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Genetic dissection of the predisposition to feather pecking of laying hens: behavioural studies, identification of candidate genes and gene expression analyses

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The search for truth is in one way hard and in another way easy. For it is evident that no one can master it fully nor miss it wholly. But each adds a little to our knowledge of Nature, and from all the facts assembled there arises a certain grandeur.

Aristotle

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Abbreviations

A	adenosine
ABI	Applied Biosystems
AMV-RT	avian myeloblastosis virus RT
ANOVA	analysis of variance
BAC	bacterial artificial chromosome
BLAST	basic local alignment search tool
bp	base pair
C	cytosine
cDNA	complementary DNA
Chick VD	Chicken Variation Database
Cy3, Cy5	cyanine 3, cyanine 5
dATP32	deoxyadenosine 5'-[P^{32}] triphosphate
dCTP	5-Amino-propargyl-2'-deoxycytidine 5'-triphosphate
DDT	dithiotreitol
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	desoxyribonucleotriphosphate
dsDNA	double stranded DNA
EDTA	ethylendiamintetraacetat
EMBL	European Molecular Biology Laboratory
ETOH	ethanol
EST	expressed sequence tag
FRET	fluorescent resonance energy transfer
G	guanine
HFP	High Feather Pecking
kb	kilo base pairs
LFP	Low Feather Pecking
LOWESS	locally weighted regression scatterplot smoothing
MAGE-ML	microarray gene expression markup language
MAANOVA	microarray analysis of variance
MIAME	minimum information about a microarray experiment
mRNA	messenger ribonucleic acid

MMLV-RT	moloney murine leukaemia virus RT
NCBI	National Center for Biotechnology Information
PCR	polymerase chain reaction
QTL	quantitative trait loci
RNA	ribonucleic acid
ROX	rhodanium derivative (6 carboxy-x-rhodamine)
rpm	rounds per minute
RT-PCR	reverse transcription PCR
S	sedimentation pattern
SNP	single nucleotide polymorphism
SSC	saline sodium citrate buffer
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TEMED	N', N', N', N', tetramethylethylendiamin
Tris	Tris (hydroxymethyl) aminomethane

The names of genes and most of the abbreviations are explained in the text.

1 Introduction and goals

1.1 Introduction

Feather pecking is an important economic as well as welfare problem in poultry industry, especially in non-cage housing systems. However, due to its complex nature, the causes of this trait have not been found yet. Both endogenous (genetic and physiological control mechanisms) and environmental (feeding, density and housing conditions) factors should be considered, but neither of them is easy to establish. The main objective of this dissertation was to identify and investigate candidate genes responsible for feather pecking. However, it is very difficult to measure feather pecking in individual egg-laying hens in non-cage housing conditions, or to adequately estimate multiple and variable environmental influences. Therefore, in an attempt to identify the genetic predisposition to that complex phenotype, the trait "feather pecking" was reduced to a less complex one. Specifically, the working hypotheses were as followed:

- Feather pecking is a redirected exploratory behaviour; and the lack of opportunity for normal exploration may trigger off aggressive feather pecking
- Genetic differences in the tendency for exploratory behaviour are responsible for the differences in the predisposition to feather pecking
- Differences in the intrinsic exploratory activity can be best measured in freshly hatched chicks in a well-defined and easily controlled environment

The existence of differences in the behaviour of the two observed lines (Low versus High Feather Pecking) would be conducive in defining the opposite phenotypes of the exploratory behaviour and obtaining DNA/RNA for further molecular analyses of putative candidate genes.

1.2 The goals of this dissertation were

To define phenotypes in a simple behavioural experiment through:

- Comparing the number of exploratory episodes (locomotory activity) between two lines of chickens that differ in their tendency for feather pecking (Low versus High Feather Pecking line)
- Collecting samples of blood and brains to be used in further molecular analyses

To set up a putative candidate genes list using:

- Literature search
- Microarray experiments

To carry out molecular analyses of candidate genes by:

- Sequencing the coding regions of candidate genes to identify polymorphisms
- Establishing and optimising RNA isolation from chicken brains
- Establishing and optimising the microarray method and data analyses
- Analysing the differences in gene expression of candidate genes between two experimental lines using microarrays and quantitative realtime RT-PCR

2 Literature review

2.1 Description and definition of feather pecking behaviour

Feather pecking is one of the biggest problems in chicken housing, as it causes economic losses and damages to the animal welfare in poultry breeding (Blokhuis and Wiepkema, 1998; Huber-Eicher and Wechsler, 1997; Riedstra and Groothuis, 2002). The phenomenon of feather pecking can be defined in many ways. Following van Hierden et al. (2002, 2004a) feather pecking behaviour ranges from mild pecking (gentle feather pecking) to vigorous pulling of the feathers of conspecifics (severe feather pecking), which may result in a damage to plumage and a loss of feathers, increasing chicken susceptibility to further injury, such as wounds of the skin. According to the same author (van Hierden et al., 2004b) severe feather pecking can ultimately lead to cannibalism and death. A similar classification was proposed by Kjaer and Vestergaard (1999), who also reported the same two types of feather pecking. Bilcik and Keeling (2000) distinguished three kinds of pecking:

- Gentle feather pecking – exploratory pecks with no reaction of recipient bird
- Severe feather pecking – forceful pecks, when feather is being pulled out, recipient bird is moving away
- Aggressive pecking – always severe and fast, mainly at the head

2.2 Environmental and endogenous factors involved in feather pecking

2.2.1 Size of the flock

One of the possible environmental factors causing feather pecking is the size of the flock. Keeling et al. (2003) assumed that hierarchical social structure that is normally noticeable in smaller groups, breaks down in larger flocks. Animals become less aggressive and more tolerant, which would confirm the opinion of Nicol et al. (1999) that aggressive pecking occurs more often in smaller groups at the lowest stocking densities. In the very early findings, however, feather pecking was considered to be a consequence of living in bigger groups (Hughes and Duncan, 1972).

2.2.2 *Social transmission*

Another behavioural phenomenon that should be considered is the possible social transmission of feather pecking. Two ways in which social transmission occurs are known: imitation and stimulus enhancement (Galef, 1988; Nicol, 1995). Imitation involves copying the exact motor pattern of the tutor. Stimulus enhancement draws the attention of the observer to previously irrelevant features (Zeltner et al., 2000). Several studies have shown that chickens are able to modify their behaviour when observing conspecifics. Consequently, social transmission could be a possible mechanism in spreading feather pecking. An experimental concept based on the introduction of chicks with high frequency of feather pecking confirms this theory, as it increases the general feather pecking rate and reduces foraging behaviour frequencies (Zeltner et al., 2000). The authors did not distinguish between severe and gentle feather pecking. Similar behaviour was observed by McAdie and Keeling (2002) who compared Low and High Feather Pecking lines. In that case social transmission of gentle feather pecking was mostly noticed in cages, not in pens and only between older animals. This might have implications for a possible effect of age.

2.2.3 *Foraging behaviour*

Foraging behaviour involves the search and intake of food (Hurnik et al., 1995). According to a hypothesis of Huber-Eicher and Wechsler (1997, 1998) feather pecking should be treated as a redirected foraging behaviour. However, Blokhuis and Arkes (1984) claimed that feather pecking is a redirected ground pecking behaviour related to foraging behaviour.

To avoid feather pecking pens should be enhanced with some environmental enrichments. Different reactions to various kinds of supplied foraging materials lead to the conclusion that not only the quantity but also the quality of environmental enrichments is important for the prevention from feather pecking (Huber-Eicher and Wechsler, 1998). Easy access to bundles of straw rather than shredded straw and polystyrene blocks rather than beads reduce feather pecking. One of the practical solutions for the problem of aggressive feather pecking is providing cages or pens with randomly located strings in different colours. Animals from two investigated strains pick strings (especially white and yellow ones) sooner and more often than any other stimuli, for example beads or chains (Jones et al., 2000; Jones et al., 2002a). Strings also seem to be long-term stimuli attracting birds even after 122 days (Jones, 2002b). Early exposure to wood shavings could also be protective

because of its influence on feather pecking and foraging behaviour. Birds that had an early, even if only short, contact with wood shavings develop a relatively stronger ground pecking and less feather pecking behaviour (Nicol et al., 2001).
Another theory concerns the causes of feather pecking in relation to abnormal dustbathing behaviour (Vestergaard et al., 1993, 1997). Aggressive pecks are more common during feeding and gentle pecks are mostly received while dustbathing (Leonard et al., 1995).

2.2.4 Housing systems

The development and the strength of feather pecking may also depend on the housing system and conditions. According to the results obtained by Huber-Eicher and Sebo (2001) feather pecking can be reduced when raising laying chicks in aviary systems with access to the litter like wood shavings from day one on. Feather pecking may be reduced when chicks are allowed to go outside the cote (outdoor runs) as observed by Bestman and Wagenaar (2003). Appleby (1998) suggested that modified cages could be a good solution for aggressive behaviour, but it seems to be out of the question soon, since the European Commission approved the directive that banns the battery cages as of 2012 (Sedlackova et al., 2004). Most of the findings tend to point out to enrichments and housing conditions rather than to the housing systems themselves (Huber-Eicher and Sebo, 2001; Potzsch et al., 2001).

2.2.5 Light intensity

Light intensity is one of the factors causing feather pecking (Kjaer and Vestergaard, 1999). The number of gentle pecks is higher in low light intensity, which could be explained as a compensatory behaviour of chicks exploring their environment. Low light level had no clear effect on pecking behaviour during the laying period. Moreover, according to Kjaer (2000) medium heavy strains (Lohmann Brown and ISA Brown) show an increase in feather pecking activity towards the end of the day.

2.2.6 Genetic factors and the effect of breed and sex

Cockerels delivered and received more directed pecks than females (Leonard et al., 1995). The presence of cockerels in the flock could also reduce feather pecking and increase desirable outdoor movements (Bestman and Wagenaar, 2003). However Oden et al. (1999)

found no significant differences in feather pecking between single-sexed and mixed-sexed groups.

Kjaer and Sorensen (1997) proposed that selection of birds with low or high tendency for feather pecking is possible. Indeed, in many studies the High and Low Feather Pecking lines were compared regarding the behavioural or the genetic factors (Albentosa et al., 2003; Kjaer, 2000; Kjaer and Sörensen, 1997; Kjaer et al., 2001; McAdie and Keeling, 2002; McKeegan and Savory, 2001; Rodenburg et al., 2002; Rodenburg et al., 2003). However, Albentosa et al. (2003) disagreed with this theory and claimed that hens cannot be easily ranked in behavioural “types” according to some behavioural test responses.

Heritability of feather pecking is scaled between h^2=0.07 (Bessei, 1986) and h^2=0.38 (Kjaer and Sorensen, 1997). The heritability of gentle feather pecking was estimated by Rodenburg et al., (2003) at the level of h^2=0.12 and h^2=0.15 at 6 and 30 weeks of age, respectively. A multi-strain experiment carried out by Hocking et al. (2004) compared genetic correlations. Twelve commercial and thirteen traditional strains of laying hens were included. Genetic correlations between behavioural traits and feather, skin score and mortality were very low and labile between sociality, skin lesions and culling. That led the authors to the conclusion that there is no strong genetic correlation between feather pecking and other behavioural traits.

Feather pecking could also be considered as a result of different pigmentation. According to Keeling et al. (2004) birds suffer more drastic feather pecking when the colour of their plumage is a result of the expression of the wild recessive allele at *PMEL17* (gene, which controls plumage melanisation). The presence of the pigmentation victims in the flock increased pecking behaviour.

Jensen et al. (2003) did not find any Quantitative Trait Loci (QTL) for feather pecking. However, they claimed that the plumage condition, a measure for the severeness of feather pecking could be associated with a QTL coinciding with the colour gene “Dominant white”. Putative QTLs for gentle feather pecking were identified by Buitenhuis et al. (2003a) on chicken chromosome (GGA) 10 at 6 weeks of age and on GGA1 and GGA2 at 30 weeks of age. All found QTLs were only suggestive. Considering severe feather pecking, a significant QTL was found on GGA2. For receiving gentle feather pecking a QTL was identified on GGA1 (significant) and GGA5 (suggestive) at 6 and 30 weeks of age, respectively (Buitenhuis et al., 2003b).

2.2.7 *Stress and frustration*

Frustration considered as an omission of an expected reward may influence the development and targeting of pecking behaviour (Rodenburg et al., 2004). In general birds that show more feather pecking behaviour have lower social motivation (Jones et al., 1995) and seem to be more fearful (Vestergaard et al., 1993). Jones et al. (1995) reported that predispositions for feather pecking might be dependent more on the underlying social motivation than on fearfulness.

2.2.8 *Nutritional effects and feather eating*

Nutritional factors should be taken into consideration as a possible cause of aggressive behaviour. Savory et al. (1999) and van Hierden et al. (2004b) suggested that feather pecking could be reduced by L-tryptophan used as a supplement of the diet. Kjaer and Sörensen (2002) investigated the influence of methionine and cystine but found no significant correlation.

A strong association between feather eating and feather pecking at the individual level was found by McKeegan and Savory (2001). Peckers ate and manipulated the feather relatively more often when compared to non-peckers. No differences in feather pecking development were found regarding different protein sources (McKeegan et al., 1999; McKeegan et al., 2001).

2.2.9 *Neurotransmitters and hormones correlated with feather pecking*

Van Hierden et al. (2004a) proposed that in the same way as in psychopathological disorders in humans, the serotonergic system might be responsible for feather pecking. They compared Low and High Feather Pecking lines and found, that indeed low serotonergic neurotransmission was associated with feather pecking. Dopamine is also considered to be a neurotransmitter, which might be involved in feather pecking. Birds from the High Feather Pecking line have a dopamine (DA) system of higher sensitivity than chicks from the Low Feather Pecking line. Birds from the High Feather Pecking line show more behavioural response to apomorphine treatment (van Hierden et al., 2005). It manifests itself as an increase in the locomotory activity and a stereotyped behaviour. Kjaer et al. (2004) also supported the hypotheses that feather pecking behaviour in adult hens can be influenced by the dopamine system. They demonstrated that feather pecking

was significantly reduced when haloperidol (D2 receptor antagonist) was administered. Cheng et al. (2003) indicated that dopamine is involved in controlling the behaviour and productivity of the chicken.

A group of hormones which play an important role in the response to stress and fear are the corticosteroids (Korte, 2001). Two lines that differ in the predispositions to feather pecking also tend to do so in the plasma level of corticosterone and noradrenalin observed during resting and manual restrain conditions. Plasma corticosterone levels in Low Feather Pecking line were significantly higher during resting conditions and restraint when compared with the High Feather Pecking line. In response to manual restraint the High Feather Pecking line showed significantly larger plasma noradrenalin response. No difference in level of adrenaline was found (Korte et al., 1997). Feather pecking may also increase as a response to a high level of corticosterone (El-lethey et al., 2001).

2.3 Exploratory behaviour

"Behaviour is the action that alters the relationship between an organism and its environment. It may occur as a result of an external stimulus, an internal stimulus or, more often, as a mixture of both types of the stimuli" (Kimball, 2005). In this study, feather pecking is assumed to be a redirected exploratory behaviour. According to Crusio (2001) "exploratory behaviour is evoked by novel stimuli and consists of behavioural acts and postures that permit the collection of information about new objects and unfamiliar parts of the environment". Mice spend a lot of time exploring the environment enriched with some novel stimuli (Crusio, 2001). Growing pigs also explore their environment orally. This, in turn, could lead to tail biting. However, the motivation could be foraging as well as exploratory (Day et al., 1995). Newberry (1999) proved that young broilers spend much more time exploring areas enriched with some essential or supplementary resources. Inter-strain differences in exploratory behaviour in rats during open field test were observed by Berrettini et al. (1994). Albentosa et al. (2003) claim that with age birds become less fearful and reveal stronger exploratory behaviour.

2.4 Analyses of candidate genes

As Fitzpatrick and Sokolowski (2004) noted that when a gene of interest and its function are conserved across a number of species including model and non-model organisms, such

a candidate gene is valuable and should be investigated in additional organisms. Consequently, molecular analyses of candidate genes, including polymorphism search and gene expression analyses, were carried out in the current project.

2.4.1 Polymorphism analysis

Single nucleotide polymorphisms (SNPs) are the most common type of variation in the human genome (Wang et al., 1998). SNPs are more stable and more frequent, when compared with microsatellites and as such are more suitable for association studies (Brookes et al., 1998; Schaid, 2004). The sequence variation might be directly related to the phenotypic trait (Ben-Dor et al., 2004). SNPs are found to be very useful as pharmacogenetic indicators and markers for complex diseases gene mapping (Mein et al., 2000).

SNPs based on sequences of broiler, layer and Chinese slikie in the number of 2.8 million were described by International Chicken Map Consortium (Wong et al., 2004). Out of these variant sites 90% were indicated as true SNPs and 70% as common ones that are segregated in many domestic breeds. The mean nucleotide diversity was estimated as about five SNPs per kilobase. “Chicken Variation Database” (ChickVD) contains a total of 3.1 million DNA sequences variants (Wang et al., 2005).

2.4.2 Gene expression analysis

Five methods are in common use for the quantification of transcription: northern blotting, *in situ* hybridisation, RNase protection assays, reverse transcription polymerase chain reaction (RT-PCR) and microarray (Bustin, 2000). Moody (2001) presented an overview of methods for investigating gene expression and focused on those techniques, which enable to analyse more than one single gene, i.e. subtractive hybridisation, differential display and serial analysis of gene expression.

Enard et al. (2002), who reported results from cDNA microarrays made from human sequences, suggested that there are large differences between humans and chimpanzees in patterns of gene expression in the brain, although their genomes are around 99% identical. Furthermore, Robinson and Ben-Shahar (2002) suggested that changes in gene regulation might be involved in the evolution of social behaviour across the animal kingdom.

2.5 *Microarray technique*

The vast amount of literature about microarrays was best commented by Mills et al. (2001) in the first words of their paper: "Gasp! Why another review about DNA micoarrays?" Many possibilities given by microarray experiments and different ways of handling them make this subject still very popular.

2.5.1 *Principles of a microarray experiment*

The microarray method was first mentioned by Schena et al. (1995) who used microarray technique to measure the expression of 45 Arabidopsis genes. The principles of a microarray experiment, based on Brown and Bolstein (1999), are presented in Figure 2.1.

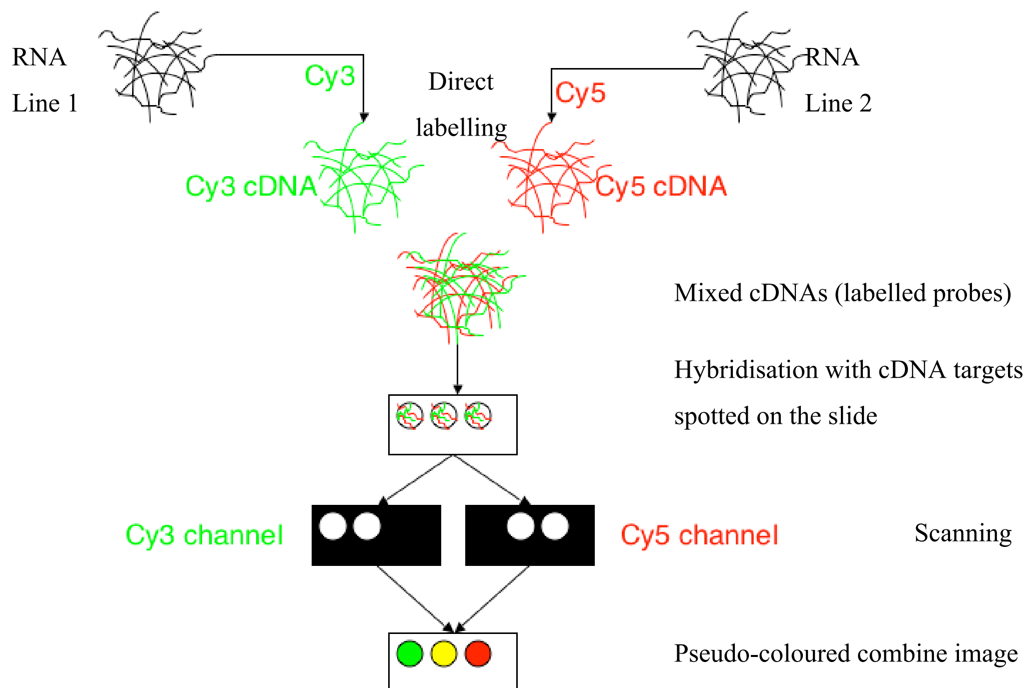

Figure 2.1: Principles of gene expression analysis using a microarray: RNA isolated from the tissue of two lines (Line 1 and Line 2) is labelled directly with Cy3 and Cy5 during the reverse transcription. Differently labelled cDNAs are subsequently combined and hybridised over night with a corresponding sequence on the microarray slide. After hybridisation the slide is washed and scanned twice to obtain two images corresponding to two used dyes. Images are analysed by available software.

2.5.2 Advantages of the microarray technique

Microarray technology enables to identify the expression levels of thousands of genes simultaneously, which means that the genetic analysis can be made on a huge scale. This relatively new technique can be used for (Gershon, 2002):

- Detection of chromosomal deletions (using comparative genome hybridisation)
- Genotyping for DNA variants like SNPs
- Detection of the probable functions of newly discovered genes by comparison with the expression patterns of known genes
- Determining new categories of genes and finding the key players in signalling pathways
- Profiling disease like autism or Alzheimer's as well as AIDS

A global view of gene expression and further biological processes using microarray should enrich the traditional analysis of the genome (Lander, 1999). The need for expression profiling using microarrays was also pointed by Cheung and Spielman (2002). MacBeath (2002) accented the possibility of creating protein-detecting microarrays.

2.5.3 Designing and processing the microarray experiment

Three wide-used designs of the microarray experiment were described by Churchil (2002):

- Dye swap in which two arrays are used to compare two samples labelled with two dyes. Each sample is labelled with each dye once
- In a loop design each sample is spotted twice on two slides, labelled with a different dye and compared with two other samples
- Reference sample design, where all samples are compared with the reference sample

A relatively new approach was reported by (Woo et al., 2005) who proposed a three-colour microarray experiment using a loop design as being more efficient and powerful.

A microarray experiment is a very complex process and can be divided into three main parts: sample preparation ("the front end"), array generation and sample analysis ("middleware") and data handling and interpretation ("the back end") (Bowtell, 1999).

According to Churchill (2002) the following sources of variation can be distinguished in a microarray experiment:

- **Biological**: depending on the genetic and environmental factors and also weather the sample is pooled or treated as individual
- **Technical**: inducted during RNA extraction, labelling reaction and hybridisation of the sample
- **Measurement error**: associated with reading the fluorescence signals

The major source of variability during microarray experiments is a gene specific dye bias (Cox et al., 2004, Martin-Magniette et al., 2005). Keer and Churchill (2001) and Dombkowski et al. (2004) also noticed this trend. Almost 20% of the final conclusions might be subjective using an approach of single dye orientation (Dombkowski et al., 2004). The gene specific dye bias can be constant within arrays but can differ across them (Martin-Magniette et al., 2005). Cy5 dye, used for labelling, is very sensitive to the light and ozone and gives weaker signals than Cy3 dye, which might otherwise be interpreted as a lower level of gene expression. cDNAs incorporate dyes with different efficiency and hybridise at different rates (Kerr and Churchill, 2001). Moreover, Cox et al. (2004) suggest that Cy3 and Cy5 dyes show different degree of quenching. Although in each microarray experiment two animals can be compared on a single two-colour slide, a so-called dye swap should also be applied, in which the same samples are compared again, but labelled in the opposite way.
The accuracy of the microarray experiment results depends on many other systematic variations. Those mostly quoted ones are array surface chemistry, RNA isolation, slide printing, labelling method, hybridisation propriety, scanner introduced bias, image analysis (Ding and Wilkins, 2004). Basically each step of the microarray experiment can be a source of different irregularities. Raw data normalisation, which can be defined as "rescaling and correction of the data sets prior to comparison" (Ding and Wilkins, 2004), is necessary. Normalisation is also defined as "adjustment of microarray data for effects which arise from variations in the technology rather than from biological differences between the RNA samples or between printed probes" (Smyth and Speed, 2003).

2.5.4 *Statistical analysis of the microarray experiment*

The most commonly discussed problems of microarray experiments are data normalisation, transformation and methods of statistical analysis (Dudoit et al., 2000; Quackenbush, 2001; Quackenbush, 2002; Tarca et al., 2005; Tsai et al., 2003; Yang et al., 2005). The image analysis can be carried out using different available software for example Spotfinder (www.tigr.org), ImaGene (www.biodiscovery.com) or, used in the current project, *Spot* software (http://experimental.act.cmsi.csiro.au/Spot/index.php, Beare and Buckley, 2004). Normalised data must undergo statistical analysis. One of the interactive environments used in the experiment was R/MAANOVA package. MAANOVA stands for MicroArray ANalysis of Variance and R is a freely available language and environment for statistical computing and graphics. That tool makes it possible to check the quality of the microarray data and to visualise them, to carry out the normalisation and Analysis of Variance (ANOVA) for both fixed and mixed effects models and to make cluster analysis (www.r-project.org, Wu and Churchill, 2005; Wu et al., 2005). The R/MAANOVA approach has already been used by other researches, including Dong et al. (2005), Mikheeva et al. (2004) and Woo et al. (2005).

A number of researches (Ball et al., 2004; Ball et al., 2002; Spellman et al., 2002; Stoeckert et al., 2002) postulated that a standard system for formatting and storing the data needed to be established. Brazma et al. (2001) defined the minimum information on a microarray experiment (MIAME) that "describes the minimum information required to ensure that microarray data can be easily interpreted and that results derived from its analysis can be independently verified". Such a document should include not only the extracted data but also information about different steps of a microarray experiment: design of the experiment and the array itself; conditions during RNA extraction and hybridisation; labelling method; measurements like images; quantification and specification; normalisation. Bassett et al. (1999) suggested five categories of essential information. They include: contact information, hybridisation targets for each spot, details of used cells or tissues, mRNA transcripts quantitation and statistical significance. Spellman et al. (2002) proposed microarray gene expression markup language (MAGE-ML), which would help to exchange information between users.

2.6 Realtime quantitative RT-PCR

"RT-PCR is an *in vitro* method for enzymatically amplifying defined sequences of RNA" (Bustin, 2000; Rappolee et al., 1988) and "for reliable detection and measurement of products generated during each cycle of polymerase chain reaction" (Ginzinger, 2002). This method was reported for the first time by Higuchi et al. (1993). The sensitivity of this procedure is such that it permits the detection of a specific mRNA in a single cell (Dale and Schantz, 2003). It is the most sensitive of the quantification methods (Wang and Brown, 1999) and can be used to compare the levels of mRNAs in different sample populations, to characterise patterns of mRNA expression, to discriminate between closely related mRNAs, and to analyse RNA structure (Bustin, 2000). Since an end-point quantification is not so reliable, realtime quantitative RT-PCR enables to measure PCR products at a point in which the reaction is in the exponential phase (Ginzinger, 2002). The two commonly used reverse transcriptases are Avian myeloblastosis virus reverse transcriptase (AMV-RT) and Moloney murine leukaemia virus reverse transcriptase (MMLV-RT). Bustin (2000) proposed that MMLV-RT may be a better choice if the aim of the experiment is the amplification of full-length cDNA molecules because native MMLV-RT and engineered derivatives (Kotewicz et al., 1988) reveal significantly less RNaseH activity than AMV-RT (Gerard et al., 1997), where even reduced RNaseH activity can interfere with the synthesis of long amplicons (DeStefano et al., 1991).

2.6.1 Detection methods and chemistries

There are different methods and chemistries that may be used to obtain a fluorescent signal from the synthesis of product in quantitative RT-PCR. According to Giulietti et al. (2001) the following methods can be distinguished:

- TaqMan Probes in which three oligonucleotides are used: a forward primer, a reverse primer and dual-labelled probe with reporter and quencher dyes. This approach is to use fluorescent resonance energy transfer (FRET) – in free form fluorescence emission of the reporter dye is absorbed by the quenching dye. Once the reporter and quencher are separated (for example by enzymatic activity of Taq polymerase) an increase in reporter fluorescence emission can be observed
- Molecular Beacons using a stem-and-loop structure of the probe that changes after hybridisation to the target and the emission of the fluorescence starts

- Scorpions involving two oligonucleotides: a primer and a fluorescent molecule that combines the primer and probe function (fluorophore and quencher are separated after annealing)
- Hybridisation Probes, where four oligonucleotides are involved: two primers and two probes, one being with donor fluorophore and the other with acceptor fluorophore, and energy being transferred by acceptor dye to allow the other one to dissipate fluorescence at a different wavelength (the amount of fluorescence is directly proportional to the amount of the generated target DNA)
- SYBR® Green being a DNA-binding dye that incorporates into double stranded DNA (dsDNA) and starts emitting fluorescence (this method can be used with any pair of primers for any target)

2.6.2 Quantification of realtime RT-PCR

Realtime results can be analysed using both absolute and relative quantification. Absolute quantification relates the PCR signal to the number of input copy using a calibration curve, while relative quantification measures the relative change in mRNA expression level (Pfaffl, 2004b). According to Giulietti et al. (2001) quantification can be based on a standard curve or threshold cycle comparison. Ginzinger (2002) assumed that absolute quantification is a misnomer. According to the author, two methods can be distinguished: relative quantification made in respect of a reference gene and standard-curve quantification. A standard curve can be constructed for example on plasmid dsDNA, *in vitro*-transcribed RNA, *in vitro*-synthesised ssDNA and cDNA sample expressing the target gene (Giulietti et al., 2001). Normalisation to a housekeeping gene is one of the most popular methods for correcting of the realtime results due to differences in the input of RNA amount as well as the efficiency of reverse transcriptase (Bustin, 2000; Giulietti et al., 2001; Pfaffl et al., 2004a). Only a few of the housekeeping genes were critically evaluated, and among them, *GAPDH*, ß-glucuronidase and 18S ribosomal RNA were successfully used (Ginzinger, 2002; Pfaffl, 2001).

