

# Functional Investigation Of Candidate Genes Affecting Feather Pecking In Chickens

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## Introduction

Feather pecking is a major welfare problem in egg production. It may be caused by endogenous (genetic and physiological) and environmental (feeding, density and housing conditions) factors. There are obvious differences in propensity to feather pecking between pure lines and commercial hybrids of laying hens. Indeed, in many studies so-called High Feather Pecking line (HFP) and Low Feather Pecking line (LFP) were compared regarding behavioural or genetic factors (Rodenburg et al., 2003; Kjaer, 2009; Flisikowski et al., 2009). Heritability estimated for feather pecking varies between  $h^2=0.07$  (Bessei, 1984) and  $h^2=0.38$  (Kjaer and Sørensen, 1997).

QTL (Quantitative Trait Loci) analysis is an important step in uncovering the genetic background of behavioural traits. Buitenhuis et al. (2003a,b; 2004) identified several QTL for performing or receiving gentle and severe pecks at different age. Furthermore, molecular analyses carried out for feather pecking have helped to define putative candidate genes. According to Keeling et al. (2004), chickens suffered more drastic feather pecking when the colour of their plumage is a result of the expression of the wild recessive allele at *PMEL17*. Two further candidate genes, i.e. *DRD4* and *DEAF1*, were suggested by Flisikowski et al. (2009). The panel of candidate genes including *TPHI* and *PRKGI* was generated and explored by Wysocki (2007). Despite the number and variety of experiments performed over past years, it is still not possible to define a genetic background of feather pecking. A possible reason is the complex nature of this trait, but also the lack of homogenous approach in carried out studies.

The main goal of the current experiment was to select candidate genes and explore their expression comparing HFP and LFP. In order to do that a list of putative candidate genes was created after literature search and signaling pathway analysis. Selected genes were subject to QPCR (Quantitative PCR) using brain tissue collected from HFP and LFP.

## Material and methods

**Animals.** A total of 24 (12 per each group) laying hens selected for low (LFP) and high (HFP) level of feather pecking were used in this study. The founder line was a White Leghorn and birds were selected for 8 generations. The latest estimated heritability was  $h^2=0.3$  (Baes, Grashorn and Bennewitz, personal communication).

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**Tissue Collection and RNA Preparation.** Brains from two investigated lines were collected at the approximate age of 2 years and stored in RNALater solution (Qiagen). Next all samples were homogenised using FastPrep system (MP Biomedicals) in addition of QIAzol Lysis reagent (Qiagen). The only disadvantage of this homogenisation method was the limited amount of tissue that can be homogenised in one tube (about 1g). An average brain of a chicken is 3-4g. Consequently each brain was divided into 3-4 pieces and the lysates were combined thereafter. The lysate was either directly used for extraction or stored at -80°C. For the RNA extraction a RNeasy Lipid Tissue Midi Kit was used (Qiagen). This technology is based on the selective binding properties of a silica-gel membrane. Maximal 1 mg of RNA molecules longer than 200 bases can be adsorbed to the membrane. The eluted RNA was stored at -80 °C.

**Evaluation of RNA.** Nanodrop (Peqlab) and Bioanalyzer (Agilent) were used to evaluate the quantity and quality of extracted RNA. The measurement using Nanodrop Spectrophotometer 2000c (Peqlab) was carried out to estimate the amount and the purification (260/280 nm ratio) of RNA. Evaluation using Bioanalyzer (Agilent) gives an opportunity to calculate RIN (RNA Integrity Number) which is capable of assessing RNA quality better than ribosomal 28S/18S ratios (Schroeder et al., 2006). In order to estimate the integrity of RNA the microcapillary electrophoretic RNA separation is used. The concentration of measured samples was above 1µg/µL and the RIN was higher than 8 which indicates almost intact total RNA.

**RT and QPCR.** Using total RNA isolated from brains of the experimental animals, single-stranded cDNA was synthesised by reverse transcription (Stratagene) and used for QPCR amplification. Specific primers designed on the exon-exon junction were used to avoid unspecific products due to amplification of genomic DNA. Amplified fragments were shorter than 200 bp. QPCR reactions were performed with CFX96 Real-Time PCR Detection System (BioRad) using SYBR Green dye (Stratagene). All runs included the *GAPDH* and *ACTB* as reference genes to enable the relative quantification of transcription. Each run was sampled in two technical repetitions.

