Institut für Nutztierwissenschaften der Universität Hohenheim

Fachgebiet für Genetik und Züchtung landwirtschaftlicher Nutztiere

Prof. Dr. Jörn Bennewitz

Genetic Analyses of Feather Pecking and related Behavior Traits of Laying Hens

DISSERTATION

Zur Erlangung des Doktorgrades

der Agrarwissenschaft

vorgelegt von

VANESSA LUTZ

M. Sc. (Agr)

aus St. Johann

Baden-Württemberg

Hohenheim 2016

Die Dissertation wurde mit dankenswerter Unterstützung der Deutschen Forschungsgemeinschaft (DFG) angefertigt.

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GENERAL INTRODUCTION

Feather pecking is a well-known multi-factorial behavior problem in laying hens, which causes welfare problems, plumage damages and economic losses (Blokhuis and Van der Haar, 1989; Huber-Eicher and Wechsler, 1997; Blokhuis and Wiepkema, 1998; Rodenburg et al., 2008; Wysocki et al., 2010). It occurs in nearly all husbandry systems and is characterized by non-aggressive pecks directed towards the plumage of other hens (Kjaer et al., 2001). Feather pecking leads to feather damages and feather losses. Poor feather cover usually increase feed consumption and feed conversion rate due to a higher heat loss (Blokhuis and Wiepkema, 1998; Tauson and Svensson, 1980). In addition, pulling out feathers causes pain and leads to skin injuries, which increase the risk of cannibalism (Keeling, 1995). The importance of feather pecking and cannibalism on the economics and welfare in layer flocks is increasing as several EU countries announced a ban of beak trimming. The underlying mechanisms are not well understood, but physiological, nutritional as well as genetic factors are known to influence this trait (Wysocki et al., 2010). Aggressive pecking is clearly distinguishable from feather pecking. Aggressive pecks are delivered in an upright body posture and are mainly directed towards the head of the recipient birds (Bilcík and Keeling, 1999; Kjaer et al., 2001). A number of environmental conditions, physiological, nutritional as well as genetic and epigenetic factors are known to influence feather pecking (Su et al., 2005; Van Krimpen et al., 2005; Kjaer and Bessei, 2013; Wysocki et al., 2010; de Haas et al., 2014).

The main objektive of the present study was to study the genetic foundation of behaviour traits, especially feather pecking behaviour, and to infer ethological interrelationship between certain traits of laying hens. The data of two divergently selected lines for feather pecking behaviour were available, and additionally a large F2-cross, set up from these divergently selected lines, was established. Chickens of a White Leghorn layer line were divergently selected for high and low feather pecking for 11 generations. The selection started in the Danish Institute of Animal Sciences, Foulum, Denmark, for the first six generations (0-5) (Kjaer et al., 2001). Thereafter, five rounds of selection took place at the Institute of Animal Science, University of Hohenheim, Germany. The common base population of both lines was established in 1995 and derived from a foundation stock, which was created in 1970 as a control population in the Scandinavian selection and cross-breeding experiment of Liljedahl et al. (1979), see also Kjaer et al. (2001) and Su et al. (2005). Across all generations, the two lines showed a consistent difference in the mean trait value. In addition, the estimated overall

F_{ST} index, i.e. a measure of genetic differentiation, showed a value of 0.15 in generation 10. The large F2-cross was established from the 10th selection generation, and a comprehensive data collection of behaviour and performance traits of 960 hens was performed. These two big data sets were used in the following five research chapters.

In **chapter one**, a quantitative genetic analysis of fear traits and feather pecking as well as aggressive pecking using data from the large F2-cross was performed. The number of chicken with trait records varied between 867 and 912. Fear was recorded by the tonic immobility test, the open field activity and the emergence box test. These were recorded at a juvenile and adult age. The behavior traits feather pecking and aggressive pecking were recorded in groups of 36 to 40 animals at the age of 27 weeks. The actor and the receiver were recorded. The genetic parameters were estimated using a linear mixed model and inference about the relationship between the traits was done using genetic and phenotypic correlations.

In **chapter two**, we used generalized linear mixed models to estimate variance components, heritabilities of feather and aggressive pecking from three different observation periods. Each group of hens was observed in 21 sessions of 20 min each, distributed over three consecutive days. The number of recorded pecks and received pecks, respectively, in each 20 min observation period (short period) and in each observation day (medium period) formed two observation periods, which were analyzed with a Poisson model. The third observation period included the accumulated number of recorded pecks over the entire observation period of 420 min (long period), resulting in one observation per hen, and was analysed using a linear mixed model.

The object of **chapter three** was to analyze the interrelationship between feather pecking and feather eating as well as general locomotor activity using structural equation models. Such models allow to separate causal effects of phenotypes from the genetic and environmental correlations among traits. For the analysis, we used the collected data from the large F2-cross experiment. In total 897 chickens with records in all three traits were available. At the age of 18 weeks the general locomotor activity was recorded in groups of 185 to 275 birds using an electronic transponder. At the age of about 19 weeks, the feather eating test in individual cages were performed. We defined a structural equation model in which feather eating and general locomotor activity trigger feather pecking and feather eating affects general locomotor activity.

In **chapter four,** we performed a quantitative genetic analysis and mapped signatures of selection in the two divergently selected lines for feather pecking behavior. The two lines

were selected for low or high feather pecking for 11 generations. Pedigree and feather pecking records were available for the last six generations of both lines. From the 11th generation 75 birds, 41 high feather peckers and 34 low feather peckers were genotyped using the Illumina 60K chicken Infinium iSelect chip. A total of 57 636 SNPs were detected and after control checks, 33 228 remained for the statistical analyses. For the quantitative genetic analyses, we used a standard mixed linear model and a Poisson model; and, an F_{ST}-based approach was used to map selection signatures.

In **chapter five,** we used the data from the large F2-cross experiment to perform a genome-wide association study for discovering associations between SNP markers and feather pecking behavior, and to confirm the detected selection signatures reported in chapter four. Totally 817 F2-hens were genotyped with the Illumina 60K chicken Infinium iSelect chip. A total of 57.636 SNPs were detected and after quality control checks, 29 376 remained in the data set. We used single marker association analysis and a Poisson model. Additionally, a differential gene expression analysis was performed in order to identify potential and functional candidate genes.

The thesis ends with a general discussion and a summary.

