

## Research report

## Brain monoamine levels and behaviour of young and adult chickens genetically selected on feather pecking



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## ARTICLE INFO

## Article history:

Received 7 February 2017

Received in revised form 7 March 2017

Accepted 8 March 2017

Available online 24 March 2017

## Keywords:

Severe feather pecking

Genetic selection

Laying hen

Serotonin

Dopamine

Development

Chicken

Brain monoamines

Hyperactivity

Poultry welfare

## ABSTRACT

Severe feather pecking (SFP) in chickens is a detrimental behaviour with possibly neurochemical deficits at its base. Recent neurological studies depicted conflicting results on the role of serotonin (5-hydroxytryptamine, 5-HT) and dopamine (DA) in the development and display of feather pecking. We studied brain monoamine levels and behaviour in domestic chickens divergently genetically selected on feather pecking behaviour, the Low Feather Pecking (LFP) and High Feather Pecking (HFP) lines, both at a young age and when adult, to elucidate the role of 5-HT and DA in feather pecking. Also pecking behaviour and the behavioural response to challenging test situations was determined. At 8 weeks of age, HFP had lower 5-HT and DA turnover in several brain areas than LFP, whereas these differences had disappeared or were even reversed at 25 weeks of age. Line differences in central monoamine activity were found both in emotion-regulating and motor-regulating areas. As expected from previous generations, HFP exceeded LFP in most types of pecking at other birds, including severe feather pecking. Furthermore, HFP responded more actively in most behavioural tests conducted, and seem more impulsive or (hyper)active in their way of coping with challenges. This paper shows different developmental trajectories of the neurochemical systems (5-HT and DA) for chickens divergently selected on feather pecking behaviour, and a remarkable reversion of differences in monoamine activity at a later stage of life. Whether this is a cause or consequence of SFP needs further investigation.

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## 1. Introduction

Severe feather pecking (SFP), i.e. the pecking at and pulling out of feathers of group mates, is a detrimental behaviour in birds [1]. SFP can easily evolve to skin pecking and cannibalism resulting in mortality of recipients and is therefore a considerable welfare problem in laying hens on commercial poultry farms. SFP in chickens has multiple risk factors, many of which are related to the environment of the birds [2,3] and their social setting [4–6]. In addition, (genetically influenced) individual characteristics that determine how animals respond to their environment influence the tendency to develop SFP [7]. Characteristics that have been associated with SFP are high fearfulness and anxiety [4,8–11], (hyper)activity [12], and

a proactive coping style [13]. Although either of these characteristics (fearfulness, proactive coping or hyperactivity) may predispose chickens to develop SFP, the underlying neural mechanisms for SFP are not clear.

Neurochemical deficits might lie at the base of the SFP problem, and several genetic studies point to a role of the brain monoamines serotonin (5-hydroxytryptamine, 5-HT) and dopamine (DA) (e.g. [14,15]). Pharmaceutical studies have shown that haloperidol—a dopamine D2 receptor agonist known to increase dopamine release when acutely administered [16] – reduces SFP in adult chickens [17]. Moreover, chronic dietary supplementation with the 5-HT precursor tryptophan, leading to enhanced 5-HT neurotransmission, decreased feather pecking (FP) in young chickens [18], whereas the 5-HT<sub>1A</sub> autoreceptor agonist S-15535, inhibiting 5-HT release, increased the incidence of FP in young chicks [19] and adult hens [20]. Initial brain analyses pointed out that young chickens displaying high FP incidences had lowered serotonergic and dopaminergic turnover compared to chickens with low FP [19,21,22].

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Recent brain analyses in adult hens, however, provided seemingly conflicting results: phenotypically and genotypically selected adult high feather peckers had a higher 5-HT turnover [6,23] and a higher DA metabolism [24] than low feather peckers. Thus, relationships between FP tendencies and brain neurochemical levels may vary between ages, but also SFP behaviour itself is not constantly present in chickens. Young chickens predominantly perform gentle feather pecking (GFP), whereas most SFP incidences are recorded when laying hens reach reproductive maturity [3,6,25]. Moreover, GFP in young chicks is generally not a good predictor of SFP in adult hens and not all animals will perform SFP [3,26,27]. Importantly, with one exception [28], previous brain studies have all been performed in lines not directly selected on SFP but on related traits such as productivity [21], or mortality [24].

Genetic selection on SFP behaviour itself has resulted in divergent feather pecking lines, called the high feather pecking (HFP) and low feather pecking (LFP) lines [29]. These lines do not only differ in SFP, but also in other behavioural and physiological characteristics such as general (hyper)activity [12,30] and heart rate variability in response to stress [31]. In order to more clearly study the role of 5-HT and DA in SFP, this study aimed to compare these monoamines in brain areas involved in both emotional regulation and motor control between HFP and LFP hens, both when young and early into lay. This was combined with behavioural tests and observations to learn more on the animals' behavioural characteristics. During observations, the prevalence of gentle, severe, aggressive and toe pecking was recorded.

## 2. Materials and methods

### 2.1. Ethical statement

The experiment was approved by the Animal Care and Use Committee of Wageningen University, and in accordance with Dutch legislation on the treatment of experimental animals the ETS123 (Council of Europe 1985) and the 86/609/EEC Directive. Animals were visually checked daily for signs of wounds as a consequence of SFP to react immediately when animal welfare was compromised.

