearful (i.e.

376 vocalized less) compared to CON and more fearful (i.e. vocalized later) compared to HFP birds. In the

377 tonic immobility (TI) test at adolescent age, HFP birds were less fearful (i.e. shorter TI duration)

378 compared to CON and LFP birds as long TI durations have been related to high fearfulness (Forkman

379 et al., 2007; Jones, 1996). Further, LFP birds were more fearful (i.e. longer TI duration) compared to

380 HFP birds and seem to be more fearful (i.e. tended to have longer TI duration) compared to CON

381 birds at adult age. In general, HFP birds appeared less fearful compared to CON and LFP birds in all

382 behavioural tests, especially at young ages. For the first time, we show that CON and LFP birds did

383 not differ in fearfulness at young ages, but LFP birds seem to be more fearful compared to CON birds

384 at adult ages. Overall, selection for FP can alter behavioural characteristics other than FP (i.e.

385 fearfulness) at young and adult ages. Selection against FP seems to alter fearfulness at an adult age.

386 These results are consistent with previous findings where young (< 16 weeks) HFP chicks were

387 indicated as being less fearful compared to LFP chicks (Kops et al., 2017) and where responses of

388 adult (> 33 weeks) HFP birds suggest that they were less fearful compared to LFP birds (Bögelein et

389 al., 2014; de Haas et al., 2010). However, Rodenburg et al. (2010) found no differences in fearfulness

390 between the HFP and LFP line at an adult age (> 25 weeks) when housed in conventional cages. In

391 other experimental and commercial lines, high FP (indicated by actual FP behaviour or feather

392 damage) has been related to high fearfulness (high vs. low FP line: Jones et al., 1995 (< 5 weeks);

393 Rodenburg et al., 2004; White Leghorn vs. Rhode Island Red: Uitdehaag et al., 2008 (> 23 weeks))

394 and de Haas et al. (2014) found the same relation in commercial flocks (ISA Brown and Dekalb

395 White). Even though cause and effect can be discussed in some of these studies, it indicates that

396 genetic correlations between FP and fearfulness might have opposite directions in different genotypes.

397 Thus, findings from the FP selection lines should be used with caution when developing control

398 and/or prevention methods that are to be applied in commercial settings. Furthermore, the responses

399 seen in the behavioural tests in the present study might not only be affected by fear. Fear-related

400 responses are complex and it is unlikely that a particular behaviour is only related to fear (Forkman et

401 al., 2007). Several other factors could have influenced birds' responses, such as coping style, activity,

402   exploration and social motivation (Forkman et al., 2007; Jones, 1996; Koolhaas et al., 1999). For

403   example, in the NE and OF test social isolation can also induce vocal responses, especially in isolated

404   young chicks that seek safety by calling for conspecifics (Gallup and Suarez, 1980; Jones et al.,

405   1995).

406   Previous studies have indicated that FP might be related to coping style (de Haas et al., 2010; Jensen

407   et al., 2005; Kops et al., 2017; Korte et al., 1997; van Hierden et al., 2002). Coping style is defined as

408   a coherent set of behavioural and physiological stress responses which is consistent over time and

409   situations (proactive vs. reactive, Koolhaas et al., 1999). Although we did find a consistent difference

410   in behavioural responses between lines over time, with HFP birds showing a more proactive coping

411   style than LFP and CON birds, physiological responses should be considered as well. Kjaer and

412   Guémené (2009) showed that HFP birds had higher corticosterone levels after manual restraint

413   compared to LFP birds, while CON birds had intermediate corticosterone levels, suggesting that HFP

414   birds are more reactive and LFP birds are more proactive. However, preliminary results showed no

415   difference in corticosterone levels between the HFP and LFP lines after manual restraint (van der Eijk

416   et al., 2017). Furthermore, HFP birds had a higher heart rate and lower heart rate variability compared

417   to LFP birds (Kjaer and Jørgensen, 2011), suggesting that HFP birds are more proactive and LFP

418   birds are more reactive. Thus, there is inconsistency between behavioural and physiological findings

419   with regard to coping style in the FP selection lines and further research is needed to indicate whether

420   HFP and LFP birds can be classified into different coping styles. Studies should include behavioural,

421   physiological and neuroendocrine characteristics as coping styles differ in these aspects (Koolhaas et

422   al., 1999).