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SUMMARY

The main objective of the present study was to study the genetic foundation of behaviour traits, especially feather pecking behaviour, and to infer ethological interrelationship between certain traits of laying hens. The data of two divergently selected lines for feather pecking behaviour was available, and additionally a large F2-cross, set up from these divergently selected lines, was established. Chickens of a White Leghorn layer line were divergently selected for high and low feather pecking for 11 generations. The selection started in the Danish Institute of Animal Sciences, Foulum, Denmark, for the first six generations (0-5). Thereafter, five rounds of selection took place at the Institute of Animal Science, University of Hohenheim, Germany. The large F2-cross was established from the 10^{th} selection generation, and a comprehensive data collection of behaviour and performance traits of 960 hens was performed. These two data sets were used for the following five research chapters.

In **chapter one**, a quantitative genetic analysis of fear traits and feather pecking as well as aggressive pecking using data from the large F2-cross was performed. Fear was recorded by the tonic immobility test, the open field activity and the emergence box test. These were recorded at a juvenile and adult age. Behavior traits as feather pecking and aggressive pecking were recorded in groups of 36 to 40 animals at the age of 27 weeks. The genetic parameters were estimated using a linear mixed model. Aggressive pecking showed the highest heritability (0.27) followed by feather pecking (0.14). The fear test traits showed heritabilities in the range of 0.07 to 0.14. The appreciable genetic correlation between fear traits and feather pecking was tonic immobility at juvenile age (r_g=0.27).

In **chapter two** we used dispersed Poisson models to estimate variance components, heritabilities of feather and aggressive pecking of different observation periods. The short period included the number of feather pecks in 20 min and the medium period was the summed bouts within one day. The results showed that modelling the data as repeated observations (short and medium period) and analysing them with a dispersed Poisson model is a suitable option to separate the important permanent environment effects from the additive animal effects and to account for the non-normal distribution of the data.

The objective of **chapter three** was to analyze the interrelationship between feather pecking and feather eating as well as general locomotor activity using structural equation models. The estimated heritabilities of feather eating, general locomotor activity and feather pecking were 0.36, 0.29 and 0.20, respectively. The genetic correlation between feather pecking and feather eating (general locomotor activity) was 0.17 (0.04). A high genetic correlation of 0.47 was

estimated between feather eating and general locomotor activity. The recursive effect from feather eating to feather pecking was $\hat{\lambda}_{FP,FE}$= 0.258, and from general locomotor activity to feather pecking $\hat{\lambda}_{FP,GLA}$= 0.046. These results imply that an increase of feather eating leads to an increased feather pecking behavior and that an increase in general locomotor activity results in a higher feather pecking value.

The objective of **chapter four** was to perform a quantitative genetic analysis and to map signatures of selection in two divergent laying hen lines selected for feather pecking behaviour. In the selection experiment, lines were selected for low or high feather pecking for 11 generations. Pedigree and phenotypic data were available for the last six generations of both lines for the statistical analysis with a standard mixed linear model and a Poisson model. The mixed linear model failed to analyse the low feather pecker data because of the large number of 0s in the observation vector. The Poisson model fitted the data well and revealed a small but continuous genetic trend in both lines. From the 11^{th} generation 75 birds, 41 high feather peckers and 34 low feather peckers were genotyped using the Illumina 60K chicken Infinium iSelect chip. An F_{ST}-based approach was used to map selection signature. We detected 17 genome-wide significant SNPs with a F_{ST}-value of 1, i.e. alleles were divergently fixed in the two lines, which are mostly located on chromosome 3 and 4, and a number of additional significant SNPs with a p-value of $\leq 5x10^{-4}$ and $\leq 5x10^{-5}$, respectively. Based on the assumption that selection affects several consecutive SNPs, 13 clusters were identified.

In **chapter five,** we used the data from the large F2-cross experiment to perform a genome-wide association study for feather pecking and aggressive pecking behaviour, to combine the results of this GWAS with the results from the selection experiment (chapter four) in a meta-analysis, and to link the results to those obtained from a differential gene expression study. 817 F2-hens were genotyped with the Illumina 60K chicken Infinium iSelect chip. We used single marker association analysis and a Poisson model. We detected four genome-wide significant SNPs for aggressive pecking delivered, but none for feather pecking and aggressive pecking received. However, a number of significant SNPs at $p \leq 5x10^{-5}$ were mapped for feather pecking and aggressive pecking received. In the meta analysis we identified nine genome-wide significant SNPs for feather pecking delivered, which were localized in chromosomal clusters (3 Mb). A previously conducted differential gene expression analysis provided eight significantly differential expressed genes within the feather pecking associated chromosomal clusters.

The thesis ends with a general discussion.

ZUSAMMENFASSUNG

Das Ziel der vorliegenden Arbeit war die Untersuchung der genetischen Fundierung von Verhaltensmerkmalen, insbesondere bei dem Merkmal Federpicken, und die Ableitung ethologischer Beziehungen zu anderen Merkmalen bei Legehennen. Hierfür standen Daten von zwei auf Federpicken divergent selektierten Linien und eine große F2-Population, welche aus diesen divergent selektierten Linien erstellt wurde, zur Verfügung. Hühner der Rasse White Leghorn wurden über 11 Generationen hinweg divergent auf hohes und niedriges Federpicken selektiert. Die ersten 5 Generationen wurden am Institut für Tierwissenschaften in Foulum, Dänemark, durchgeführt. Die weiteren Selektionsrunden fanden am Institut für Nutztierwissenschaften an der Universität Hohenheim, Deutschland, statt. Die große F2-Population, bestehend aus 960 Hennen, wurde aus der 10-ten Selektionsgeneration erzeugt und es wurde eine umfassende Datensammlung von Verhaltens- und Leistungsmerkmalen erhoben. Diese beiden Datensätze wurden zur Erstellung der nachfolgenden fünf Kapitel verwendet.