### 2.2. Birds and housing

White leghorn hens from the 9th generation of divergently selected lines for high feather pecking (HFP) and low feather pecking (LFP) were used (see [32,33] for details on the selection procedure). Eggs of both HFP and LFP birds were brooded and after hatch the one-day old female chicks received a neck tag with a color/number combination for identification. In total 84 female chicks were distributed over 12 pens (42 chicks/line;  $n=7$ /pen). Birds were not beak-trimmed. The chicks were housed in floor pens ( $1.9 \times 1.2$  m) covered with paper (first seven weeks) or sawdust (after week 7). Water and a commercial mash diet were provided ad libitum: a starter diet (week 1–5), a grower diet (week 6–16) and a layer diet (from week 17 onwards). Each pen had a 50 cm high perch installed and a lower perch (a block of wood) in the first seven weeks. Continuous light was given the first week, then 18 h of light (week 2) followed by 13 h (week 2–3), and 10 h of light (week 4–15). From 17 weeks of age onwards, the light period was extended by 1 h per week, until the birds had 16 h of light between 2.00 am–6.00 pm at 23 weeks of age, in line with commercial practice. In the first two weeks, three chicks turned out to be male and four chicks had died. In week 8, each group was reduced by one chicken and the brains of these chickens were dissected and stored ( $n=6$ /line) (referred to as young). The group size was now 65 animals ( $n=32$  LFP;  $n=33$  HFP,  $n=5$  or 6 per pen). At an age of 10 weeks, the pullets were moved to a new animal facility. In week 23, two hens per pen were

selected for microdialysis, as described elsewhere [28]. In week 25, 25 animals ( $n=12$  LFP;  $n=13$  HFP) were culled and the brains were dissected and stored (referred to as adult).

### 2.3. Behavioural observations and tests

Birds were individually subjected to six behavioural tests, which are described below. In addition, pecking behaviours were scored between two and 16 weeks of age. Order of testing and observations was always balanced for lines and pens. The experimenter was blind to the allocation of lines to the different pens.

#### 2.3.1. Pecking observations

Pecking behaviour of each individual bird was weekly observed from week 2 till week 16 with exception of week 9 and 10. Each observation lasted 25 min. At the start of each observation, the experimenter sat in front of the pen and waited for 5 min until starting with behavioural recordings. Frequencies of gentle feather pecking (GFP), severe feather pecking (SFP), toe pecking, and aggressive pecking were recorded at individual level. GFP was defined as light pecks given at the feathers; SFP was vigorous pecking and/or the pulling of feathers resulting in feather damage and/or removal; toe pecking was pecking directed at toes of others with risk of damaging the skin; and aggressive pecking consisted of forceful pecks at the head [34]. Pecking behaviours were averaged over weeks 2–8 for young birds, and weeks 10–16 for older birds, and expressed as frequencies per hour.

#### 2.3.2. Behavioural tests

*Isolation test.* At an age of 8–9 days, each chick was subjected to an isolation test, carried out on two consecutive days. The chick was put in a round bucket (diameter 28 cm) outside the home pen, but in the same room. For 2 min, the latency to move and to vocalize, the number of vocalizations and escape attempts were recorded.

*Runway test.* At an age of 15–16 days, each chick was subjected to a runway test, carried out on two consecutive days. The wooden runway of  $160 \times 20$  cm was 25 cm high and had a start box ( $20 \times 20$  cm) and a goal box ( $20 \times 20$  cm) at both ends of the runway closed with a steel mesh door. Three female stimulus chickens of similar age, which were not part of the experiment, were placed in the goal box. The tested chick was placed in the start box, and after 1 min, the mesh door was removed and the chick was given 5 min to get to its conspecifics. Latency to move, time to reach the goal box, frequency and latency of vocalizing and number of defecations were recorded.

*Novel object test.* At 23 days of age, the response to a novel object was tested. The novel object was a wooden block ( $5 \times 5 \times 2$  cm) wrapped with colored tape (red, yellow, white, and green) which was placed on the floor of the pen. The experimenter stood in front of the pen and recorded the latency of each bird to approach the object at a distance (radius) of 25 cm. Birds that did not approach the object within the maximum test time of 5 min, were given the maximum time score. As many birds did not approach the object within 25 cm or closer during the test, approaching at 25 cm was also scored as a binary variable (yes/no).

*Human approach test.* At 31 days of age, a human approach test was conducted. One experimenter squatted in front of the opened door of the pen and stretched her arm inside the pen while the gaze was averted (head turned away) (Welfare Quality: Assessment Protocol for Poultry). Another observer stood aside and recorded the latency of each animal to approach the person's hand at a distance (radius) of 25 cm. Birds that did not approach the person within the maximum test time of 5 min, were given the maximum time score. Also, the binary variable of approaching the person at 25 cm (yes/no) within the total observation time of 5 min was scored.

**Open Field test.** Each bird was individually subjected to an open field (OF) test for 5 min at an age of 16 weeks (see [11] for details). Birds were tested on two consecutive days. A square 1.25 × 1.25 m observation pen operated as OF. Three walls and the flooring were wooden and one wall was see-through perspex. The ceiling was covered with wire mesh. Behaviour was scored from a video-screen in an adjacent room by two persons using The Observer software package (Noldus Information Technology B.V., Wageningen, The Netherlands). Two cameras were placed, one in front of the see-through window and one above the OF. Durations and latencies to walk, stand, sit and vocalize [35] were recorded, as well as the number of vocalizations and yawns. Chicks were transported to and from the OF in a cardboard box.

**Manual restraint.** At 17 weeks of age, each hen was individually subjected to a manual restraint (MR) test, using a method previously described [8]. In short, a hen was placed on her right side on a table covered with cardboard, with the right hand of the experimenter covering the hen's trunk and the left hand gently stretching the hen's legs. Hens were retained in this position for 5 min. Consecutive struggles were scored as escape attempts, after which the hen was brought back in start position. The frequency and latency to vocalize were also recorded. All hens were tested at the same day by two observers and the test was situated in a room adjacent to their home pens.