3 Materials and Methods

3.1 Behavioural experiments

Behavioural experiments were carried out in May and July 2004 to compare the exploratory behaviour of freshly hatched chicks from two commercial chicken lines representing opposite phenotypes with respect to the incidence of feather pecking: High Feather Pecking (HFP) line versus Low Feather Pecking (LFP) line. Although six experiments were carried out, only experiments #1 and #2 constitute the subject of the formal statistical analysis.

3.1.1 Animals

In the experiments, the Lohmann Selected Leghorn represented the HFP line, and the Lohmann Brown represented the LFP. According to the information provided by Lohmann Tierzucht GmbH (Preisinger R., personal communication) the HFP is a female-line from their regular breeding programme, white-feathered and laying white-shelled eggs. The line has been selected for over 50 years as a closed population with an annual generation interval. Every year between 80 and 100 cockerels are selected and mated to 800 or 1000 females to reproduce the next generation. The LFP is the D-line of the Lohmann Brown. It is a white-feathered White Rock breed, laying brown-shelled eggs. The gene pool has been closed for 40 years and selected in a way similar to the LSL line. The White Rock bird is about 200 to 300 g heavier comparing with the White Leghorn. All eggs, 1800 from each line, were supplied by Lohmann Tierzucht GmbH. Such a large number of eggs was necessary to obtain in each experiment chicks, which would be at the same stage of the hatching process.

3.1.2 Hatching conditions

The common 21-day hatching routine was applied (Table 3.1) in a Petersime 126 incubator that was disinfected prior to the experiment. Eggs were turned 90° every 2 hours. All the experimental conditions were checked daily.

Table 3.1: Description of the environmental hatching conditions.

Day	ºC (°F) [1]	ºC (°F) [2]	Air-ventilation (Levels: 0-5)[3]
1	37.8 (100)	29.2 (84.5)	0.5
2-18	37.5 (99.5)	29.4 (85)	1.5
18-21	36.9 (98.5)	32.2 (90)	2.5
21	36.6 (98)	33.3 (92)	3-4

[1] dry bulb temperature

[2] wet bulb temperature

[3] maximal capacity (at the level 5) is 400 m^3 / h

3.1.3 Environmental conditions during the behavioural experiments

The room in which the behavioural experiments were carried out was isolated from the incubator, supplied with artificial light and the temperature was maintained at the level appropriate for small chicks after hatching (around 36ºC). Cameras recording the movements of the animals were fixed to the ceiling. Regular 76.5 x 53.5 x 11.5 cm hatching boxes were used for observations in each of the six experiments, hence termed "observation boxes".

3.1.4 Experiment #1 and #2

The designs for experiment #1 and #2 were identical. Ten freshly hatched chicks from each line (HFP and LFP) were placed in a single observation box at the same time. Chicks from one line were marked with blue colour to differentiate them from the chicks from the other line. The recordings were carried out using 2 cameras per 4 observation boxes simultaneously. During each experiment 12 observation boxes were used (4 observation boxes in 3 repetitions). Experiments #1 and #2 were carried out in May and July 2004, respectively.

3.1.5 *Experiments #3-#6*

Experiment #3 was identical to experiments #1 and #2 except for the fact that each box was subdivided into two sections by means of a paper wall with a 15 x 15 cm wide passage in the middle. All chicks were placed in one subdivision of the box only. The set up of experiment #4 was identical to that in experiments #1 and #2 except for the addition of some environmental enrichments such as silver paper or pieces of coloured plastic straws. Those two experiments were carried out in May 2004. Experiment #5 was similar to experiments #1 and #2 except for the fact that only three chicks per line were placed in each box and each chick was marked with a different, randomly selected colour. In experiment #6, five chicks from each line were used. The chicks were line-specifically labelled: two different colours were used. Experiments #5 and #6 were carried out in July 2004. The behaviour of the animals in all six experiments was recorded on a videotape (25 minutes). Additional details for each experiment are presented in Table 3.2.

Table 3.2: Technical details of the behavioural experiments.

Experiment	No of boxes	Month
#1	12	May
#2	12	July
#3	4	May
#4	4	May
#5	7	July
#6	2	July

3.1.6 *Scoring exploratory episodes*

The activity of freshly hatched chicks was measured by manual scoring. Independent observers watched the recording twice, each time focusing on one line, looking for out-of-the-group movements i.e. exploratory episodes, such as the chick walking out of the group of other chicks for a noticeable distance. The out-of-the-group movement and the return to the group were scored with one point each. The recordings of May experiment were shown to three observers who counted the exploratory episodes for the whole length of 25 minutes. In July experiment the whole 25-minute videotape was shown to one observer

(experiment #2a), while two observers counted exploratory episodes that occurred in the first 10 minutes (experiment #2b). In total 240 animals per line were observed.

3.1.7 Data transformation

Before the statistical analysis was carried out, the quality of the data was checked. The exploratory episodes that were noticed in the experiments were not normally distributed. In order to assure unbiased P-values in the ANOVA model, the data were transformed using either the normal logarithm or the square root transformation of the original data, depending on the shape of the diagnostic plots of the corresponding ANOVA model (Table 3.3).

Table 3.3: Type of transformation used for the data obtained in behavioural experiments #1, #2a and #2b.

Experiment	No. of observers	Duration of the observation period	Type of transformation applied
#1	3	25 minutes	ln
#2a	1	25 minutes	square root
#2b	2	10 minutes	square root

3.1.8 Statistical analysis

After transformation, all the data were analysed using the ANOVA model with the following effects: line, repetition-box and observer. All the analyses were carried out using the open source software R (www.r-project.org). The following model was applied:

$$y_{ijkl} = \mu + line_i + observer_j + repbox_k + e_{ijkl}$$

y_{ijkl}	Observed values (exploratory episode measured in defined time)
μ	Mean
$line_i$	Fixed line effect (i = 1, 2) 1 = HFP line 2=LFP line

$observer_j$	Fixed observer effect (j = 1, 2, 3 in May; j = 1, 2 in July) [1]
$repbox_k$	Fixed repetition x box effect (k = 1, 2…12); 3 repetitions and 4 boxes per repetition
e_{ijk}	Residual error

[1] In the statistical analysis of the data from experiment #2a (one observer, 25 minutes of observation) the same model was applied but the fixed observer effect was excluded

3.2 Identification of candidate genes through literature search

Searching for candidate genes involved in feather pecking was a multi-step process that included:

- Entering the keywords into the Internet databases such as PubMed (www.ncbi.nlm.nih.gov) and GeneCards Homepage (www.genecards.org) to find papers and web sites correlated with the investigated trait
- Carrying out a Google search (www.google.com)
- Screening the obtained papers and web sites for new keywords and names of putative candidate genes that might be responsible for feather pecking or any other related aggressive behaviour in chickens or other species
- Repeating the search process including new keywords found in the previous search

Since there were no specific clues to the causes of feather pecking, the first two keywords entered into the databases were “feather pecking” and “exploratory behaviour” because the working hypothesis of the project was that feather pecking was a redirected exploratory behaviour. The first papers and the web sites that were found did not provide the expected information about potential candidate genes. However, they enriched the number of keywords that could be entered into databases during further searches. Those words included for example “cannibalism”, considering the fact that severe feather pecking could lead to the consumption of tissues between conspecifics, or “foraging behaviour” following the hypothesis that feather packing might be a redirected foraging behaviour (Huber-Eicher and Wechsler, 1998). Each of the subsequent searches provided terms that could be used as new keywords and that narrowed the searching area. Other keywords such as the names of human psychiatric diseases (for example obsessive-compulsive disorders)

and names of neurotransmitters involved in behaviour and stress response (for instance serotonin and epinephrine) were also taken into consideration during the literature search. The keywords list was thus updated regularly and its final version numbered forty-three (Appendix 9.4).

3.3 Acquiring and processing sequences of candidate genes

Two databases were used to obtain chicken sequences of the analysed candidate genes: NCBI (http://www.ncbi.nlm.nih.gov) and Ensembl (www.ensembl.org). In order to acquire sequences and subsequently process the candidate genes three main steps must be made:

- Obtaining the mRNA sequences
- Defining the exon/intron borders
- Obtaining the intronic sequences

The chicken mRNA sequences were obtained by entering human symbols or full names of candidate genes in the NCBI (http://www.ncbi.nlm.nih.gov/entrez/DB=nucleotide) Nucleotide database. For almost all candidate genes the chicken mRNA reference or the predicted sequence was readily available. In the case of *ACADS* gene two ESTs, namely BU107469.1 and BU373053.1 were used. ESTs were found by entering the human *ACADS* sequence into the NCBI ESTs database (http://www.ncbi.nlm.nih.gov/entrez/DB=nucest). After that, exon/intron borders were defined by comparing the chicken sequence with the human sequence (using NCBI BLAST tool: www.ncbi.nlm.nih.gov/BLAST) and the human exon/intron structure.

Designing the primer pair in the neighbouring introns seemed to be the most efficient way to amplify the coding regions, which were the main target of the polymorphism analysis. Although the mRNA sequence of the desired gene was easily available from the databases, the intronic sequence had to be obtained by standard sequencing or direct sequencing of BAC DNA. The planned BAC direct sequencing was ended after the first run carried out for *HOXB8*, when the first draft of the chicken genome sequence had been available from public databases. This simplified the procedure of acquiring information on the intronic sequence. The mRNA sequences of candidate genes were simply BLASTed against the chicken genomic sequence in Ensembl database using BLAST tool (www.ensembl.org/Gallus_gallus/blastview). Subsequently, the contig (part of the genomic sequence) with the highest percentage of the identity was exported as a fasta file

(format for unaligned sequence). The sequence was divided according to the exon/intron borders.

Ensembl database develops very fast, and currently sequences for all candidate genes, including the exon/intron structure, are available. However, the gene structure of the investigated candidate genes was changing over the duration of this project. *TPH1* has 10 exons in the human sequence and the same exon structure was expected in the chicken. According to the information available in Ensemble database, chicken *TPH1* has 11 exons and the first, not translated exon is 96 bases long. Before December 2005, the same exon was 54 bases long. Another example of such changes in the gene structure is *TPH2*. Human *TPH2* has 11 exons. The same structure in chicken *TPH2* had been presented in Ensemble until December 2005. Currently, between the former exons 3 and 4 there is the additional, 10 bases long exon. None of the changes in the gene structure that were introduced after the candidate gene had been analysed, was taken into consideration.

The same procedures to acquire and analyse the sequences were applied for candidate genes identified in the microarray experiment.

A table that shows the positional and structural information, including the accession numbers of the reference sequences available in NCBI and Ensemble databases for all candidate genes, is presented in Appendix 9.6.

3.4 Isolation and evaluation of genomic DNA

3.4.1 Isolation of genomic DNA from blood

In the course of experiment #1 blood samples were collected from twenty animals per each line (HFP and LFP line), and were subsequently mixed with anticoagulant EDTA. The initial volume of 200 µl of chicken blood was diluted with 20-30 ml of distilled water, 3 ml of 1.8% NaCl and centrifuged for 30 minutes at 8ºC (speed 3000 Upm). The precipitate was mixed with 30 ml of NP40 and centrifuged for 30 minutes under the same conditions. The pellet was resuspended with 2.5 ml PK 1x buffer, 200 µl 10% SDS, 50 µl Proteinase K and incubated overnight at 55ºC with moderate shaking. Thereafter, the lysate was centrifuged with 2 ml of NaCl (30 minutes, 3000 Upm, 8ºC). Further on, the supernatant was mixed with 10 ml of isopropanol by inversion in 15 ml Falcone tubes. The precipitated DNA was transferred into new 1.5 ml tubes and washed three times with 70% alcohol by centrifuging 10 minutes in 10 000 rpm. DNA was diluted in 200 µl of TE buffer (at 55ºC).

3.4.2 Evaluation of genomic DNA

The result of DNA isolation was tested by carrying out the 1% agarose gel electrophoresis. For this purpose, first 1% gel of 40 ml was prepared by mixing 0.4 g of agarose with 40 ml of 0.5x TBE buffer. The mixture was heated in a microwave oven to melt agarose and then cooled to 65ºC. Following this, 0.4 µl ethidium bromide were added. Before loading on the gel, 2 µl of DNA samples were mixed with 5 µl of the loading buffer (6x Orange Loading Solution, MBI Fermentas, St.Leon-Rot, Germany). The electrophoresis was carried out for 30 minutes in 100 V in 0.5x TBE buffer, the samples were separated together with Lambda DNA/HindIII marker (MBI Fermentas, St.Leon-Rot, Germany). Samples that appeared as one band in a size larger than 23 130bp were considered an expected, high molecular weight DNA which confirmed a successful DNA isolation.

More precise estimations of the DNA concentration were carried out using spectrophotometer (Ultraspec III, Pharmacia, USA) by measuring the absorbance at 260 nm (A_{260}). The absorbance of 1 unit at 260 nm corresponds to 50 µg of DNA per ml. Following the Qiagen protocol (Qiagen, Hilden, Germany) DNA was diluted in TE buffer in 1:50 dilution ratio and the concentration of the sample was calculated according to the following formula:

Concentration (µg/ml) = 50 µg x A_{260} x dilution factor

Total yield (µg) = concentration x volume of sample (ml)

All the samples of DNA extracted from the experimental lines were brought up to an approximate concentration of 25 ng/µl by diluting in TE buffer.

Besides the DNA samples from two experimental lines, DNAs supplied by AVIANDIV project (AVIANDIV, EC contract No BIO4-CT98-0342, Weigend, S. (co-ordinator), Tixier-Boichard, Groenen, M. A. M., M., Vignal, A., Hillel, J., Wimmers, K., Burke, T., Mäki-Tanila, A. http://w3.tzv.fal.de/aviandiv/index.html) were used. This panel included ten different breeds and lines. Since the volume of DNA samples obtained from AVIANDIV project was approximately 20 µl, the concentration measures were carried out using fluorometer (DyNA Quant 200; Hoefer Pharmacia Biotech Inc., San Francisco, CA, USA). This method of DNA quantification requires only 2 µl of DNA mixed with 500 µl of a low range (for measures of 10-500 ng/µl) buffer. Only samples with the concentration of min 15 ng/µl were used.

3.5 *Panel used for polymorphism analysis*

Panel (Table 3.4) of twelve breeds and lines was created using DNA extracted from two experimental lines (LFP and HFP) and DNA of ten lines and breeds supplied by AVIANDIV project.

Table 3.4: Chicken breeds panel used for SNP searching.

Breed name	Short	Origin
Red Jungle Fowl *	RJF	Thailand/Wild
Malay*	MAL	Asia
Cochin *	COC	Asia
Fayoumi *	FAY	Egypt
Marans *	MAR	France
Tr. Naked Neck *	TNN	Hungary
Iceland landrace *	ICL	Iceland
Line Sarcoma Susc *	LSS	USA/Germany
Green legged Partridge *	GLP	Poland
New Hampshire *	NH	USA
HFP line	LSL	Lohmann Tierzucht Germany
LFP line	LB	Lohmann Tierzucht Germany

*AVIANDIV, EC contract No BIO4-CT98-0342, Weigend, S. (co-ordinator), Tixier-Boichard, Groenen, M. A. M., M., Vignal, A., Hillel, J., Wimmers, K., Burke, T., Mäki-Tanila, A. http://w3.tzv.fal.de/aviandiv/index.html

3.6 *Polymerase chain reaction (PCR)*

3.6.1 *Standard PCR*

A standard PCR reaction of 20 µl volume containing 50 ng genomic DNA, 1x Qiagen PCR buffer, 1.5 mM $MgCl_2$, 200µM of each nucleotide, 5 pmol of each forward and reverse primer (MWG Biotech, Ebersberg, Germany), 0.5 units of Qiagen Taq Polymerase (Qiagen, Hilden, Germany) was performed using T-Gradient Thermocycler (Biometra, Göttingen, Germany) under the following conditions: initial denaturing at 95°C for 3 minutes; followed by 30 cycles at 94°C for 30 seconds, 60°C for 1 minute, 72°C for 1 minute and the final extension at 72°C for 3 minutes. PCR products were separated

together with GeneRuler™100bp DNA Ladder® (MBI Fermentas, St.Leon-Rot, Germany) on 1.5% agarose gel.

3.6.2 PCR products purification

The purification of PCR products was carried out using filtration plates (MultiScreen™PCR, Millipore, Eschborn, Germany). The whole PCR product was diluted on the filtration plate in 50 µl of distilled water and filtered by Millipore vacuum manifold (Eschborn, Germany). Each sample was resuspended in 40 µl of 10 mM Tris/HCl.

3.6.3 Primer design for standard PCR

Primers were designed using the Primer3 software available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi (Rozen and Skaletsky, 2000). Standard settings were applied. The optimal melting temperature was estimated at 60ºC and the optimal primer size was 20 bases. All used primers were synthesised by MWG Biotech (Ebersberg, Germany). The sequences of primers for standard PCR are presented in Appendix 9.1.

3.6.4 PCR optimisation

All new primer systems were tested in three reactions:

- Standard PCR, plain
- Addition of 1 µl of 5% dimethyl sulfoxide
- Addition of 4 µl of Qiagen Q-solution (Qiagen, Hilden, Germany)

The thermal conditions were identical comparing to standard PCR (chapter 3.6.1).
Gradient PCR was applied when there was no amplification or in case of unspecific products in all three test reactions. Annealing temperature varied between 54ºC and 66ºC.

3.7 BAC cloning procedure

The CHORI-261 Chicken BAC Library supplied by the Children's Hospital Oakland Research Institute (BACPAC Resources, Oakland, CA, USA) was used. The library contains four 22x22 cm nylon filters. Detailed information about the BAC library is available at: http://bacpac.chori.org/chicken261.htm. Screening the BAC library was

performed for two genes: *HOXB8* (PCR product used for probe preparation: ~1100bp; primers: 3172up/3173dn) and *TPH1* (PCR product used for probe preparation: ~1200bp; primers: 3199up/3200dn).

3.7.1 Dot-Blot as positive control

The obtained PCR products were diluted in 1:10, 1:100 proportion and spotted (2 µl) together with an undiluted sample onto nylon membranes (Hybond-N+, Amersham Biosciences, Freiburg, Germany). After saturation with 0.4 N NaOH for 5 minutes the membrane was shaken moderately for 1 minute in 5x SSC buffer and placed in a hybridisation tube (roller bottle).

3.7.2 Labelling

The PCR products were radiolabelled with deoxyadenosine 5'-(P^{32}) triphosphate (Amersham Biosciences, Freiburg, Germany). The probes for the two analysed genes were mixed together. 40 ng of PCR product (5 µl, maximum 21µl) was denaturised for 5 minutes at 99ºC together with 16µl of water and 5 µl of primer mix (random nanomer primers) - final volume was 26 µl. Next 5 µl 10x reaction buffer, 4 µl each of dGTP, dCTP and dTTP, 2 µl Klenow fragment (1 unit/µl) and 5 µl dATP32 were added to the reaction on ice. The reaction was incubated for 15 minutes at 37°C and then stopped by adding 10 µl of 0.2 M EDTA (pH 8.0). The radiolabeled probes were denatured at 95°C for 7 minutes.

3.7.3 Hybridisation

The BAC library filters were pre-hybridised for 30 minutes at 67°C in roller bottles together with dot-blot controls: one filter per bottle with 20 ml of Church buffer (Church and Gilbert, 1984) composed of: 5% SDS, 1 mM EDTA, 0.341 M Na_2HPO_4 and 0.159 M NaH_2PO_4. Following this, 14 µl of radiolabeled probes were added to each bottle and hybridised at 67°C overnight (14-16 hours).

The filters were washed three times for 20 minutes at 63ºC: twice in 2x SSC and once in 0.5x SSC + 0.1% SDS. Finally, each filter was rinsed in 2x SSC, wrapped in a household plastic film and placed together with a medical X-ray film NewRX (FUJIFILM Medical Systems, Stamford, CT, USA) in cassettes. In the next step, the X-ray films were exposed

for a minimum of five hours at –80°C. Positive signals from the filters were assigned to the clone names following the library documentation. The numbers of ordered clones are presented in Appendix 9.3.

The colony PCR was applied to check whether the ordered clones contained the right insert. The randomly chosen single bacteria colonies were boiled for one minute at 99ºC with 50 µl of water, thereafter, the standard PCR procedure was applied with the primer pair that was previously used to generate the probe for BAC screening.

3.7.4 BAC DNA isolation

Clones that were tested positively after colony PCR were streaked out on luria broth (LB) agar plates containing 12.5 µg/ml of chloramphenicol (Roche Diagnostics, Mannheim, Germany) and incubated overnight at 37°C. Single colonies of each clone were used for starter culture incubation for 8 hours at 37°C (vigorous shaking with 300 rpm) in 3 ml LB medium (with additional 3 µl of chloramphenicol). Then, 500 µl of the pre-culture was inoculated with 100 ml LB medium (including 100µl chloramphenicol) and incubated for 14 hours at 37°C (shaking with 300 rpm). The cell pellet was used for BAC DNA preparation by Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany). Pellet was resuspended with 12 ml of P1 buffer and mixed with 12 ml of P2 buffer. After 5 minutes of incubation, 12 ml of P3 buffer were added, the mixture was incubated on ice for 15 minutes and centrifuged for 30 minutes at 13 000 rpm (4ºC). Lysate was filtered and mixed with 25 ml of isopropanol. Pellet was resuspended in 500 µl of 10 mM Tris-Cl and adjusted to 5 ml with QBT buffer. Probes were centrifuged at 12 000 rpm for 10 minutes, the supernatant was transferred onto the column and let to flow through. Thereafter, the columns were washed twice with 10 ml of QC buffer. DNA was eluted with 1 ml of warm (70ºC) QF buffer. Then, 3.5 ml of isopropanol were added and the mixture was centrifuged for 60 minutes at 5000 rpm. DNA pellet was diluted in 500 µl of 10 mM Tris-Cl and incubated over night. Afterwards, samples were transferred onto the microfilter (Microcon, Millipore, Eschborn, Germany) and centrifuged for 15 minutes. The microfilters were washed with 500 µl of 10 mM Tris-Cl. During the final elution step, the microfilters were turned upside-down and washed with 250µl of 10mM Tris-Cl.

3.7.5 Direct sequencing of BAC DNA:

For direct sequencing 0.5 µl (MWG Biotech, Ebersberg, Germany) of the PCR primer were used together with 5.5 µl of BAC DNA and 4 µl BigDye of terminator cycle sequencing ready reaction kit v2.0 (Applied Biosystems Division, Foster City, CA, USA). The following temperature conditions were used: initial denaturing for 5 minutes at 96°C, followed by 100 cycles at 96°C for 20 seconds, 57°C for 10 seconds, and 60°C for 4 minutes. Electrophoresis was carried out on the polyacrylamide gel (subchapter 3.8.3).

3.8 Sequencing reaction

3.8.1 Sequencing reaction using the Sanger method (Sanger et al., 1977)

A 10-20 ng of purified PCR product (maximum of 5,5 µl, necessary for long fragments and weak PCR products) were mixed with 4 µl BigDye of terminator cycle sequencing ready reaction kit v2.0 (Applied Biosystems Division, Foster City, CA, USA) and 0.5 µl of the forward or reverse PCR primer (2.5 pmol). Alternatively a maximum of 7.5 µl of PCR product, 2 µl of BigDye and 0.5 µl of primer were applied. The final volume of 10 µl reaction was filled up with water if necessary. The sequencing reaction was performed in T-Gradient Thermocycler (Biometra, Göttingen, Germany) under the following conditions: initial denaturing at 96°C for 15 seconds, followed by 35 cycles including 10 seconds in 96°C, 5 seconds in 50°C, and 4 minutes in 60°C.

3.8.2 Cleanup of sequencing reaction

The gel filtration of the sequencing reaction was applied using the MultiScreen filtration plate (MAHVN4510; Millipore, Eschborn, Germany) filled in with 45 µg (per each column) of Sephadex G-50 Fine (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) and 300 µl of distilled water. The plate was left for incubation at room temperature for 3 hours and then centrifuged for 7 minutes at 980 rpm. The sequencing reaction mixture was transferred onto the Sephadex columns and the plate was centrifuged for 5 minutes in the same conditions. The samples were dried in a vacuum centrifuge (SpeedVac Plus, SC110A; Thermo Savant, Holbrook, NY, USA) and dissolved in 2 µl of loading buffer (formamide coloured with dextranblue, Fluka, Buchs, Switzerland). Immediately before loading the gel, all samples were denaturised at 96ºC for 2 minutes.

3.8.3 Polyacrylamide sequencing gel preparation

The sequencing gels were prepared using 21 g of urea (Roth, Karlsruhe, Germany), 8.4 ml of 30% acrylamide/bisacrylamide (29:1), 20 ml of water (HPLC grade, Roth, Karlsruhe, Germany), 6.0 ml of 10x TBE buffer, 20 µl of TEMED and 300 µl of 10% ammonium per sulphate (APS). An average run lasted 10 hours using ABI 377 (Applied Biosystems, Foster City, CA, USA) sequencer.

3.8.4 Sequencing data analysis

Base calling, sequence alignment and polymorphism detection were made using the *Phred/Phrap/Polyphred* software (Ewing and Green, 1998b; Ewing et al., 1998a; Nickerson et al., 1997). Sequences viewing was done by *Consed* software (Gordon et al., 1998).

3.9 Tissue homogenisation; isolation and evaluation of RNA

3.9.1 Tissue homogenisation

In experiment #2 the brains of freshly hatched chicks were collected. The dissection was carried out using a scalpel and the brains were stored immediately after in the RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany). The following homogenisation methods were applied:

- A stator rotor dispersion tool was used together with Polytron PT 1200 (Kinematica, Luzerne, Switzerland)
- The "syringe" method was applied using a 5 ml syringe (B.Braun, Meisungen, Germany)
- The "syringe-needle" method was carried out using a 5 ml syringe and 0.70 x 30 mm needle (B.Braun, Meisungen, Germany)
- FastPrep system (MP Biomedicals, Eschwege, Germany) based on mortar and pestle approach; this method was chosen as the most efficient one and was used for all homogenisations

The FastPrep system uses tubes with small beads of matrix. Samples are placed in impact-resistant 2.0 ml tubes (supplied by MP Biomedicals) containing an optimised lysing matrix D (preferred for animal tissues homogenisation) and lysis buffer (or Trizol reagent depending on used isolation method). In approximately 40 seconds, the figure-8 vertical,

angular motion of the FastPrep Instrument caused the lysing matrix particles to impact the tissue from all directions simultaneously. After homogenisation the sample was centrifuged at the maximum speed (13 000 x g) and the supernatant was transferred into a new tube for further isolation. The only disadvantage of this homogenisation method was the limited amount of tissue that can be homogenised in one tube (about 200 mg). An average brain of a freshly hatched chick is 1g. Consequently each brain was divided into 5-6 pieces and the lysates were combined thereafter. The lysate was either directly used or stored at –80ºC.

3.9.2 RNA isolation

3.9.2.1 Using RNeasy® Maxi Kit (Qiagen, Hilden, Germany)

The Qiagen Kit RNA isolation column method was chosen to carry out the total RNA extraction from all 20 brains (10 per each line). This technology is based on the selective binding properties of a silica-gel-based membrane, which, as claimed by the supplier, efficiently removes DNA. Maximal 6 mg of RNA molecules longer than 200 bases can be adsorbed to the membrane.

Total RNA isolation RNeasy® Maxi Kit (Qiagen, Hilden, Germany) contains: DEPC water, RLT buffer, RW 1 and washing RPE buffers. All steps were performed at room temperature. After homogenisation, the resulting tissue lysate, adjusted to a volume of 15 ml with RLT buffer, was centrifuged at 5000 x g for 10 min. The supernatant was mixed with 1 volume of 70% ethanol and shaken. The sample was loaded onto an RNeasy® Maxi Kit column, where the total RNA binds to the silica-gel based membrane, and placed into a centrifuge tube. The column was centrifuged at 5000 x g for 5 min. The flow-through was discarded. Following this, the washing steps were carried out, 15 ml of RW1 buffer was added to the column and centrifuged at 5000 x g for 5 min. After discarding the flow-through, 10 ml of RPE buffer were added and the column was centrifuged at 5000 x g for 2 min and in repetition for 10 minutes to dry up the membrane. The flow-through was discarded. To elute RNA, the column was transferred into a new collection tube and 1 ml of RNase-free water was added onto the column. The tube was left for 1 min and then centrifuged at 5000 x g for 3 min. The elution step was repeated using the first elute. The eluted RNA was stored at -80 °C.