423   The present and previous studies show that HFP birds had more active responses to several

424   behavioural tests compared to LFP birds (Bögelein et al., 2014; de Haas et al., 2010; Kops et al.,

425   2017). For the first time, we show that HFP birds had more active responses to several behavioural

426   tests compared to CON birds. Kjaer (2009) showed that HFP birds had higher home-pen locomotor

427   activity compared to LFP and CON birds. Similar results were found in an individual NE test where

428   HFP birds walked a longer distance than LFP birds (de Haas et al., 2017a). Kjaer (2009) suggested

429   that FP in the HFP line might be linked to changes in intrinsic motivation, which either directly or

430   indirectly leads to higher locomotor activity and could thus be a result of a genetically based

431   hyperactivity disorder. When HFP birds are indeed more active in general because of changes in their

432   intrinsic motivation this might result in a more active response to any type of behavioural test. A

433   higher general level of activity in the behavioural tests may suggest that HFP birds are less fearful

434   while this might not be the case. Even responses to the TI test, which is considered a validated test for

435   fearfulness (Forkman et al., 2007), might be affected by activity and/or coping style. Especially when

436   birds have their eyes open but remain lying down during a TI test, latency to self-righting might be

437   more related to activity and/or coping style than to fearfulness as was suggested in pigs by Erhard and

438   Mendl (1999). The comparable responses of LFP and CON birds indicate that selection against FP

439   might not alter fearfulness or intrinsic motivation. Based on the present findings we suggest that

440   activity and/or coping style might overrule fearfulness within the HFP line, suggesting a complex

441   interplay between fearfulness, activity and coping style that might play a role in the development of

442   FP. Such an interplay between fearfulness, activity and coping style has been suggested before to

443   affect behavioural responses of calves to several behavioural tests (van Reenen et al., 2005, 2004).

444   4.2. Phenotype Effects in the HFP Line

445   The present findings give first indications that birds which differ in actual FP behaviour (i.e. FP

446   phenotypes) within the same genetic line (HFP line) seem to differ in fearfulness. Previous studies

447   either found a positive (Vestergaard et al., 1993), negative (Jensen et al., 2005) or no relation

448   (Bögelein et al., 2014) between fearfulness and actual FP behaviour. Here we show that feather

449   peckers tended to show more flight attempts compared to victims, while victims showed more flight

450   attempts compared to neutrals in the NE test. In the OF test, feather peckers tended to walk more

451   compared to neutrals. These findings suggest that feather peckers were less fearful (i.e. tended to

452   show more flight attempts) compared to victims at young age and less fearful (i.e. tended to walk

453   more) compared to neutrals at adolescent age. Neutrals seem to be more fearful (i.e. less flight

454   attempts) compared to victims at young age and compared to feather peckers (i.e. tended to walk less)

455   at adolescent age. These findings suggest that victims were more fearful compared to feather peckers

456 and neutrals more fearful compared to feather peckers and victims. The higher fearfulness in victims

457 might be a consequence of being feather pecked as also indicated by earlier studies (Hughes and

458 Duncan, 1972; Rodenburg et al., 2010). It should be noted, that we found no phenotype effects in the

459 TI test, which is considered a validated test for fearfulness (Forkman et al., 2007). Yet, we did find

460 phenotype effects in the NE and OF test, where behavioural responses could also be related to coping

461 style, activity, etc. (Forkman et al., 2007; Jones, 1996; Koolhaas et al., 1999). A similar line of

462 reasoning, as for the differences seen between the FP selection lines, might be true for the differences

463 seen between feather peckers and other FP phenotypes. Feather peckers might be more active in

464 general and have a more proactive coping style compared to other FP phenotypes. In order to classify

465 FP phenotypes into a certain coping style physiological responses should be considered as well. First

466 indications have been found that phenotypes can differ with regard to their physiology. Brunberg et al.