#### 2.4. Brain regions investigated and tissue preparation

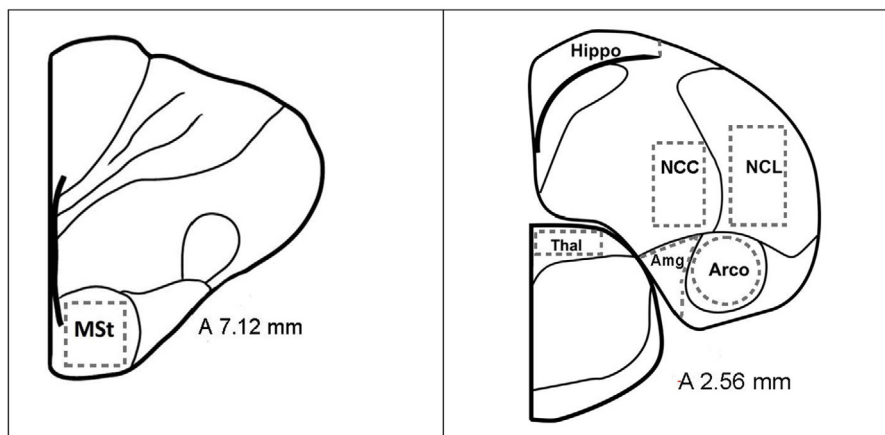
**Brain regions.** Seven regions of interest were selected because of their involvement in emotional and motor regulation (Fig. 1). The diencephalic dorsal thalamus connects with telencephalic areas and disinhibition of the thalamus will affect goal-directed behaviour [36]. The telencephalic medial striatum (MSt) is together with the nucleus accumbens considered the limbic avian ventral striatum known to play a role in reward [37,38]. The hippocampus is considered the memory and learning area in both mammals and birds [39]. Being a large associative area in the chickens' brain, the nidopallium has a potential role in guiding motor actions and decision making [40–42]. The nidopallium is subdivided in the caudolateral nidopallium (NCL) with frontal-like executive functions [43] and the limbic caudocentral nidopallium (NCC) [44,45]. Both the NCL and the NCC have reciprocal projections to the arcopallium intermedium, a somatosensory area, and the arcopallium mediale, a limbic region [44,46,47]. Together with other subnuclei that surround the arcopallium intermedium, the arcopallium mediale is

considered the birds' amygdala [41,48,49]. Therefore, in the paper 'arcopallium' refers to the arcopallium intermedium and 'amygdala' refers to the sampled arcopallium mediale.

**Brain tissue preparation.** Brains of young (8 weeks of age) and adult hens (25 weeks of age) were removed and immediately deep frozen in n-heptane put on dry ice and stored at  $-80^{\circ}\text{C}$  (protocol by [21]). Slicing of brains was executed in a cryostat (Frigocut Jung Mod.700) under cold conditions ( $-10^{\circ}\text{C}$ ). Slice thickness was 400  $\mu\text{m}$ . The seven regions of interest were located using the brain atlas for 2-week-old chickens [50], with considering literature on the avian brain [44,46,51] and also taking into account that brain size had increased in the 25 week old hens. Fig. 1 is a schematic drawing depicting the location of the medial striatum (MSt), dorsal thalamus (Thal), hippocampus (Hippo), NCC, NCL, arcopallium (Arco), and amygdala (Amg). The gray dotted shapes illustrate the cutting lines per brain area (carefully cut with a scalpel). Tissue samples were taken from multiple slices ("A" is anterior to the interaural line and the abbreviations reflect those in the atlas [50]: (Medial striatum (A7.56 – 5.68 mm, MSt) including the nucleus accumbens (A8.08 – 7.56 mm, Acb), dorsal thalamus (A2.80 – 1.36 mm; DPe, DMA, DIA, DLA), hippocampus (A4.24 – 1.60 mm; Hi1, Hi2, PHiM, PHiL, PHiL1, PHiL2, and PHiA), NCC (A3.04 – 1.36 mm; lateral parts of the caudal island of the nidopallium, NClF), NCL (A3.04 – 2.56 mm), arcopallium (A3.28 – 2.08 mm; amygdala core regions 1–4), and the amygdala (A2.80 – 1.60 mm; amygdaloid Taenial nucleus; ATn). Brain samples of the left and right hemisphere were taken together and analyzed as one. Brain samples were weighed before analyzed.

#### 2.5. Determination of monoamines and metabolite content of brain tissue by HPLC

Concentrations of central serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), and dopamine (DA) with corresponding metabolites 3-methoxytyramine (3-MT), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA), and also noradrenaline (NA) in the brain tissue were measured by high-performance liquid chromatography (HPLC) with electrochemical detection (ECD) after acid deproteinization as previously described [23]. Monoamine concentrations are expressed as nmol/g. Turnover levels of serotonin (5-HIAA/5-HT) and dopamine ((DOPAC+HVA+3-MT)/DA) were calculated as an index for the activity of the serotonergic and dopaminergic system [52,53]; high levels indicate a higher monoamine neurotransmission.



**Fig. 1.** Coronal schematic views of the chicken brain illustrating the medial striatum, thalamus, hippocampus, NCL, NCC, arcopallium, and amygdala. The schematic views of the left hemisphere of a chicken's brain. Depicted are the medial striatum (MSt) at 7.12 mm anterior to the interaural line (A), and the thalamus (Thal), hippocampus (Hippo), NCL, NCC, arcopallium (Arco), and amygdala (Amg) at 2.56 mm anterior to the interaural line (B). Chicken brains were sampled from both the left (shown here) and right hemisphere (not shown); the gray dotted shapes illustrate the cutting lines per brain area.

**Table 1**  
Gentle feather pecking, severe feather pecking, toe pecking, and aggressive pecking in young chickens and adolescent hens of the LFP and HFP line.

Behaviour (freq/bird/h) <sup>a</sup>	Young (age 2–8 weeks)			Adolescent (age 11–16 weeks)		
	LFP (n = 40)	HFP (n = 41)	P	LFP (n = 32)	HFP (n = 33)	P
Gentle feather pecking	4.77 ± 1.54	5.98 ± 1.42	ns	2.50 ± 0.53	7.20 ± 1.77	**
Severe feather pecking	0.41 ± 0.31	0.94 ± 0.40	*	0.09 ± 0.05	0.98 ± 0.33	**
Toe pecking	0.26 ± 0.10	0.59 ± 0.14	*	0.03 ± 0.03	0.09 ± 0.06	ns
Aggressive pecking <sup>b</sup>	–	–	–	0.01 ± 0.01	0.17 ± 0.05	**

<sup>a</sup> Average of number of pecks per bird per hour when young (age 2–8 weeks; 175 min total observation time per bird) and older (age 11–16 weeks; 150 min total observation time per bird).

<sup>b</sup> Aggressive pecks only occurred during observations between weeks 13–16. \*\*\* $P < 0.01$ ; \* $P < 0.05$ ; ns non-significant.