In **Kapitel eins** werden mit den Daten der F2-Population eine quantitativ genetische Analyse von Furchtmerkmalen und Federpicken sowie aggressivem Picken durchgeführt. Furcht wurde mittels der Tonischen Immobilität, Open Field Aktivität und dem Emerge Box Test erfasst. Diese wurden sowohl im juvenilen als auch adulten Alter durchgeführt. Die Verhaltensmerkmale Federpicken und aggressives Picken wurden in Gruppen von 36 bis 40 Tieren im Alter von 27 Wochen aufgezeichnet. Die genetischen Parameter wurden mit einem linear gemischten Modell geschätzt. Aggressives Picken zeigte die höchste Heritabilität (0.27), gefolgt von Federpicken (0.14). Die Furchttestmerkmale zeigten Heritabilitäten zwischen 0.07 und 0.14. Die einzige nennenswerte genetische Korrelation zwischen den Furchtmerkmalen und Federpicken ist die tonische Immobilität im juvenilen Alter (r_g=0.27).

In **Kapitel zwei** werden unter der Verwendung eines Poisson Modells Varianzkomponenten und Heritabilitäten der Merkmale Federpicken und aggressivem Picken in unterschiedlichen Zeitperioden geschätzt. Die kurze Zeitperiode beinhaltet die Anzahl Federpicks pro 20 min und die aufsummierten Pickwerte über einen Tag ergab die mittlere Zeitperiode. Die Ergebnisse zeigten, dass die Modellierung der Daten als wiederholte Beobachtungen (kurze und mittlere Zeitperiode) und die Auswertung mittels Poisson Modell eine geeignete Methode darstellt, um wichtige permanente Umwelteffekte vom additiven Tiereffekt zu trennen.

Das Ziel in **Kapitel drei** ist mittels Strukturgleichungsmodellen die Beziehung zwischen Federpicken und Federfressen, sowie der allgemeinen Bewegungsaktivität, zu analysieren. Die geschätzten Heritabilitäten von Federfressen, der allgemeinen Bewegungsaktivität und des Federpickens waren 0.36, 0.29 und 0.20. Die genetische Korrelation zwischen Federpicken und Federfressen (allgemeine Bewegungsaktivität) betrug 0.17 (0.04). Eine hohe genetische Korrelation mit 0.47 konnte zwischen Federfressen und der allgemeinen Bewegungsaktivität geschätzt werden. Der rekursive Effekt von Federfressen auf Federpicken war $\hat{\lambda}_{FP,FE}$= 0.258, und von der allgemeinen Bewegungsaktivität zum Federpicken lag bei $\hat{\lambda}_{FP,GLA}$= 0.046. Diese Ergebnisse implizieren, dass ein erhöhtes Federfressverhalten zu einer Erhöhung des Federpickens führt und das eine gesteigerte allgemeine Bewegungsaktivität in einer höhere Federpickanzahl resultiert.

Das Ziel von **Kapitel vier** ist die Durchführung einer quantitativ genetischen Analyse und die Kartierung von Selektionssignaturen bei zwei divergent selektierten Legehennenlinien auf Federpickverhalten. In diesem Selektionsexperiment wurden die Linien über 11 Generation hinweg auf hohes und niedriges Federpicken selektiert. Das Pedigree und die Phänotypdaten der letzten sechs Generationen beider Linien standen für die statistische Auswertung mit einem linear gemischten Modells und einem Poisson Modell zur Verfügung. Das linear gemischte Modell eignete sich nicht zur Auswertung der Niedrigpickerdaten auf Grund der zu hohen Anzahl an Null-Werten im Beobachtungsvektor. Das Poisson Modell passte sich den Daten besser an und lieferte einen kleinen, aber kontinuierlichen genetischen Trend in beiden Linien. Aus der 11-ten Generation wurden 75 Tiere, davon 41 Hochpicker und 34 Niedrigpicker, mit dem Illumina 60K chicken Infinium iSelect chip genotypisiert. Zur Kartierung von Selektionssignaturen wurde ein F_{ST} basierten Ansatz verwendet. Es konnten 17 genomweit signifikante SNPs mit einem F_{ST}-Wert von 1 detektiert werden, das heißt die Allele sind an diesem SNP divergent fixiert. Die meisten dieser SNPs sind auf Chromosom 3 und 4 lokalisiert. Des Weiteren konnte eine Anzahl an signifikanter SNPs mit einem p-Wert von $\leq 5x10^{-4}$ und $\leq 5x10^{-5}$ kartiert werden. Basierend auf der Annahme, dass eine Selektion mehrere aufeinanderfolgende SNPs beeinflusst, konnten 13 Cluster identifiziert werden.

In **Kapitel fünf** werden die Daten des F2-Kreuzungsexperimentes verwendet um eine genomweite Assoziationsanalyse der Merkmale Federpicken und aggressivem Pickverhalten durchzuführen. Die Ergebnisse dieser Assoziationsanalyse wurden mit den Ergebnissen des Selektionsexperiments (Kapitel vier) in einer Metaanalyse kombiniert, um diese mit denjenigen aus einer differentiellen Genexpressionsanalyse in Zusammenhang zu bringen.

Hierfür wurden 817 F2-Hennen mit dem Illumina 60K chicken Infinium iSelect chip genotypisiert. Es wurde eine Single-Marker-Assoziationsanalyse durchgeführt und ein Poisson Modell verwendet. Es wurden vier genomweit signifikante SNPs für das Ausführen von aggressivem Picken detektiert, aber keine für Federpicken oder das Erhalten von aggressivem Picken. Jedoch konnte eine Reihe an signifikanten SNPs mit $p \leq 5x10^{-5}$ für das Ausführen von Federpicken und für das Erhalten von aggressivem Picken kartiert werden. In der Metaanalyse wurden neun genomweit signifikante SNPs für Federpicken identifiziert, welche in chromosomalen Clustern (3 Mb) lokalisiert waren. Die differentielle Genexpressionsanalyse lieferte 8 von 750 untersuchten Genen, welche ein genomweit signifikant unterschiedliches Expressionslevel zeigten.

Die Dissertationsschrift endet mit einer kapitelübergreifenden Diskussion.

CHAPTER ONE

Quantitative genetic analysis of traits related to fear and feather pecking in laying hens

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Abstract

Feather pecking is a well known problem in flocks of laying hens. It is partially controlled by genetics. Fear is frequently reported to be related with feather pecking. The present study reports the result from a quantitative genetic analysis of feather pecking and three fear test traits in laying hens. Fear was recorded by the tonic immobility test, the open field activity and the emergence box test. These were recorded at a juvenile and adult age of the hens. The heritability of feather pecking was 0.16, and in the range between 0.07 and 0.14 for the fear test traits. Genetic correlations between fear measured in the juvenile and in the adult age point to different but correlated traits. Tonic immobility measured early in life was moderately correlated with feather pecking and might be used as a breeding criterion to reduce feather pecking.