467 (2011) identified differences in brain gene expression when comparing feather peckers to victims and

468 control birds. Furthermore, phenotypes were shown to differ in serotonergic neurotransmission

469 parameters in several brain areas, although no or small differences were found in dopaminergic

470 neurotransmission parameters (Kops et al., 2013). However, Daigle et al. (2015) found no differences

471 in corticosterone or whole blood serotonin levels after manual restraint between phenotypes. First

472 indications have been found that phenotypes can differ in activity. Feather peckers walked a longer

473 distance than victims in a NE test (de Haas et al., 2017b), suggesting that feather peckers are more

474 active. Furthermore, Newberry et al. (2007) found that birds that performed more foraging behaviour

475 when young were more likely to become feather peckers as adults, indicating that feather peckers

476 might be more active. To shed more light on whether FP phenotypes differ in activity levels and

477 whether they can be classified into different coping styles, further research is needed.

478 A limitation in our study is that we observed FP behaviour for a limited amount of time which might

479 have led to FP behaviour not being observed. However, continuous observation is impractical. Daigle

480 et al. (2015) showed that around half of the birds were classified with the same phenotype at three out

481 of five ages, suggesting that birds are able to switch phenotypes and are not consistent over time.

482 Unfortunately, we could not identify phenotype consistency as several birds (specifically feather

483 peckers and neutrals) were sacrificed during the experiment for other purposes. However, the strength

484 of this study was that we identified phenotype effects using the most recent FP phenotype

485 categorization that was based on FP observations closest to a particular behavioural test. We

486 emphasize the importance of identifying FP phenotypes as they seem to differ in their responses to

487 several behavioural tests.

488 **5. Conclusion**

489 Feather pecking genotypes and feather pecking phenotypes within the same genetic line differ in their

490 responses to several behavioural tests at both young and adult ages. The high FP line and feather

491 peckers within the high FP line showed more active responses, suggesting lower fearfulness.

492 Selection for FP has been effective in increasing FP behaviour and altering other behavioural

493 characteristics (i.e. activity, fearfulness), whereas selection against FP has been less effective in

494 reducing FP and altering other behavioural characteristics.

495 High FP seems to be related to low fearfulness, which is opposite to what has been found in other

496 experimental and commercial lines. This stresses the need for further research into the genetic and

497 phenotypic correlations between FP and fearfulness in various populations of chickens.

498 Activity and/or coping style might overrule fearfulness within the high FP line, suggesting a complex

499 interplay between fearfulness, activity and coping style that might play a role in the development of

500 FP.

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644

645   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.

646

647 Table 2. Feather pecking behaviour (exploratory feather pecking (EFP), stereotyped feather pecking

648 (StFP) (bouts) and severe feather pecking(SFP)) of the high (HFP), control (CON) and low feather

649 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

650 Average number of pecks or bouts per bird per hour (age 3-4 weeks: 60 min total observation time per

651 bird; age 8-9, 12-13, 15-16 and 28-28 weeks: 30 min total observation time per bird). Differing

652 superscript letters (a,b) indicate significant differences (P < 0.05) between lines.

653   Table 3. The number (and percentage) of hens per phenotype category (feather pecker (P), feather

654   pecker-victim (P-V), victim (V) and neutral (N)) within the high (HFP), control (CON) and low

655   feather pecking (LFP) lines based on the number of severe feather pecks (SFP) given or received at

656   different ages.

| Criteria	P	P-V	V	N
	Give > 1 SFP	Give > 1 SFP	Give 0 or 1 SFP	Give 0 or 1 SFP
	Receive 0 or 1 SFP	Receive > 1 SFP	Receive > 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%)