## 2.6. Statistical analysis

SAS version 9.2 was used for statistical calculations (SAS Inst. Inc., Cary, NC, USA). Data of behavioural tests were analyzed using mixed models containing the fixed effect of line (HFP vs. LFP) and the random effect of pen within line. In the case of the isolation test, runway test and open field test, also test day was added as a fixed effect. Observer was added as a fixed effect for the manual restraint. Binary data were analyzed with a generalized mixed model with a logit link function. Brain measurements were analyzed with a mixed model containing the fixed effect of line (HFP vs. LFP). Prior to analysis, if needed, variables were logarithmically (durations in the behavioural tests and brain monoamine measurements) or square root (frequencies in behavioural tests) transformed to obtain normality of residuals. Data are presented as (untransformed) means ± SEM. The assumption of normality was invalid for frequencies of pecking behaviour, even after transformation, and therefore the non-parametric Kruskal Wallis test was applied.

## 3. Results

### 3.1. Pecking observations

At a young age, HFP chicks showed more severe feather pecks (Kruskal Wallis,  $\chi^2 = 5.0$ ,  $P < 0.05$ ) and toe pecks (Kruskal Wallis,  $\chi^2 = 5.2$ ,  $P < 0.05$ ) than LFP chicks (Table 1). HFP and LFP chicks did not differ in gentle feather pecks (see Table 1). Also the percentage of birds showing severe feather pecks at least once during observations was higher in HFP (39%) than LFP (17.5%) birds ( $F_{1,79} = 4.4$ ,  $P < 0.05$ ), and the same held for toe pecking (48.8 vs. 25.0% of birds,  $F_{1,79} = 4.8$ ,  $P < 0.05$ ), whereas the percentage of birds showing gentle feather pecks did not differ (90.2 vs. 95.0%).

As adolescent, HFP still showed more severe feather pecks than LFP (Kruskal Wallis,  $\chi^2 = 12.6$ ,  $P < 0.01$ ), and also more gentle feather pecks (Kruskal Wallis,  $\chi^2 = 10.8$ ,  $P < 0.01$ ) and aggressive pecks (Kruskal Wallis,  $\chi^2 = 8.6$ ,  $P < 0.01$ , Table 1). Frequency of toe pecking did not differ between lines at this older age. Also more HFP than LFP birds showed severe feather pecking (54.5% vs. 15.6%,  $F_{1,63} = 9.7$ ,  $P < 0.01$ ), gentle feather pecking (97.0 vs. 78.1%,  $F_{1,63} = 4.0$ ,  $P = 0.05$ ), and aggressive pecking (30.3 vs. 3.1%,  $F_{1,63} = 5.8$ ,  $P < 0.05$ ), whereas the percentage birds displaying toe pecking as adolescents did not significantly differ between lines (HFP: 9.1%; LFP: 3.1%).

### 3.2. Behavioural tests

The results of the behavioural tests are summarized in Table 2.

**Isolation test.** Chicks of the HFP line vocalized ( $F_{1,10} = 5.0$ ,  $P < 0.05$ ) and walked sooner ( $F_{1,10} = 6.1$ ,  $P < 0.05$ ) in the isolation test than LFP chicks. Lines did not differ in the number of vocalizations in this test.

**Runway test.** LFP and HFP chicks did not differ in the latency to vocalize or walk in the social runway test. Time to reach the goal box after the start of the test tended to be shorter in HFP than in

LFP ( $F_{1,10} = 3.9$ ,  $P < 0.10$ ). When the latency to walk was subtracted from this measure (i.e. latency to goal box–latency to walk) the line difference was, however, not significant (LFP vs. HFP:  $38.5 \pm 10.90$  vs.  $22.8 \pm 7.35$  sec).

**Novel object test.** HFP chicks were faster in the 25 cm radius than LFP ( $F_{1,10} = 5.3$ ,  $P < 0.05$ ), and a higher percentage of HFP chicks (83.3%) approached the novel object to a distance of 25 cm or closer compared to the LFP chicks (52.5%, ( $F_{1,10} = 5.33$ ,  $P < 0.05$ )).

**Human approach test.** Compared to LFP, HFP entered the 25 cm radius faster ( $F_{1,10} = 11.0$ ,  $P < 0.01$ ), and twice as much HFP chicks (90.5%) approached the human to a distance of 25 cm or closer compared to LFP chicks (45.0%, ( $F_{1,10} = 11.5$ ,  $P < 0.01$ )).

**Open Field test.** In the open field, lines did not differ in the number of steps or vocalizations, or in the latency to vocalize. The HFP hens yawned more often than the LFP ( $F_{1,10} = 5.1$ ,  $P < 0.05$ ).

**Manual restraint.** In the manual restraint, chickens of the HFP line vocalized sooner ( $F_{1,10} = 15.3$ ,  $P < 0.01$ ) and more often ( $F_{1,10} = 8.6$ ,  $P < 0.05$ ) than the LFP. HFP struggled later ( $F_{1,10} = 5.6$ ,  $P < 0.05$ ) and tended to struggle less frequently ( $F_{1,10} = 54.7$ ,  $P < 0.10$ ) than the LFP.

### 3.3. Serotonergic line differences in young and adult chickens

In general, young HFP had lower serotonergic levels in most brain areas compared to young LFP (8 weeks of age), while these differences appeared less prominent in adult chickens (25 weeks of age) (Fig. 2). Five of the seven brain areas in young chickens depicted