Key words: Feather pecking, Fear, Laying hens, Genetic parameters

Introduction

Feather pecking is a well-known multi-factorial behavior problem in laying hens, which causes welfare as well as economic problems (Blokhuis and Wiepkema 1998; Rodenburg et al. 2008; Wysocki et al. 2010). It relates to an abnormal behavior pattern and is characterized by non-aggressive pecks directed towards the plumage of other hens (Kjaer et al. 2001). Aggressive pecking is clearly distinguishable from feather pecking. Aggressive pecks are delivered in an upright body posture and are mainly directed towards the head of the recipient birds (Bilcík and Keeling 1999; Kjaer et al. 2001). For both traits a low to moderate heritability was frequently reported (Kjaer and Sørensen 1997; Rodenburg et al. 2003, 2004; Su et al. 2005; Bennewitz et al. 2014). Thus, breeding against these behavior anomalies may be feasible. However, these traits can be seen as so called hard-to-measure traits, for which a data collection is difficult to implement in a routine breeding scheme. An alternative would be to breed for a trait that shows a high genetic correlation with these traits, but it is easier to record. In this context feather damage and feather condition score have been suggested (Bilcík and Keeling 1999) and investigated (Biscarini et al. 2010; Brinker et al. 2014) based on the observations that damage to the plumage is related to feather pecking behavior.

Some studies found that hens with a higher pecking activity showed also a higher level of fear (Jones 1996; Vestergaard et al. 1993; Jensen et al. 2005). Forkman et al. (2007) reviewed fear test traits for different species. For poultry common fear tests are tonic immobility (TI), open field (OF) (Forkman et al. 2007), and emergence box (EB) test (Jones and Mills 1983). The

estimated heritabilities for TI were between 0.08 and 0.32 (Campo and Carnier 1993; Agnvall et al. 2012; Recoquillay et al. 2013). For the trait OF heritabilities in the range of 0.15-0.60 were estimated (Rodenburg et al. 2003, 2004; Agnvall et al. 2012). In a recent study, Bögelein et al. (2014) investigated the phenotypic relationship between fear traits and feather pecking traits using data from a large F2 cross, which was established from two divergently selected feather pecking lines. They found almost no phenotypic correlation between pecking behavior and fear test traits.

Fear traits are supposed to be easier to record than feather pecking traits. Hence, if fear traits show a high genetic correlation to feather pecking traits, they might be included as breeding goals in order to breed indirectly for lower pecking hens (Rodenburg et al. 2004). A prerequisite is the knowledge of genetic correlations between feather pecking and fear traits. Therefore, the aim of the present study was to perform quantitative genetic analysis of fear traits and feather pecking traits using data from the large F_2-cross reported in Bögelein et al. (2014) and Bennewitz et al. (2014). The traits were analysed univariately to obtain heritability estimates. Genetic correlations between the traits were estimated from pairwise bivariate analyses.

Material and methods

Animal population

The research protocol was approved by the German Ethical Commission of Animal Welfare of the Provincial Government of Baden-Wuerttemberg, Germany. Starting from a founder generation, two lines were divergently selected for low and for high feather pecking, resulting in an low-feather-pecking line (LFP) and in a high-feather-pecking line (HFP). Selection took place for five generations at the Danish Institute of Animal Science (Kjaer et al. 2001) and then for five additional generations at the Institute of Animal Husbandry and Breeding, University Hohenheim, Germany. From these two selected lines five cocks and ten hens of each line were used to generate 10 F_1-families. Thereby each HFP cock was crossed with 2 LFP full-sib hens and vice versa. Ten F_1-cocks were selected to generate the F_2-families. Each cock was mated with 8 F_1-hens four times by using artificial insemination technique. This resulted in four hatches for each F_1-hen, with three weeks in between two hatches. Finally 960 F_2-chickens were generated. The number of chicken with trait records varied between 867 and 912. An F_2-Design consisting of large families was established, because it enables mapping feather pecking genes using either linkage or association mapping.

Feather pecking

At the age of 26 weeks the pullets were moved into pens of group size between 36 and 42 individuals. With the age of 27 weeks the behavior traits were recorded. For the traits feather pecking and aggressive pecking the actor and the receiver were observed. The ethogram was generated according to Savory (1995) and Bessei et al. (2013) and was as follows. Feather pecking was defined as a non aggressive behavior and includes forceful pecks, sometimes with feathers being pulled out and the recipient hen tolerates or moves away. Aggressive pecking was defined as fast pecks towards the head and the body of conspecifics and occurred during a fight among hens. The hen being attacked moved away and could have tissue damage. For the behavior observations the hens were marked with numbered plastic batches on their back. Each pen was visually observed for 20 minutes per observer for three consecutive days. Hatch three and four were observed twice for three consecutive days. The total number of observers varied between five and seven persons per observation day.

Fear tests

The fear tests traits TI, OF, and EB were recorded on day seven, eight and nine, respectively (i. e. in the juvenile status of the hens) as well as 40 weeks of age, when the hens were adult. This is indicated by the suffix j and a, respectively. TI_j and TI_a were induced by turning the individual on its back in a cradle. The duration of TI was recorded when the bird turned around, but was finished latest after 180 seconds. For the EB test (EB_j and EB_a) the box measured 23x23x20cm (length x breadth x height) with an opaque lid and a trapdoor with a size of 10 cm^2. Each pullet or hen was taken for 60 seconds in this box with darkness. After this time the trapdoor was opened for 180 seconds and the time was recorded till the head of the chick passed the door. For the OF test (OF_j and OF_a) the chickens were taken in the middle of a box measured 1m^2. Then the numbers of steps were recorded over a period of 180s.