**Statistical analyses.** Relative gene expression levels were calculated using delta-delta Ct (threshold cycle) method (CFX96 Real-Time PCR Detection System software, BioRad). For each gene a sample with a minimum Ct value was calculated as 1. In order to discover a difference in gene expression between two investigated lines Analysis of Variance ([www.r-project.org](http://www.r-project.org)) was carried out separately for every candidate gene. A linear model included line as the factor of interest. Additionally a False Discovery Rate q-value was estimated for each test.

## Results and discussion

In this study candidate genes were identified using a literature search followed by signaling pathway analysis. The main criteria were a relation to the feather pecking, but also to the other behavioural disorders, activity, exploration and foraging behaviour. The final list of putative candidate genes included: *ACADS*, *ADRB2*, *CRHR1*, *DRD4*, *GPR17*, *MBP*, *PRKG1*,

*SLC6A3*, *TPH1* and *TPH2*. For example *DRD4*, the dopamine receptor D4, is associated with temperament and behaviour. Fidler et al. (2007) reported significant association of *DRD4* polymorphisms with early exploratory behaviour in a passerine bird species (*Parus major*). It has been revealed that dopaminergic and serotonergic systems are involved in feather pecking (van Hierden et al., 2002). Following these findings Flisikowski et al. (2009) hypothesized that variation in the *DRD4* gene might be associated with pecking behaviour in laying hens. *PRKG1* encodes cGMP-dependent protein kinase type 1 and was also suggested as an interesting candidate gene for feather pecking by Wysocki (2007). In the fruit fly (*Drosophila melanogaster*) and in honeybee (*Apis mellifera*), the cGMP dependent protein kinase is encoded by the foraging gene (for), which has been identified as a major gene involved in food-search behaviour (Sokolowski, 1980; Ben-Shahar, 2005). Foraging behaviour involves the search and intake of food and feather pecking might be a redirected form of foraging behaviour (Blokhuis, 1986; Huber-Eicher and Wechsler, 1997). The first results are very promising. After the statistical analysis, four genes showed a significant difference in expression, namely *DRD4*, *MBP*, *PRKG1* and *SLC6A3*, between HFP and LFP (Table 1).

**Table 1: Results of the QPCR analysis. Genes that are marked in bold show a significant difference in expression between HFP and LFP.**

	HFP	LFP	P-value	q-value
<b>DRD4</b>	<sup>a</sup> <b>0.94±0.20</b>	<b>1.23±0.33</b>	<b>0.0152</b>	<b>0.0873</b>
<b>SLC6A3</b>	<b>1.13±0.24</b>	<b>0.93±0.16</b>	<b>0.0229</b>	<b>0.0947</b>
<b>PRKG1</b>	<b>1.00±0.17</b>	<b>0.85±0.14</b>	<b>0.0284</b>	<b>0.1114</b>
<b>MBP</b>	<b>0.97±0.22</b>	<b>1.19±0.24</b>	<b>0.0349</b>	<b>0.1145</b>
GPR17	1.17±0.29	0.97±0.20	0.0557	0.1520
ADRB2	0.88±0.32	0.70±0.19	0.1099	0.1706
CRHR1	0.87±0.25	0.72±0.19	0.1194	0.1832
ACADS	1.12±0.25	1.05±0.13	0.4418	0.5523
TPH2	0.86±0.41	0.91±0.33	0.6657	0.7397
TPH1	0.20±0.53	0.15±0.41	0.8050	0.8050

<sup>a</sup> Relative gene expression averaged across 12 samples; ± Standard Deviation

Among the first seven genes listed in Table 1 around 20% are expected to be type one error, showing that some effects are true. There is no tendency for over or under expression between two investigated lines.

To increase a statistical power we might carry out QPCR for four differently expressed candidate genes using a larger number of animals. Another future step is a microarray experiment. Gene expression differences between HFP and LFP will be compared using Chicken Gene Expression Microarray (Agilent). The large amount of data will be used to identify new candidate genes and perform pathway analysis with Ingenuity software ([www.ingenuity.com](http://www.ingenuity.com)).

## Conclusion

Because feather pecking remains an important issue, this study was undertaken with an attempt to elucidate the genetic foundation of this complex trait, and it can be concluded that it has indeed, revealed some interesting differences in gene expression between HFP and LFP. Among ten investigated genes four were differentially expressed. Most likely, more than one, single gene causes feather pecking due to the complexity of this trait. The carried out study is a part of a larger project that will be continued using a wide range of techniques and materials. We hope that additional analysis and future experiments will lead to a successful genetic dissection of feather pecking.

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