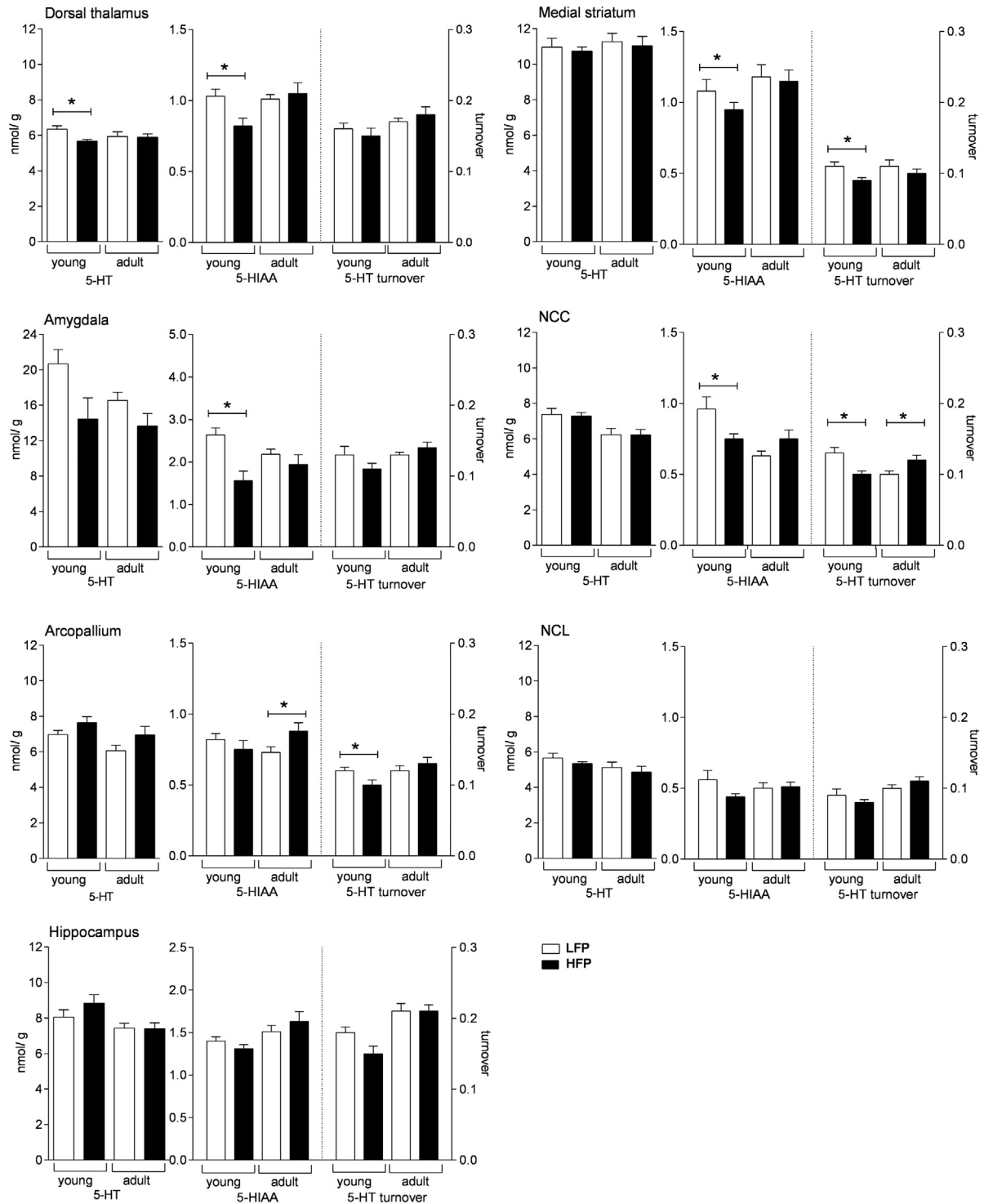
**Table 2**  
Behaviour in the social isolation test, runway test, novel object test, human approach test and manual restraint test in chickens of the LFP and HFP line.

Variables	LFP	HFP	P
<i>Isolation test (8–9 days)</i>			
Latency to vocalize (s)	25.3 ± 3.7	14.6 ± 3.5	*
Number of vocalizations	37.5 ± 5.3	54.2 ± 8.2	ns
Latency to walk (s)	35.7 ± 5.9	12.7 ± 3.5	*
<i>Runway test (15–16 days)</i>			
Latency to vocalize (s)	16.2 ± 4.7	19.6 ± 8.0	ns
Number of vocalizations	54.1 ± 5.2	56.1 ± 4.3	ns
Latency to walk	23.6 ± 10.8	13.2 ± 6.8	ns
Latency to goal box	62.2 ± 14.8	36.0 ± 12.1	+
<i>Novel object test (23 days)</i>			
Latency to 25 cm radius	178.5 ± 19.5	118.4 ± 18.1	*
<i>Human approach test (31 days)</i>			
Latency to 25 cm radius	202.4 ± 18.6	60.3 ± 14.4	**
<i>Open field test (16 weeks)</i>			
Latency to vocalize (s)	94.5 ± 15.6	80.8 ± 15.8	ns
Number of vocalizations	66.5 ± 12.6	61.8 ± 10.3	ns
Number of steps	31.3 ± 5.0	33.5 ± 5.8	ns
Number of yawns	0.4 ± 0.4	2.6 ± 0.9	*
<i>Manual restraint (17 weeks)</i>			
Latency to vocalize (s)	144.6 ± 18.4	71.2 ± 15.0	**
Number of vocalizations	23.2 ± 3.7	57.2 ± 9.2	*
Latency to struggle (s)	173.3 ± 17.6	230.3 ± 16.6	*
Number of struggles	2.0 ± 0.4	1.2 ± 0.4	+

Ages are given in between brackets. \*\* $P < 0.01$ ; \* $P < 0.05$ ; + $P < 0.10$ ; ns non-significant.