Data editing and statistical analyses

The statistical analysis was performed using linear mixed models. The number of recorded pecks for feather pecking (feather pecks delivered, FPD) and aggressive behavior (aggressive pecks delivered, APD) were standardized to an observation period of 420 minutes. The model for these two pecking traits was

$$y = Xb + Z_{pen}pen + Z_a a + e$$

where *y* is the vector of observations, *b* is a vector of fixed hatch effects; *pen* is a vector with random pen effects, *a* is a vector with the random additive-genetic effects of the individuals, *X*, Z_{pen} and Z_a are corresponding design matrixes and *e* denotes for the residual term. It is known that the observer has a significant effect (Bennewitz et al. 2014). However, because the number of feather pecks and aggressive pecks was summed up over the entire observation period, the effect of the observer was not included in the model. An alternative approach to consider the observer effect would be to pool the information of the observers and then apply a modified mixed model approach, as described by Biscarini et al. (2008). Since this would not be a straightforward analysis, it was not applied in this study. The covariance structure of the random animal effect was $\text{var}(a) = A * \sigma_a^2$, with *A* being the numerator relationship matrix and σ_a^2 the additive genetic variance. The variance of the random residual effect was $\text{var}(e) = I * \sigma_e^2$, where *I* denotes the identity matrix and σ_e^2 is the residual variance. The variance of the random pen effects was $\text{var}(pen) = I * \sigma_{pen}^2$, where σ_{pen}^2 is the pen variance. The identity matrix was considered a good approximation since all pens had more or less the same size.

The histograms of the recorded fear traits are presented in Figures 1 to 3. The bar at 180 sec for the TI (Fig. 1) traits represents the proportion of hens that did not turn around within the data collection period. Similarly, the bar at 180 sec for EB (Fig. 2) denotes the proportion of hens that did not move the head out of the box. The data set can be seen as a truncated data set, because data collection was stopped at 180 sec. In order to account for this, we transformed the observations for these traits into 0/1 traits. For TI, birds received a 1 (0) if they turned around within 180 seconds (did not turned around within 180 seconds). For EB, birds received a 1 (0) if they moved their head out of the box within 180 seconds (did not move the head out of the box). Then, the TI and EB traits were modelled as binomial traits in the model shown above, but without the random pen effect, because they were partly collected before the hens were transferred into the pens. In order to do so we applied a generalised linear mixed model with a probit link function. The residual variance was fixed at 1.0. The OF traits (Fig. 3) are also not normally distributed, but a truncation could not be observed. Therefore, this trait was modelled as a normally distributed trait.

Univariate analyses were performed in order to estimate the heritability of the traits. Pairwise bivariate analyses were conducted in order to calculate genetic and phenotypic correlations

using the same models. The statistical analyses were performed using ASReml software (Gilmour et al. 2006).

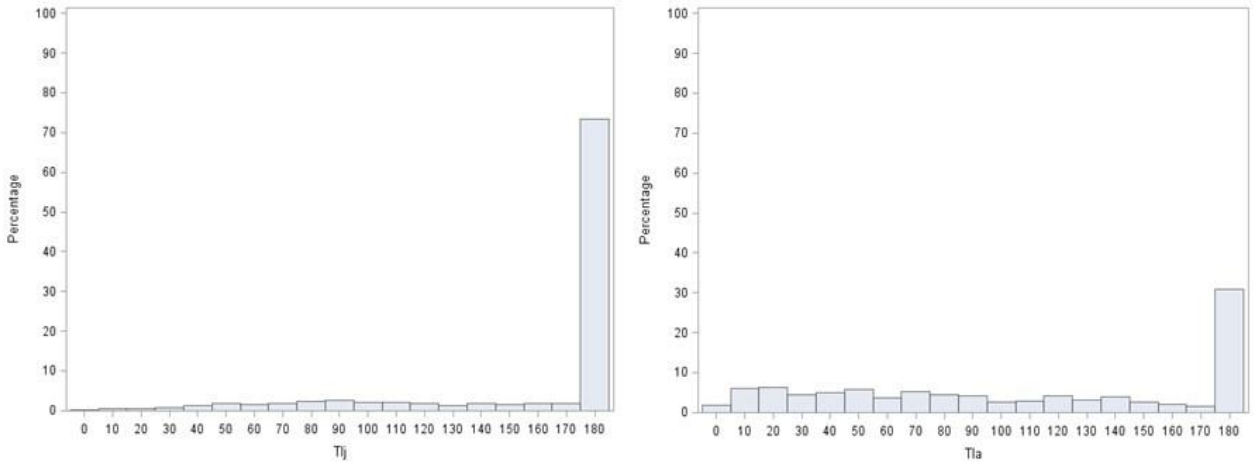

Fig.1 Histogram of tonic immobility at juvenile age (TI_j) and adult age (TI_a). The measured duration of tonic immobility is shown. The bar at 180 seconds represents the proportion of hens that did not turned around within the data collection period.

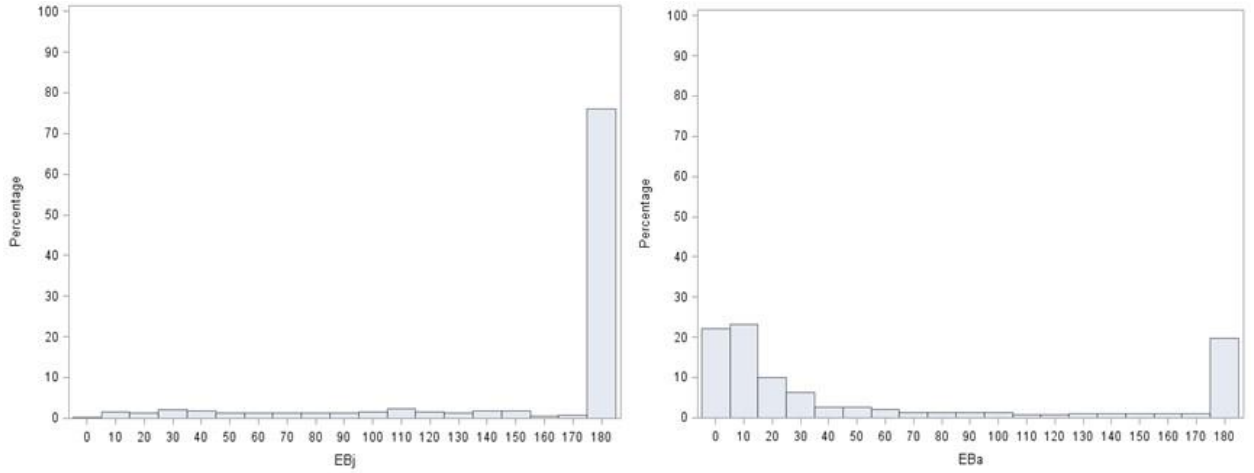

Fig 2 Histogram of emerge box at juvenile age (EB_j) and adult age (EB_a). The measured duration till the head moved out of the box is shown. The bar at 180 seconds represents the proportion of hens that did not passed the door within the data collection period.