3.9.2.2 Using Trizol reagent (Invitrogen, Karlsruhe, Germany)

The second tested method was based on a phenol-chlorophorm isolation using Trizol reagent (Invitrogen, Karlsruhe, Germany). The tissue was homogenised together with Trizol (1 ml per 100 mg of the tissue) and incubated for 10 minutes at room temperature. Next, the lysate was centrifuged for 10 minutes, 12 000 x g at 4ºC. Supernatant was transformed into a new tube, mixed with chloroform (0.2 ml of chloroform per each 1 ml of Trizol) and incubated for 10 minutes at room temperature. After 15-minute centrifugation (12 000 x g, 4ºC), the supernatant was mixed with isopropanol (0.5 ml, per each ml of Trizol) and centrifuged in the same conditions for other 8 minutes. The supernatant was discarded and the pellet was washed in 75% ETOH (at least 1 ml per each 1 ml of Trizol). The washing step was repeated twice. After the last washing step the used tube was dried up at room temperature and RNA was resuspended in 300µl of RNase-free water.

3.9.3 Evaluation of RNA

Samples of total RNA should show two distinct, major bands of high molecular weight derived from the ribosomal RNA (28S and 18S ribosomal RNA in a ratio of 2:1). Therefore the quality of the eluted RNA was analysed using 1.2 % agarose-formaldehyde gel electrophoresis (all buffers used for preparation and running of the formaldehyde-agarose gel were prepared according to Qiagen instructions, which are presented in Appendix 9.5). For this purpose, first 1.2% FA gel of 50 ml was prepared by mixing 0.6 g of agarose with 5 ml 10x FA gel buffer. Further, RNase-free water was added to the mixture to fill up to the total volume of 50 ml. Following this, 1 ml of 37% (12.3 M) formaldehyde and 0.5 µl ethidium bromide were added to the mixture. RNA samples were prepared to load onto the FA gel by adding 2 µl of 5x RNA loading buffer to 6 µl of total RNA. The samples were mixed and incubated at 60ºC for 5 min. After incubation, the samples were chilled on ice and separated on the FA gel together with High Range RNA ladder® marker (MBI Fermentas, St.Leon-Rot, Germany). The run was performed for 1.5 hours, 50 V in 1x FA Gel Running Buffer.

The concentration of RNA was determined by the spectrophotometer (Ultraspec III, Pharmacia, USA) measurement of the absorbance at 260 nm (A_{260}). The absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (in pH=7 conditions). Following the

Qiagen protocol (Qiagen, Hilden, Germany) RNA was diluted in 1:50 dilution ratio and the concentration of the sample was calculated as follows:

Concentration (µg/ml) = 40µg x A_{260} x dilution factor

Total yield (µg) = concentration x volume of sample (ml)

The measurements were also performed at A_{280}. The purity of RNA was rated on the basis that A_{260}/A_{280} ratio should be within 1.9 to 2.1

3.10 RT-PCR

3.10.1 First-strand cDNA synthesis

The reverse transcriptase reaction was performed using First Strand cDNA Synthesis Kit from Fermentas (MBI Fermentas, St.Leon-Rot, Germany), which contains:

- DEPC water
- dNTP mix 10mM
- 5x Reaction Buffer
- Oligo$(dT)_{18}$ primer (0.5 µg/µl)
- Moloney Murine Leukemia Virus reverse transcriptase (M-MuLV RT)
- RiboLock (Ribonuclease Inhibitor)

Approximately 1 µg of total RNA was mixed with 1 µl of Oligo $(dT)_{18}$ primer and filled up with DEPC water to the final volume of 11 µl. Mixture was incubated for 5 minutes at 70ºC. Thereafter, 4 µl of 5x reaction buffer, 1 µl RiboLock and 2 µl of dNTP mix were added. After incubation for 5 minutes at 37ºC 2 µl of M-MuLV RT were added. The reaction was incubated for 60 minutes at 37ºC and stopped by heating for 10 minutes at 70ºC. The amplification of the first strand cDNA was carried out using standard PCR procedure (chapter 3.6.1) but 35 cycles rather than 30 and 10 not 5 pmol of each primer were used. To avoid the amplification of genomic DNA both, the forward or reverse, primer were designed to span the junction of the two exons (Figure 3.1). All sequences of the used primers are presented in Appendix 9.2.

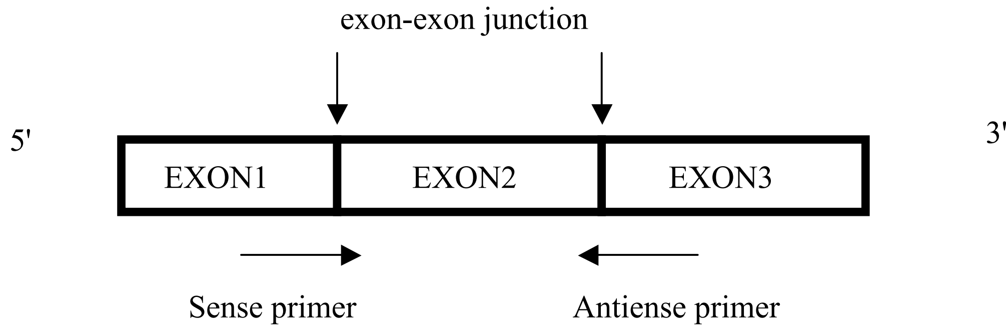

Figure 3.1: Primer design for RT-PCR reaction.

3.10.2 Realtime quantitative RT-PCR

RealTime quantitative PCR amplification reactions were carried out using an ABI Prism® 7000 Cycler-Detection System (Applied Biosystems Division, Foster City, CA, USA) and Platinium® SYBR® Green qPCR SuperMix UDG with ROX (Invitrogen, Karlsruhe, Germany). The 25 µl reaction was composed of: 12.5 µl of SuperMix, 0.4 µl of each reverse and forward primer (4 pmol), 3µl of cDNA template (transcribed from 1µg of total RNA and diluted 1:10) and water to adjust to the final volume. The thermal cycling programme was based on three stages: 50 °C for 2 minutes, 95 °C for 2 minutes, and 45 cycles of 95 °C for 15 sec, 60 °C for 30 sec, and 72 °C for 30 sec. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), a housekeeping gene, was used as a reference gene. Eight animals per each line (LFP and HFP line) were analysed. Each sample was set up in three technical replicates. After each run, the dissociation curve analysis was carried out for the experimental and the housekeeping gene to check for unspecific amplification. Samples should yield only one clear peak at the melting temperature of the amplicon. In case of non-specific products, different melting temperatures and lower concentration of primers were tested and, if the results were still not improved, a new pair of primers had to be designed.

The obtained raw data included all the values of threshold cycles (Ct), average across technical replicates, for experimental and reference gene for each sample. The Ct value of experimental gene was divided by the Ct value of the reference gene. The non-parametric Wilcoxon Rank Sum Test was applied to determine whether the gene expression differed between HFP and LFP lines (Wilcoxon, 1945). This test was chosen because the data were

not normally distributed. P-values were corrected for multiply testing using Bonferroni-Holm sequential procedure (Holm, 1979).

3.11 Microarray experiment

3.11.1 RNA concentration with RNeasy® MiniElute Cleanup kit (Qiagen, Hilden, Germany)

The concentration of RNA used for the microarray experiment was increased to 45 µg. The initial volume was 200 µl. Samples were mixed with 700 µl of RLT buffer and 500 µl of 96% ethanol. The mixture was applied onto the column and centrifuged for 15 seconds at 8000 x g. Thereafter, a silica-gel-based membrane was washed twice: with 500µl of RPE buffer and 500 µl of 80% ethanol by centrifuging at 8000 x g for 15 seconds and 2 minutes, respectively. The column was centrifuged for 5 minutes at the maximum speed (13 000 x g) to dry the membrane. RNA was eluted with 10 µl of RNase-free water by centrifugation for 1 minute at the maximum speed.

3.11.2 Array used for the microarray experiment

A Chicken Neuroendocrine cDNA array spotted by ARK-Genomics, consisting of 4 800 chicken clones, was used. Each clone was spotted at minimum three repetitions (www.ark-genomics.org). Altogether there were 16 416 spots. The detailed information was obtained from the microarray supplier ARK-Genomics (Talbot R., personal communication). The source clones for the array were taken from four separate subtracted libraries. All four libraries were made from hypothalamic tissue, obtained from short day exposed birds or long day exposed birds and from laying hens and broody hens. 1000 clones from each library were printed onto the array. The array was supplemented with genes previously identified as being expressed in chicken brain. A search of the EMBL database yielded 750 genes. The control spots included on the array were: salmon sperm DNA, calf Thymus DNA and 3 Arabidopsis negative controls. A “Landing Lights” of Cy3 dye coupled to streptavidin were printed in the top left hand corner of each block (Figure 3.2) to assist grid alignment. The structure of the array is presented in Figure 3.2.

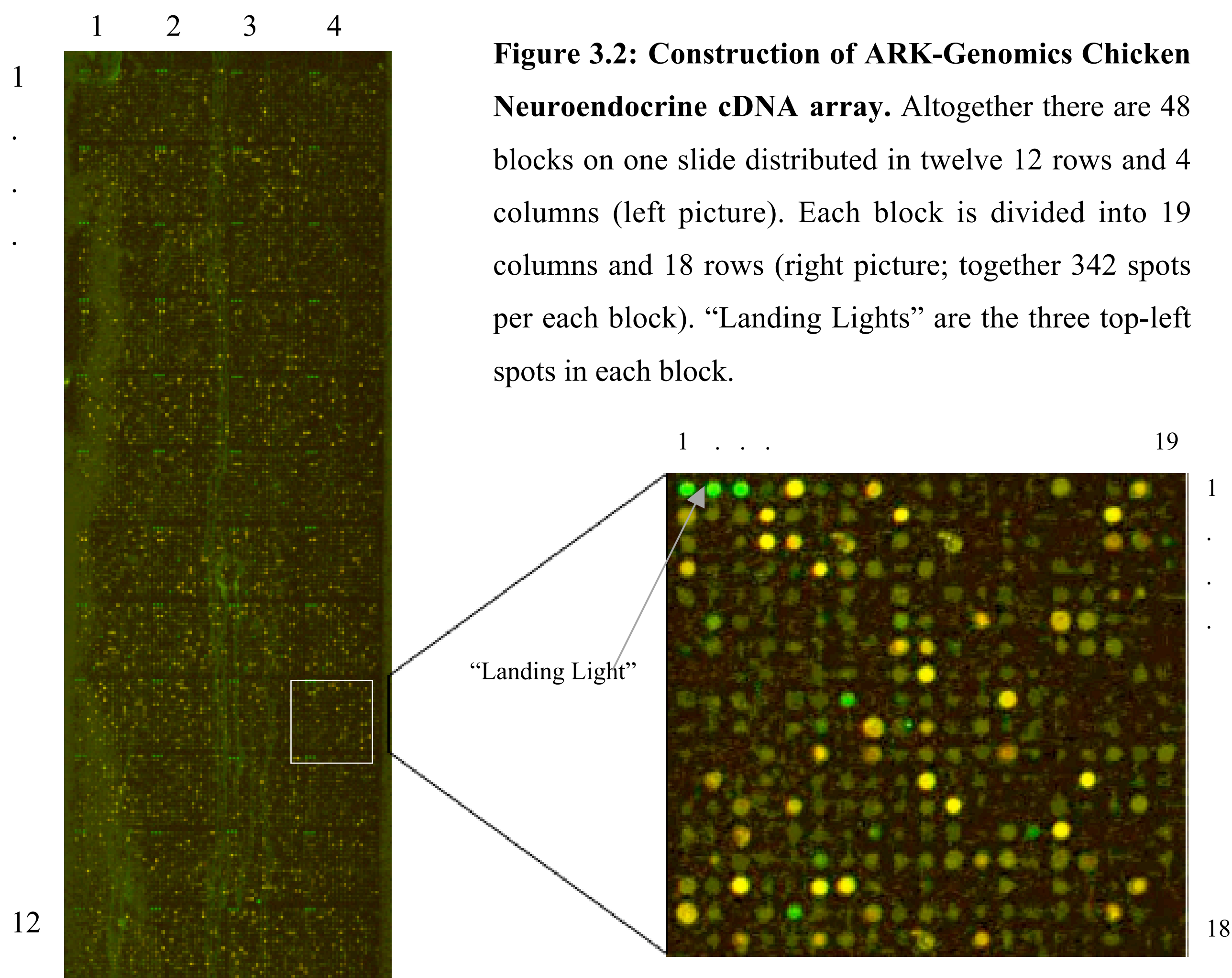

Figure 3.2: Construction of ARK-Genomics Chicken Neuroendocrine cDNA array. Altogether there are 48 blocks on one slide distributed in twelve 12 rows and 4 columns (left picture). Each block is divided into 19 columns and 18 rows (right picture; together 342 spots per each block). "Landing Lights" are the three top-left spots in each block.

3.11.3 Design of the microarray experiment

In the microarray experiment the loop design was applied (Kerr and Churchill, 2001). This design was chosen because it was more efficient when compared with the reference design on the same number of slides (Churchill, 2002). The proposed design compares three animals per line (six individuals in total) on six slides. On each slide, labelled cDNAs from two animals representing opposite lines (LFP versus HFP) are hybridised. To counteract the dye bias inherent to a two-colour microarray experiment, each individual was spotted twice onto the different slides where it was hybridised with different individuals from the other lines and labelled once with each dye (Cy3 and Cy5). Therefore the loop design is robust to the dye bias and enables the comparison of a single individual with two others. Figure 3.3 illustrates the loop design used to compare animals from Low (LFP) and High Feather Packing lines (HFP) symbolised with circles. Numbers inside the circles refer to

individual numbers, where individuals 1,3 and 5 belong to the HFP line and 2,4 and 6 to the LFP line. Arrows indicate microarray slides. The green and red parts of the arrow correspond to the Cy3 and Cy5 dyes, respectively. Such a loop can be repeated, using preferably, a new set of animals. Moreover, it is possible to extend the loop adding additional slides between animals, where other pairs of used individuals are hybridised.

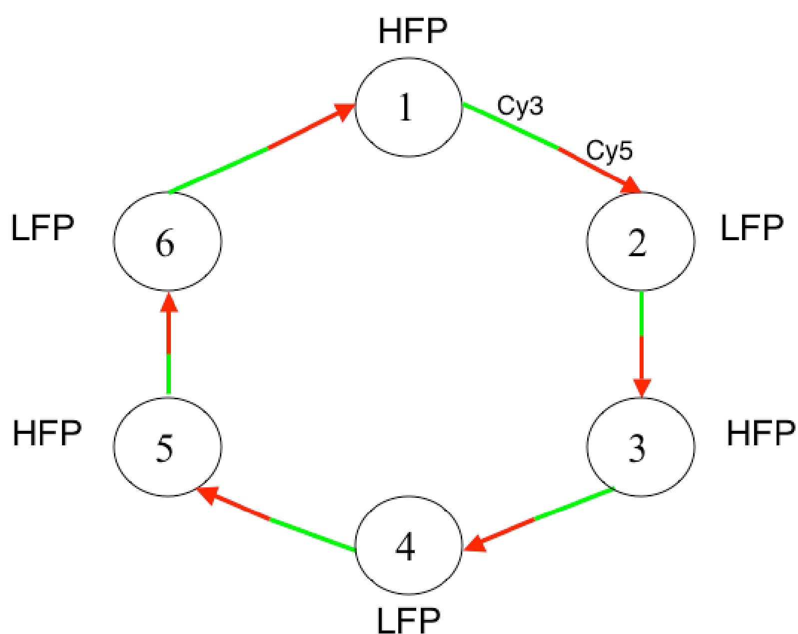

Figure 3.3: Microarray experiment carried out as a loop design.

3.11.4 Labelling

Labelling reactions were carried out with CyScribe First-Strand cDNA Labelling kit, the direct labelling system (Amersham Biosciences, Munich, Germany). A maximum of 45 µg of total RNA was transcribed into fluorescently labelled cDNA. Ten µl of total RNA were mixed with 1 µl of anchored oligo-dT primer and incubated for 5 minutes at 70ºC. After that, the reaction was incubated for 10 minutes at room temperature, to let the primer anneal with the RNA template. The reaction was spanned down and the following components were added: 4 µl of 5x CyScript buffer, 2 µl of 0.1 M DDT, 1 µl of dCTP nucleotide mix, 1 µl of dCTP CyDye-labelled nucleotide (Cy3 or Cy5) and 1 µl of CyScript reverse transcriptase. The final reaction volume of 20 µl was incubated for 1.5 hours at 42ºC. After incubation each reaction was checked by standard PCR using primers

designed for housekeeping gene to prove that proper cDNA synthesis occurred. The fluorescence properties of both dyes (Cy3 and Cy5) can be strongly affected by exposure to the light and ozone. Therefore, all steps should be made quickly and the samples must be light protected.

3.11.5 Degradation of RNA

To remove the rest of RNA, templates of 2 µl of 2.5 M NaOH were added and the reaction mixture was incubated for 15 minutes at 37ºC. Thereafter, 10 µl of 2 M HEPES free acid were added to each reaction and mixed gently.

3.11.6 Purification of labelled cDNA with CyScribe GFX Purification kit (Amersham Biosciences, Munich, Germany)

The labelled unpurified mixture was added onto the GFX column together with capture buffer. The column was centrifuged for 30 seconds at 13 000 x g. After that 600 µl of washing buffer were added to each column and centrifuged for 30 seconds at 13 000 x g. The washing step was repeated three times. The columns were dried by centrifugation for additional 10 seconds. Further, 60 µl of pre-warmed elution buffer (65ºC) were added onto the column and centrifuged for 3 minutes at 13 000 x g. The elution step was repeated twice. The purified reaction mixture was frozen or used directly for the preparation of the hybridisation reaction.

3.11.7 Microarray hybridisation protocol

The labelled Cy3 and Cy5 cDNA samples were dried using a vacuum centrifuge (SpeedVac Plus, Savant) and were next dissolved in 37.5 µl of nuclease free water per reaction. cDNA samples were denaturised for 2 minutes at 95ºC, combined and cooled on ice. Next, 75 µl of CyScribe microarray buffer (Amersham Biosciences, Munich, Germany) and 150 µl of 100% Formamide (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) were added. The final volume of 300 µl hybridisation mixture was pipetted onto the microarray slide. To avoid drying up and to ensure the even distribution of the hybridisation reaction, Hybidization Slide (1.9 x 6.0 cm GeneFrame, Peqlab, Erlangen, Germany) covered with a cover slip (Peqlab, Erlangen, Germany) were used.

Hybridisation was performed in a hybridisation chamber (Peqlab, Erlangen, Germany) for 16-18 hours at 42ºC.

3.11.8 Washing the slides

Hybridised slides were washed three times: once using 1x SSC 0.2% SDS for 10 minutes, and twice in 0.1x SSC 0.2% SDS for 10 minutes. Both washing buffers were preheated up to 42ºC. After that, slides were dipped into distilled water for 10 seconds. The slides were placed in 50 ml Falcon tubes and dried by centrifuging for 5 minutes at 1500 rpm.

3.11.9 Scanning slides and images analysis

Each slide was scanned with the GMS 418 Array Scanner (Affymetrix, Germany). The photo excitation was 532 nm (green laser) and 635 nm (red laser) for Cy3 and Cy5, respectively. The proper calibration ensured the optimal signal-to-noise response. The GMS 418 Array Scanner enables to choose different percentage of the gain and laser power. The gain, which is the photomultiplicator voltage, controls sensitivity of the scanner. The percentage of the gain should not be too high; otherwise the bright spots will be saturated. During scanning the gain was set up to 60% or 70%, which allowed maximising weaker signals, and keeping the stronger ones onscale. Laser power was set up to 60%. Too low laser power may not ensure an optimal signal-to-background performance. Following Yang et al. (2000) too high laser power helps to generate stronger signals but, simultaneously, more noise. Scanning steps can be repeated to obtain the best images. All images were saved as 16-Bit TIFF files. More detailed information about the used scanner, and other possible setting options, can be found in the GMS 418 Array Scanner User Guide available at www.ifg-izkf.uni-muenster.de.
Microarray image analyses were carried out using *Spot* software (http://experimental.act.cmsi.csiro.au/Spot/index.php, Beare and Buckley, 2004). The analysis of the two images obtained from red and green channels can be divided into three steps (Yang et al., 2000):

- **Addressing**, which includes setting up the parameters, creating the template that captures information about overall position of the array, size of blocks and their position, and grid detection

- **Segmentation** process that divides an image into different regions, which allows calculating intensities of foreground and background. A so-called *spot mask* is being created that contains foreground pixels for a given spot
- **Information extraction** includes background and total intensities measurements; adjusting the annotation information to each spot and creating output files

The *Spot* starting window is presented in Figure 3.4A. The batch, a file that contains the images of red and green channels, had to be created. Next, the parameters file was set up. The parameters file contains the information about the geometry of the slide and block; the grid search tolerance was set up to zero.
Filtering and rotation correction were not used in the analysis. For grid finding, a large search was selected. The large search uses the tolerance parameters defined in the parameters file and the location of the template to control the way in which the grid is determined. GOGAC (globally optimal geodesic active contours) method was chosen as a segmentation method. That method created a closed contour of optimal integrated edge strength, produced a quality measure and performs serial segmentations for each spot independently. The gal file (supplied by ARK-Genomics) was used as an annotation file. The gal file (Gene Array List) includes the names of all clones, genes and controls spotted onto the slide. The used gal file had to be corrected considering that some of the blanks were not included by the supplier.
The template was created by pointing to the top-left spot (first "Landing Light") in each block and the rightmost spot in the rightmost block on the combined (from red and green channels) image. The software estimated the overall grid location in the analysed image and performed small adjustments in rows and columns.
Subsequently, the *Spot* software performed segmentation. During that process an image was divided into different regions, which enabled to calculate the intensities for each spot. These intensities were measured for each pixel within the defined spot area and averaged thereafter.
The final step of the analysis was the data extraction. The measures reported after the analysis and the form of the report depends on the selected output options (Figure 3.4B). These measures were extracted as a "spot" file. The combined image of both dyes, the images of the segmentation and the grid parameters are saved as TIFF files.

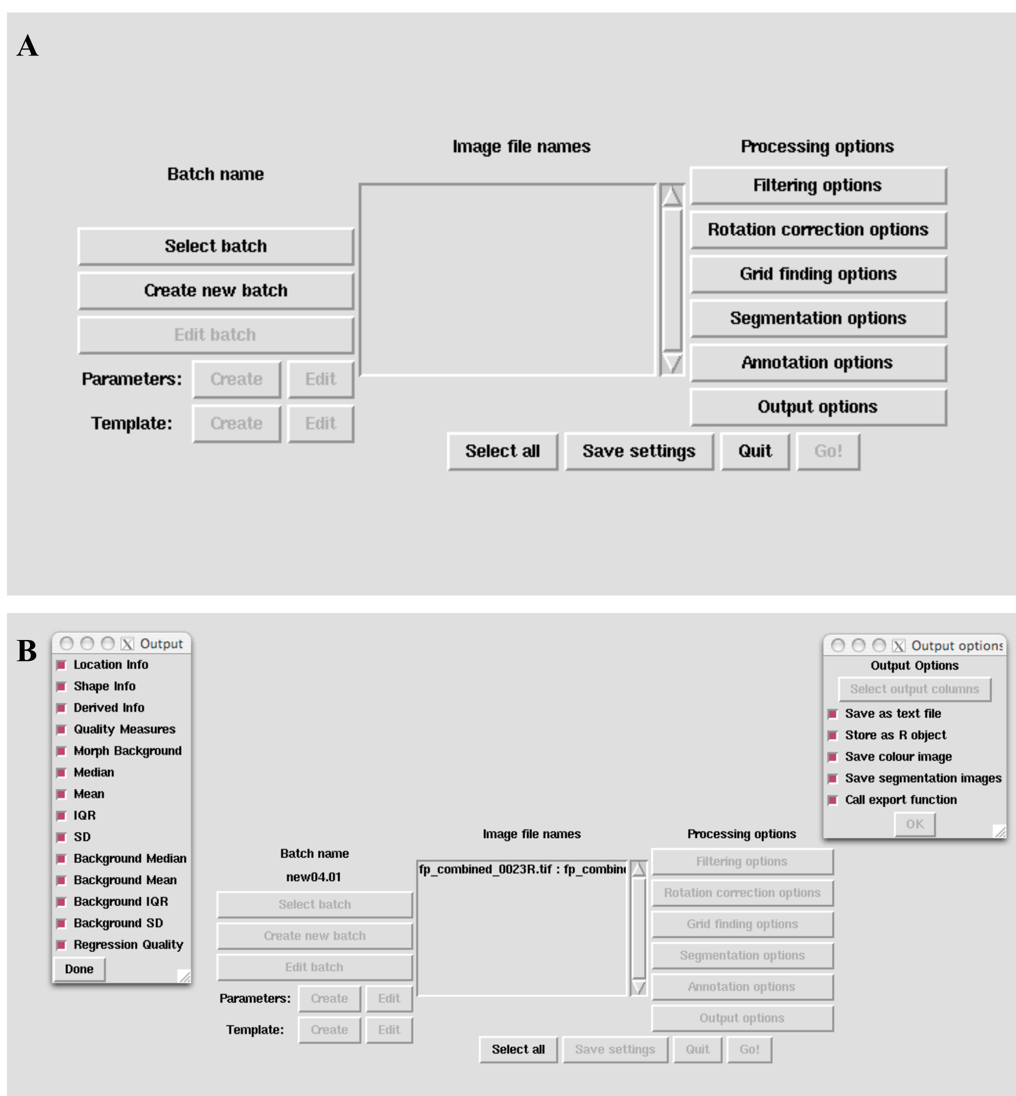

Figure 3.4: ***Spot*** **software. A.** Screen shot of the starting window. **B.** Detailed view of the output options window.

After the first analyses, the mismatch between the red and green channel images was detected. A shift of 4 pixels between Cy3- and Cy5-image caused during scanning the slides was corrected using the following R script written by *Spot* software programmer Michael Buckley (CSIRO):

```
X <- imloadtiff("batchname_combinedpicture.tif")
R <- X[[1]]
G <- X[[2]]
imsetpixtype(R, 1, IM.UINT2)
imsetpixtype(G, 1, IM.UINT2)
```

```
r <- R[,5:6748]
g <- G[,1:6744]
imsetpixtype(R, 1, IM.UINT2)
imsetpixtype(G, 1, IM.UINT2)
imsavetiff(r, "batchname_Cy5R.tif")
imsavetiff(g, "batchname_Cy3G.tif")
```

3.11.10 Data normalisation and statistical analyses

3.11.10.1 LOWESS normalisation and clone-by-clone mixed model using R/MAANOVA package (Wu and Churhill, 2005)

For the purpose of using the R/MAANOVA package for slide quality check and for statistical analyses, an input file had to be created. First, a single input file for all sides of the microarray experiment was created using median values of the total intensity for the red and green channels extracted from the spot files of all six slides. The first few columns of the input file contained the grid location of each spot and ID according to the gal file. “Landing Lights” and blanks were flagged and excluded from the statistical analysis. Background intensity was not taken into consideration.

LOWESS (locally weighted regression scatterplot smoothing) normalisation was carried out for raw data obtained from the microarray image analysis using R/MAANOVA package (Wu and Churchill, 2005). Imbalances between Cy 3 and Cy 5 dyes cause a phenomenon, which is called “dye-bias”. LOWESS corrects the dye-bias by carrying out a locally weighted linear regression with the log (Cy3*Cy5) being the dependent and log (Cy3/Cy5) being the independent variable. The residuals from the LOWESS model, which represent the normalised data, are centred around zero value. Intensity specific dye effects are largely removed.

The normalised data were analysed by the following clone-by-clone mixed model using the R/MAANOVA package (Wu and Churchill, 2005):

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

y	Log_{10} transformed and normalised intensity measurements			
μ	Overall mean			
A_i	Fixed array effect	(i = 1,2,…,6)		
D_j	Fixed dye effect	(j = 1,2)	1 = Cy3	2 = Cy5
L_k	Fixed line effect	(k = 1,2)	1 = HFP line	2=LFP line
I_l	Random individual effect	(l = 1,2,…,6)		
e_{ijklm}	Residual error			

A mixed model was carried out for each replicate within each clone. The first term in the model captured the overall average intensity. *A* and *L* indicate the fixed effects of the array and line, respectively. *D* indicates the clone specific dye bias, necessary to perform clone-by-clone local normalisation since the LOWESS method takes care of the global normalisation of the entire intensity data set in the microarray experiment. *I* is considered as a variation due to individuals and represents a random effect.

The P-values were obtained for each of the replicates and the mean of P-values across replicates within each clone was calculated.

3.11.10.2 Alternative statistical analysis using two interconnected linear mixed models ("normalisation" and "gene" model) as proposed by Wolfinger et al. (2001)

The used raw data include the medians of the total intensity for each spot excluding "Landing Lights" and blanks. Statistical analyses were carried out using R/nlme package, which is recommended for linear and nonlinear mixed effects models (Pinheiro et al., 2006).

Wolfinger et al. (2001) proposed a two-step procedure. In the first step a global and array specific normalisation is carried out with a linear mixed model which is applied to the whole data set.