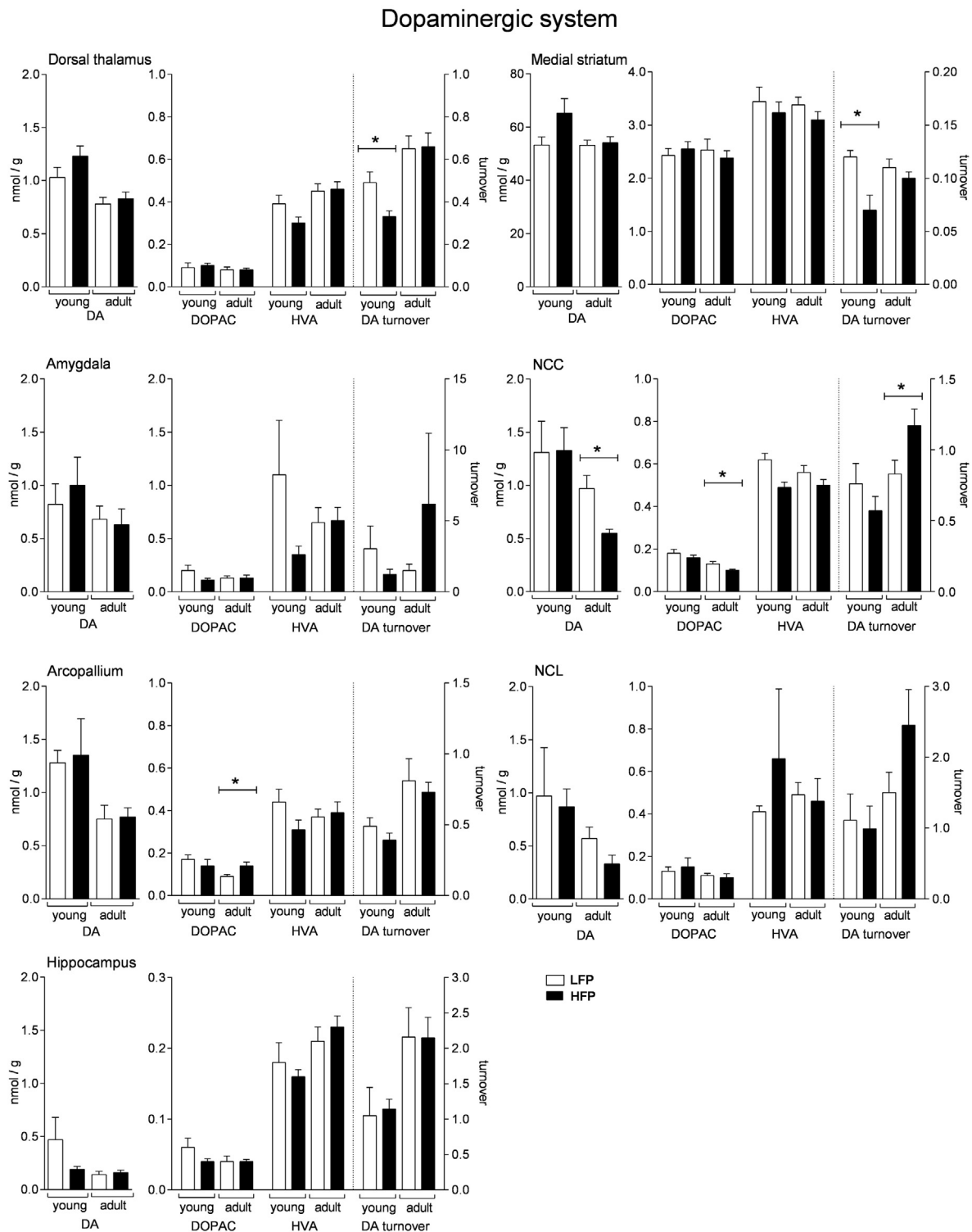
### Serotonergic system



**Fig. 2.** Serotonergic concentrations in seven brain areas of young (8-week old) chickens (LFP:  $n=6$ , HFP:  $n=6$ ) and adult (25-week old) chickens (LFP:  $n=12$ , HFP:  $n=13$ ) of the low feather pecking (LFP, white bars) and high feather pecking (HFP, black bars) lines. Of each brain area, the two most left panels show concentrations of 5-HT and 5-HIAA in nmol/g and the right panel shows the turnover ratio. \* $P < 0.05$ .

significant line differences for either 5-HT, its metabolite (5-HIAA), or the turnover ratio (5-HIAA/5-HT). That is, young HFP had lower 5-HT in the dorsal thalamus ( $F_{1,10} = 9.1, P < 0.05$ ), and also lower 5-HIAA in the dorsal thalamus ( $F_{1,10} = 8.1, P < 0.05$ ), the medial striatum ( $F_{1,10} = 7.5, P < 0.05$ ), the amygdala ( $F_{1,10} = 15.7, P < 0.05$ ), and the NCC ( $F_{1,10} = 12.9, P < 0.01$ ). Also, compared to young LFP,

young HFP had a lower 5-HT turnover ratio in the medial striatum, ( $F_{1,10} = 7.3, P < 0.05$ ), the NCC ( $F_{1,10} = 8.2, P < 0.05$ ), and in the arcopallium ( $F_{1,10} = 5.1, P < 0.05$ ). In contrast, in 25-week old chickens almost all significant serotonergic effects in similar brain areas had disappeared; except for 5-HIAA in the arcopallium ( $F_{1,21} = 4.4, P < 0.05$ ) and 5-HT turnover ratio in the NCC ( $F_{1,21} = 4.7, P < 0.05$ ).



**Fig. 3.** Dopaminergic concentrations in seven brain areas of young (8-week old) chickens (LFP:  $n=6$ , HFP:  $n=6$ ) and adult (25-week old) chickens (LFP:  $n=12$ , HFP:  $n=13$ ) of the low feather pecking (LFP, white bars) and high feather pecking (HFP, black bars) lines. Of each brain area, the two most left panels show concentrations of DA and metabolites DOPAC and HVA in nmol/g and the right panel shows turnover ratio. \* $P < 0.05$ .

Opposite to the results at a young age, adult HFP had higher 5-HT turnover levels compared to adult LFP.

#### 3.4. Dopaminergic line differences in young and adult chickens

Dopaminergic line differences were found in both young and adult chickens (Fig. 3). Young 8-week old HFP chicks had a lower

DA turnover in both the dorsal thalamus ( $F_{1,10} = 7.6$ ,  $P < 0.05$ ) and medial striatum ( $F_{1,10} = 8.0$ ,  $P < 0.05$ ) compared to young LFP. In the 25-week old chickens these particular areas did not depict line differences, whereas the arcopallium and NCC did. That is, adult HFP had lower DA ( $F_{1,21} = 10.6$ ,  $P < 0.01$ ) and lower DOPAC ( $F_{1,21} = 10.6$ ,  $P < 0.01$ ) compared to adult LFP in the NCC. In contrast with results at a younger age, DA turnover in this area was significantly higher

in adult HFP than in adult LFP ( $F_{1,21} = 5.2$ ,  $P < 0.05$ ). HFP had also significantly higher levels for DOPAC in the arcopallium ( $F_{1,21} = 6.7$ ,  $P < 0.05$ ).

