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Identification of candidate genes for feather pecking in chickens: evidence from behavioral and gene expression analyses

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Feather pecking is an important economic and welfare problem in poultry industry, especially in non cage housing systems. To facilitate uncovering a possible genetic predisposition for this complex trait, we consider it as redirected exploratory behavior, whereby the lack of opportunity for normal exploration might represent the trigger leading to aggressive feather pecking. In our study, we investigated two chicken lines that differed in their tendency to feather pecking (High versus Low Feather Pecking lines) using a combination of quantitative behavioral observation experiments and gene expression analysis. First, we observed the exploratory movements (locomotory activity) of individuals of the two lines in reduced, well-defined environment immediately after hatching. Independent observers counted short-term (10-25 minutes) exploratory movements of 480 animals divided in groups of twenty chicks, ten per each line. Observations revealed that a significantly higher ($P<0.001$) number of exploratory movements were made by newly hatched chicks from the High Feather Pecking line. Secondly, RNA was isolated from the brains of three individuals per line and investigated using two-color microarray experiments based on the Chicken Neuroendocrine array from ARK-Genomics. A loop design was applied. The statistical analysis was carried out using a clone by clone mixed model approach, which was applied to each technical replicate: $y_{ijkl} = \mu + A_i + D_j + L_k + I_l + e_{ijkl}$. The first term in the model captures the overall average intensity. *A, D* and *L* indicate fixed effects of array, dye and line, respectively. *I* is considered as a variation due to individuals and represents a random effect. P-values for each clone were calculated as an average of P-values across technical replicates. Four genes met significance criteria and were selected for

further detailed analysis by RealTime quantitative RT-PCR using eight individuals per line, in three technical repetitions. The GAPDH housekeeping gene was used as reference gene for relative quantification of transcription. Three of the four genes analyzed demonstrated significant ($P<0.01$) differences in expression level between the high and low feather pecking lines and therefore represent strong candidate genes, the expression of which might be affected by cis-regulatory DNA variation.

Key words: laying hens; feather pecking; exploratory behavior; microarray; gene expression.

Introduction

Establishing the causes of feather pecking, which are unknown to date, presents several difficulties. First, the reasons causing each behavior might be different. Although both genetic and environmental (e.g., feed composition, type of light, stock density) factors should be considered, both are problematic. The complexity of the traits hinders molecular analysis of the genetic factors. Moreover, it is very difficult to measure feather pecking in individual egg-laying hens in free-range housing conditions and also to adequately consider multiple and variable environmental influences. We attempted to uncover the genetic background of the complex trait of feather pecking by following the hypothesis that feather pecking represents redirected exploratory behavior, with the corollary assumption that the predispositions for exploratory behavior are linked to predispositions for severe feather pecking. According to this model, we observed the exploratory movements (locomotory activity) of two lines of chickens that differed in their tendency to feather pecking (low versus high) to define behavioral phenotypes. To ensure both the least bias in the measurements of exploratory behavior and to avoid different environmental influences, observations were made in reduced, well-defined conditions immediately after hatching. As determined by independent observers, the High Feather Pecking line displayed significantly higher spontaneous exploratory behavior irrespective of time of observation. As such, exploratory activity can be considered to be a good indicator for an increased risk of severe feather pecking. This simple experiment was also an essential, foundational step for collecting material to compare the two lines in further molecular analysis focused mostly on gene expression studies. RNA isolated from chicken's brains of opposite lines, obtained during experiments, was further on compared considering the gene expression profile using microarray technique. Results were confirmed by RealTime quantitative RT-PCR. As can be seen, our approach combines different techniques and methods, which is necessary for the full molecular study of a complex, destructive behavior such as feather pecking in chickens.