The following normalisation model was applied:

$$y_{ijk} = \mu + D_i + A_j + (DA)_{ij} + e_{ijk}$$

y_{ijk}	Log_{10} transformed intensity measurements		
μ	Overall mean		
D_i	Fixed dye effect	(i = 1,2) 1 = Cy3	2 = Cy5
A_j	Fixed array effect	(j = 1,2…,6)	
$(DA)_{ij}$	Dye by array interaction		
e_{ijk}	Residual error		

In the second step, a clone specific mixed model accounts for the clone specific dye bias and other design effects such as the array effect. Moreover, the line effect is estimated and a significance test is carried out in order to test the line differences in gene expression. The random effect of technical *R(A)* replicates was included.

The "gene model" was as follows:

$$y_{ijklm} = \mu + D_i + A_j + L_k + R(A)_{jl} + e_{ijklm}$$

y_{ijklm}	Log_{10} transformed and normalised intensity measurements			
μ	Overall mean			
D_i	Fixed dye effect	(i = 1,2)	1 = Cy3	2 = Cy5
A_j	Random array effect	(j = 1,2,…,6)		
L_k	Fixed line effect	(k = 1,2)	1 = HFP line	2=LFP line
$R(A)_{il}$	Random effect of the technical replicate, nested within an array			
e_{ijklm}	Residual error			

4 Results

4.1 Results of the behavioural experiments

4.1.1 Experiment #1

Ten freshly hatched chicks per each line (LFP and HFP line) were collected, placed in an observation box and recorded for 25 minutes (Figure 4.1). The observers who counted exploratory episodes did not know which line was HFP and which was LFP. As was expected, HFP showed a significantly higher number of exploratory episodes (Figure 4.2A). Moreover, there was no significant inter-observer variation (Figure 4.2B). The results of the statistical analysis are presented in Table 4.1.

Figure 4.1: Pictures of observation boxes. The chicks with the blue marking belong to the HFP line. The picture on the left shows a partial view of two boxes. In the picture on the right most of the observation box can be seen with a group of twenty chicks (ten per each line).

4.1.2 Experiment #2

The setup of experiment #2 was the same as that of experiment #1. Although the activity of the HFP line was found to be lower than that in experiment #1, the number of observed exploratory episodes was found to be still significantly higher in the HFP line (Figure 4.3). Again, inter-observer variation was not significant (data not shown). The results of the statistical analysis are presented in Table 4.1.

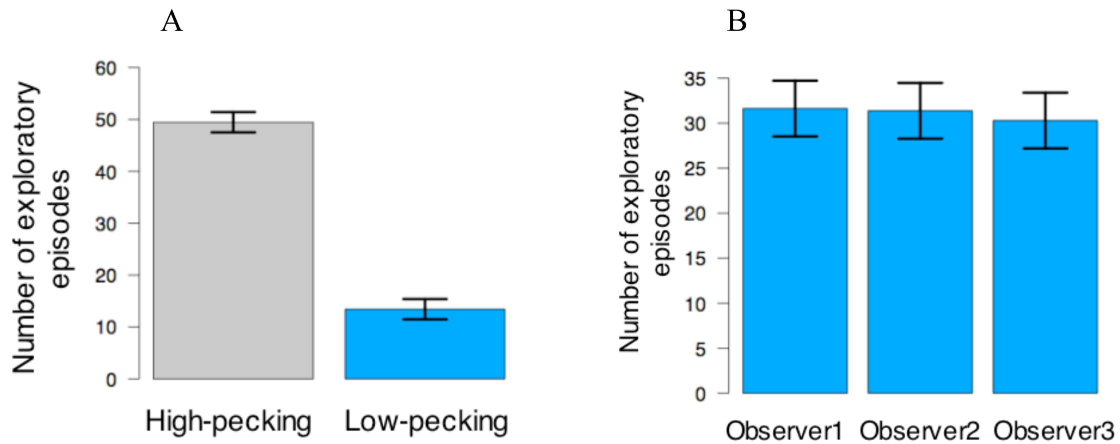

Figure 4.2: Mean (± standard error) of the number of exploratory episodes observed in freshly hatched chicks of two lines by three observers over 25 minutes (experiment #1). **A**. Variation between lines. **B**. Variation between observers.

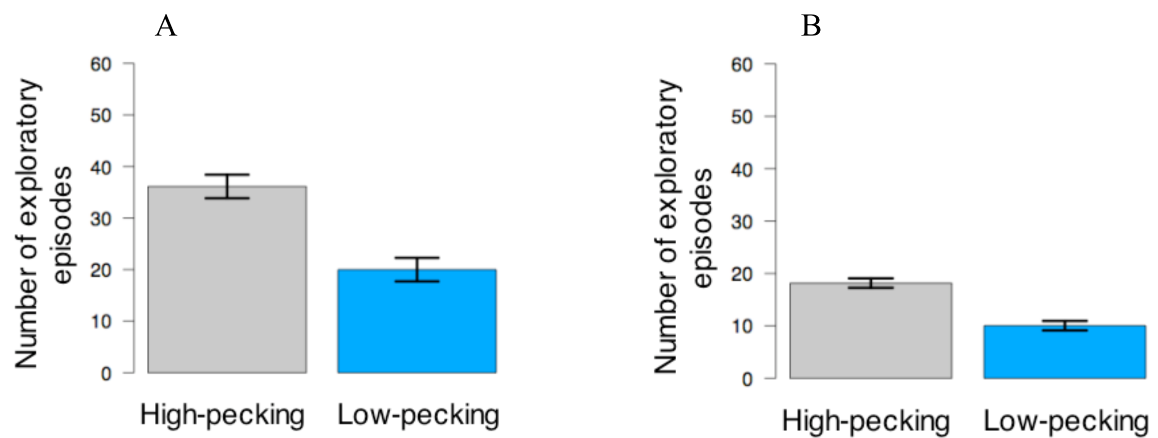

Figure 4.3: Mean (± standard error) of number of exploratory episodes observed in freshly hatched chicks of two lines. A. 25-minute observations by one observer (experiment #2a). **B.** 10-minute observations by two observers (experiment #2b).

Table 4.1: Results of the ANOVA of number of exploratory episodes counted in experiment #1 (May 2004) and in experiments #2a and #2b (July 2004).

Experiment #1[1]	**Sum Sq**	**df**	**F value**	**Pr(>F)**
Intercept	93.799	1	574.697	< 2.2e-16
Line	30.684	1	187.995	< 2.2e-16
Observer	0.200	2	0.613	0.545
Box	13.272	11	7.392	1.106e-07
Residuals	9.303	57		
Experiment #2a[2]				
Intercept	80.339	1	171.396	1.303e-09
Line	18.646	1	39.780	1.406e-05
Box	40.667	11	5.784	0.00078
Residuals	7.031	11		
Experiment #2b[3]				
Intercept	61.194	1	194.262	1.281e-15
Line	15.353	1	48.737	4.721e-08
Observer	0.694	1	2.204	0.147
Box	28.673	11	8.275	8.215e-07
Residuals	10.710	34		

[1] 25-minute observations by three observers in May

[2] 25-minute observations by one observer in July

[3] first 10-minute of observations by two observers in July

4.1.3 Experiments #3-#6

Additional experiments carried out in May and July 2004 provided more information about the exploratory behaviour of freshly hatched chicks.

Experiment #3 was a more extreme test for checking the tendency for exploratory episodes. Freshly hatched chicks were placed in a subdivision of the box that had a passage to an empty subdivision. Very few individuals went through the hole to reach the empty subdivision; however, of the five passages observed, four were made by chicks from the HFP line. The main aim of experiment #4 was to check differential interest between the two lines to the stimuli added to each box. Birds from both lines showed no interest in pecking at either silver balls or pieces of plastic straw. Experiments #5 and #6 were identical in the basic setup with experiments #1 and #2, but a reduced number of

individuals were used. During experiment #5, which had the smallest group sizes (three chicks per line), it was noticed that chicks showed almost no exploratory episodes, staying in one corner of the box most of the time. In fact, despite being placed individually in different parts of the box initially, the chicks quickly formed a single group and hardly ever left it. In experiment #6, the addition of only two chicks per line (compared with experiment #5) caused a noticeable increase in chicks' activity and the trends were similar to those observed in experiments #1 and #2.

4.2 Characteristics and analyses of candidate genes identified through the literature search

4.2.1 Characteristics of candidate genes – results of the literature search

Each candidate gene was identified using a different combination of keywords. Consequently, it was difficult to define which of the used keywords was the most meaningful for which candidate gene. Some publications, published after 2004 are included in the gene description. However, they confirmed the right choice of the candidate genes, they did not help to identify them.

Seven candidate genes were selected after the literature search, namely *HOXB8*, *TPH1*, *TPH2*, *ACADS*, *CRHR1*, *PRKG1* and *PRKG2* and are briefly described in the following paragraphs.

HOXB8 (Homeobox B8) is a member of the homeobox gene family. Genes containing homeobox sequences encode nuclear proteins with regulatory functions (Acampora et al., 1989). Mutant mice show excessive pathologic grooming behaviour that is comparable to human obsessive-compulsive behaviour like trichotillomania (Greer and Capecchi, 2002).

TPH1 is one of two genes for a rate-limiting enzyme in biosynthesis of serotonin. It uses (6*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) and dioxygen cofactors along with the dietary essential amino acid substrate L-tryptophan (L-Trp) in generating 5-hydroxy- L-tryptophan that is converted to 5-hydroxytryptamine (5-HT or serotonin) by aromatic amino acid decarboxylase. Serotonergic neurotransmission is changed in depression, aggression and obsessive-compulsive disorders in humans (Wang et al., 2002). It is also involved in the regulation of sleep, anxiety and food intake (Walther et al., 2003). The most important finding is that feather pecking is triggered by low serotonin neurotransmission in chickens (van Hierden et al., 2004a).

TPH2 – the gene for the second TPH isoform is exclusively expressed in brain. Mice brain

stem total RNA samples from wild type mice revealed much higher (about 150 times) *TPH2* than classical *TPH1* mRNA in ribonuclease protection assays (RPAs) experiments reported by Walther et al. (2003). *TPH2* is also considered as a candidate gene for ADHD (attention-deficit hyperactivity disorder) in humans (Sheehan et al., 2005).

CRHR1 encodes the corticotropin releasing hormone receptor 1. Hypothalmic corticotropin releasing hormone (CRH) is responsible for the regulation of pituitary ACTH secretion and mediates the behavioural and autonomic responses to stress through an interaction with type 1 plasma membrane receptors (CRHR1) located in pituitary corticitrophs and the brain (Aguilera et al., 2004). According to investigations made by Smith et al. (1998), lack of *CRHR1* in mice displays reduced anxiety. The role of CRHR1 antagonists as novel antidepressant drugs was reported by Müller and Wurst (2004).

ACADS – encodes acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain. It is a member of acyl-Coenzyme A dehydrogenases (ACADs) family of mitochondrial enzymes that initial rate-limiting step in the ß-oxidation of fatty acyl-CoA (Zhang et al., 2002). Tafti et al. (2003) claim that *ACADS* affects only theta oscillation during sleep. At the same time he did not exclude its role in the exploratory behaviour.

The last identified candidate gene was ***PRKG1,*** encoding cGMP-dependent protein kinase type 1. In the fruit fly *Drosophila melanogaster*, 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 (de Belle et al., 1989). Sokolowski (1980) discovered that there are two naturally occurring food-search behaviour variants in *Drosophila melanogaster*: rover and sitter, being more and less active, respectively, considering foraging behaviour. Additionally, this difference in the foraging behaviour is observed during both the larval and adult stages (Osborne et al., 1997). In honey bees (*Apis mellifera*) the role of *PRKG1* in behavioural polymorphism was demonstrated by Ben-Shahar et al. (2002 and 2003).

PRKG2 was also investigated given that it is an ubiquitous brain protein (de Vente et al., 2001) compared to PRKG1 which is restricted to Purkinje cells. *PRKG2*, encoding cGMP-dependent protein kinase type 2, was found to be more active in the brain of rats, than *PRKG1* (de Vente et al., 2001). *PRKG1* and *2* are strong candidate genes since Huber-Eicher and Wechsler (1997) considered feather pecking as redirected foraging behaviour.

4.2.2 BAC cloning of the selected candidate genes

First, a BAC library was screened for *HOXB8* and *TPH1*. Thirty-one clones were chosen and subsequently tested for the presence of the expected insert. Positive results were

obtained for eleven clones, four for *HOXB* and seven for *TPH1*. BAC DNA was isolated from eight clones, four containing the *HOXB8* and four containing *TPH1*.

Twenty clones did not contain the desired *HOXB8* or *TPH1* specific inserts. The identity of *TPH1* and *TPH2* for overall sequence in humans has been reported to be 71% (McKinney et al., 2005) and 73% when aligning the mRNA sequences of *TPH1* and *TPH2* in chickens (NM_204956 for *TPH1* and NM_001001301 for *TPH2*). Thus, it is very likely that some of the false positives actually represented *TPH2*-specific clones. *HOXB8* belongs to the homeobox genes family, which has approximately 39 members (Lufkin, 2003). However, since the intronic sequence was used to generate the probe, it seams unlikely that some of the strong signals obtained after screening the BAC library might have derived from other members of the homeobox family. The complete list of all thirty-one clones is presented in Appendix 9.3.

BAC DNA was used for FISH (fluorescence in-situ hybridisation) performed by Dr. Felix Habermann (TUM, Chair of Animal Breeding, data not shown). Signals were detected on chicken chromosome (GGA) 5 for *TPH1* confirming the previous results (Habermann et al., 2001), and on the GGA 27 for *HOXB8*. The assignment of *HOXB8* is in conflict with Ladjali-Mohammedi et al. (2001), who assigned *HOXB8* to GGA 3. However, recent BLAST analyses based on the *HOXB8* reference sequence and the annotated chicken draft sequence support the assignment to GGA27.

HOXB8-sepcific BAC DNA isolated from two clones was also used for direct sequencing with primers 3253dn and 3254up (primer sequences are available in Appendix 9.1). The obtained sequences confirmed the *HOXB8*-specifity of the two clones (data not shown).

FISH and direct sequencing using isolated BAC DNA were carried out in March 2004. Since the chicken genome sequence became available from public databases in the same month, there was no further use for the BAC DNA or screening the BAC library for the remaining candidate genes.

4.2.3 Polymorphism analysis

Polymorphism analysis was carried out for five candidate genes: *HOXB8*, *TPH1*, *TPH2*, *ACADS*, *CRHR1*. *PRKG1* and *PRKG2* were added to the candidate genes list as the latest, and therefore were not included in the polymorphism analysis. For each gene a minimum of seven individuals across the panel of twelve breeds and lines were used to search for SNPs. The DNA of individuals from both experimental lines (HFP and LFP lines) and Red Jungle Fowl were always sampled for each gene. All polymorphisms found within the

investigated coding regions were silent. Examples of two substitutions in *TPH1* and a deletion in *ACADS* are shown in Figure 4.4 (indicated with red arrows on the screenshots obtained from the Consed software).

A

B

Figure 4.4: Exemplary sequence traces displaying. **A.** Deletion in *ACADS*, exon 1. **B.** Two substitutions in *TPH1*, exon 2.

Information regarding each SNP, including its position and the primer pair (sequences are available in Appendix 9.1) used for PCR and sequencing reaction is presented in Table 4.2. The genotypes of animals from the sequenced lines and breeds are shown in Appendix 9.7.

Table 4.2: Sequence variation identified in five candidate genes.

SNP number	Locus	Exon	Alleles	Position in mRNA (ref. seq.)	PCR primers: up/dn	Reference sequence
1	*HOXB8*	2	G/T	738	3170/3171	NM_204911
2	*TPH1*	1	G/A	118	3564/3565	NM_204956
3		2	C/T	271	3566/3567	
4		2	C/T	274		
5		2	C/T	341		
6		2	G/A	355		
7		8	T/A	1066	3578/3579	
8		8	C/T	1085		
9		10	C/T	1381	3582/3583	
10	*TPH2*	2	C/A	174	3542/3543	NM_001001301
11		2	T/G	207		
12		3	A/G	321	3544/3545	
13		6	T/C	642	3548/3549	
14		8	T/C	991	3552/3553	
15	*ACADS*	1	GCG del	41	3969/3970	NM_001006193
16		2	G/C	157	3969/3970	
17		3	T/C	376	3369/3370	
18		5	C/T	514	3373/3374	
19	*CRHR1*	3	C/T	422	3450/3451	NM_204321
20		4	C/T	518	3452/3453	
21		4	C/T	536		
22		5	T/C	600	3454/3455	
23		6	T/C	749	3456/3457	
24		7	C/T	869	3458/3459	
25		7	C/T	896		
26		9	A/G	1049	3462/3463	

The main aim of the presented polymorphism analysis was to find one or more mutations that change the amino acid sequence of the gene product. When the identified polymorphisms were silent, the next exon or gene was analysed. Because all found polymorphisms were silent, no further analyses, such as genotyping large number of animals, were performed.

The phylogenetic analysis was carried out by Dr. Olaf R.P. Bininda-Emonds (TUM, Chair of Animal Breeding). The goal of this analysis was to obtain a phylogenetic supertree (Figure 4.5) of the twelve breeds and lines from the panel used for polymorphism analysis so as to infer the evolutionary history of the breeds and polymorphisms in the candidate genes. The supertree was based on the genetic distance information available at the AVIANDIV project homepage http://w3.tzv.fal.de/aviandiv/index.html. Two breeds, New Hampshire and Malay had to be excluded, due to no genetic distance information available.

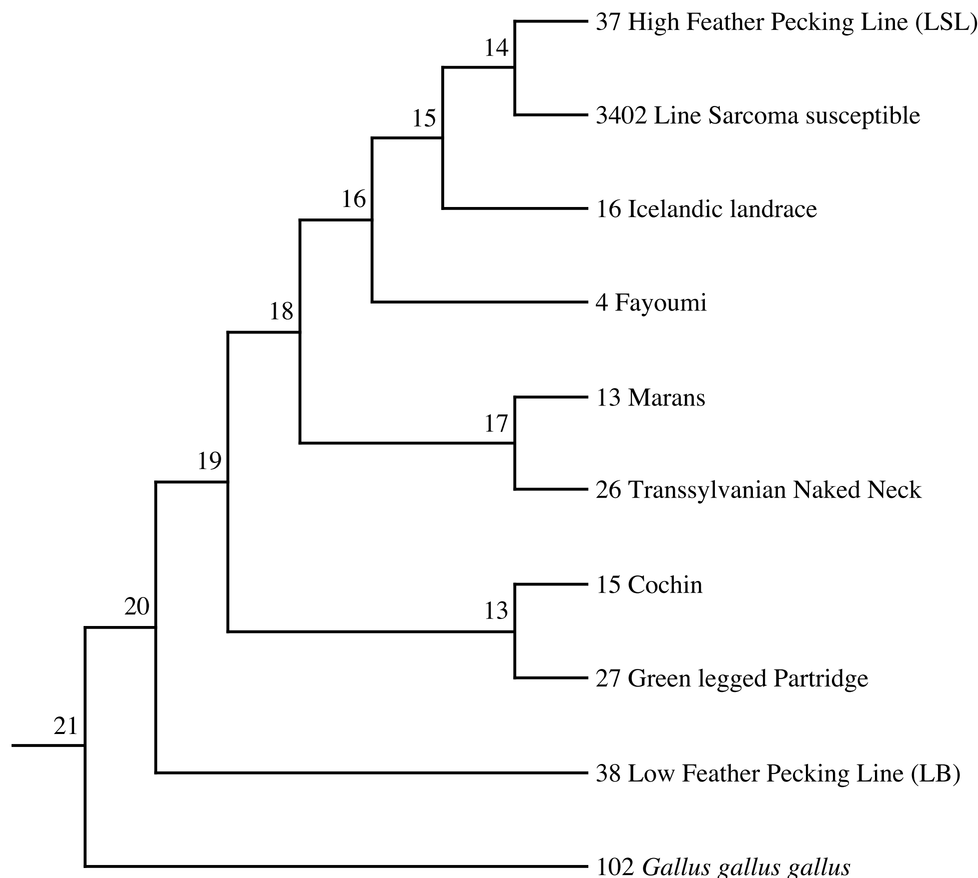

Figure 4.5: **The structure of the supertree, including lines and breeds that were previously used for polymorphism analysis.** Numbers placed directly on the tree refer to id numbers of the nodes. Numbers of breeds and lines stay in line with the AVIANDIV project numbering system.

As can be seen from Figure 4.5, HFP and LFP lines are not closely related genetically. Giving this, it cannot be excluded that the observed genetic differences between these two lines are associated with some traits other than the tendency towards feather pecking or

exploratory behaviour.

4.2.4 RT-PCR

Using total RNA isolated from brains of the experimental animals, single-stranded cDNA was synthesised by reverse transcription and used for PCR amplification with primers specific for seven candidate genes. Optimisation of RNA isolation is presented in subchapter 4.3.1. First RT-PCR was carried out for *TPH1* with primers 3195up/3196dn (sequences are available in Appendix 9.1) flanking about 800bp of genomic DNA and about 200bp of cDNA products. As can be seen in Figure 4.6, the results of the amplification included cDNA and genomic DNA, which appeared as two bands of the expected size. This indicates DNA contamination of RNA.

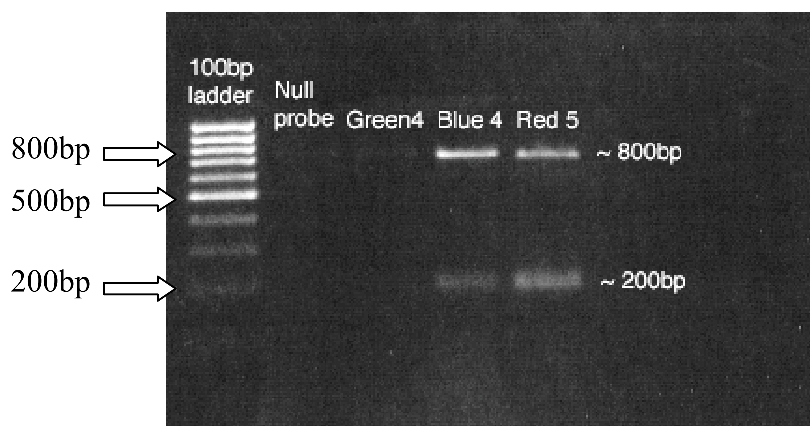

Figure 4.6: RT-PCR results in the 1.5% agarose gel. The 100 bp DNA Ladder® was used as size standard. Two products indicate the DNA contamination. PCR failed for Green 4 animal.

The supplier of the RNeasy isolation kits claims that the used silica-gel-based membrane technology prevents DNA contaminations. However, the obtained RNA does not confirm this, being still contaminated with DNA. To avoid the amplification of genomic DNA, special primers were designed for the experiment. Each primer had to span the exon-exon junction of the cDNA to avoid non-specific products due to DNA contamination.

The results of the RT-PCR performed for seven candidate genes are presented in Figure 4.7. The information about the expected product sizes and sequences of used primers (marked in Figure 4.7) is presented in Appendix 9.2. Bands of the expected size indicate the expression of all genes in the chicken brain. Differences in the amount of PCR may reflect different expression levels but it is more likely that they are a technical artefact. Control PCR carried out using genomic DNA with the same primers set did not yield any amplification confirming the proper design of the primers (data not shown).

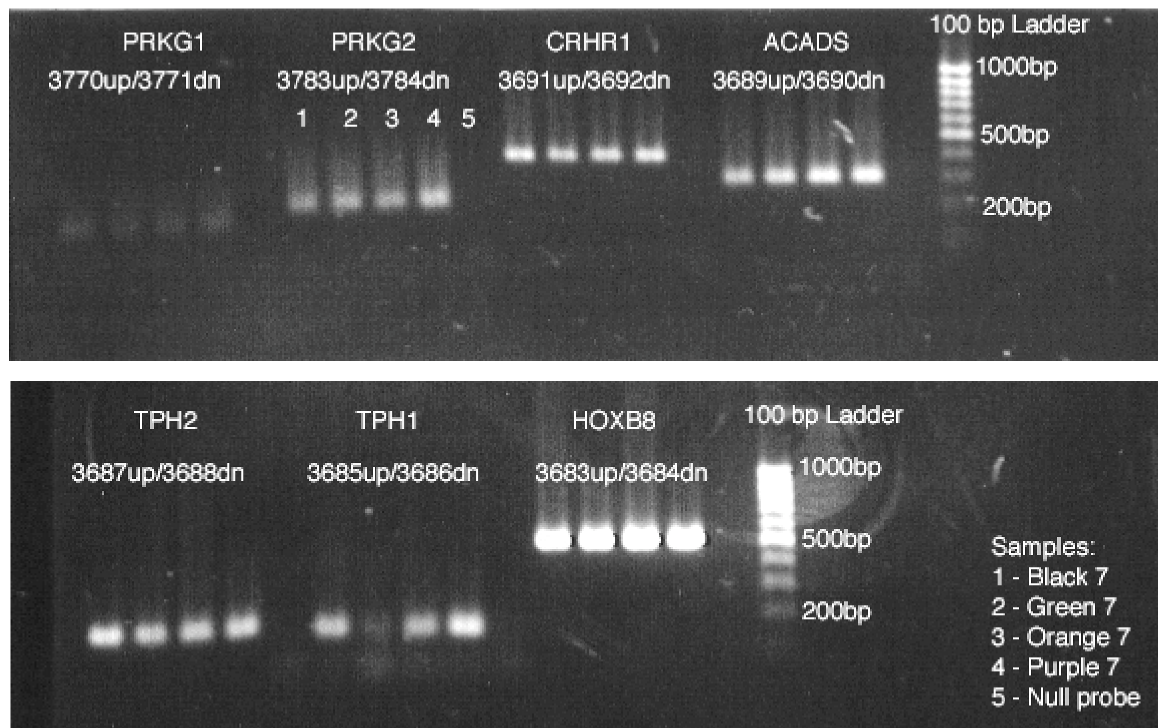

Figure 4.7: Results of RT-PCR for seven candidate genes: *PRKG1*, *PRKG2*, *CRHR1*, *ACADS*, *TPH2*, *TPH1*, *HOXB8*. All candidate genes were expressed in the chicken brains of four individuals (samples). The RT-PCR products were separated in the 1.5% agarose gel. The DNA 100 bp Ladder® was used as size standard.

Positive results obtained for all genes after RT-PCR confirm their status as functional candidate genes. Such a result was, in fact, expected since all those genes are expressed in human brain (see GeneCard database www.genecards.org). Since RT-PCR confirms the appropriate design of primer pairs and the expression of selected genes, it can be considered as a good preparation step for subsequent realtime quantitative RT-PCR.

4.2.5 Expression analysis by realtime quantitative RT-PCR

Realtime quantitative RT-PCR was carried out for all candidate genes identified through literature search: *HOXB8*, *TPH1*, *TPH2*, *ACADS*, *CRHR1*, *PRKG1* and *PRKG2*. Brain derived cDNA samples of eight individuals per each line (LFP and HFP line) were used in realtime quantitative RT-PCRs. The specificity of the amplification products was assessed by dissociation curve analysis. Two peaks (Figure 4.8A) indicate unspecific amplification

of the *ACADS* gene, most likely in the form of a primer artefact (so called primer dimer). The redesign of the primers led to specific amplification as can be seen in Figure 4.8B.