No line differences in NA were found in any of the brain areas (not shown), except in the NCL. There, young HFP ( $0.24 \pm 0.13$ ) had significantly higher NA compared to LFP ( $0.17 \pm 0.12$ ,  $F_{1,10} = 6.3$ ,  $P < 0.05$ ), whereas HFP ( $0.08 \pm 0.04$ ) had lower NA than LFP when adult ( $0.23 \pm 0.04$ ,  $F_{1,21} = 6.3$ ,  $P < 0.05$ ).

#### 4. Discussion

This study demonstrates differences in 5-HT and DA metabolism between young chickens from a low feather pecking (LFP) vs. a high feather pecking (HFP) line, which were, notably, absent or even reversed at an adult age. The lines also showed striking differences in behaviour.

##### 4.1. Pecking behaviour

Behavioural observations in this study demonstrate line differences in all types of allopecking investigated (gentle and severe feather pecking, toe pecking and aggressive pecking) even though the LFP and HFP lines have been selected on their level of severe feather pecking (SFP) only [32]. Only GFP at a young age was, at a similar frequency, observed in almost all birds in both lines which can be explained by the social explorative character of that type of pecking [54]. Line differences in allopecking behaviour were similar to those observed in previous studies with these lines [55–58]. Also, a higher proportion of HFP chicks pecked others compared to LFP chicks, which might partly explain the higher levels of allopecking in the HFP line. Thus, divergent selection on SFP affects general allopecking activity.

##### 4.2. Behavioural tests comparing lines

The behavioural responses of the birds in the different tests conducted may reflect different underlying traits and motivations, including fearfulness (reviews by [59,60]). In several studies, HFP has been related to high fearfulness and anxiety [5,8,27,61–64]. The isolation test and the runway test examine a bird's fear of social isolation and the sooner or more chicks vocalize or walk (to others), the more motivated they are to join their conspecifics and to avoid detection by predators [65]. HFP birds had a shorter latency to vocalize and move when isolated and they tended to move faster to their conspecifics in the runway. This active behaviour may suggest that HFP are more fearful than LFP. Conflicting with these results, however, is the lack of line differences in activity in the open field test, a validated measure of fearfulness in chickens [59]. Moreover, almost all HFP were fast in approaching the novel object/person compared to only half of the LFP approaching, which would indicate lower fearfulness in HFP in this specific test situation. Strikingly, all these tests show that HFP respond faster and more actively than the LFP. Kjaer [12] showed a higher general locomotor activity in HFP chickens than in LFP ones, which was not a consequence of increased feather pecking (performing or receiving); rather it seemed a trait-specific result of selection for SFP behaviour. In line with this, we suggest that differences in the way the birds cope with challenging conditions, i.e. a more active coping style, (hyper)activity and/or impulsivity, rather than fearfulness, might underlie the fast and active responses of the HFP observed in our behavioural tests. Previously, it has been suggested that behavioural styles of coping with aversive events and fearfulness can be seen as two independent dimensions of stable trait characteristics [66].

##### 4.3. The serotonergic and dopaminergic system in young and adult LFP and HFP hens

In this study, concentrations of 5-HT, DA, and their metabolites were measured in seven brain areas of chickens originating from the HFP and LFP selection lines. At 8 weeks of age, young HFP had lower 5-HIAA concentrations and/or serotonin turnover ratios, compared to young LFP in all brain areas except the NCL and hippocampus. Also, HFP had a lower DA turnover in the dorsal thalamus and the medial striatum when young. This is in agreement with previous literature on 5-HT and DA activity in the rostral forebrain of young chickens differing in FP [21]. At 25 weeks of age, lines differed less prominent, but still serotonergic and dopaminergic differences were found in the NCC and the arcopallium. Strikingly, several differences at a young age had disappeared or were even reversed. At an adult age, the HFP had higher DA and 5-HT turnover in the NCC as compared to the LFP. This fits with our previous findings in other hens with a propensity to perform SFP [23,24]. It is also in line with the more active tone of 5-HT release in adult birds of the currently studied SPF line, as measured with microdialysis [28].

##### 4.4. Monoaminergic systems in young chickens divergently selected on severe feather pecking

The current study demonstrates that young LFP and HFP lines predominantly differ in serotonergic activity, and to a lesser extent, dopaminergic activity in the dorsal thalamus, medial striatum, amygdala, NCC and arcopallium. These brain areas are involved in guiding motor actions and decision-making by integrating sensorimotor, motivational and emotional information [40–42,67]. Furthermore, studies in birds have shown that these brain areas are innervated by both serotonergic and dopaminergic fibers [51,68,69,71]. Lesions in, for instance, the limbic medial striatum impaired the suppression of immediate reward seeking behaviour in chicks [72]—thus made chickens more impulsive—and lesions in the limbic amygdala (to recall, the amygdala sampled here refers to the arcopallium mediale) increased fear in quails [73]. The arcopallium seems also involved in anxiety, although lesions in the arcopallium (intermediate part) decreased fear in chicks [72]. Moreover, the arcopallium is a somatosensory area involved in motor output [41,48,49]. Most strong serotonergic differences between the lines were found in 8-week-old chicks. At that age, the brain is still in development, and this phase of brain maturation is characterized by high neuroplasticity. This lasts until chicks are 10 weeks of age [74,75]. The shaping of brain networks is coordinated by 5-HT, but 5-HT is also essential for synapse formation during adulthood [76,77]. Abnormal concentrations of 5-HT can have a long-lasting effect on brain circuitries and with that, on the behavioural output [76]. For instance, mice lacking brain 5-HT (by a mutation on the tryptophan hydroxylase 2 (Tph2) gene responsible for the 5-HT producing enzymes) have abnormalities of the serotonergic circuitry formation in several rostral brain areas; increasing the risk to develop anxiety [78]. Recently, it was shown that 5-HT administration in the embryonic phase and also in the post-hatch period altered aggressiveness and fearful behaviour later in life, up to adolescence [79,80]. In addition, genetic factors, stress and other environmental influences such as social isolation, in general affect neuroplasticity [81,82] and modulate monoaminergic pathways in the chick's forebrain [83]. Overwhelming evidence from human studies shows a role for 5-HT and early deficits in frontal-striatal-thalamic brain areas in neurological/psychiatric disorders such as schizophrenia, anxiety disorders, impulse control disorders, compulsive disorders, and attention deficit/hyperactivity disorders (ADHD) [35,84–89]. Children with ADHD have a delay in cortical, prefrontal, maturation [90]. The hyperactive and impulsive