Material and methods

Behavioral experiment

The experiments provided in May and July 2004 were based on the comparison of exploratory behavior in chicks from two commercial chicken lines representing opposite behavioral phenotypes with respect to the incidence of feather pecking: Low Feather Pecking line (LFP) versus High Feather Pecking (HFP) line. High Feather Pecking line is Lohmann Selected Leghorn (LSL) female-line from a regular breeding program. The Low Feather Pecking line is the D-line of Lohmann Brown (LB). Ten chicks from each line were placed in a single box and recorded for 10-25 minutes. All chicks from a randomly chosen line were marked with a blue color. Videos were watched independently by different observers, in part to assess if any bias in the measurement of exploratory movements existed. Moreover, each movie was watched twice, once for each line.

RNA isolation

All brains were collected after the behavioral experiment and stored in RNAlater solution (Qiagen, Hilden, Germany). Homogenization and disruption of the tissues were carried out with the Fast Prep FP120 (matrix D, MP Biomedicals, Germany). RNA was isolated using Qiagen Maxi Kit (Qiagen, Hilden, Germany). After each isolation, both the quality and quantity of the obtained RNA was checked with formaldehyde-agarose gel and the spectrophotometer.

Microarray experiments – global gene expression analysis

Our experiments Chicken Neuroendocrine array spotted by ARK-Genomics (www.ark-genomics.org) and consisting of 4,800 clones was used. On each slide, we compared cDNA extracted from animals from each of our two experimental lines (i.e., the High versus Low Feather Pecking lines). Each individual was labeled with one of two color of cyanine dye (Cy3-dCTP, Cy5-dCTP) and its expression for a given genes was compared with that of the other line. A loop design presented on Figure 1 was applied following Churchill (2002). After scanning, images were analyzed by Spot software (http://spot.cmis.csiro.au/spot). Further statistical analyses were provided with R/MAANOVA package (www.r-project.org) that includes data normalization, array quality check and statistical analyses. Statistical analyses were done according to the given fixed model:

$$y_{ijkl} = \mu + A_i + D_j + L_k + I_l + e_{ijkl}$$

The first term in the model captures the overall average intensity. *A, D* and *L* indicate fixed effects of

array, dye and line, respectively. *I* is considered as a variation due to individuals and represents a random effect.

y	transformed intensity data
μ	overall average intensity
A_i	array (i = 1…6)
D_j	dye (j = 1…2) 1 = Cy3 2 = Cy5
L_k	line (k = 1…2) 1 = HFP line 2=LFP line
I_l	individual (l = 1…6)
e_{ijkl}	residual error

Considering applied model usual F statistics test was performed. P-values for each clone were calculated as an average of P-values across technical replicates.

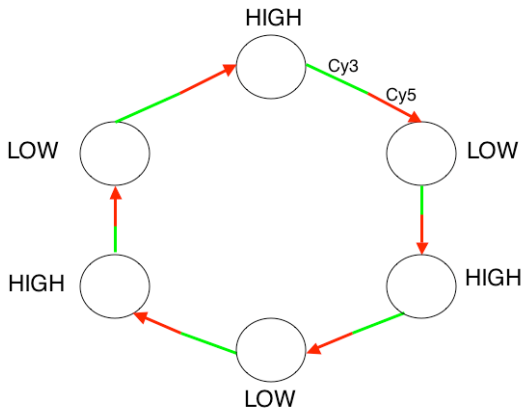

Figure 1: Microarray experiment loop design. Circles mark animals from Low (LOW) and High (HIGH) Feather Pecking lines. The green and red parts of the each arrow correspond to Cy3 and Cy5 dye, respectively. As can be noticed each animal has to be labeled with both dyes and compared with two other animals from opposite line. All together three animals per each line were analyzed. Loop design helps to provide an efficient microarray experiment.

RealTime quantitative RT-PCR

RealTime quantitative PCR amplification reactions were carried out by an ABI Prism® 7000 Cycler-Detection System using Platinium® SYBR® Green qPCR SuperMix UDG with ROX (Invitrogen, Karlsruhe, Germany). All four selected candidate genes were investigated using eight individuals per line; all runs included the *GAPDH* housekeeping gene as reference gene to enable the relative quantification of

transcription and each run was sampled in three technical repetitions. To determine if the gene expression in one chicken line was shifted with respect to that in the other, the non-parametric Wilcoxon Runk Sum Test was used under the null hypothesis that the distribution functions in two compared populations are identical.