657
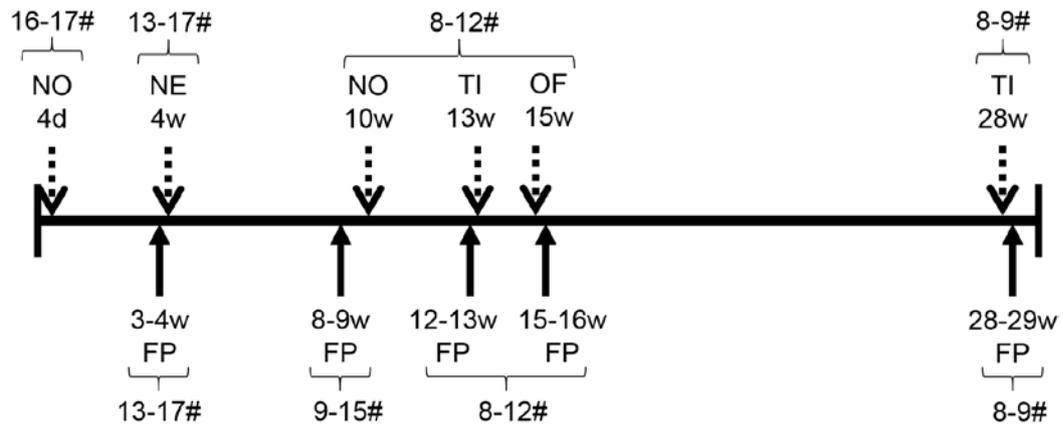
Figure 1. Timeline of feather pecking observations (below line) and behavioural tests (above line)

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.
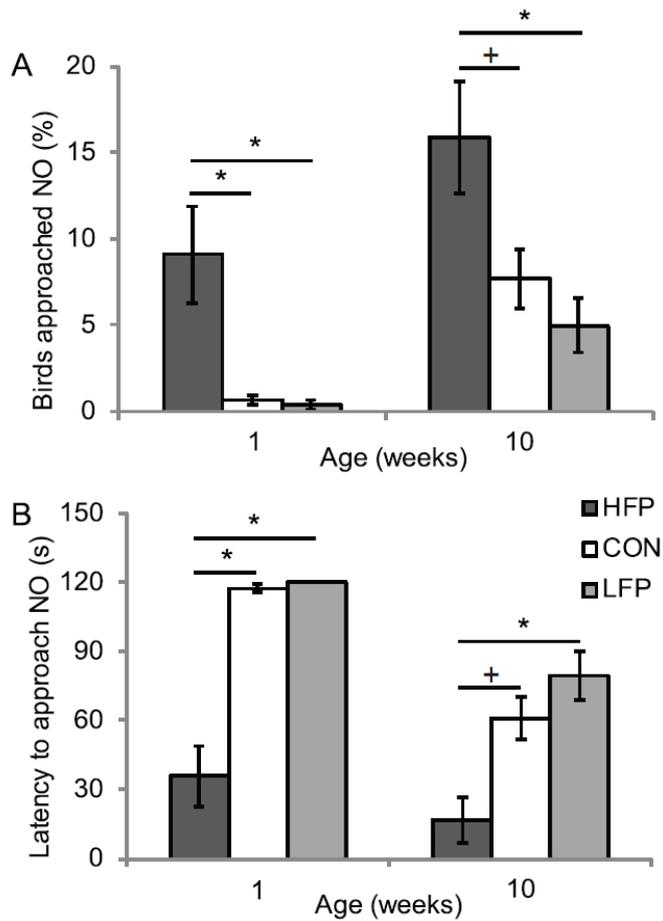
662

663   Figure 2. A) Mean percentage (± SEM) of birds approaching the novel object (NO) and B) mean

664   latency (± SEM) for three birds to approach the NO in the NO test at 4 days (indicated as 1 week of

665   age) and 10 weeks of age for the high (HFP, n = 8), control (CON, n = 8) and low feather pecking