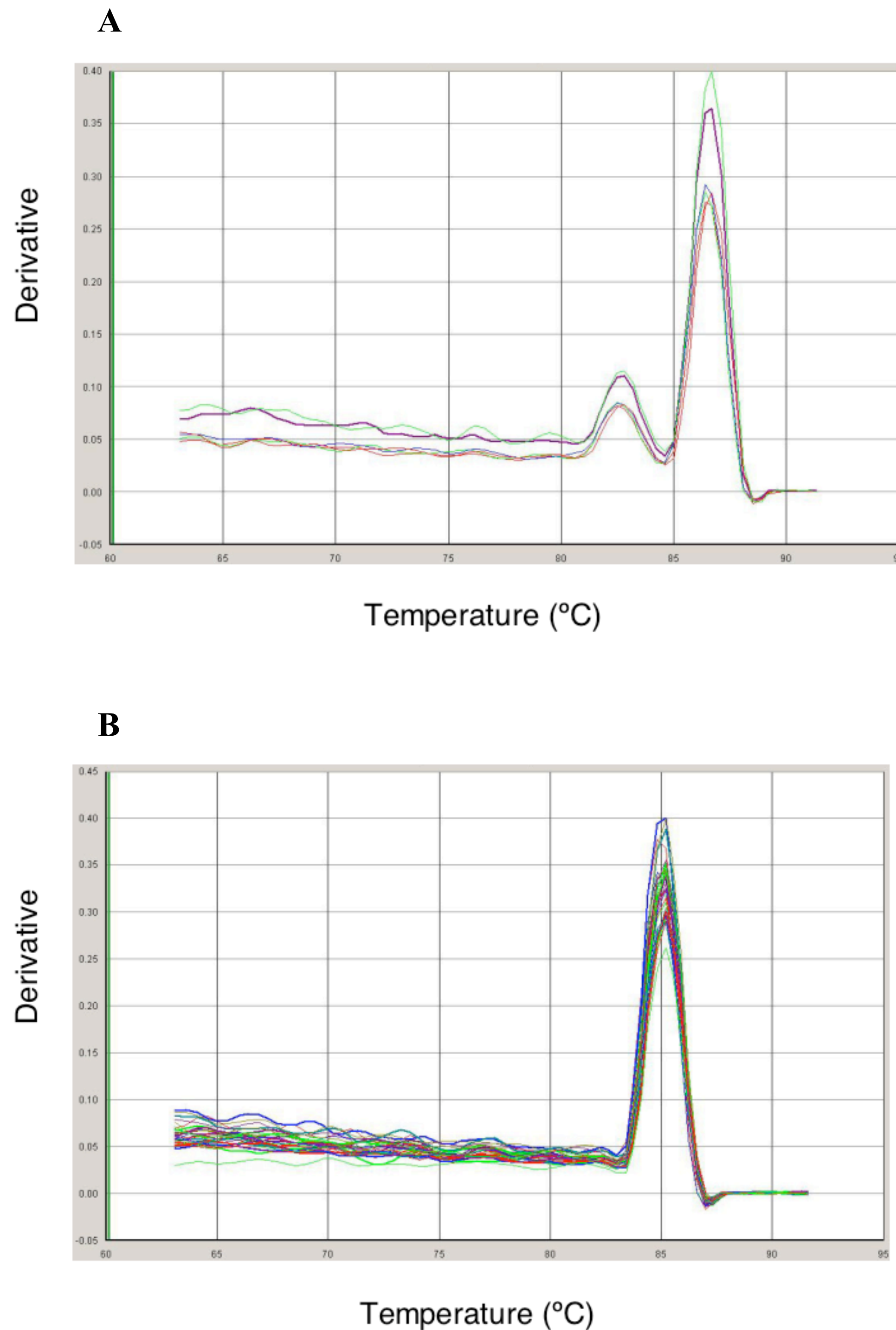

Figure 4.8: Dissociation curve of the *ACADS* amplification product. A. Two peaks indicate an unspecific product using primers: 3689up/3690dn (sequences shown in Appendix 9.2) **B.** A single peak indicates specific amplification with redesigned primers 4003up/4004dn (sequences shown in Appendix 9.2)

After the statistical analysis, two genes showed a highly different expression, namely *TPH2* and *PRKG1* (Table 4.3), even after Bonferroni-Holm correction (Table 4.4). In both genes the expression was lower in the HFP line. The expression did not significantly differ among the five other candidate genes. However, a tendency for under expression in HFP line was constant across all candidate genes.

Table 4.3: Results of the realtime quantitative RT-PCR. Genes that are marked in bold show a significant difference in expression between HFP and LFP lines.

Gene	R[1] HFP[2]	R LFP[3]	P-value[4]
HOXB8	2.2120 ± 0.0805	2.1982 ± 0.0883	0.5737
TPH1	1.8225 ± 0.1032	1.7915 ± 0.0693	0.7209
TPH2	**1.5359 ± 0.0218**	**1.4916 ± 0.0199**	**0.0006**
ACADS	1.5784 ± 0.0328	1.5624 ± 0.0210	0.3282
CRHR1	1.6561 ± 0.2658	1.5868 ± 0.1977	0.7984
PRKG1	**1.6626 ± 0.0358**	**1.6140 ± 0.0152**	**0.0060**
PRKG2	2.2120 ± 0.0349	2.1982 ± 0.0304	0.0721

[1] averaged ratio = Ct (threshold cycle) of the experimental gene divided by Ct of the housekeeping gene

[2] High Feather Pecking line

[3] Low Feather Pecking line

[4] P-values from Wilcoxon Rank Sum Test

±: Standard Deviation

Table 4.4: Results of Bonferroni-Holm correction. The highly different expression of two genes *TPH2* and *PRKG1* was confirmed (bold marked).

Gene	Ranked P-values	Significance threshold
TPH2	**0.0006**	**0.05 / 7 = 0.0071**
PRKG1	**0.0060**	**0.05 / 6 = 0.0083**
PRKG2	0.0721	0.05 / 5 = 0.0100
ACADS	0.3282	0.05 / 4 = 0.0125
HOXB8	0.5737	0.05 / 3 = 0.0167
TPH1	0.7209	0.05 / 2 = 0.0250
CRHR1	0.7984	0.05 / 1 = 0.0500

4.3 Microarray analysis of gene expression

4.3.1 Optimisation of RNA isolation

This section compares the quality of RNA obtained in different homogenisation and isolation processes. The quality of RNA is an important factor in gene expression analyses, especially in microarray experiments. Therefore, different methods of homogenisation of chicken brains and isolation of total RNA were tested.

4.3.1.1 Homogenisation of the brain tissue using a stator rotor dispersion tool and isolation by Trizol method

The brain tissue is clammy and very tender. Even after freezing in liquid nitrogen, the tissue was winding around the dispersing aggregate and the obtained RNA was insufficient for any analysis (data not shown).

4.3.1.2 Homogenisation of the brain tissue using a syringe and isolation by Trizol method

Pieces of brain tissues were forced through a syringe and RNA was isolated using the Trizol reagent. Figure 4.9 shows a higher intensity of the 18S ribosomal RNA (rRNA) in comparison with 28S rRNA band and the smear. This indicates RNA degradation. Moreover, the third band which corresponds to low weight molecular RNA ought not be as bright as the rRNA fractions obtained after properly carried out RNA isolation. To sum up, the quality of RNA obtained in this method was inadequate.

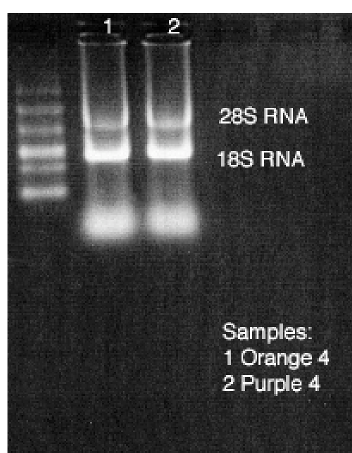

Figure 4.9: Total RNA isolated using the Trizol reagent after homogenisation with a syringe. Samples were separated in the 1.2 % agarose-formaldehyde gel. High Range RNA ladder® was used as size standard.

4.3.1.3 Homogenisation of the brain tissue using syringe and needle and isolation by the RNeasy® Mini Kit

To improve homogenisation, a 0.7 mm needle was used together with the syringe. The decision to use the RNeasy isolation kit instead of the Trizol reagent was based on the claim of the supplier of the kit that the silica-gel-based membrane technology efficiently removes the contaminating DNA. Furthermore, using RNeasy® kit is less toxic than using Trizol, which contains phenol.

The results are shown in Figure 4.10. Two distinct bands show the proper intensities, with 28S rRNA band being stronger than 18 rRNA band (in ratio 2:1). There is no visible smear resulting from degradation of RNA and no third band. Since the RNA was isolated from whole brains, the maxi-version of RNeasy® kit was necessary if the isolation was to be carried out in one tube.

Figure 4.10: **Total RNA isolated using the RNeasy Mini Kit after homogenisation with a syringe and a needle.** All samples were separated in the 1.2 % agarose-formaldehyde gel together with the High Range RNA ladder®, which was used as size standard. No degradation of RNA occurred.

4.3.1.4 Homogenisation of RNA using the FastPrep system and isolation by RNeasy® Maxi Kit

The syringe and needle method produced satisfactory quality RNA but it was time consuming. Therefore the FastPrep system based on mortar and pestle approach (described

in subchapter 3.9.1) was used for subsequent homogenisations. This method shortened the process of homogenisation but the quality of the isolated RNA reminded high.

4.3.1.5 Evaluation of RNA

The obtained RNA samples were measured with the spectrophotometer. This procedure provides accurate information about the quantity and quality of RNA. The absorbance values of the RNA samples at 260 nm and 280 nm, the A_{260}/A_{280} absorbance ratios, calculated concentrations per µl and total yield are shown in Table 4.5. A_{260}/A_{280} ratio of between 1.83-2.1 indicates a very high degree of purity (ideal ratio is between 1.9 and 2.1)

Table 4.5: Concentration and purity of isolated RNA.

Animal (sample)	**Phenotype**	**A_{260}**	**A_{280}**	**Estimated purity A_{260}/A_{280}**	**Concentration µg per µl**	**Total yield[3] (µg)**
Orange6	[1]HFP	0.32	0.17	1.88	0.64	640
Red6	HFP	0.40	0.19	2.00	0.80	800
Purple6	HFP	0.30	0.15	2.00	0.60	60
Black6	[2]LFP	0.42	0.20	2.10	0.84	840
Green6	LFP	0.37	0.18	2.05	0.74	740
Blue6	LFP	0.40	0.20	2.00	0.80	800
Black7	HFP	0.24	0.12	2.00	0.40	400
Green7	HFP	0.24	0.13	1.85	0.40	400
Blue7	HFP	0.26	0.14	2.10	0.44	440
Orange7	LFP	0.24	0.12	2.00	0.40	400
Red7	LFP	0.33	0.17	1.85	0.40	400
Purple7	LFP	0.26	0.13	1.85	0.44	440
Blue5	HFP	0.51	0.24	2.10	1.00	1000
Orange5	LFP	0.60	0.28	2.10	1.20	1200
Red5	LFP	0.21	0.11	1.90	0.42	420
Purple5	LFP	0.13	0.07	1.85	0.26	260
Black4	LFP	0.16	0.08	2.00	0.32	320
Green4	HFP	0.15	0.08	1.87	0.30	300
Blue4	HFP	0.19	0.09	2.10	0.38	380
Red4	HFP	0.22	0.12	1.83	0.44	440

[1]HFP: High Feather Pecking line

[2]LFP: Low Feather Pecking line

[3]yield – calculated according to procedure presented in Material and Methods (*subchapter 3.9.3*)

4.3.1.6 Amount of RNA needed for successful hybridisation

According to the labelling protocol from Amersham, the initial 25 µg of total RNA per animal should be sufficient for a successful hybridisation reaction. That means that the intensity signals should be clear and strong, and evenly distributed across the array. As can be seen from Figure 4.11 (left picture), the signals were very weak, except for the "Landing Lights", which are recognizable unlike the other spots. A higher amount of total RNA was added during the subsequent labelling reaction. An amount of 45µg of total RNA clearly increased the strength of the signals (Figure 4.11, right picture).

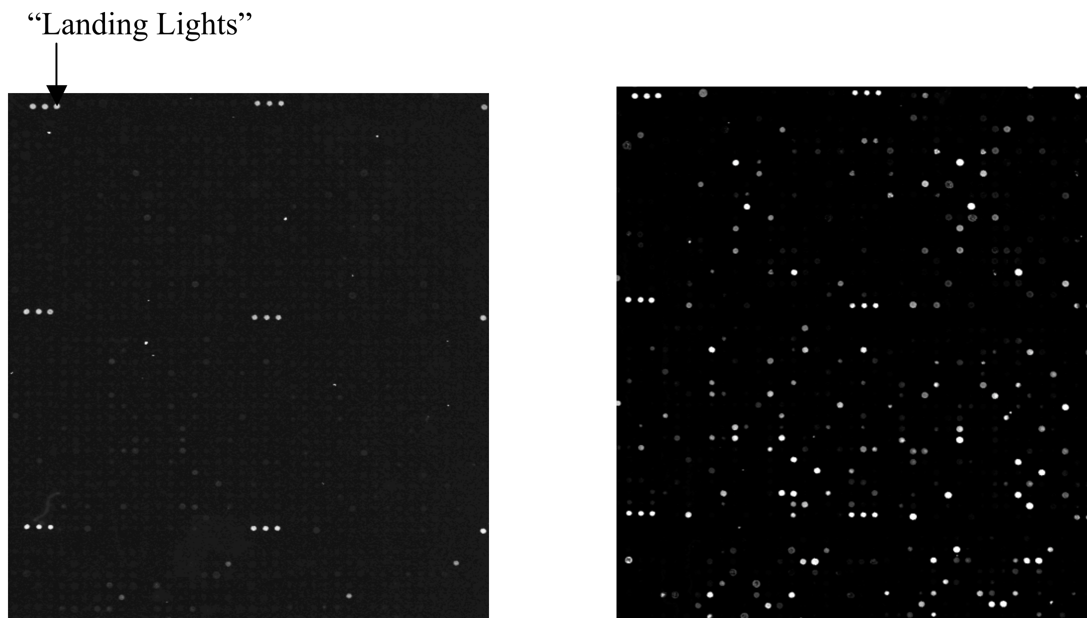

Figure 4.11: Comparison of two slides obtained using different amounts of total RNA for hybridisation in two microarray experiments: 25µg (*left*) and 45µg (*right*). The higher amount of initial total RNA increases the strength of signals.

4.3.2 Processing of microarray images

The two black-and-white images that were obtained for each slide after scanning the Cy3 and Cy5 signals, respectively, were processed with the R-based software *Spot* (http://experimental.act.cmsi.csiro.au/Spot/index.php). The first step included combining the two images and pseudo-colouring of the signals. Figure 4.12 (left) shows a partial combined image. It turned out, that the Cy5-image was shifted 4 pixels down in relation to the Cy3-image. An R-script provided by the curator of *Spot* (Michael Buckley) allowed correcting the pixel shift (Figure 4.12, right).

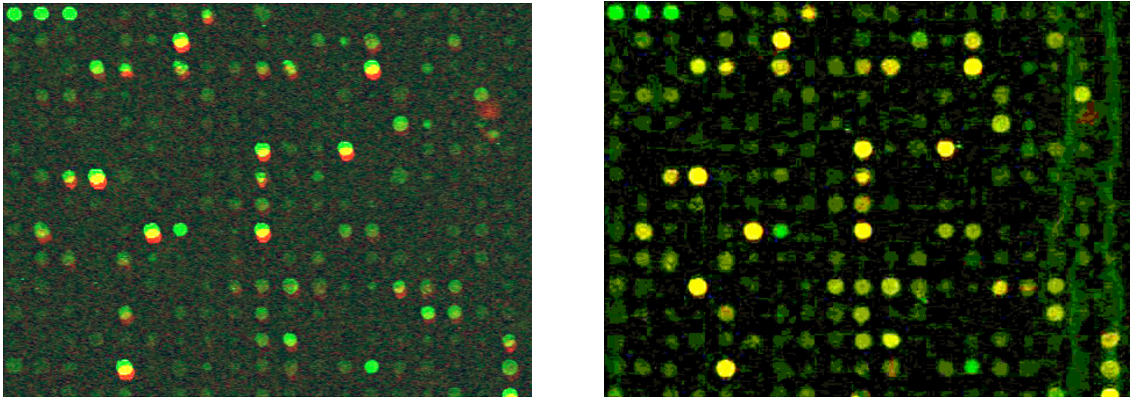

Figure 4.12: Partial combined and pseudo-coloured pictures of the red (Cy3) and green (Cy5) channels before correcting the pixel shift (*left*) and after correction (*right*). The three intense spots in the upper left corner are the so called "Landing Lights". They result from Cy3-labelled spots.

The spots were addressed semi-automatically using *Spot* on the basis of a template with an array structure and manually. Figure 4.13 (left) shows a partial picture of the grid-overlay after the spot-addressing step. Further, the borders of each spot were defined by a segmentation process (Figure 4.13, right). *Spot* assesses the pixel characteristics both inside and outside the boundary of each spot (background). A partial output of *Spot* is shown in Table 4.6. The *Spot* output, a file for each slide, represents the raw data set of the microarray analysis. Mean and median pixel intensities were calculated for each spot.

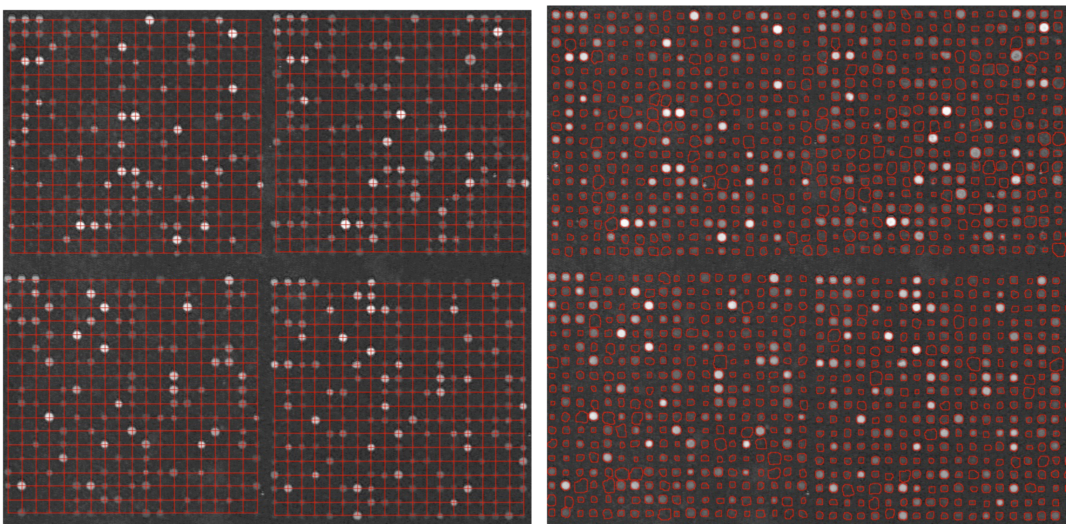

Figure 4.13: Partial grid (*left*) - and segmentation (*right*) overlays generated by *Spot*.

Table 4.6: Partial *Spot* output. *Rmean, Rmedian, Gmean, Gmedian* stand for median and mean pixel intensity within each spot for the R- (*R*) and G-(*G*) channel, respectively.

Spot	Rmean	Rmedian	Gmean	Gmedian
0	13.407	12.0	165.292	188.0
1	14.448	13.0	147.459	163.0
2	12.750	11.0	122.580	131.5
3	11.298	12.0	21.561	21.0
4	63.986	67.0	68.520	74.0
5	10.909	10.5	20.704	20.0
6	17.711	17.5	27.555	27.0
7	21.788	21.0	44.653	43.5
...	...	...	...	...
16416	...	...	...	...

4.3.3 Checking slides quality

The quality of each slide was checked using R/MAANOVA package. For illustration purposes different qualities of two analysed slides are described below (Figure 4.14 A: Array 1 and B: Array 5). Both images show strong signals as compared to the control “Landing Lights”, but differ greatly in respect to the background level. The lower quality of Array 5 was confirmed by different tests available from MAANOVA package. One was the arrayview function that plots a simple portray of spatial patterns of the array considering standardised $\log_{10}$ ratios for all spots, shown in different colours. The main objective of arrayview was to check the quality of the hybridisation reaction and the washing procedure. Each rectangle corresponds to one spot. Figure 4.14 illustrates two images obtained after the arrayview test (Figure 4.14 C: for Array 1 and D: for Array 5). Red rectangles correspond to the “Landing Lights” and to the blank spots that are not taken into consideration during statistical analyses. Dark areas reflect either the effects of incorrect hybridisation or mistakes made during washing of the slide or the effects of both (Figure 4.14 C: for Array 1 and D: for Array 5). This phenomenon could have different origins. For example air bubbles, dust, wrong temperature and insufficient washing of the buffer, or an unevenly distributed hybridisation reaction all over the slide. Another possible reason could be the drying up of the hybridisation reaction due to the fact that not recognisable, dark areas appeared in the often close proximity of the corners or the borders of the slide. The reason for that could be an open cover slip.

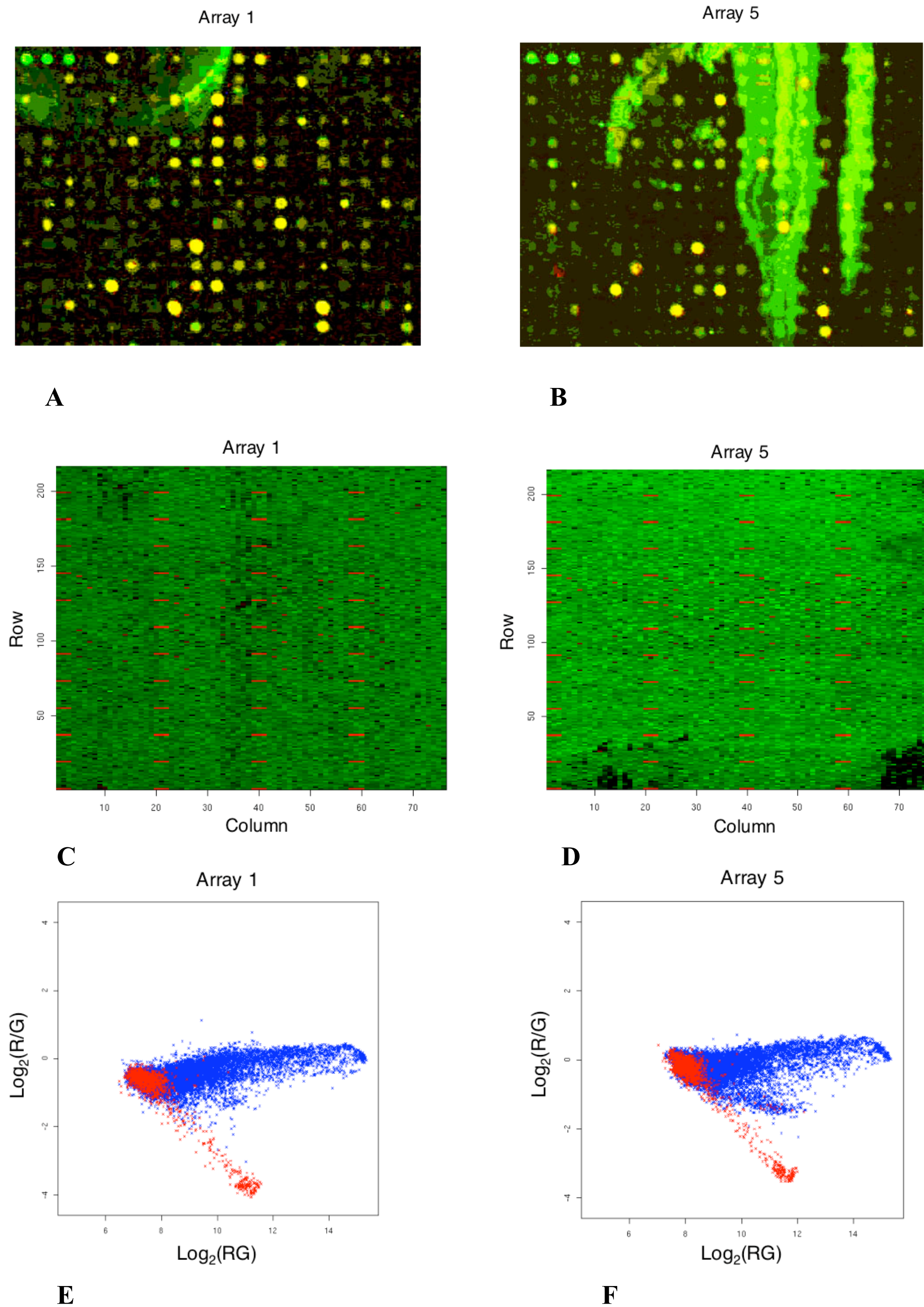

Figure 4.14: Two microarray slides showing different qualities (for description see page 75).

Figure 4.14: Two examples of microarray slides, which reveal different qualities. A. Combined image of Array 1 – the low background indicates the good quality of the slide. **B.** Combined image of Array 5 – the large smear worsens the quality of the slide. **C.** Arrayview plot of Array 1 – the small number of dark rectangles confirms the properly provided hybridisation and washing steps. **D.** Arrayview plot of Array 5 – the large dark areas indicate mistakes made during washing or hybridisation, due to which the intensity values could not be obtained from those spots. **E.** R-I plot for Array 1 – points are not scattered around the y-0 horizontal line but are more concentrated in comparison with the R-I plot for Array 5. **F.** R-I plot for Array 5.

The data within dark coloured parts of the slide are missing. The lower quality Array 5 has definitely more dark coloured rectangles when compared to Array 1.
The other test was a ratio intensity (R-I) plot, which plots the log ratio $\log_2(R/G)$ versus the log intensity $\log_2(R*G)$. This test is another good indicator of an array quality. It is a tool used to visualise the intensity-dependent effects. Each point represents one spot. As can be seen, the points are not ideally scattered around the y-0 horizontal line (Figure 4.14 E for Array 1 and F for Array 5). This is because at that stage of the microarray experiment the normalisation was not carried out. However, the points on R-I plot for Array 1 are more centred when compared with Array 5. This indicates a better quality of Array1. Red points reflect the flagged spots which were excluded from the statistical analysis.

4.3.4 Normalisation of the data

The global LOWESS (locally weighted regression scatterplot smoothing) normalisation was applied using R/MAANOVA package. Figure 4.15 shows the ratio-intensity (R-I) plot before (A) and after normalisation (B) for the data obtained across all arrays. Red points, which can be seen in Figure 4.15(A), mark the regression line fitted by LOWESS method as described in Materials and Methods, subchapter 3.11.10. After LOWESS normalisation performed as presented in Figure 4.15(B), points were approximately centred around zero. This means that the data were corrected for differences between the intensities across all clones, measured on the red and green channel. The majority of the intensity specific dye effects had been removed from the entire data set, even though they were not particularly large initially. The normalised data were subsequently used for statistical analysis.

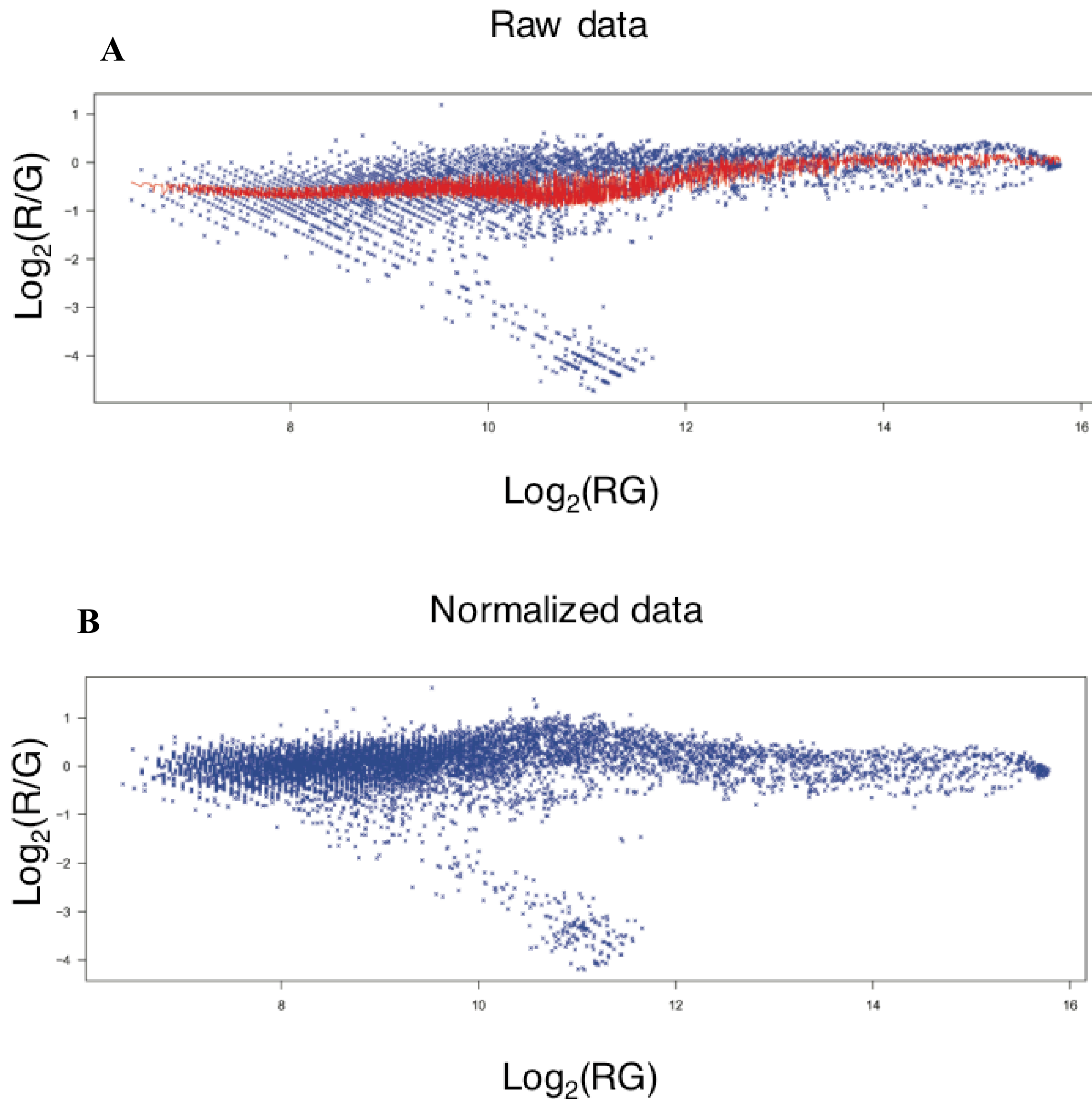

Figure 4.15: Ratio-Intensity plot for intensity measures obtained for all slides. A. Before LOWESS normalisation. **B.** After LOWESS normalisation.