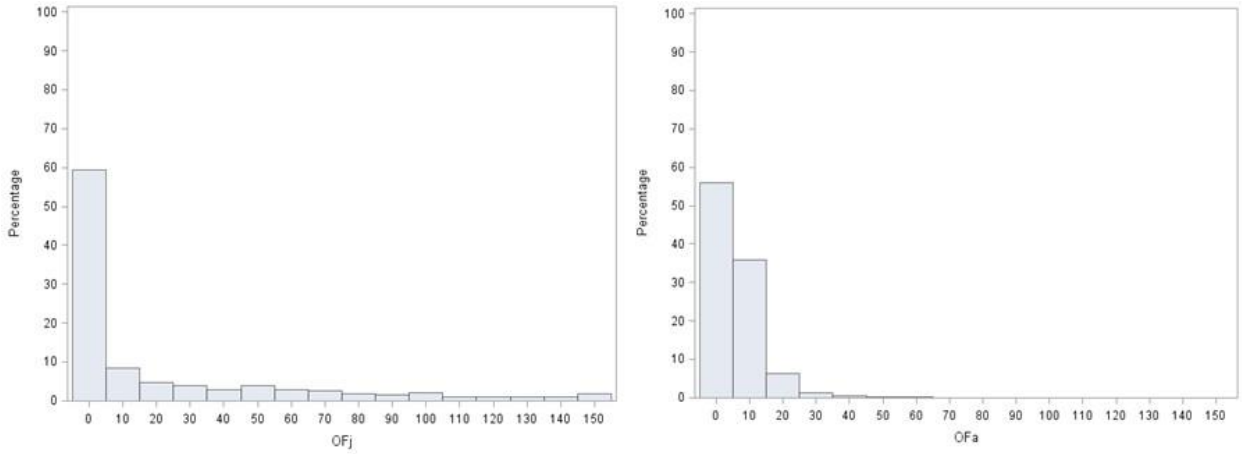

Fig 3 Histogram of open field test at juvenile age (OF_j) and adult age (OF_a). The number of steps within 180 seconds is shown.

Results

The summary statistics of the edited traits are shown in Table I. The duration of TI_j and EB_j was in average shorter than for TI_a and EB_a, respectively. The average number of steps in the OF test were higher in the juvenile stage compared to the adult stage. The maximum number of steps recorded in the juvenile stage was 312; the number of steps recorded in the adult stage was 56. Compared to the adult stage, the maximum number of steps in the juvenile stage was in general higher. The maximum number of FPD is 198, whereas the maximum number of APD is 85.

The additive genetic variance, residual variance and heritability of each trait are shown in Table II. The trait APD showed the highest heritability (0.27) followed by FPD (0.14). The fear test traits showed heritabilities in the range of 0.07 (OF_a) to 0.14 (OF_j, EB_a, TI_j). The heritability of OF_j and TI_j is higher compared to OF_a and TI_a, respectively. For EB the opposite pattern was observed.

Table I Traits, trait abbreviations (Abbr), number of animals (N), mean, standard deviation (SD), minimum (min) and maximum (max) of the observed traits after standardization

Trait	Abbr	Unit	N	mean	SD	min	max
Tonic immobility juvenile	TI_j	Binary coded	939	0.31	0.46	0	1
Tonic immobility adult	TI_a	Binary coded	939	0.72	0.45	0	1
Open field juvenile	OF_j	No. of steps	869	22.80	40.29	0	312
Open field adult	OF_a	No. of steps	892	5.74	6.43	0	56
Emerge box juvenile	EB_j	Binary coded	939	0.28	0.45	0	1
Emerge box adult	EB_a	Binary coded	939	0.81	0.39	0	1
Feather pecks delivered	FPD	No. bouts	938	13.66	25.85	0	198
Aggressive pecks delivered	APD	No. bouts	938	5.41	7.59	0	85

The results from pairwise bivariate analyses are shown in Table III. The traits FPD and APD showed a low phenotypic (0.09) and a moderate genetic (0.2) correlation. The phenotypic correlations between the TI, OF and EB fear traits measured at juvenile and at adult stage were close to zero. This was not the case for the genetic correlations, which were high with estimates of 0.9 (0.59, 0.86) for the two TI traits (OF traits, EB traits). The phenotypic correlations across the three fear trait complexes were close to zero. The genetic correlations were all positive and ranged between 0.04 (TI_j and OF_j) and 0.63 (OF_a and EB_a).

Table II Estimated additive genetic variance (σ_a^2), pen variance (σ_{pen}^2), residual variance (σ_e^2), heritability (h^2) and standard error (in parenthesis) as well as statistical distribution and link function used to analyse the traits, for the traits, results from univariate analyses

Trait[1]	Distribution	Link	σ_a^2	σ_{pen}^2	σ_e^2	h^2
APD	normal	identity	15.26	0.50	40.81	0.27
			(4.81)	(0.53)	(3.52)	(0.07)
FPD	normal	identity	93.93	<0.001	573.89	0.14
			(40.84)		(38.12)	(0.06)
OF_j	normal	identity	211.20		1290.12	0.14
			(100.57)		(91.11)	(0.06)
OF_a	normal	identity	5.55		35.13	0.07
			(2.51)		(2.37)	(0.05)
EB_j	binomial	probit	0.11		1.00[2]	0.10
			(0.06)			(0.05)
EB_a	binomial	probit	0.16		1.00	0.14
			(0.07)			(0.05)
TI_j	binomial	probit	0.16		1.00	0.14
			(0.07)			(0.05)
TI_a	binomial	probit	0.13		1.00	0.11
			(0.06)			(0.05)

[1]For trait abbreviation see Table I.

[2]The residual variance was fixed to 1.0, because of the use of the probit link function.

The phenotypic correlations between behavior traits FPD and APD and the fear test traits are all close to zero. A genetic correlation equal or above 0.2 was found between FPD and TI_j, APD and TI_j, APD and OF_a. In addition, APD and EB_j showed a negative genetic correlation of -0.22.