component of ADHD behaviour seems under the control of 5-HT also mediating dopaminergic functioning [91]. 5-HT is known to directly affect locomotor activity as well [92,93]. Therefore, it is speculated that the general higher behavioural activity of young HFP birds in the different tests might be related to their low activity of the serotonergic system and corresponding affected neuronal circuits.

#### 4.5. Monoaminergic systems in adult chickens divergently selected on severe feather pecking

At the age of 25 weeks, line differences in adult birds were restricted to the NCC and the arcopallium, and in the opposite direction as observed in young birds. HFP laying hens had both higher 5-HT and DA turnover ratios in the NCC and higher DOPAC and 5-HIAA levels in the arcopallium compared to LFP. This latter area also showed similar DA-metabolite differences in lines selected on low mortality as a consequence of SFP and cannibalism [23]. Remarkably, the NCC and arcopallium are strongly connected [41,42,94] and the NCC predominantly displays limbic connectivity [44,45] while the arcopallium processes somatosensory information [68–70,95]. Therefore deficits in the monoaminergic system in these brain areas have the potential to affect both the emotional perception and behavioural output. Indeed, increased DA levels can induce stereotypies [96] and deficits in the control of posture and motor activity [97]. Similar to the arcopallium [98] dopaminergic terminals are abundant in the NCC and activate primarily D1-receptors [68], thereby possibly increasing spike densities of pre-activated neurons [95,99]. In our study, the higher DA turnover in the NCC of HFP compared with LFP hens, although DA concentrations were not different, suggests that HFP have a higher metabolic DA activity. Previously, it was shown that chickens with a higher SFP-propensity showed a higher increase in locomotor response after acute administration of apomorphine, a DA-D<sub>1</sub> and D<sub>2</sub> receptor agonist, than birds with the lower propensity to SFP - reflecting a more sensitive dopaminergic system in the former [22]. Consequently, the higher DA activity might lead to a more active motor output system in HFP, underlying hyperactivity. It is suggested that these higher levels in adult hens are responsible for more persistent pecking behaviour. Yawning during the open field test was significantly increased in HFP in comparison to LFP birds and this comfort-like behaviour might be a marker of recovery from acute stress [100]. Yawning is under the control of the serotonergic and dopaminergic system, as increased activity of these systems—as seen in the adult HFP—has a facilitating effect on yawning [101].

This paper shows a clear inversion of neurochemical markers over developmental time: where young high feather pecking hens are characterized by a low serotonergic and dopaminergic turnover, adult HFP hens have higher turnover values of these monoaminergic systems. For LFP the opposite accounts, the monoaminergic turnover is first higher in young, than lower in the adults. The fast, perhaps even impulsive reactions of HFP in various tests, fit in the behavioural profile of individuals with a hyperactivity disorder. It is suggested here that this enhanced locomotor activity is caused by lower serotonergic activity affecting neural circuits at a young age. Interpretation of the present data in relation to monoaminergic release are difficult. Monoamines and metabolites in punches do not elucidate whether the differences found are due to an altered phasic or tonic response, to altered reuptake by monoamine transporters or to altered negative feedback by somatodendritic 5-HT<sub>1A</sub> autoreceptors. Recently, however, we showed that adult HFP had a higher tonic release in the caudal arcopallium, as measured by microdialysis [28]. It is speculated that these HFP birds activate their (impaired) serotonergic system by increasing locomotor activity thereby increasing 5-HT activity as seen later in life [93,102], but more research is needed on the causation of this

neurochemical shift during development and how hyperactivity can be related with SFP via the serotonergic and dopaminergic systems.

In conclusion, this paper shows that divergent genetic selection on SFP has changed the serotonergic and dopaminergic system with possible implications for the underlying motivation for displaying SFP. Similar to previous studies in young chickens, low monoamine turnover ratios are related to HFP behaviour; suggesting that the brain of HFP chicks is prone for certain behavioural deficits, such as the increased general behavioural (hyper)activity. We hypothesize that selection for severe feather pecking behaviour has indirectly resulted in changes of predominantly the serotonergic system early in life, with long-term behavioural and neurological consequences later in life. Remarkably, differences between the low and high feather pecking lines in activity of neurochemical systems (5-HT and DA) observed at a young age were reversed at an adult age. Whether this is a cause or consequence of the behavioural differences between the lines - SFP and (hyper)activity - needs further investigation. Furthermore, SFP behaviour seems closely related with a high locomotor activity which is in line with previous findings. More research is needed to further specify the role of the developing monoaminergic systems in SFP and the accompanying hyperactivity and SFP and vice versa.

#### Acknowledgements

Eggs from the feather pecking selection lines used in the present study were brooded with help of Bernd Riedstra, Behavioural Biology, Groningen, the Netherlands. We would like to thank Bas Rodenburg, Monique Ooms, Fleur Bartels, Ger de Vries-Reilingh, and Mike Nieuwland from Wageningen University and Farrah Bannink from Hogeschool Utrecht for their technical assistance and help with the behavioral observations. We also thank the staff of experimental farm “De Haar” for their excellent animal care. This study is part of the project “Preventing feather pecking in laying hens: from principle to practice (no: 827.09.020) which is financially supported by the program ‘The Value of Animal Welfare’ of the Netherlands Organization for Scientific Research (NWO) and the Ministry of Economic Affairs. The authors have no conflict of interest to declare.

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