4.3.5 Statistical analysis carried out using clone-by-clone mixed model for each technical replicate

In order to perform a statistical analysis, a linear mixed model was created and fitted to the data using R/MAANOVA package. Each technical replicate within each clone was analysed separately. P-values for each clone were calculated as an average of P-values

across the technical replicates. Eight clones with the lowest mean P-values (P<0.1) were selected (Table 4.7).

Table 4.7: Clones with the smallest P-values.

Clone name	P-value
C0003909B24	0.040
C0003910F16	0.044
C0004092L14	0.047
C0003910F7	0.050
C0003910I21	0.057
C0000078M13	0.082
C0003908K23	0.089
C0003908K6	0.091

4.3.6 Identification of differentially expressed candidate genes between HFP and LFP line according to the results of the statistical analysis

The eight clones selected by statistical modelling were sequenced by the microarray supplier ARK-Genomics (http://www.ark-genomics.org/). Sequences obtained from ARK-Genomics were subsequently BLASTed against the chicken genome sequence in Ensembl database (www.ensembl.org) and in human and chicken NCBI Nucleotide (including EST) databases (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=nucleotide). A significant match was not always found. For some of the sequences there was a complementarity between the clone and the human, but not the chicken, genomic sequence. All sequences with an average length of approximately 1000 base pairs were subject to a BLAST search. Subsequently four genes, namely *APOA1*, *TLR7*, *HSC70* and *MBP*, were selected as candidate genes. The choice was based on a combination of their known function, and the availability of the chicken genomic sequence for them. Table 4.8 shows the clones and their P-values, the sequences of which were used to identify candidate genes.

Table 4.8: Three clones the sequences of which covered the four candidate genes.

Clone name	P-value	Candidate gene
C0003910F7	0.050	*APOA1* *TLR7*
C0003908K23	0.089	*HSC70*
C0003908K6	0.091	*MBP*

The four candidate genes chosen based on the microarray experiment are characterised on the basis of available literature as follows:
APOA1 gene The apolipoprotein A-1 is the major protein component of high density lipoprotein (HDL) in plasma. In mammals the *APOA1* is expressed in liver and intestine. Additionally, in chicken, the synthesis of *APOA1* mRNA occurs in kidneys, ovaries, testes, brain, lungs, skeletal and heart muscles (Lamon-Fava et al., 1992). The level of *APOA1* mRNA increases in skeletal muscles dramatically during the hatching process which could be the result of yolk absorption (Rajavashisth et al., 1987). Vollbach et al. (2005) suggested that variants of *APOA1* might be one of the factors responsible for Alzheimer's disease.
TLR7 encodes the Toll-like receptor 7 which belongs to Toll-like receptor family and plays a fundamental role in pathogen recognition and activation of innate immunity. Its principal function is acting in the first line effector cells in the avian host defence system against bacterial, viral, and parasitic infections (Kogut et al., 2005).
HSC 70 encodes heat shock cognate 70 protein, a member of a heat shock protein family. As reported by Pizarro et al. (2003) the increase of mRNA and proteins of *HSC70* in rats was correlated with learning (not stress) and memory related changes. Chaperone *HSC70* protects the cell from the heat shock and other environmental stresses (de la Rosa et al., 1998).
MBP the myelin basic protein encoded by this gene is a major constituent of the myelin sheath of oligodendrocytes and Schwann cells in the nervous system. Because of the occurrences of autism anti-*MBP* was investigated by Singh et al. (1993) for its possible role. *Shiverer* mice, which lack a substantial part of *MBP* gene, have no myelin in their central nervous system, and exhibit a neurologic dysfunction with the onset of shivers, then of tonic seizures, and early death (Kimura et al., 1998). Conversion from normal to shivering type by *MBP* antisense cDNA in transgenic mice was noticed by Katsuki et al. (1988).

4.3.7 *Alternative statistical analysis carried out using two interconnected linear mixed models ("normalisation" and "gene model") proposed by Wolfinger et al. (2001)*

In order to optimise the statistical analysis, an alternative approach was applied. Two interconnected linear models were used:

- Normalisation model which basically corrects for experiment-wide and array specific intensity differences induced by dye effects
- Gene model, which is the clone-by-clone linear mixed model. In this approach the random effect of technical replicates was included

The first ten clones and genes selected on the basis of the lowest P-values are shown in Table 4.9.

Table 4.9: Ten clones and genes with the lowest P-values.

Clone / gene name	P-value	Ranking position
C0000091D6	2,70E-06	1
C0004091H6	1.88E-05	2
C0003910M10	4.32E-05	3
C0003909L21	4.54E-05	4
ADRB2 (*Canis f*amiliaris)	5.88E-05	5
C0004092O4	9.26E-05	6
Chicken mRNA for neuropilin	0.000149509	7
C0000092N23	0.000163786	8
C0000076D4	0.000192795	9
C0000086C19	0.000208048	10

No clone or gene defined in the statistical analysis using two interconnected linear mixed models was investigated at that stage because that method was applied in the last phase of the project. However, already within the first ten clones and genes with lowest P-values, an interesting new candidate gene, i.e. the gene encoding adrenergic receptor, beta 2 *ADRB2 (Canis familiaris)* appeared. An analysis of dog (NM_001003234.1) and chicken (ENSGALG00000002808) mRNA sequences showed an identity of more than 85%. Therefore, further realtime analysis of the differences in expression of this gene in HFP and LFP lines ought to be carried out.

4.3.8 Comparison between P-values obtained from different statistical analyses

Two different statistical analyses were carried out:

- LOWESS normalisation followed by a mixed clone-by-clone model carried out for each technical replicate (subchapter 4.3.5)
- Two interconnected linear mixed models (subchapter 4.3.7)

To compare and contrast those two approaches to a statistical analysis, P-values were compared (Table 4.10). The clones were chosen according to P-values obtained after the clone-by-clone linear mixed model for each technical replicate. The ranking was based on P - values obtained according to the interconnected linear mixed models. Seven out of the previously selected eight clones were placed within 6% of the clones with the lowest P-values.

Visual evaluation of the performance of normalisation of the LOWESS approach compared with the global normalisation model (Wolfinger et al., 2001) indicated that both methods remove the dye induced intensity effects satisfactorily (data not shown). However, substantially lower P-values obtained after the approach employing two linear mixed models demonstrated an increased power achieved by the joint analysis of all three technical replicates for each clone. The mixed model approach where each technical replicate was analysed separately with subsequent calculation of the mean P-values produced higher P-values because neither the separate analysis of technical replicates nor the calculation of mean P-values was statistically effective.

Table 4.10: Comparison between P-values for 8 clones considering two kinds of statistical analysis.

Clone name	P-value [1]	P-value [2]	Ranking position[3]
C0003909B24	0.040	0.004	103
C0003910F16	0.044	0.005	120
C0004092L14	0.047	0.014	287
C0003910F7	0.050	0.005	108
C0003910I21	0.057	0.001	31
C0000078M13	0.082	0.036	641
C0003908K23	0.089	0.007	151
C0003908K6	0.091	0.002	46

[1] P-values calculated using method presented in subchapter 4.3.5

[2] P-values calculated using two interconnected linear mixed models (subchapter 4.3.7)

[3] Ranking based on the statistical analysis incorporating two interconnected linear mixed models (subchapter 4.3.7)

4.3.9 Confirmation of differential gene expression by realtime quantitative RT-PCR

In order to increase the accuracy of the results obtained in the microarray experiment and to quantify the exact level of the gene expression a realtime quantitative RT-PCR was carried out for four candidate genes: *HSC70*, *MBP*, *APOA1* and *TLR7*. The set up of the realtime quantitative RT-PCR and the approach to the statistical analysis were the same as in subchapter 4.2.5. The results of the statistical analysis are shown in Table 4.11.

Table 4.11: Results of the realtime quantitative RT-PCR. Genes that are marked in bold show significant differences in the expression between HFP and LFP line.

Gene	R[1] HFP[2]	R LFP[3]	P-value[4]
HSC70	**1.1026 ± 0.0283**	**1.0661 ± 0.0186**	**0.0047**
MBP	**1.0710 ± 0.0339**	**1.0405 ± 0.0087**	**0.0104**
APOA1	**1.1215 ± 0.0302**	**1.0691 ± 0.0144**	**0.0011**
TLR7	1.8985 ± 0.7821	1.9024 ± 0.7832	0.9015

[1] averaged ratio = Ct (threshold cycle) of the experimental gene divided by Ct of the housekeeping gene

[2] High Feather Pecking line

[3] Low Feather Pecking line

[4] P-values from Wilcoxon Rank Sum Test

±: Standard Deviation

Out of the four candidate genes that were chosen in the microarray experiment, three were differently expressed in the two investigated chicken lines: *HSC70*, *MBP* and *APOA1*. The differences were still significant after Bonferroni-Holm correction (Table 4.12). Moreover, the tendency for under expression in HFP line in those genes stays in line with the results obtained by realtime RT-PCR carried out for seven candidate genes identified after literature search.

Table 4.12: Results of Bonferroni-Holm correction. The highly different expression of three genes *APOA1, HSC70, MBP* was confirmed (bold marked).

Gene	**Ranked P-values**	**Significance threshold**
APOA1	**0.0011**	**0.05 / 4 = 0.0125**
HSC70	**0.0047**	**0.05 / 3 = 0.0167**
MBP	**0.0104**	**0.05 / 2 = 0.0250**
TLR7	0.9015	0.05 / 1 = 0.0500

5 Discussion

5.1 General approach

The objective of this dissertation was to identify and investigate candidate genes responsible for feather pecking. The general approach is outlined in Figure 5.1.

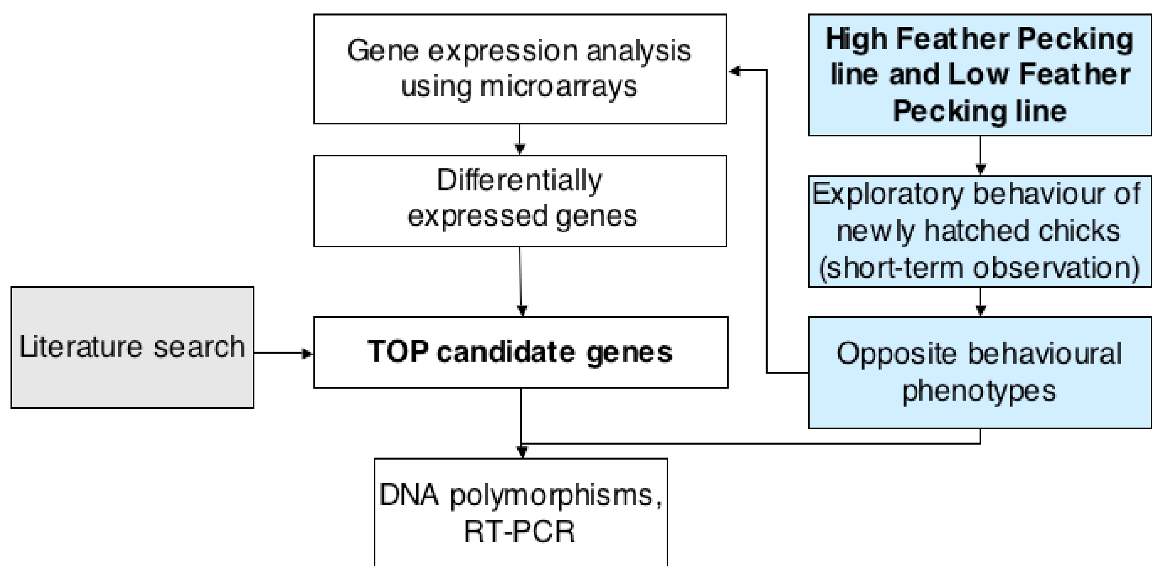

Figure 5.1: General approach. The three main parts of the study were: a behavioural experiments, candidate genes search and molecular analyses of the identified candidate genes.

It has been found that the two lines that differed in the tendency for feather pecking (LFP and HFP line) also differed in the tendency for exploratory behaviour measured in freshly hatched chicks. The two lines also showed differences in the level of gene expression in the brain tissue.

5.2 *Results of the behavioural experiments*

The design of the behavioural experiments, the basis of which was a comparison of two lines that differed in the tendency for feather pecking, had already been applied by different authors (Albentosa et al., 2003; Kjaer, 2000; Rodenburg et al., 2002). The approach to the experiment, however, was novel, as there had been no experiment in which the exploratory behaviour was measured by the number of exploratory episodes of freshly hatched chicks under the hypothesis that feather pecking was a redirected exploratory behaviour. As was expected the chicks from the HFP line showed a significantly higher number of exploratory episodes. Moreover, this tendency was identified across all the repetitions in experiments #1 and #2. The findings reveal a strong positive correlation between the propensity for exploratory behaviour in the freshly hatched chicks and the predispositions to feather pecking. This, in turn, supports the hypothesis that feather pecking is indeed a redirected exploratory behaviour.

As found in experiment #4, chicks in very small groups show almost no exploratory episodes. Thus, it seems very likely that there exists a threshold group size that encourages exploratory behaviour in chicks. It still remains unclear whether a larger group size provides sufficient security to permit short episodes of exploratory behaviour and/or whether larger groups engender the feeling of “crowding”. Support for the former explanation comes from the observation during the experiments that the natural behaviour of freshly hatched chicks in the incubator was to put their heads in their shells or, at least, to stay close to other individuals.

The environmental factors during experiments were reduced and well-defined. The light intensity had to be changed. Chicks hatched in a dark incubator, which makes the observations impossible or very difficult. Therefore, for observation purposes, the freshly hatched chicks were moved to the bright observation room. Such a change could have influenced the concentration of melatonin which depends on the light intensity and is higher during dark periods. According to Saito et al. (2005) there is a connection between stress response in neonatal chicks and melatonin concentration. High concentration of melatonin might be associated with lower stress, longer sleep during night and can block the stress responses in freshly hatched chicks placed in a novel open space (Saito et al., 2005). The correlation between melatonin concentration, stress and exploratory behaviour was not investigated in this study but it seems that it would be worth investigating it further in the future. For example the time of observations could be extended and genes involved in melatonin synthesis could be considered as potential candidate genes.

Despite the fact that the difference in exploratory behaviour between the observed lines has unknown origins, the working hypothesis that feather pecking is a redirected exploratory behaviour cannot be rejected. Moreover, because the environmental factors were reduced and well-defined for both lines, it can be assumed that the behavioural difference observed in the experiments was largely due to genetic factors.

5.3 Identification of candidate genes

Candidate genes were identified through literature search and in the microarray experiment. In either case the final decision which gene should be investigated was a matter of choice. Considering the scope of the research, the Neuroendocrine Chicken cDNA array seemed to be a perfect choice to identify candidate genes in microarray experiments. An ideal array according to Holloway et al. (2002) should be characterised by unique sequences, minimal cross-hybridisation and a comprehensive representation of the expressed fraction (including splices variants) of the genome. After the initial statistical analysis eight clones met the significance threshold and their sequences were BLASTed against the chicken genome sequence. The absence of similarity between them can be explained by the following example. Savolainen et al. (2005) reported that out of 12 549 unique transcripts generated from four chicken cDNA libraries constructed of brain and testis tissue dissected from Red Jungle Fowl and White Leghorn, 32.9% showed no significant match to gene/protein database and 13.1% to dbEST. The authors found that 180 unmatched transcripts contained the coding regions which corresponded to the number of putative novel chicken genes. One way to increase the accuracy of the results would be to switch from cDNA to oligonucleotide microarray with known sequences. Oligonucleotide arrays are more reliable than cDNA ones because short fragments of 50-70 nucleotides are included. This allows checking individual or particular genes in more repetitions on one slide and consequently obtaining more accurate results. Unfortunately at the time of this study the chicken oligonucleotide array had not yet been on the market. The Affymetrix Chicken GeneChip, although available, was not used due to its speciality and high costs.

The huge amount of data constituted the biggest challenge in the microarray experiment. As was mentioned before only eight clones were selected after initial statistical analysis. In order to increase the statistical power, a new alternative joint analysis of all three technical replicates for each clone was carried out. More residual degrees of freedom to estimate the

parameters led to smaller standard errors and therefore to lower P-values. P-values of technical replicates did not have to be aggregated anymore. So far there was no further analysis carried out for clones and genes obtained after the alternative statistical approach. One of the identified genes, namely *ADRB2*, is a promising candidate gene and it would be worth working on it further. It is responsible for mediating the physiological and the psychological responses to environmental stressors (Diatchenko et al., 2006). Savory and Kostal (2002) suggested a role of adrenorecoptors in the stereotyped oral behaviour.
A large number of data obtained after the microarray experiment has not yet been fully analysed due to the time deficiencies. The following could be of a particular interest:

- Analysis of the clones and genes identified using the two interconnected linear mixed models, alternative statistical approach
- Adding new individuals or more slides to extend the designed loop
- Crating a new loop and combining the data for statistical analysis
- Clustering, considered as a "wide variety of unsupervised methods for organising multivariate data into groups with roughly similar patterns" (Slonim, 2002)
- Reanalysing the data after background subtraction

5.4 Molecular analyses of selected candidate genes

5.4.1 SNP search

The searching for SNPs in the first five candidate genes did not produce any satisfactory results. Although some SNPs were found in the coding regions, none of them revealed an amino acid changing mutation. Therefore, further research in candidate genes was directed to gene expression analyses. Nevertheless, it would be interesting to focus on:

- SNP search in coding regions in the remaining six candidate genes
- Investigation of promoter regions, especially of the candidate genes that differed significantly in their expression
- Genotyping carried out on a larger number of animals from HFP and LFP lines

5.4.2 Realtime quantitative RT-PCR

The microarray technique helped to identify candidate genes and offered a general idea about gene expression in the investigated lines, while the realtime quantitative RT-PCR

analysis confirmed the results of the microarray experiment and enabled quantification of the exact level of the gene expression difference.

Five of the eleven candidate genes were differently expressed (*TPH2*, *PRKG1*, *APOA1*, *MBP* and *HSC70*). The expression was lower in the HFP line. As can be seen from their characteristics, all those genes are related to behavioural and neurological disorders, or stress response, which consequently confirmed their status as strong candidate genes. Moreover, a tendency for lower gene expression in the HFP line was observed in almost all analysed candidate genes.

To obtain more accurate data the gene expression could be investigated in specific brain regions. One of the conceptions was to divide chicken brain with respect to its anatomical parts, and carry out separate RNA isolations. However, this would drastically increase the costs and the duration of the microarray experiments and realtime quantitative RT-PCR.

5.5 Conclusions

The two main conclusions that can definitely be drawn after the analysis of the obtained results are:

- There is a significant difference in exploratory behaviour between the HFP and LFP line
- There is a significant difference in gene expression of five candidate genes and there is a general tendency for lower expression of almost all analysed candidate genes in the HFP line

Most likely, more than one, single gene causes feather pecking due to the complexity of this trait. A cluster analysis of the data obtained in micorarray experiments would probably give a more precise answer as to which group of genes, rather than a single gene, might be responsible for feather pecking. Moreover, the phylogenetic analysis has shown, that the investigated chicken lines are not closely related genetically. This means that the differences in gene expression between two lines may be correlated with other traits as well, and thus the role of differentially expressed candidate genes in feather pecking or exploratory behaviour may be even more difficult to prove. Nevertheless, 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 the behaviour and gene expression, which would be worth studying in more detail in the future.

6 Summary

6.1 Summary

Feather pecking is a complex trait which may be caused by endogenous factors (i.e. genetic factors or, physiological control mechanisms) or environmental factors (feeding, density, housing conditions). The objective of this study was to identify and characterise candidate genes responsible for feather pecking in laying hens. In this study, in order to facilitate the determination of a possible genetic predisposition to that complex trait, feather pecking was considered to be a redirected exploratory behaviour occurring when there is no opportunity for normal exploration, which may consequently trigger off severe feather pecking. Differences in intrinsic exploratory behaviour can be best measured in freshly hatched chicks in a well-defined and easily controlled environment.

The exploratory behaviour of two chicken lines differing in the tendency to feather pecking (High versus Low Feather Pecking) was investigated. Observations of the freshly hatched chicks in a well-defined environment showed differences in the locomotory activity taking the form of exploratory episodes of the chicks from the two investigated lines. The number of exploratory episodes was significantly higher in the chicks from the High Feather Pecking line ($P<0.001$). Blood and brain samples were collected and used for further molecular analyses.

Seven candidate genes (*HOXB8*, *TPH1*, *TPH2*, *ACADS*, *CRHR1*, *PRKG1*, *PRKG2*) were identified from literature. The coding regions of five candidate genes (*HOXB8*, *TPH1*, *TPH2*, *ACADS*, *CRHR1*) were screened for single nucleotide polymorphisms (SNPs). All found SNPs were silent.

RNAs isolated from chicken brains of Low and High Feather Pecking lines were subjected to two-colour microarray experiments using the Chicken Neuroendocrine array from ARK-Genomics. A loop design was applied. A statistical analysis was carried out using a clone-by-clone mixed model approach, applied to each technical replicate. P-values for each clone were calculated as an average of P-values across technical replicates. Eight clones met the significance criteria and, on the basis of their sequences BLASTed against the chicken genome sequence, four candidate genes (*HSC70*, *MBP*, *APOA1*, *TLR7*) were

selected for further detailed analysis. To increase a statistical power the joint analysis of all three technical replicates for each clone was applied. The clones and genes obtained after this alternative statistical analysis were not further analysed.
Realtime quantitative RT-PCR carried out for all eleven candidate genes revealed a different expression for five of them (P<0.05): *TPH2*, *PRKG1*, *HSC70*, *MBP*, *APOA1*.

The two main conclusions that can be drawn in this study are:

- There is a significant difference in exploratory behaviour between the High Feather Pecking and Low Feather Pecking line (in line with the hypothesis of feather pecking being redirected exploratory behaviour)
- There is a significant difference in gene expression of five candidate genes. A general tendency for lower expression of almost all analysed candidate genes in the High Feather Pecking line was observed during realtime quantitative RT-PCR

This study was conducted as a comprehensive effort to elucidate the genetic foundations of the complex trait feather pecking. The results of the study have revealed some very interesting differences in the behaviour and gene expression in the two observed lines (High and Low Feather Pecking) which should be further analysed in the future.

6.2 *Zusammenfassung*

Federpicken ist ein komplexes Merkmal, welches durch angeborene Faktoren wie genetische Prädisposition oder physiologische Regelmechanismen und Umweltfaktoren wie Fütterung, Platzangebot und andere Haltungsbedingungen verursacht wird. Ziel dieser Untersuchung war es, potentielle Kandidatengene für das Federpicken bei Leghennen zu identifizieren und zu charakterisieren. Um die mögliche genetische Prädisposition zu dieser komplexen Eigenschaft feststellen zu können, wurde als Grundlage für diese Studie angenommen, dass Federpicken ein umorientiertes Erkundungsverhalten darstellt, welches auftritt wenn kein normales Erkundungsverhalten möglich ist. Die Unterschiede im angeborenen Erkundungsverhalten können am besten bei frisch geschlüpften Kücken in einer gut definierten und leicht kontrollierbaren Umgebung gemessen werden.

Untersucht wurde das Erkundungsverhalten bei zwei Hennenlinien, die sich durch die Veranlagung zum Federpicken unterscheiden (hohe bzw. niedrige Veranlagung zum Federpicken). Die Beobachtungen frisch geschlüpfter Küken aus beiden Linien in einer gut definierten Umwelt haben Aktivitätsunterschiede in der Lokomotion, gemessen anhand der Art der Erkundungsverhaltenepisoden, aufgezeigt. Die Zahl der Erkundungsverhaltenepisoden war bedeutend höher bei den Küken aus der Linie mit hoher Veranlagung zum Federpicken ($P<0.001$). Es wurden weiterhin Blut- und Hirnproben entnommen um molekulargenetische Analysen durchzuführen.

Sieben potentielle Kandidatengene wurden aufgrund der Literatur (*HOXB8*, *TPH1*, *TPH2*, *ACADS*, *CRHR1*, *PRKG1*, *PRKG2*) identifiziert. In der Protein-kodierende Region von fünf Kandidatengenen (*HOXB8*, *TPH1*, *TPH2*, *ACADS*, *CRHR1*) wurde nach Polymorphismen gesucht. Alle identifizierten Polymorphismen hatten keinen Einfluss auf die Aminosäuresequenz.

RNA aus Kükengehirnen der beiden Linien wurde einem Mikroarray-Experiment mit dem neuroendokrinen Array von ARK-Genomics unterzogen. Dazu wurde das Loop-Design genutzt. Zur statistischen Analyse wurde ein "clone-by-clone" gemischtes Model gewählt, welches für jedes technische Replikat verwendet wurde. Die P-Werte für jeden Klon wurden als Mittelwert des P-Wertes der technischen Replikate berechnet. Acht Klonen

erreichten die Signifikanzgrenze. Mit Hilfe ihrer Sequenz, die gegen das Hühnergenom geBLASTet wurde, konnten vier Gene (*HSC70*, *MBP*, *APOA1*, *TLR7*) für die weitere Untersuchung ausgewählt werden. Um die statistische Power der Auswertung des Microarray - Experimentes zu erhöhen wurde eine gemeinsame Analyse der drei technischen Replikate durchgeführt. Die dadurch erhaltenen Klone und Gene wurden allerdings nicht weitergehend untersucht.
Realtime RT-PCR Analysen mit allen elf Kandidatengenen, ergaben eine unterschiedliche Expression für fünf dieser Gene ($P<0.05$): *TPH2*, *PRKG1*, *HSC70*, *MBP*, *APOA1*.

Aus der oben beschriebenen Untersuchung können zwei grundsätzliche Schlussfolgerungen gezogen werden:

- Es besteht ein wesentlicher Unterschied im Erkundungsverhalten zwischen Tieren der Linien mit hoher und niedriger Veranlagung für das Federpicken. Dies stützt die Hypothese, dass es sich beim Federpicken um ein umorientiertes Erkundungsverhalten handelt.
- Es gibt signifikante Unterschiede in der Genexpression von fünf Kandidatengenen. Eine generelle Tendenz zu einer erniedrigten Expression von nahezu allen untersuchten Kandidatengenen in der Linie mit hoher Veranlagung für das Federpicken wurde bei der Real time quantitative RT-PCR festgestellt.

Das Bestreben dieser Studie war es in einem umfassenden Ansatz die genetische Grundlage für das komplexe Merkmal des Federpickens zu untersuchen. Die gewonnenen Ergebnisse zeigen sehr interessante Unterschiede im Verhalten und in der Genexpression zwischen den beiden Linien mit hoher und niedriger Veranlagung für das Federpicken, welche in Zukunft weitergehend untersucht werden sollten.

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8 Bibliography

Acampora, D., D'Esposito, M., Faiella, A., Pannese, M., Migliaccio, E., Morelli, F., Stornaiuolo, A., Nigro, V., Simeone, A., and Boncinelli, E. (1989). The human HOX gene family. Nucleic Acids Res *17*, 10385-10402.

Aguilera, G., Nikodemova, M., Wynn, P. C., and Catt, K. J. (2004). Corticotropin releasing hormone receptors: two decades later. Peptides *25*, 319-329.

Albentosa, M. J., Kjaer, J. B., and Nicol, C. J. (2003). Strain and age differences in behaviour, fear response and pecking tendency in laying hens. Br Poult Sci *44*, 333-344.

Appleby, M. C. (1998). Modification of Laying Hen Cages to Improve Behavior. Poultry Science *77*, 1828–1832.

Ball, C. A., Brazma, A., Causton, H., Chervitz, S., Edgar, R., Hingamp, P., Matese, J. C., Parkinson, H., Quackenbush, J., Ringwald, M.*, et al.* (2004). Submission of microarray data to public repositories. PLoS Biol *2*, E317.

Ball, C. A., Sherlock, G., Parkinson, H., Rocca-Sera, P., Brooksbank, C., Causton, H. C., Cavalieri, D., Gaasterland, T., Hingamp, P., Holstege, F.*, et al.* (2002). Standards for microarray data. Science *298*, 539.

Bassett, D. E., Eisen, M. B., and Boguski, M. S. (1999). Gene expression informatics—it's all in your mine. Nat Genet *21*, 51-55.

Beare, R., and Buckley, M. (2004). Spot: cDNA Microarray Image Analysis Users Guide. CSIRO Mathematical and Information Sciences.

Ben-Dor, A., Hartman, T., Karp, R. M., Schwikowski, B., Sharan, R., and Yakhini, Z. (2004). Towards optimally multiplexed applications of universal arrays. J Comput Biol *11*, 476-492.

Ben-Shahar, Y. (2005). The foraging gene, behavioral plasticity, and honeybee division of labor. J Comp Physiol A *191*, 987–994.

Ben-Shahar, Y., Leung, H. T., Pak, W. L., Sokolowski, M. B., and Robinson, G. E. (2003). cGMP-dependent changes in phototaxis: A possible role for the foraging gene in honey bee division of labour. J Exp Biol *206*, 2507-2515.

Ben-Shahar, Y., Robichon, A., Sokolowski, M. B., and Robinson, G. E. (2002). Influence of gene action across different time scales on behaviour. Science *296*, 741-744.

Berrettini, W. H., Harris, N., Ferraro, T. N., and Vogel, W. H. (1994). Maudsley reactive and non-reactive rats differ in exploratory behavior but not in learning. Psychiatr Genet *4*, 91-94.