Table III Phenotypic (above the diagonal) and the genetic (below the diagonal) correlation between traits as well as their standard errors (in parenthesis), results from bivariate analyses

Trait[1]	TI_j	TI_a	OF_j	OF_a	EB_j	EB_a	FPD	APD
TI_j	-	0.06	<0.01	0.07	0.10	0.07	0.03	-0.04
		(0.04)	(0.04)	(0.04)	(0.03)	(0.04)	(0.04)	(0.04)
TI_a	0.90	-	0.09	0.07	0.06	0.08	0.06	0.05
	(0.16)		(0.04)	(0.04)	(0.04)	(0.03)	(0.04)	(0.04)
OF_j	0.04	0.26	-	0.05	0.25	0.04	-0.03	0.01
	(0.36)	(0.37)		(0.04)	(0.04)	(0.04)	(0.04)	(0.04)
OF_a	0.40	0.12	0.59	-	0.01	0.22	0.02	0.06
	(0.31)	(0.35)	(0.27)		(0.04)	(0.04)	(0.04)	(0.04)
EB_j	0.37	0.05	0.21	0.31	-	0.06	-0.04	-0.02
	(0.35)	(0.39)	(0.37)	(0.35)		(0.03)	(0.04)	(0.04)
EB_a	0.60	0.36	0.51	0.63	0.86	-	0.11	0.03
	(0.26)	(0.34)	(0.30)	(0.22)	(0.3)		(0.04)	(0.05)
FPD	0.27	0.04	0.03	0.08	0.15	-0.03	-	0.09
	(0.35)	(0.35)	(0.32)	(0.32)	(0.37)	(0.33)		(0.04)
APD	0.20	0.17	-0.04	0.82	-0.22	0.13	0.20	-
	(0.30)	(0.29)	(0.27)	(0.20)	(0.30)	(0.28)	(0.26)	

[1]For trait abbreviation see Table I.

Discussion

The aim of the present study was to perform quantitative genetic analyses to estimate heritability of fear traits and phenotypic and genetic correlations between fear test traits, aggressive pecking and feather pecking behavior. The standard errors of the variance

components were small for the results from the univariate analysis (Table II), but larger for bivariate analysis (Table III). This implies that the structure and size of the experiment is sufficient for the estimation of genetic parameters in univariate analysis, but the results from the bivariate analyses have to be interpreted with some cautions due to the larger standard errors.

Heritabilities

Data from a large F2-cross, set up from lines selected divergent on feather pecking, were used. As discussed in Bennewitz et al. (2014), it can be expected that the distribution of gene frequencies is not strongly U-shaped but closer to intermediate values, which increases additive genetic variance. This might be true especially for genes involved in feather pecking, but also for correlated traits due to correlated selection response as well as for uncorrelated traits due to genetic drift that operated within the lines. On the other hand, it might be that some genes are lost due to selection and drift within the selection lines before the cross was established. Nevertheless, the low to medium heritability of FPD (0.14, see Table II) is in agreement with literature reports (Rodenburg et al. 2003, 2004; Kjaer and Sørensen 1997; Kjaer et al. 2001). Note that Bennewitz et al. (2014) found lower heritabilities for feather pecking using in the same data set. They applied an overdispersed Poisson model and defined the heritability entirely on the link scale. In the present study the heritability was defined on the observed scale, and hence a comparison of the two heritability estimates is not valid. For the trait APD a medium heritability was estimated (0.27, see Table II). Recoquillay et al. (2013) found a higher heritability for aggressive pecks in Japanese Quail. In contrast, Rodenburg et al. (2003) found almost no heritability for APD. The expression of dominance and aggression in chicken flocks depend on the circumstances of the observations. Siegel (1978) showed that the incidence of aggression encounters in chicken flocks declined within about 30 days after the groups were assembled. Difference between groups and between individuals may not be identified after this time period. In the present study, the aggressive pecking observations started one week after the birds were transferred to the observation pens, and according to Siegel (1978), within the informative period.

Low heritabilities in the range of 0.07-0.14 were estimated for the fear test traits (Table II). Rodenburg et al. (2003, 2004) reported higher heritabilities for OF in the range of 0.15 to 0.49, depending mainly on the age of the hens. A higher heritability of OF was also estimated by Agnvall et al. (2012). For the trait TI a low heritability was estimated, which is in agreement with Agnvall et al. (2012). In contrast, higher heritabilities for the trait TI were

reported by Campo and Carnicer (1993), and in Japanese Quail by Recoquillay et al. (2013). The inconsistency of the estimated heritabilities might depend on the different definition of the traits, the recording methods, the age of the animals, the statistical model applied and, as discussed above, on the study population.

Genetic correlations and correlated selection response

The low phenotypic correlation between FPD and APD (Table III) is supported by McKeegan and Savory (1999), Kjaer and Sørensen (2002) and Bessei et al. (2013). This implies that individuals which deliver feather pecking do not necessarily deliver aggressive pecking and vice versa. On the other hand, they are genetically correlated (Table III), and hence selection against feather pecking reduces aggressive pecking as well.

Several studies proved that divergent selected lines on feather pecking show different fear response (Vestergaard et al. 1993; Jones et al. 1995; Jensen et al. 2005). A moderate genetic correlation was estimated between TI_a and FPD as well as APD (Table III). Hence, based on our results, TI at juvenile age might be used as a predictor for feather and aggressive pecking in adult hens and TI_j may be used for selection against pecking behavior. The expected correlated selection response (*CR*) for FPD when selection on TI_j can be approximated using the deterministic equation $CR_y = i * h_x * h_y * r_g * \sigma_{PY}$, where suffix *x* (*y*) denotes for trait FPD (TI_j), *i* is the selection intensity for trait TI_j (i. e. the trait selected for), h_x (h_y) is the square root of the heritability of trait *x* (*y*) , r_g is the genetic correlation between trait *x* and *y*, and σ_{PY} the phenotypic standard deviation of trait *y* (Falconer and Mackay, 1996, p 317). If the 50% top ranked individuals for TI_j are selected to breed the next generation, this would result in a selection intensity of $i = 0.798$ (Appendix 1 in Falconer and Mackay, 1996). Inserting this figure as well as corresponding figures from the Table II and III in the deterministic equation shown above result in an expected correlated selection response for FPD of 0.83 per generation, when selecting on TI_j. In contrast, a direct selection on FPD with the same selection intensity leads to an expected selection response of 3.29 per generation. Hence, selecting for TI_j is inefficient in reducing FPD. It is important to note that breeding against feather pecking, either indirect or direct, could result in a reduced genetic gain for the number of eggs laid, since the genetic correlations between feather pecking and egg production is positive (Recoquillay et al. 2013; Bennewitz et al. 2014), unless both traits are properly accounted in the breeding objective.