666   (LFP, n = 8) lines. [+] show tendencies (P < 0.1) and * show significant differences (P < 0.05) between
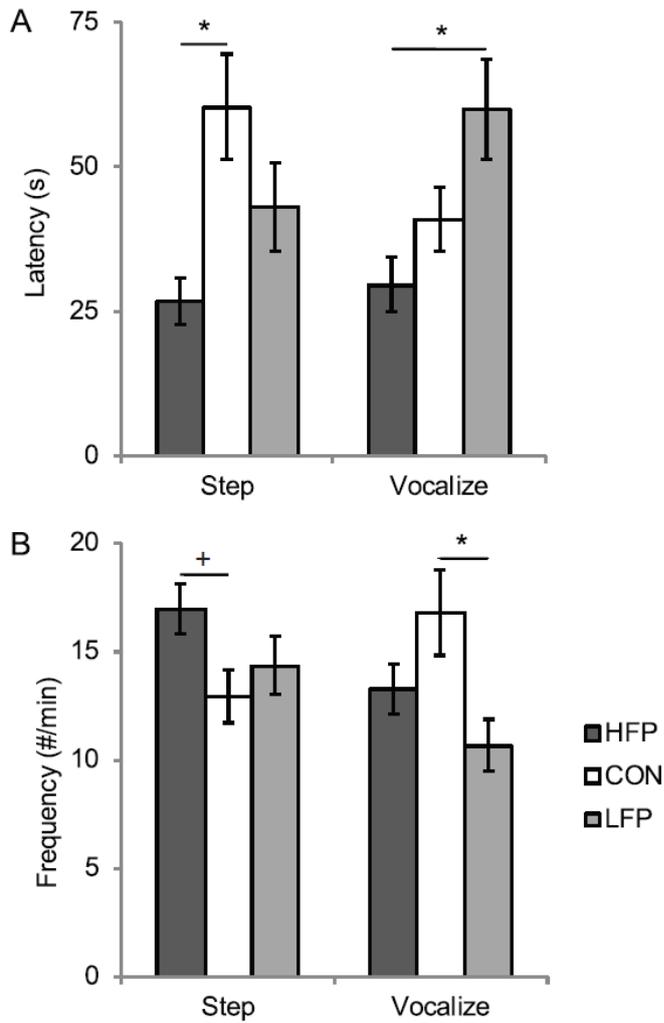
667   lines.
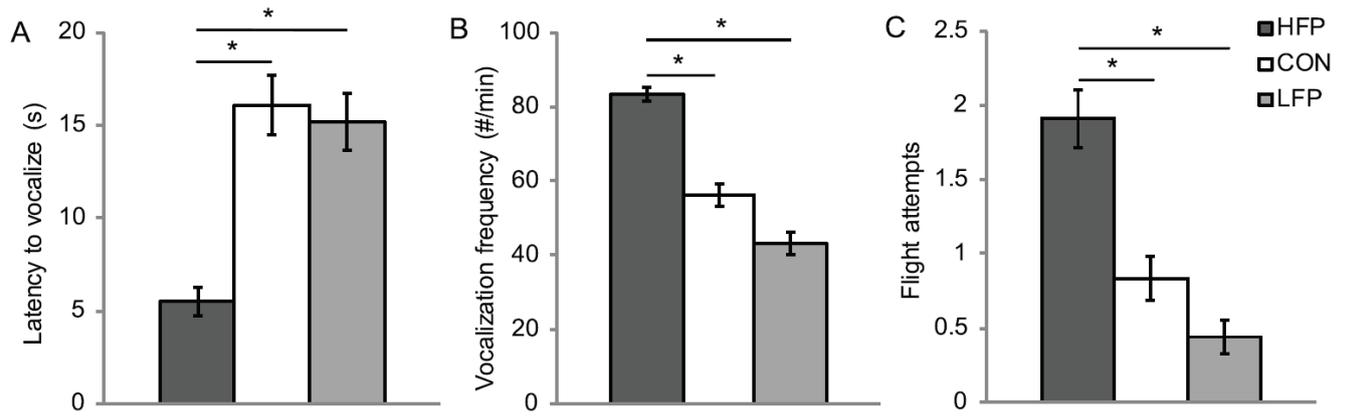
668

669   Figure 3. A) Mean latency (± SEM) to vocalize, B) mean vocalization frequency (± SEM) and C)

670   mean number of flight attempts (± SEM) in the novel environment test at 4 weeks of age for the high