Bessei, W. (1986). Pecking and feather loss – genetical aspects. Zootecnica Int, 56-59.

Bestman, M. W. P., and Wagenaar, J. P. (2003). Farm level factors associated with feather pecking in organic laying hens. Livst Prod Sci *80*, 133-140.

Bilcik, B., and Keeling, L. J. (2000). Relationship between feather pecking and ground pecking in laying hens and the effect of group size. Appl Anim Behav Sci *68*, 55-66.

Blokhuis, H. J., and Arkes, J. G. (1984). Some observations on the development of feather-pecking in poultry. Appl Anim Behaviour Sci *12*, 145–157.

Blokhuis, H. J., and Wiepkema, P. R. (1998). Studies of feather pecking in poultry. Vet Q *20*, 6-9.

Bowtell, D. D. (1999). Options available--from start to finish--for obtaining expression data by microarray. Nat Genet *Jan;21(1 Suppl) Erratum in: Nat Genet 1999 Feb;21(2)*, 25-32.

Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C. A., Causton, H. C.*, et al.* (2001). Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet *29*, 365-371.

Brookes, A., Landegren, U., Syvänen, A.-C., Issacson, A., and Gyllensten, U. (1998). SNP attack on complex traits. Nature Genetics Editorial - first International Meeting on Single Nucleotide Polymorphism and Complex Genome Analysis Skokloster, Sweden, 29 August-1 September, 1998 *20*, 217-218.

Brösamle, C., and Halpern, M. E. (2002). Characterization of Myelinationin the Developing Zebrafish. Wiley-Liss, Inc *39*.

Brown, P. O., and Botstein, D. (1999). Exploring the new world of the genome with DNA microarrays. Nat Genet *21*, 33-37.

Buitenhuis, A. J., Rodenburg, T. B., Siwek, M., Cornelissen, S. J. B., Nieuwland, M. G. B., Crooijmans, R. P. M. A., Groenen, M. A. M., Koene, P., Bovenhuis, H., and van der Poel, J. J. (2003b). Identification of Quantitative Trait Loci for Receiving Pecks in Young and Adult Laying Hens. Poultry Science: *82*, 1661-1667.

Buitenhuis, A. J., Rodenburg, T. B., van Hierden, Y. M., Siwek, M., Cornelissen, S. J., Nieuwland, M. G., Crooijmans, R. P., Groenen, M. A., Koene, P., Korte, S. M.*, et*

al. (2003a). Mapping quantitative trait loci affecting feather pecking behavior and stress response in laying hens. Poult Sci *82*, 1215-1222.

Bustin, S. A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology *25*, 169-193.

Cheng, H. W., Singleton, P., and Muir, W. M. (2003). Social stress differentially regulates neuroendocrine responses in laying hens: I. Genetic basis of dopamine responses under three different social conditions. Psychoneuroendocrinology *28*, 597-611.

Cheung, V. G., and Spielman, R. S. (2002). The genetics of variation in gene expression. Nat Genet *32*, 522-525.

Church, G. M., and Gilbert, W. (1984). Genomic sequencing. Proc Natl Acad Sci U S A *81(7)*, 1991-1995.

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

Cox, W. G., Beaudet, M. P., Agnew, J. Y., and Ruth, J. L. (2004). Possible sources of dye-related signal correlation bias in two-color DNA microarray assays. Anal Biochem *331*, 243-254.

Crusio, W. E. (2001). Genetic dissection of mouse exploratory behaviour. Behav Brain Res *125*, 127-132.

Dale, J. W., and Schantz, M. (2003). From Genes to Genomes. John Wiley and Sons Ltd, Italy, pp.39, 227-228, 231-234.

Day, J. E. L., Kyriazakis, I., and Lawrence, A. B. (1995). The effect of food deprivation on the expression of foraging and exploratory behaviour in the growing pig. Appl Anim Behav Sci *42*, 193-206.

de Belle, J. S., Hilliker, A. J., and Sokolowski, M. B. (1989). Genetic localization of foraging (for): A major gene for larval behaviour in Drosophila melanogaster. Genetics *123*.

de la Rosa, E. J., Vega-Nunez, E., Morales, A. V., Serna, J., Rubio, E., and de Pablo, F. (1998). Modulation of the chaperone heat shock cognate 70 by embryonic (pro)insulin correlates with prevention of apoptosis. Proc Natl Acad Sci U S A *95*, 9950-9955.

de Vente, J., Asan, E., Gambaryan, S., Markerink van Ittersum, M., Axer, H., Gallatz, K., Lohmann, S. M., and Palkovits, M. (2001). Localization of cGMP- dependent protein kinase type II in rat brain. Neuroscience *108*, 27-49.

DeStefano, J. J., Buiser, R. G., Mallaber, L. M., Myers, T. W., Bambara, R. A., and Fay, P. J. (1991). Polymerization and RNase H activities of the reverse transcriptases from avian myeloblastosis, human immunodeficiency, and Moloney murine leukemia viruses are functionally uncoupled. Journal of Biological Chemistry *266*, 7423-7431.

Diatchenko, L., Anderson, A. D., Slade, G. D., Fillingim, R. B., Shabalina, S. A., Higgins, T. J., Sama, S., Belfer, I., Goldman, D., Max, M. B.*, et al.* (2006). Three major haplotypes of the 2 adrenergic receptor define psychological profile, blood pressure, and the risk for development of a common musculoskeletal pain disorder. Wiley-Liss, Inc.

Ding, Y., and Wilkins, D. (2004). The Effect of Normalization on Microarray Data Analysis. DNA and Cell Biology *23*, 635-642.

Divne, A. M., and Allen, M. (2005). A DNA microarray system for forensic SNP analysis. Forensic Sci Int *154*, 111-121.

Dombkowski, A. A., Thibodeau, B. J., Starcevic, S. L., and Novak, R. F. (2004). Gene-speci¢c dye bias in microarray reference designs. FEBS Letters *560*, 120-124.

Dong, H., Wade, M., Williams, A., Lee, A., R., G., Douglas, G. R., and Yauk, C. (2005). Molecular insight into the effects of hypothyroidism on the developing cerebellum. Biochemical and Biophysical Research Communications *330,* 1182–1193.

Dudoit, S., Yang, Y. H., Callow, M. J., and Speed, T. P. (2000). Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments. Statistica Sinica.

El-lethey, H., Jungi, T. W., and Huber-Eicher, B. (2001). Effects of feeding corticosterone and housing conditions on feather pecking in laying hens (Gallus gallus domesticus). Physiol Behav *73*, 243-251.

Enard, W., Khaitovich, P., Klose, J., Zollner, S., Heissig, F., Giavalisco, P., Nieselt-Struwe, K., Muchmore, E., Varki, A., Ravid, R.*, et al.* (2002). Intra- and interspecific variation in primate gene expression patterns. Science *296*, 340-343.

Ewing, B., and Green, P. (1998b). Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res *8(3)*, 186-194.

Ewing, B., Hillier, L., Wendl, M. C., and Green, P. (1998a). Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res *8(3)*, 175-185.

Fitzpatrick, M. J., and Sokolowski, M. B. (2004). In search of food: Exploring the evolutionary link between cGMP-dependent protein kinase (PKG) and behaviour. Integr Comp Biol *44*, 28-36.

Galef, B. G. (1988). Imitation in animals: history, definition, and interpretation of data from the psychological laboratory. In: Social Learning: Psychological and Biological Perspectives (Ed by T R Zentall & B G Galef), 3-28.

Gerard, G. F., Fox, D. K., Nathan, M., and D'Alessio, J. M. (1997). Reverse transcriptase: The use of cloned Moloney murine leukemia virus reverse transcriptase to synthesize DNA from RNA. Molecular Biotechnology *8*, 61-77.

Gershon, D. (2002). Microarray technology: an array of opportunities. Nature *416*, 885-891.

Ginzinger, D. G. (2002). Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp Hematol *30*, 503-512.

Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R., and Mathieu, C. (2001). An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods *25*, 386-401.

Gordon, D., Abajian, C., and Green, P. (1998). Consed: A Graphical Tool for Sequence Finishing. Genome Res *8*, 195 - 202.

Greer, J. M., and Capecchi, M. R. (2002). Hoxb8 is required for normal grooming behavior in mice. Neuron *33*, 23-34.

Gupta, R., Truong, L., D., B., Chafik, D., Modafferi, E., and Hung, C. T. (2005). Shear stress alters the expression of myelin-associated glycoprotein (MAG) and myelin basic protein (MBP) in Schwann cells. Journal of Orthopaedic Research *23*, 1232-1239.

Habermann, F. A., Biet, P., Fries, R. (2001). Physical mapping of the genes encoding tryptophan hydroxylase and insulin to chicken chromosome 5. Anim Genet *32(5), 319-321*.

Higuchi, R., Fockler, C., Dollinger, G., and Watson, R. (1993). Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology *11*, 1026-1030.

Hiroi, R., McDevitt, R. A., and Neumaier, J. F. (2006). Estrogen Selectively Increases Tryptophan Hydroxylase-2 mRNA Expression in Distinct Subregions of Rat Midbrain Raphe Nucleus: Association between Gene Expression and Anxiety Behavior in the Open Field. BIOL PSYCHIATRY *in press*.

Hocking , P. M., Channing, C. E., Robertson, G. W., Edmond, A., and Jones, R. B. (2004). Between breed genetic variation for welfare-related behavioural traits in domestic fowl. Appl Anim Behav Sci *89*, 85-105.

Holm, S. (1979). A simple sequentially rejective multiply test procedure. Scand J Stat *6, 65-70.*

Holloway, A. J., van Laar, R. K., Tothill, R. W., and Bowtell, D. D. L. (2002). Options available—from start to finish—for obtaining data from DNA microarrays II. Nature Genetics *32*, 481-489.

Huber-Eicher, B., and Sebo, F. (2001). Reducing feather pecking when raising laying hen chicks in aviary systems. Appl Anim Behav Sci *73*, 59-68.

Huber-eicher, B., and Wechsler, B. (1997). Feather pecking in domestic chicks: its relation to dustbathing and foraging. Anim Behav *54*, 757-768.

Huber-eicher, B., and Wechsler, B. (1998). The effect of quality and availability of foraging materials on feather pecking in laying hen chicks. Anim Behav *55*, 861-873.

Hughes, B. O., and Duncan, I. J. (1972). The influence of strain and environmental factors upon feather pecking and cannibalism in fowls. Br Poult Sci *13*, 525-547.

Hurnik, J. F., Webster, A. B., and Siegel, P. B. (1995). Dictionary of Farm Animal Behavior, second edition. Iowa State University Press.

Jensen, P., Keeling, L., Schütz, K., Andersson, L., Kerje, S., Carlborg, Ö., and Jacobson, L. (2003). Feather pecking in poultry – phenotypic correlations and QTL-analysis in F2-intercross between red jungle fowl and white leghorn layers. Proceedings of the 37th ISAE International Congress In Ferrante V (Ed) Fondazione Iniziative Zooprofilattiche e Zootecniche, Brescia, Abano Terme Italy, June 24-28, 68.

Jones, R. B. (2002b). Practical solutions for feather pecking. Farm Animal Welfare: Curren resaerch and future directions.

Jones, R. B., Blokhuis, H. J., and Beuving, G. (1995). Open-field and tonic immobility responses in domestic chicks of two genetic lines differing in their propensity to feather peck. Br Poult Sci *36*, 525-530.

Jones, R. B., Carmichael, N. L., and Rayner, E. (2000). Pecking preferences and pre-dispositions in domestic chicks: implications for the development of environmental enrichment devices. Appl Anim Behav Sci *69*, 291-312.

Jones, R. B., McAdie, T. M., McCorquodale, C., and Keeling, L. J. (2002a). Pecking at other birds and at string enrichment devices by adult laying hens. Br Poult Sci *43*, 337-343.

Katsuki, M., Sato, M., Kimura, M., Yokohama, M., Kobayashi, K., and Nomura, T. (1988). Conversion of normal behavior to *shiverer* by myelin basic protein antisense cDNA in transgenic mice. Science *241*, 593-595.

Keeling, L., Andersson, L., Schutz, K. E., Kerje, S., Fredriksson, R., Carlborg, O., Cornwallis, C. K., Pizzari, T., and Jensen, P. (2004). Chicken genomics: feather-pecking and victim pigmentation. Nature *431*, 645-646.

Keeling, L. J., Estevez, I., Newberry, R. C., and Correia, M. G. (2003). Production-related traits of layers reared in different sized flocks: the concept of problematic intermediate group sizes. Poult Sci *82*, 1393-1396.

Kerr, M. K., and Churchill, G. A. (2001). Experimental design for gene expression microarrays. Biostatistics *2*, 183-201.

Kimball, J. W. (2005). Innate Behaviour. Retrieved August 01, 2005, from http://usersrcncom/jkimballmaultranet/BiologyPages/I/InnateBehaviorhtml.

Kimura, M., Sato, M., Akatsuke, A., Saito, S., Ando, K., Yokohama, M., and Katsuki, M. (1998). Overexpression of a minor component of myelin basic protein isoform (17.2 kDa) can restore myelinogenesis in transgenic *shiverer* mice. Brain Research 1998 *785*, 245 – 252.

Kjaer, J. B. (2000). Diurnal rhythm of feather pecking behaviour and condition of integument in four strains of loose housed laying hens. Appl Anim Behav Sci *65*, 331-347.

Kjaer, J. B., Hjarvard, B. M., Jensen, K. H., Hansen-Müller, J., and Naesbye Larsen, O. (2004). Effects of haloperidol, a dopamine D2 antagonist, on feather pecking behaviour in laying hens. Appl Anim Behav Sci *86*, 77-91.

Kjaer, J. B., and Sorensen, P. (1997). Feather pecking behaviour in White Leghorns, a genetic study. Br Poult Sci *38*, 333-341.

Kjaer, J. B., and Sörensen, P. (2002). Feather pecking and cannibalism in free-range laying hens as affected by genotype, dietary level of methionine + cystine, light intensity during rearing and age at first access to the range area. Appl Anim Behav Sci 21-39 *76*, 21-39.

Kjaer, J. B., Sorensen, P., and Su, G. (2001). Divergent selection on feather pecking behaviour in laying hens (Gallus gallus domesticus). Appl Anim Behav Sci *71*, 229-239.

Kjaer, J. B., and Vestergaard, K. S. (1999). Development of feather pecking in relation to light intensity. Appl Anim Behav Sci 243-254 *62*, 243-254.

Kogut, M. H., Iqbal, M., He, H., Philbin, V., Kaiser, P., and Smith, A. (2005). Expression and function of Toll-like receptors in chicken heterophils. Dev Comp Immunol *29*, 791-807.

Korte, S. M. (2001). Corticosteroids in relation to fear, anxiety and psychopathology. Neurosci Biobehav Rev *25*, 117-142.

Korte, S. M., Beuving, G., Ruesink, W., and Blokhuis, H. J. (1997). Plasma catecholamine and corticosterone levels during manual restraint in chicks from a high and low feather pecking line of laying hens. Physiol Behav *62*, 437-441.

Kotewicz, M. L., Sampson, C. M., D'Alessio, J. M., and Gerard, G. F. (1988). Isolation of cloned Moloney murine leukemia virus reverse transcriptase lacking ribonuclease H activity. Nucleic Acids Research *16*, 265-277.

Ladjali-Mohammedi, K., Grapin-Botton, A., Bonnin, M. A., LeDouarin, N. M. (2001). Distribution of HOX genes in the chicken genome reveals a new segment of conservation between human and chicken. Cytogenet Cell Genet *92:157–161 (2001).*

Lamon-Fava, S., Rajeswari, S., Ferrari, S., Rajavashisth, T. B., Lusis, A. J., and Karathanasis, S. K. (1992). Evolutionary distinct mechanisms regulate apolipoprotein A-I gene expression: differences between avian and mammalian apoA-l gene transcription control regions. Journal of Lipid Research *33*.

Lander, E. S. (1999). Array of hope. Nat Genet *21*, 3-4.

Leonard, M. L., Horn, A. G., and Fairfull, R. W. (1995). Correlates and consequences of allopecking in White Leghorn chickens. Appl Anim Behav Sci *43*, 17-26.

Lufkin, T. (2003). Hox genes and embryonic development. In Nature Encyclopedia of the Human Genome, D. N. Cooper, ed. (London, Nature Publishing Group), pp. Chapter 46.

MacBeath, G. (2002). Protein microarrays and proteomics. Nat Genet *32*, 526-532.

Martin-Magniette, M. L., Aubert, J., Cabannes, E., and Daudin, J. J. (2005). Evaluation of the gene-specific dye bias in cDNA microarray experiments. Bioinformatics *21*, 1995-2000.

McAdie, T. M., and Keeling, L. J. (2002). The social transmission of feather pecking in laying hens: effects of environment and age. Appl Anim Behav Sci *75*, 147-159.

McKeegan, D. E., and Savory, C. J. (2001). Feather eating in individually caged hens which differ in their propensity to feather peck. Appl Anim Behav Sci *73*, 131-140.

McKeegan, D. E., Savory, C. J., MacLeod, M. G., and Mitchell, M. A. (1999). Is development of pecking damage in layer pullets influenced by dietary protein source? Br Poult Sci *40 Suppl*, S14-15.

McKeegan, D. E., Savory, C. J., MacLeod, M. G., and Mitchell, M. A. (2001). Development of pecking damage in layer pullets in relation to dietary protein source. Br Poult Sci *42*, 33-42.

McKinney, J, Knappskog, P. M., Haavik, J. (2005). Different properties of central and peripheral forms of human tryptophan hydroxylase. J Neurochem *92(2), 311-320.*

Mein, C. A., Barratt, B. J., Dunn, M. G., Siegmund, T., Smith, A. N., Esposito, L., Nutland, S., Stevens, H. E., Wilson, A. J., Phillips, M. S*., et al.* (2000). Evaluation of single nucleotide polymorphism typing with invader on PCR amplicons and its automation. Genome Res *10*, 330-343.

Mikheeva, S., Barrier, M., Little, S. A., Beyer, R., Mikheev, A. M., Kerr, K., E., P., and Mirkes, P. E. (2004). Alterations in Gene Expression Induced in Day-9 Mouse Embryos Exposed to Hyperthermia (HS) or 4-Hydroperoxycyclophosphamide (4CP): Analysis Using cDNA Microarrays. Toxicological Sciences *79*, 345-359.

Mills, J. C., Roth, K. A., Cagan, R. L., and Gordon, J. I. (2001). DNA microarrays and beyond: completing the journey from tissue to cell. NATURE CELL BIOLOGY *3*, 175-178.

Moody, D. C. (). Genomes techniques. An overviw of methods for the study of gene expression. J Anim Sci *79, 128-135*.

Muller, M. B., and Wurst, W. (2004). Getting closer to affective disorders: the role of CRH receptor systems. Trends Mol Med *10*, 409-415.

Newberry, R. C. (1999). Exploratory behaviour of young domestic fowl. Appl Anim Behav Sci *63*, 311-321.

Nickerson, D. A., Tobe, V. O. a., and Taylor, S. L. (1997). PolyPhred: automating the detection
and genotyping of single nucleotide substitutions using fluorescence-based resequencing. Nucleic Acids Res *25(14)*, 2745-2751.

Nicol, C. J. (1995). The social transmission of information and behaviour. Appl Anim Behav Sci *44*, 79-98.

Nicol, C. J., Gregory, N. G., Knowles, T. G., Parkman, I. D., and Wilkins, L. J. (1999). Differential effects of increased stocking density, mediated by increased flock size, on feather pecking and aggression in laying hens. Appl Anim Behav Sci *65*, 137-152.

Nicol, C. J., Lindberg, A. C., Phillips, A. J., Pope, S. J., Wilkins, L. J., and Green, L. E. (2001). Influence of prior exposure to wood shavings on feather pecking, dustbathing and foraging in adult laying hens. Appl Anim Behav Sci *73*, 141-155.

Oden, K., Vestergaard, K. S., and Algers, B. (1999). Agonistic behaviour and feather pecking in single-sexed and mixed groups of laying hens. Appl Anim Behav Sci *62*, 219-231.

Osborne, K. A., Robichon, A., Burgess, E., Butland, S., Shaw, R. A., Coulthard, A., Pereira, H. S., Greenspan, R. J., and B., S. M. (1997). Natural behaviour polymorphism due to a cGMPdependent protein kinase of Drosophila. Science *277*, 834-836.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res *29*, e45.

Pfaffl, M. W. (2004b). A-Z of quantitative PCR. Quantification strategies in real-time PCR. International University Line (IUL), La Jolla, CA, USA Editor: Bustin, SA *Chapter 3*.

Pfaffl, M. W., Tichopad, A., Prgomet, C., and Neuvians, T. P. (2004a). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechnol Lett *26*, 509-515.

Pinheiro, J., Bates, D., DebRoy, S., Deepayan, S. (2006). The nlme Package. Linear and nonlinear effects models.

Pizarro, J. M., Haro, L. S., and Barea-Rodriguez, E. J. (2003). Learning associated increase in heat shock cognate 70 mRNA and protein expression. Neurobiol Learn Mem *79*, 142-151.

Potzsch, C. J., Lewis, K., Nicol, C. J., and Green, L. E. (2001). A cross-sectional study of prevalence of vent pecking in laying hens in alternative systems and its associations with feather pecking, management and disease. Appl Anim Behav Sci *74*, 259-272.

Quackenbush, J. (2001). Computational analysis of microarray data. Nat Rev Genet *2*, 418-427.

Quackenbush, J. (2002). Microarray data normalization and transformation. Nat Genet *32 Suppl*, 496-501.

Rajavashisth, T. B., Dawson, P. A., Williams, D. L., Shackelford, J. E., Lebherz, H., and Lusis, A. J. (1987). Structure, Evolution, and Regulation of Chicken Apolipoprotein A-I. The Journal of Biological Chemistry *262*, 7058-7065.

Rappolee, D. A., Mark, D., Banda, M. J., and Werb, Z. (1988). Wound macrophages express TGF-alpha and other growth factors in vivo: analysis by mRNA phenotyping. Science *241*, 708–712.

Riedstra, B., and Groothuis, T. (2002). Early feathre pecking as a form of social exploration: the effect of group stability on feathre pecking and tonic immobility in domestic chicks. Appl Anim Behav Sci *77*, 127-138.

Robinson, G. E., and Ben-Shahar, Y. (2002). Social behavior and comparative genomics: new genes or new gene regulation? Genes, Brain and Behavior *1*, 197-203.

Rodenburg, T., Zimmerman, P., and Koene, P. (2002). Reaction to frustration in high and low feather pecking laying hens. Behav Processes *59*, 121.

Rodenburg, T. B., Buitenhuis, A. J., Ask, B., Uitdehaag, K. A., Koene, P., van der Poel, J. J., and Bovenhuis, H. (2003). Heritability of feather pecking and open-field response of laying hens at two different ages. Poult Sci *82*, 861-867.

Rodenburg, T. B., Koene, P., and Spruijt, B. M. (2004). Reaction to frustration in high and low feather pecking lines of laying hens from commercial or semi-natural rearing conditions. Behav Processes *65*, 179-188.

Rozen, S., and Skaletsky, H. J., pp (2000). Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ *Source code available at http://fokker.wi.mit.edu/primer3/*, 365-386.

Saito, S., Tachibana, T., Choi, Y., Denbowc, D. M., and Furuse, M. (2005). ICV melatonin reduces acute stress responses in neonatal chicks. Behavioural Brain Research *165*, 197–203.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating
inhibitors. Proc Natl Acad Sci U S A *74*, 5463-5467.

Savolainen, P., Fitzsimmons, C., Arvestad, L., Andersson, L., and Lundeberg, J. (2005). ESTs from brain and testis of White Leghorn and red junglfowl:annotation, bioinformatic classification of unknown transcripts and analysis of expression levels. Cytogenet Genome Res *111*, 79-87.

Savory, C. J., and Kostal, L. (2002). The role of adrenoreceptors in control of stereotyped oral behavior in restricted-fed fowls.

Savory, C. J., Mann, J. S., and MacLeod, M. G. (1999). Incidence of pecking damage in growing bantams in relation to food form, group size, stocking density, dietary tryptophan concentration and dietary protein source. Br Poult Sci *40*, 579-584.

Schaid, D. J. (2005). Power and Sample Size for Testing Associations of Haplotypes with Complex Traits. Annals of Human Genetics *70*, 116–130.

Sedlackova, M., Bilcik, B., and Kostal, L. (2004). Feather Pecking in Laying Hens: Environmental and Endogenous Factors. Acta Vet Brno *73*, 521-531.

Sheehan, K., Lowe, N., Kirley, A., Mullins, C., Fitzgerald, M., Gill, M., and Hawi, Z. (2005). Tryptophan hydroxylase 2 (TPH2) gene variants associated with ADHD. Mol Psychiatry.

Schena, M., Shalon, D., Davis, R. W., Brown, P. O. (1995). Quantitative monitoring of geneexpression patterns with a complementary DNA microarray. Science Oct *20, 270(5235), 467-70*.

Singh, V. K., Warren, R. P., Odell, J. D., Warren, W. L., and Cole, P. (1993). Antibodies to Myelin Basic Protein in Children with Autistic Behaviour. Brain, Behavior and Immunity *7*, 97-103.

Slonim, D. K. (2002). From patterns to pathways: gene expression data analysis comes of age. Nat Genet *32*, 502-508.

Smith, G. W., Aubry, J. M., Dellu, F., Contarino, A., Bilezikan, L. M., and Gold, L. H. (1998). Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. Neuron *20*, 1093-1102.

Smyth, G. K., and Speed, T. P. (2003). Normalization of cDNA microarray data. Methods *31*, 265-273.

Sokolowski, M. B. (1980). Foraging strategies of Drosophila melanogaster: A chromosomal analysis. Behav Gen *10*, 291-302.

Spellman, P. T., Miller, M., Stewart, J., Troup, C., Sarkans, U., Chervitz, S., Bernhart, D., Sherlock, G., Ball, C., Lepage, M., *et al.* (2002). Design and implementation of

microarray gene expression markup language (MAGE-ML). Genome Biol *3*, RESEARCH0046.

Stoeckert, C. J., Causton, C. J., and Ball, C. A. (2002). Microarray databases: standards and ontologies. Nat Genet *32*, 469-473.

Tafti, M., Petit, B., Chollet, D., Neidhart, E., de Bilbao, F., Kiss, J. Z., Wood, P. A., and Franken, P. (2003). Deficiency in short-chain fatty acid beta-oxidation affects theta oscillations during sleep. Nat Genet *34*, 320-325.

Tarca, A. L., Cooke, J. E. K., and Mackay, J. (2005). A robust neural networks approach for spatial and intensity-dependent normalization of cDNA microarray data. Bioinformatics *21*, 2674–2683.

Tsai, C., Chen, Y., and Chen, J. J. (2003). Testing for differentially expressed genes with microarray data. Nucleic Acids Research *31*.

van Hierden, Y. M., de Boer, S. F., Koolhaas, J. M., and Korte, S. M. (2004a). The control of feather pecking by serotonin. Behav Neurosci *118*, 575-583.

van Hierden, Y. M., Koolhaas, J. M., and Korte, S. M. (2004b). Chronic increase of dietary L-tryptophan decreases gentle feather pecking behaviour. Appl Anim Behav Sci *71*, 84-89.

van Hierden, Y. M., Koolhaas, J. M., Kost'al, L., Vyboh, P., Sedlackova, M., Rajman, M., Jurani, M., and Mechiel Korte, S. (2005). Chicks from a high and low feather pecking line of laying hens differ in apomorphine sensitivity. Physiol Behav *84*, 471-477.

van Hierden, Y. M., Korte, S. M., Ruesink, E. W., van Reenen, C. G., Engel, B., Korte-Bouws, G. A., Koolhaas, J. M., and Blokhuis, H. J. (2002). Adrenocortical reactivity and central serotonin and dopamine turnover in young chicks from a high and low feather-pecking line of laying hens. Physiol Behav *75*, 653-659.

Vestergaard, K. S., Krujitt, K. S., and Hogan, J. A. (1993). Feather pecking and chronic fear in groups of red junglefowl: their relations to dustbathing, rearing environment and social status. Anim Behav *45*.

Vestergaard, K. S., Skadhauge, E., and Lawson, L. G. (1997). The stress of not being able to perform dustbathing in laying hens. Physiol Behav *62*, 413-419.

Vollbach, H., Heun, R., Morris, C. M., Edwardson, J. A., McKeith, I. G., Jessen, F., Schulz, A., Maier, W., and Kolsch, H. (2005). APOA1 polymorphism influences risk for early-onset nonfamiliar AD. Ann Neurol *58*, 436-441.

Walther, D. J., Peter, J., Bashammakh, S., Hörtnagl, H., Voits, M., Fink, H., and Bader, M. (2003). A unique central tryptophan hydroxylase isoform. Biochem Pharmacol *66*, 1673-1680.