Results and discussion

Behavioral evidence

Initial observations indicated highly significant differences between the two egg-laying lines in the spontaneous exploratory behavior of newly hatched chickens. In particular, chicks from the High Feather Pecking line showed a significantly higher number of exploratory movements (Figure 2). There was no significant inter-observer variation with respect to the number of movements recorded from the viewing of a particular analysis.

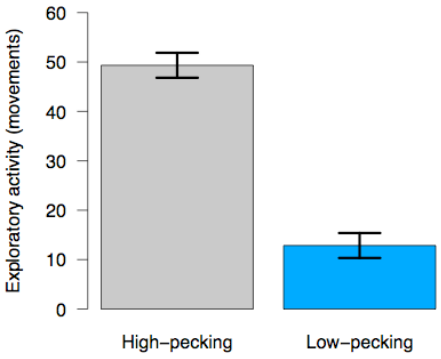

Figure 2: Number of exploratory movements made in 25 minutes as observed for the May experiment.

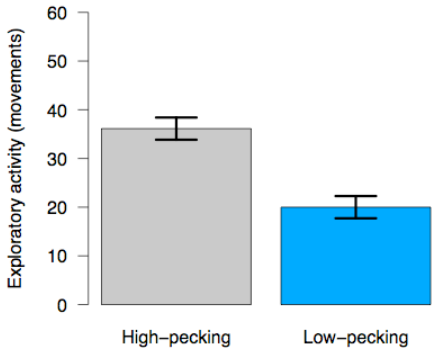

Figure 3: Number of exploratory movements made in 25 minutes as observed for the July experiment.

Although, the activity of the High Feather Pecking line was lower in July (Figure 3) comparing to May experiment, still reproducible results were obtained in all boxes for 25 minutes observations. Even the short, 10 minutes observations (provided in July, data not shown) detected a significant difference in exploratory movement between the two lines.

Gene expression analyses

The microarray slide image is the final result of multistep experiment. Different functions available in MAANOVA package enable checking of the quality of the hybridization and gridding, both within and across arrays. For example R-I plot (ratio intensity plot), which plots the log ratio versus log intensity,

represents good indicator of intensity-depended effects. All of investigated slides qualified for further analysis. After normalization using LOWESS method and statistical analyses using mentioned ANOVA model clones with the smallest P-values were chosen for sequencing that was carried out by the array producer ARK-Genomics (www.ark-genomics.org). Sequences were blasted against human and chicken sequences. Four putative candidate genes were selected for further detailed analysis based on a combination of their observed differential expression level, their known function potentially having an influence on feather pecking / exploratory behavior or another behavior-correlated trait, and the availability of the chicken genomic sequence for them. To increase the precision of the results and to quantify the exact level of gene expression quantitative PCR was performed. Results are presented in Table 1.

Table 1: Four selected genes after microarray experiment were analyzed using RealTime PCR. Three of them show significant difference in expression level (marked with *).

Gene	Ratio HFP	SE HFP	Ratio LFP	SE LFP	P-values
Gene1*	1.1026	0.0100	1.0661	0.0066	0.0047
Gene2*	1.0710	0.0120	1.0405	0.0031	0.0104
Gene3*	1.1215	0.0107	1.0691	0.0051	0.0011
Gene4*	1.8985	0.2765	1.9024	0.2769	0.9015

Ratio – number of the threshold cycle of the investigated gene divided by the threshold cycle of the reference gene **HFP** – High Feather Pecking Line **LFP** – Low Feather Pecking Line **SE** – standard error

There is a difference found between investigated Low and High Feather Pecking lines in exploratory behavior and in gene expression considering global expression pattern and analyses of selected genes. The eventual role of differentially expressed genes in exploratory behavior and in feather pecking is still being investigated.

References

Churchill, G. A. (2002). Fundamentals of experimental design for cDNA microarrays. Nat Genet *32 Suppl*, 490-495.

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