671   (HFP, n = 132), control (CON, n = 128) and low feather pecking (LFP, n = 128) lines. * show
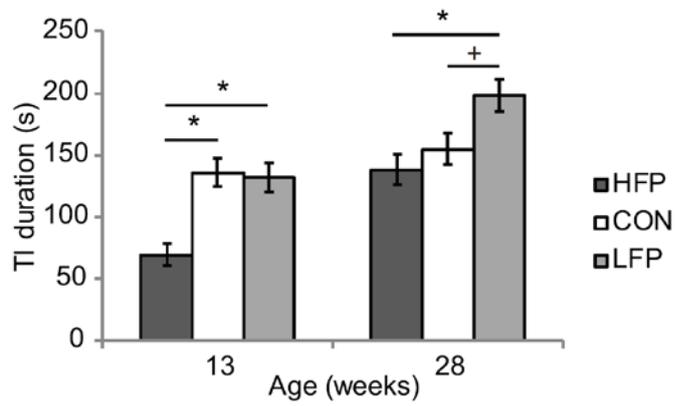
672   significant differences (P < 0.05) between lines.

673

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.

678

679   Figure 5. Mean tonic immobility (TI) durations (± SEM) in the TI test at 13 and 28 weeks of age for

680   the high (HFP, n = 88 (13 weeks) and n = 72 (28 weeks)), control (CON, n = 81 (13 weeks) and n =

681   70 (28 weeks)) and low feather pecking (LFP, n = 79 (13 weeks) and n = 63 (28 weeks)) lines. + show

682   tendencies (P < 0.1) and * show significant differences (P < 0.05) between lines.

683
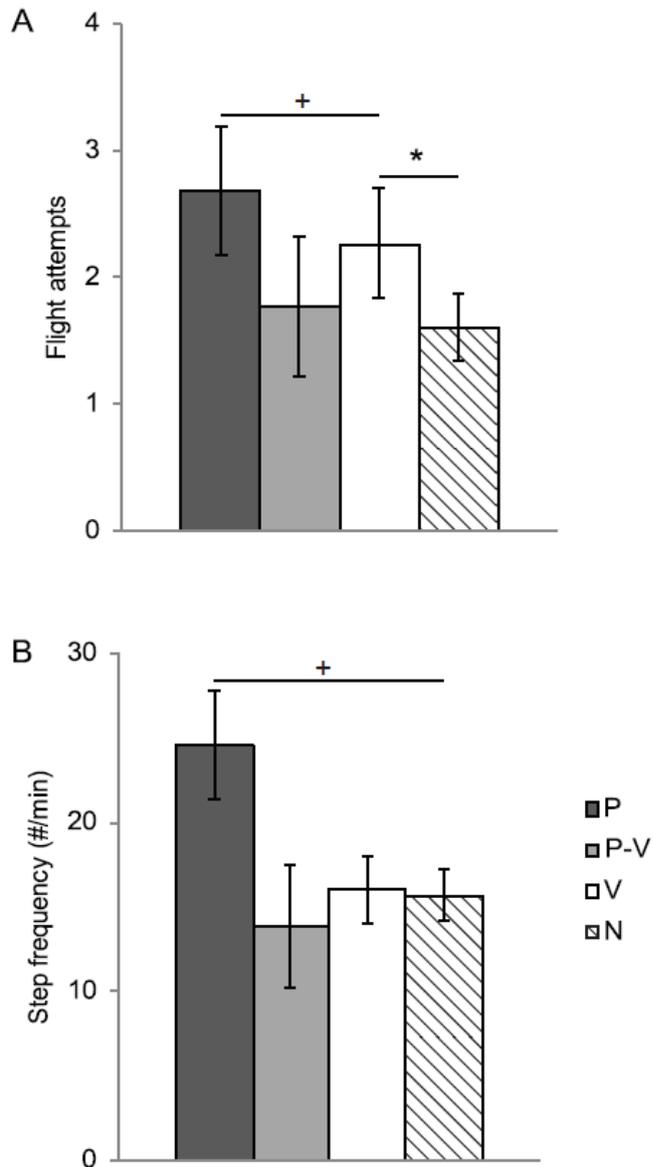
Figure 6. A) Mean number of flight attempts ($\pm$ SEM) of feather peckers (P, n = 16), feather pecker-victims (P-V, n = 13), victims (V, n = 34) and neutrals (N, n = 68) of the high feather pecking line in the novel environment (NE) test at 4 weeks of age and B) mean step frequency ($\pm$ SEM) of feather 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.