Wang, D. G., Jian-Bing Fan, C.-J. S., Anthony Berno,, Peter Young, R. S., Ghassan Ghandour,, Nancy Perkins, E. W., Jessica Spencer,, Leonid Kruglyak, L. S., Linda Hsie,, Thodoros Topaloglou, E. H., Elizabeth Robinson,, Michael Mittmann, M. S. M., Naiping Shen,, Dan Kilburn, J. R., Chad Nusbaum, Steve Rozen,, Thomas J. Hudson, R. L., Mark Chee,, and Lander, E. S. (1998). Large-Scale Identification, Mapping, and Genotyping of Single-Nucleotide Polymorphisms in the Human Genome. Science *280*, 1077-1082.

Wang, J., He, X., Ruan, J., Dai, M., Chen, J., Zhang, Y., Hu, Y., Ye, C., Li, S., Cong, L.*, et al.* (2005). ChickVD: a sequence variation database for the chicken genome. Nucleic Acids Research *33*, 438-441.

Wang, L., Erlandsen, H., Haavik, J., Knappskog, P. M., and Stevens, R. C. (2002). Three-Dimensional Structure of Human Tryptophan Hydroxylase and Its Implications for the Biosynthesis of the Neurotransmitters Serotonin and Melatonin. Biochemistry *41*, 12749-12754.

Wang, T., and Brown, M. J. (1999). mRNA quantification by real time TaqMan polymerase chain reaction: validation and comparison with RNase protection. Analytical Biochemistry *269*, 198-201.

Wilcoxon, F. (1945). Individual comparisons by ranking methods. Biometrics Bulletin *1*, 80-83.

Wolfinger, R. D., Gibson, G., Wolfinger, E. D., Bennett, L., Hamadeh, H., Bushel, P., Afshari, C., and Paules, R. S. (2001). Assessing gene significance from cDNA microarray expression data via mixed models. J Comput Biol *8*, 625-637.

Wong, G. K., Liu, B., Wang, J., Zhang, Y., Yang, X., Zhang, Z., Meng, Q., Zhou, J., Li, D., Zhang, J.*, et al.* (2004). A genetic variation map for chicken with 2.8 million single-nucleotide polymorphisms. Nature *432*, 717-722.

Woo, Y., Krueger, W., Kaur, A., and Churchill, G. A. (2005). Experimental design for three-color and four-color gene expression microarrays. Bioinformatics *21*, 459-467.

Wu, H., and Churchill, G. A. (2005b). R/MAANOVA: An extensive R environment for the Analysis of Microarray Experiments.

Wu, H., Churchill, G. A., Kerr, K., and Cui, X. (2005a). The maanova Package. Tools for analyzing Micro Array experiments.

Yang, Y. H., Buckley, M. J., Dudoit, S., and Speed, T. P. (2000). Comparison of methods for image analyses on cDNA microarray data. Technical report.

Yang, Y. H., Xiao, Y., and Segal, M. R. (2005). Identifying differentially expressed genes from microarray experiments via statistic synthesis. Bioinformatics *21*, 1084–1093.

Zeltner, E., Klein, T., and Huber-Eicher, B. (2000). Is there social transmission of feather pecking in groups of laying hen chicks? Anim Behav *60*, 211-216.

Zhang, J., Zhang, W., Zou, D., Chen, G., Wan, T., Zhang, M., and Cao, X. (2002). Cloning and functional characterization of ACAD-9, a novel member of human acyl-CoA dehydrogenase family. Biochem Biophys Res Commun *297*, 1033-1042.

9 Appendices

9.1 Primers used for PCR and sequencing

Locus	No	Position	Sequence	No	Position	Sequence	Product size	T °C	Additi-ves
HOXB8	3168	5'UTR	TTCCTCCTCCTCCGTACAAAT	3169	Intron	GAAAAGCTGGGTCGGAGAA	441	60	
HOXB8	3170	Intron	AACCTACAGCCGCTACCAGA	3171	3'UTR	GGGCTCTCTACCCCAGCAG	329	60	
HOXB8	3172	Exon1	GAGACCTCGATCCTCCGTTT	3173	Exon2	GTCGGACTTGGTGCAGTACA	1000	60	
HOXB8	3252	Intron	TACCCTGAAGGAGCCCTTTT					60	
HOXB8	3271	Intron	GTAATTGGGACGCAACGAGT					60	
HOXB8	3253	Exon1	AAATGGAAAGAGCCCAGGAG				BAC	60	
HOXB8				3254	Exon2	CACCATCACCCTGTTCCTTT	BAC	60	
TPH1	3193	Exon4	TTTTGCAGACCTGGCTATGA	3194	Exon5	CCCACAGTATTTGGTGAGCA	1696	60	
TPH1	3195	Exon5	AAAAACTTGCCCTTGCTCAC	3196	Exon6	AGGGTCCGAGCTGTGTCTAA	713	60	
TPH1	3197	Exon6	CAGTCGCTGGCTATCTGTCA	3198	Exon7	CAAAAGAGGGACATGGCCTA	622	60	
TPH1	3199	Exon7	CATGAGCTCCTAGGCCATGT	3200	Exon8	TGCACAGGCCAAACTCTACA	1198	60	DMSO
TPH1	3272	Intron5	TCCATTGCGAATCACACAGT	3273	Intron5	TTGAGAATGGCCAACATTCA	280	60	
TPH1	3274	Intron6	GCTGTGCTTCCAGCTAAACC	3275	Exon7	TCACTGTTGCCAGTTTTTGG	230	60	
TPH1	3276	Intron7	CAAGTTTCCATGCTAGGAAAAAG	3277	Intron7	CCAGCTGGGACAGAAGCTAT	650	60	
TPH1	3564	5'UTR	CCTTGTGATTCAGATCCTTGC	3565	Intron1	GGGTGCCAAGCCTACTTTTA	244	60	
TPH1	3566	Intron1	TGTTTTTGCAGAATGCCACT	3567	Intron2	GCACGCTGAGAAGAGTGGTT	299	60	
TPH1	3568	Intron2	CACGCTTGGCGTTGTTAAT	3569	Intron3	GGGGAACGAGAGAGAGAAGG	247	60	DMSO
TPH1	3570	Intron3	TTCTTTACTGCGGCTTGAAAA	3571	Intron4	CCAGGGAACATGAACCAGTC	209	60	
TPH1	3572	Intron4	CAAGGAATGTCCCTTTGTCC	3573	Intron5	ACTGTGTGATTCGCAATGGA	291	60	
TPH1	3574	Intron5	GCAGAGATGGGAACATTATGG	3575	Intron6	TGAAAATAAGTTTTCTTGCTGGT	295	60	
TPH1	3576	Intron6	TCCATCACAAAGAGAGATGAGA	3577	Intron7	CAAATTAATTAGAGTGGGATCAGTTG	250	60	Q
TPH1	3578	Intron7	GAAAGTAGAAGTGCCCCCATC	3579	Intron8	ACATGGCAGAACAGCGACA	250	60	
TPH1	3580	Intron8	TCTTCTTCATGGATTCTTTTGG	3581	Intron9	AACACAGCAAGTCAAGAATGC	250	60	DMSO
TPH1	3582	Intron9	TGACTGCACATGGAGGATACA	3583	3'UTR	TGGACCTCTCACTCTTCCATT	373	60	
TPH2	3540	5'UTR	AGCGGCGTATGAGGTAAGC	3541	Intron1	TACCTGCAGCCGCAAAGTTA	348	60	DMSO
TPH2	3542	Intron1	TGTGTCTTACTTTTGGGAAATACC	3543	Intron2	CCAACAAAGAGCCTGACCTC	283	60	
TPH2	3544	Intron2	ACCAGAGGGGACCTTCATTT	3545	Intron4	GCACTCAAAACCAGCAGTCA	590	60	
TPH2	3546	Intron4	TGGTACCAGGTAAACACTTTAATGA	3547	Intron5	GGCTGCACCAGTGGTAGTTT	279	60	
TPH2	3548	Intron5	AATTCTGCCATCTGGAGTCG	3549	Intron6	AGCCTTTCCATTCAATACGC	367	60	
TPH2	3550	Intron6	GCAGAACATTGCCTGTCCTT	3551	Intron7	TTTTCAACCTTGGCTGGTTC	295	60	
TPH2	3552	Intron7	TTGATTATGGAATTACTATGGCATT	3553	Intron8	CCAACCAAAAAGGATGACAGA	248	60	
TPH2	3554	Intron8	CACCAATTGTCATGGCTGAA	3555	Intron9	TCAAGTCAAAATGTTTGCATCC	265	60	
TPH2	3556	Intron9	TGACTGAAGAAATACCGCACA	3557	3'UTR	CCATGTCACGTGATGTCACTA	577	60	
TPH2	3963	5'UTR	TATGTTCAAGGCGAGCCATC	3964	Intron1	AACTTTCGCTGTCGTTGAGG	593	60	
ACADS	3302	Exon3	GCCAGAGCAGTTCAAAGGAG	3303	Exon4	CTGGTGAAGGGAGAAATCCA	345	60	
ACADS	3304	Exon4	TGGATTTCTCCCTTCACCAG	3305	Exon5	ATTCAGCACCCACTCGTCAC	468	60	
ACADS	3306	Exon5	ACGTCTGGATGGTGACGAGT	3307	Exon6	TTGGGTATCCGACAGTCCTC	446	60	
ACADS	3317	Exon6	TCTTCCACTGCCAACCTGAT	3318	Exon8	CGCACTCTCTAATGCCACAG	1460	60	
ACADS	3319	Exon8	GTTAGCAGACATGGCTGTGG	3320	Exon10	TACGGTAGGCCTTCAGCAGT	1180	60	
ACADS	3321	Exon1	GTTGGCGTTGCTCGTGAC	3322	Exon2	AGCTCCACCGTCTGGTACAC	281	60	Q
ACADS	3367	5'UTR	TAAAGGAACCTCCGCATTTC	3368	Intron2	ACGGAACGTCTCTTGGTACG	771	60	
ACADS	3369	Intron2	GCATGTATTTGCTGGTGCAG	3370	Intron3	CCTCACACACAGCCTTGAGA	201	60	
ACADS	3371	Intron3	CCTGGGAGTTCATCGATTGT	3372	Intron4	TTACCTTGCAACAGGAATGG	210	60	
ACADS	3373	Intron4	TGGGCAGGTGATGGTTTTAC	3374	Intron5	CCAACTGACCCACTGAACAC	240	60	
ACADS	3375	Intron5	TGGGAAGTCAACACCATTCC	3376	Intron6	TGCTTTACACAAATGCACCTC	250	60	
ACADS	3377	Intron7	TGGGAGACTGCTGTGTGTTC	3378	Intron8	TCTGCCCAGAGCTGTCACTA	210	60	
ACADS	3379	Intron9	CGTGTGCTTCCTCCAGGTAT	3380	3'UTR	CATGAGGGGCTCACACTACA	832	60	
ACADS	3967	5'UTR	GGAACCTCCGCATTTCGTA	3968	Intron1	GCTCCACCGTCTGGTACAC	500	60	DMSO
ACADS	3969	5'UTR	TAAAGGAACCTCCGCATTTC	3970	Intron2	ACGGAACGTCTCTTGGTACG	771	60	DMSO
ACADS	3971	Intron9	TGCATGCAGTGAGATTAGTTCC	3972	3'UTR	CTTCCACCATGATCCTCAGC	295	60	
CRHR1	3446	5'UTR	CTGAGCACGAGGATTTGGAG	3447	Intron1	AAAGGGGCTCACCCTCAC	295	60	
CRHR1	3448	Intron1	AACCCTCAAGGTCCTCGACT	3449	Intron2	CTGTCACTTGTGCCCCATC	234	60	
CRHR1	3450	Intron2	AGACCCCTGCCTTACTGACA	3451	Intron3	TTCCATCACCCATTCCTCAC	250	60	
CRHR1	3452	Intron3	CTCTGGGTGGTGGAGGAG	3453	Intron4	CACCAGGTCTCCAGGTCTGT	247	60	
CRHR1	3454	Intron4	AGACACCCATTGTGCTCTGA	3455	Intron5	CAGCCACCAATCAGCACTAT	248	60	DMSO
CRHR1	3456	Intron5	CCATGCTGGTCTGTTTGTTG	3457	Intron6	AGAGCAGAGGATAGGGGTGAG	283	60	
CRHR1	3458	Intron6	CCTGTGCAAGAAGACCATCA	3459	Intron7	CAGGGAATTGGGACAGAGAA	184	60	
CRHR1	3460	Intron7	TTGTCCCTGACACCTCCTGT	3461	Intron8	AGATCCAGGTCCACGTATGG	204	60	
CRHR1	3462	Intron8	GCTGTGGGAACAGAGGAGAA	3463	Intron9	GCTCATCTGATTGGGCTGTT	229	60	Q
CRHR1	3464	Intron9	GACGGTCCCCATGCTCAG	3465	Intron10	TCCTCCACATCCCTTGGT	250	60	
CRHR1	3466	Intron10	GCCTTGAGGTTGCTTCTGAG	3467	Intron11	AGTGTGAGGCTGTGGATGTG	220	60	
CRHR1	3468	Intron11	GCAGCGTGCATAGCTCAGT	3469	3'UTR	CTGCAAGTTCGTGCATCCT	299	60	
CRHR1	3965	5'UTR	GGGAGGTTATAAAAGGCAGTGA	3966	Intron1	GAGCATGAGCTGAGGACACA	298	60	

9.2 Primers used for RT-PCR and realtime quantitative RT-PCR

GAPDH	3667	Exon1/2	TATCTTCCAGGAGCGTGACC	3668	Exon3/4	CGATGCATTGCTGACAATTT	229	60
GAPDH	3669	Exon5	CACACAGAAGACGGTGGATG	3670	Exon6/7	CTTGGCTGGTTTCTCCAGAC	214	60
GAPDH	3671	Exon2	GCAGATGCAGGTGCTGAGTA	3672	Exon5	TGACTTTCCCCACAGCCTTA	400	60
ACTB	3713	Exon1/2	CAGACATCAGGGTGTGATGG	3714	Exon2/3	CATGATCTGTGTCATCTTCTCTCTG	250	60
ACTB	3715	Exon3/4	CCAGCCATCTTTCTTGGGTA	3716	Exon4/5	GTGGGGCAATGATCTTGATT	212	60
ACTB	3717	Exon2	CCCAAAGCCAACAGAGAGAA	3718	Exon3/4	CAGGACTCCATACCCAAGAAA	484	60
ACTB	3719	Exon3	AGGCTGTGCTGTCCCTGTAT	3720	Exon5	AGGGTCCGGATTCATCGTA	693	60
HOXB8	3683	Exon1	CCAGGAGTCGGACTTGGTG	3684	Exon2	GAAGGGGCTTTCTTAAGGCTA	496	60
HOXB8	3172	Exon1	GAGACCTCGATCCTCCGTTT	4006	Exon1/2	CTCCTCCGTCCAGCGGCTT	200	60
TPH1	3685	Exon5/6	CTGAAAGAGCGCACAGGTTT	3686	Exon6/7	CATGGCAGGTATCAGGCTCT	173	60
TPH1	3977	Exon1/2	TCAGGAGAAGCATGTGAACCT	3978	Exon3/4	AAACCTGGGTGGTCAGCAT	297	60
TPH2	3687	Exon1/2	CGGCCTCACCATAAACAGAT	3688	Exon2/3	CTCACATGCTTCTCCTGGAA	177	60
TPH2	3975	Exon7/8	ACCCCAGAACCGGATACAT	3976	Exon8/9	TCAGAAAGAGCATGCTTTAGTTC	248	60
ACADS	3689	Exon3/4	TCATTGCCAGCGTCAATAAT	3690	Exon5/6	CACTAATGCCCCTTGTGCTT	298	60
ACADS	3979	Exon2/3	CCGAGCAGGTGAAGAAGATG	3980	Exon4/5	ACTGCCGTTCCCTGGTTC	281	60
ACADS	4000	Exon4	TGGATTTCTCCCTTCACCAG	4001	Exon5/6	GGAATGCACTAATGCCCTTG	226	60
ACADS	4002	Exon4/5	CTCTTAGTGAACCAGGGAAC	4001	Exon5/6	GGAATGCACTAATGCCCTTG	180	60
ACADS	4003	Exon6/7	TTCAAAATTGCCATGCAAAC	4004	Exon8	GCATAGCAGCTCTCCAGGTC	220	60
ACADS	4003	Exon6/7	TTCAAAATTGCCATGCAAAC	4005	Exon8	CCACAGCCATGTCTGCTAAC	180	60
CRHR1	3691	Exon3/4	TCAGTGAGGAGAAGAGGAGCA	3692	Exon6/7	GGGGATACACCAGCCAATG	398	60
CRHR1	3973	Exon1/2	CACACAGGACCTCAGTGCAA	3974	Exon3/4	GCTTGCTCCTCTTCTCCTCA	226	60
HSC70	4111	Exon1/2	ACCAACACAGTCTTCGATGC	4112	Exon2/3	TGACTACAGCATTAGTTACAGTCTTCC	244	60
HSC70	4113	Exon4/5	TGGCTTATGGTGCAGCTGTT	4114	Exon5/6	CACGTTCTCCTTCATATACCTGA	246	60
MBP	4115	Exon3	TGTCTCACCCCGTACTCCTC	4116	Exon5/6	AGCCAGAGCCTCCCAGTTTA	206	60
MBP	4117	Exon1/2	GGGGCTTTGGCAAAGGATATA	4118	Exon2/3	AGTACGGGGTGAGACAATGT	234	60
APOA1	4119	Exon1/2	GGCAAACAGCTTGACCTGA	4120	Exon2	GGAGAACTGGTCCAGGAAGG	201	60
TLR7	4121	Exon1/2	TGCACACCGGAAAATGGTA	4122	Exon2	CTGGAGAGATACGGGGGATA	203	60
TLR7	4123	Exon1/2	CACACCGGAAAATGGTACATC	4122	Exon2	CTGGAGAGATACGGGGGATA	246	60
TLR7	4124	Exon2	TGCATCACACCACCATCAAT	4125	Exon2	GAAACATTGCATGGATTACGG	242	60
PRKG1	3166	Exon4/5	TTTTAAAAAGTGTTCCCACATTC	3767	Exon6/7	GACGTTCACCTTTCCTTTGC	161	60
PRKG1	3768	Exon5/6	TCCTTGAAGAGACACACTATGAAA	3769	Exon7/8	CTCCCATTGCAGTGCTTTTT	188	60
PRKG1	3770	Exon7/8	GCAATGGGAGGATGTCAGAA	3771	Exon9/10	GCTTCGGCTTCATATTTTGC	161	60
PRKG1	3772	Exon4	GGCTATTGATCGGCAATGTT	3773	Exon4/5	CTGGAATGTGGGAACACTTTT	100	60
PRKG1	3774	Exon4	CTCTCGTAAATGTGAAACTTTGG	3775	Exon5	CTTCAAGGACATCTGCAAGC	168	60
PRKG2	3779	Exon9/10	GCGACATGCCAAAAGAAGAT	3780	Exon11/12	TGTGCGATACAGTTTCACAATG	313	60
PRKG2	3781	Exon12/13	GCTGAGAGACAGAGGCTGCT	3782	Exon14/15	AACGGTGGACTGCCAGTAAG	327	60
PRKG2	3783	Exon14/15	TACTGGCAGTCCACCGTTCT	3784	Exon16/17	AACCATTCAACCACCTGTGC	209	60
PRKG2	3785	Exon14	ATCGGATCAGGGCAGAAAA	3786	Exon15	GCCGCCTTGTTATTGTTCTC	235	60

9.3 Clones identified after BAC screening of HOXB8 and TPH1 gene

Clone	**Gene**	**Colony PCR test**	Isolated DNA	**TUM number**
CHORI26116N3				1147
CHORI26129J16				1148
CHORI2619O9				1149
CHORI26127I18				1150
CHORI26133C22	TPH1	X	X	1151
CHORI2613B12				1152
CHORI2619A8				1153
CHORI261112G18	TPH1	X	X	1154
CHORI261118D8				1155
CHORI261116O23				1156
CHORI261110M12				1157
CHORI261133J8				1158
CHORI261126E7				1159
CHORI261129E1				1160
CHORI261125F5				1161
CHORI261101B17				1162
CHORI261160O14				1163
CHORI261172K3	TPH1	X	X	1164
CHORI261146N6				1165
CHORI261163A4	TPH1	X	X	1166
CHORI261151M13	HOXB8	X	X	1167
CHORI261180M23	HOXB8	X	X	1168
CHORI261168M11	HOXB8	X	X	1169
CHORI261168M10	HOXB8	X	X	1170
CHORI261155P20				1171
CHORI261179H22	TPH1	X		1172
CHORI26156P10				1173
CHORI26185F8	TPH1	X		1174
CHORI26171H9				1175
CHORI26171E6				1176
CHORI26165G11	TPH1	X		1177

X-positively tested clones, clones used for DNA isolation

9.4 The list of keywords used in identification of candidate genes through literature

Abnormal behaviour
Aggression
Agonistic behaviour
Antagonistic behaviour
Cannibalism
Cannibalism chickens genetics
Corticotropin
Environmental enrichment
Exploratory behaviour
Feather pecking
Feather peckers
Foraging behaviour
Frustration
Grooming
Ground pecking
Hatching
Hatching environmental
Hormone realizing
Housing systems
Laying hen chickens
Life necessities finding
Locomotor activity
Mortality chickens
Newly hatched chickens
Novelty object seeking
Novelty seeking
Obsessive compulsive disorders
Obsessive grooming
Open-field behaviour

Physiology of birds

Plumage condition

Psycho disorders

Psycho disorders animals

QTL

Serotonin

Social behaviour

Social learning

Social transmission

Stereotypic behaviour

Stress response

Theta oscillation

Uncontrollable behaviour

Uncontrollable stress

9.5 Buffers used for preparation and running the formaldehyde-agarose gel prepared according to Qiagen instructions

- 10x Formaldehyde-Agarose (FA) Gel Buffer (pH 7.0)
    - 200 mM 3-[N-morpholino] propanesulfonic acid (MOPS) (free acid)
    - 50 mM sodium acetate
    - 10 mM EDTA
- 1x FA Gel Running Buffer:
    - 100 ml 10xFA gel buffer
    - 20 ml 37% (12.3M) formaldehyde
    - 880 ml RNase-free water
- 5x RNA Loading Buffer
    - 16 µl saturated aqueous bromophenol blue solution
    - 80 µl 500 mM EDTA, pH 8.0
    - 720 µl 37% (12.3M) formaldehyde
    - 2 ml 100% glycerol
    - 3084 µl formamide
    - 4 ml 10x FA gel buffer

9.6 The positional and structural information, including the accession numbers of the reference sequences available in NCBI (**) and Ensembl (*) databases for all candidate genes

Locus	Name	Chr. location GGA	Chr. location HS	Number of exons GGA	Number of exons HS	Reference sequences
HOXB8	Homeobox B 8	27	17	2	2	NM_204911**
TPH1	Tryptophan hydroxylase 1	5	11	10	10	NM_204956** ENSGALG00000006236*
TPH2	Tryptophan hydroxylase 2 (neuronal)	1	12	11	11	NM_001001301** ENSGALG00000010198*
ACADS	Acyl-coenzyme A dehydrogenase, short chain	15	12	10	10	NM_001006193** ENSGALG00000007072*
CRHR1	Corticotropin releasing hormone receptor 1	27	17	12	13	NM_204321** ENSGALG00000000371*
PRKG1	Protein kinase, cGMP-dependent, type I	6	10	19	18	ENSGALT00000006037*
PRKG2	Protein kinase, cGMP-dependent, type II	4	4	18	18	ENSGALG00000010909* XM_426309**
HSC70	Heat shock cognate 70	24	11	9	9	NM_205003** ENSGALG00000006512*
MBP	Myelin basic protein	2	18	7	9	NM_205280** ENSGALG00000013640*
APOA1	Apolipoprotein A-1	24	11	3	4	NM_205525** ENSGALG00000007114*
TLR7	Toll-like receptor 7	1	X	2	3	NM_001011688** ENSGALP00000026726*

9.7 *The genotypes of animals from the sequenced lines and breeds, considering identified polymorphisms*

	SNP numbers																									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*
Red Jungle Fowl	G	G	C	C	C	AG	AT	CT	C	CA	T	A	C	T	GCG	G	T	CT	CT	C	T	T	T	C	C	A
Malay	T	G	CT	C	C	AG	T	C	?	?	T	A	?	?	?	?	CT	C	T	C	C	C	?	C	C	A
Cochin	GT	G	T	C	C	A	?[1]	?	T	A	T	A	C	?	?	?	C	?	?	?	?	T	?	?	?	A
Fayoumi	G	G	C	C	C	G	T	C	?	CA	T	A	T	T	GCG	G	C	C	T	C	T	T	CT	T	T	G
Marans	T	G	C	T	T	A	A	T	?	C	T	A	T	CT	GCG	?	C	?	?	C	C	?	T	C	C	A
Tr. Naked Neck	G	A	C	C	C	G	T	C	C	CA	T	A	C	T	?	C	CT	C	T	C	T	T	?	C	C	A
Iceland landrace	G	A	C	C	C	G	?	?	CT	C	T	A	?	CT	GCG	G	T	C	T	C	T	T	T	?	?	A
Line Sarcoma S.	G	G	C	C	C	G	T	C	?	C	T	G	T	T	GCG	G	T	T	T	C	T	T	T	C	C	A
Green legged P.	T	G	C	C	C	G	AT	C	C	CA	T	AG	T	CT	no del[2]	G	T	C	T	CT	T	C	T	C	C	A
New Hampshire	GT	G	CT	T	CT	A	A	T	?	C	T	G	?	T	?	?	C	T	T	C	T	T	CT	C	C	A
LSL[3]	G	G	C	C	C	G	T	C	C	C	GT	A	T	CT	GCG	G	CT	CT	T	C	T	T	CT	C	C	A
LB[4]	G	G	CT	CT	C	A	A	T	T	CA	T	A	T	CT	no del	G	CT	CT	CT	C	CT	CT	T	C	C	A

[1] result for the particular breed or line not available

[2] no deletion occurred

[3] Lohmann Selected Leghorn (High Feather Pecking line)

[4] Lohmann Brown (Low Feather Pecking line)

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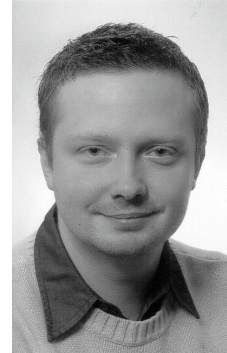

Personal Data

Age: 28

Date/Place of birth: 30.07.1978, Leszno

Family : Father Maciej, MD Mother Ewa, MD

Marital status: single

Education

- Chair of Genetics and Animal Breeding Technical University of Munich (Freising-Weihenstephan) –PhD studies
- 2003 M. Sc. Degree, Chair of Genetics and Animal Breeding – Agriculture University in Poznan
- 2001 Engineer - Faculty of Animal Breeding (Animal Husbandry)
- Since 1997 student of Faculty of Animal Breeding (University of Agriculture-Poznan). Finished with the first locate
- 1993-1997 High School in Poznan (finals with very good in biology, Polish and excellent in English)

Job experience

- Chair of Genetics and Animal Breeding Technical University of Munich (Freising-Weihenstephan), PhD student

- Dairy Cattle Management Seminar at California Polytechnic
- International Dairy Farm Training Program-one year in USA–coordinated by California Polytechnic
- Practice at polish farms and vet
- Practice: Agriculture School in Switzerland (Chateauneuf)

Trainings

- Wilde animals breeding course
- AI course in USA (ABS)
- English Language Course-BEET Language Centre (England)

Languages

- Polish – native language
- English – fluent written and spoken
- German – good written and spoken

Capabilities

- laboratory techniques : DNA and RNA isolation and handling, PCR, BAC libraries screening and BAC DNA preparation, basic skills and experience in cell culture laboratory, sequencing reaction, SNP detection, RealTime PCR, Microarrays
- knowledge of Windows tools including Office, Corel (office worker for two years during studies)
- knowledge of Mac OS X, Unix, MySQL, R
- agriculture and husbandry computer programs
- agriculture and husbandry practical skills
- driving licence

Conferences, publications

- ISAG (International Conference on Animal Genetics) Tokyo September 11-16 2004 – poster, “Identification of candidate genes for exploratory behaviour in chickens: results of a preliminary SNP analysis guided by bioinformatic methods“ Michal Wysocki, Olaf R.P. Bininda-Emonds, Michele Tixier-Boichard, Steffen Weigend, Ruedi Fries, Felix A. Habermann

- DGfZ and GfT meeting, 29/30 September 2004 in Rostock - presentation and abstract, “Identification of candidate genes for exploratory behaviour in chickens: a preliminary SNP analysis guided by bioinformatic methods. “ M. Wysocki, O.R.P. Bininda-Emonds, R. Fries, F.A. Habermann

- DGfZ und GfT meeting, 21/22 September 2005 in Berlin - presentation and abstract, “Polymorphism analysis and transcript quantification of candidate genes for feather pecking in chickens” M. Wysocki, O.R.P. Bininda-Emonds, R. Fries

- EPC 2006, 10-14 September 2006 in Verona – poster and publication in World’s Poultry Science Journal 62 supplement,, “Identification of candidate genes for feather pecking in chickens: evidence from behavioral and gene expression analyses” M. Wysocki, R. Preisinger, R. Fries

Hobby

music, film and history of the movie, dogs breeding, cooking, sport, foreign languages, traveling, fishing