Rodenburg et al. (2004) found a high genetic correlation between open field activity and pecking behavior and suggested that open field test could be used as a predictor for pecking

behavior at juvenile age. According to the estimated correlation between OF_j and FPD as well as OF_j and APD in this study (Table III), OF_j is not a predictor for feather or aggressive pecking in this data set. However, OF_a and APD are highly genetically correlated (Table III). The genetic correlations between fear test traits measured in juvenile and in adult ages point to different correlated traits (Table III). In some studies, it is proved that fear decrease with age (Hocking et al. 2001; Albentosa et al. 2003). Hocking et al. (2001) reported that the number of steps in the open field test decreases with age. This is in agreement with the results of the current study (Table II, Figure 1). The decline of fear response from juvenile to adult age is also reflected by the low phenotypic correlation between traits records measured in both ages (Table III) and also by the decline in heritability in adult age for TI and OF (Table II). It seems that fear in adult hens is motivated by different factors, and in addition, genetic differences between hens vanish in higher ages.

Behavior traits in laying hens are influenced by interactions among individuals. Models were developed that include interaction or associated effects (Bijma et al. 2007a, 2007b; Bijma 2013), and it was shown that these effects can contribute substantially to the heritable variation of survival of hens related to feather pecking and cannibalism and of plumage conditions (Ellen et al. 2008; Brinker et al. 2014). Because the size of the pens were rather large in the present study, social effects were not modelled explicitly. Alternatively, as suggested by Bijma (2013), we chose to capture shared environmental effects and associated effects by fitting a random pen effect when analysing the pecking traits. The pen variances were small (Table II), but interpreting the magnitude of associated effects is not trivial (Bijma 2013).

Conclusion

Fear traits showed a low heritability. The genetic correlation between fear measured in the juvenile and in the adult status of the hens point to different but correlated traits. Phenotypically, fear was not correlated with feather pecking or aggressive pecking. Tonic immobility in the juvenile status showed a genetic correlation of 0.27 with feather pecking and aggressive pecking, and hence might be considered as a trait in a breeding goal to indirectly reduce feather pecking. However, the expected correlated selection response for FPD when selecting for TI_j is around 75% reduced compared to a direct selection on FPD.

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CHAPTER TWO

Genetic Parameters for Feather Pecking and Aggressive Behavior in Laying Hens Using Poisson and Linear Models

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Genetic Parameters for Feather Pecking and Aggressive Behavior in Laying Hens Using Poisson and Linear Models

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Abstract: An F2-design was established from two divergent selected founder laying hen lines, which included 910 F2-hens. Each pen was observed in 21 sessions of 20 min each, distributed over three consecutive days. An animal model was applied that treated the bouts observed within 20 minutes (short period) or the summed bouts within one day (medium period) as repeated observations. A dispersed Poisson distribution was assumed. Residual variance was approximated on the link scale by the delta method. The estimated variance components were on a similar level for the two observation periods, except the approximated residual variance. This was substantially smaller for the medium period, leading to lower heritabilities on the link scale for the short period compared to the medium period (0.11 vs. 0.19 for feather pecking and 0.09 vs. 0.24 for aggressive pecking). Variance components were also estimated using a linear model.
Keywords: Feather pecking, Generalized linear model, Heritability

Introduction

Feather pecking in laying hens is characterized by non-aggressive pecks directed towards the plumage of other hens. The underlying mechanisms are not well understood, but physiological, nutritional as well as genetic factors are known to influence this trait (Wysocki et al. (2010)). Quantitative genetic analyses have revealed low to moderate heritabilities (Kjaer et al. (2001); Rodenburg et al. (2003)). Aggressive pecking is clearly distinguishable from feather pecking. Aggressive pecks are delivered in an upright body posture and are mainly directed towards the head of the recipient birds (Kjaer et al. (2001)).

Feather pecking and aggressive pecking are recorded by observation of the hens in defined time periods. The observations are count values with large proportions of zero counts. The

latter one makes it difficult and in most cases impossible to obtain a normal distribution by a data transformation. The use of generalized linear mixed models (GLMM) to analyze the data is an appropriate way to account for the non-normal distribution of the data. In a recent study we used dispersed Poisson models to estimate variance components, heritabilities and approximate genetic correlations for behavior traits in a large F2 cross of laying hens (Bennewitz et al. (2014)). The aim of the present study was to extent these analyses towards different observation periods. In addition, the data were analyzed with a standard linear model.

Materials and Methods

Data collection and editing

Starting from a founder generation, two lines were divergently selected for low respectively high feather pecking. From the 10^{th} selection generation an F1-cross was generated using an equal number of birds from each line. These were mated reciprocally with males or females from the other line in order to generate 10 F1-families. From these families an F2-cross was established, which consisted of 10 F2 paternal half-sib families with an average number of 91 female offspring, and in 910 hens in total. Behavioral data collection started at 27 weeks of age. The group size varied between 36 and 42 birds. Seven experienced observers recorded feather pecking and aggressive pecking within each pen during sessions of 20 min each. Each pen was observed for 140 minutes per day and over three consecutive days making a total of 21 sessions of 20 min. All incidences (bouts) of feather pecking and aggressive pecking were recorded. For each bout, the identity of the actor and receiver was recorded. This resulted in four behavior traits: bouts of feather pecking delivered (**FPD**), bouts of feather pecking received (**FPR**), bouts of aggressive pecking delivered (**APD**) and bouts of aggressive pecking received (**APR**). FPR showed an additive genetic variance of almost zero (Bennewitz et al. (2014)) and therefore was not considered in this study.

Data analysis using GLMM

The number of recorded pecks and received pecks, respectively, in each 20 min observation period (short period) and in each observation day (medium period), were modeled as repeated observations in the statistical analysis with the GLMMs. There were 21 repeated observations for the short period and in three repeated observations for the medium period per hen. Because the observations were count values they were initially assumed to be Poisson

distributed and were analyzed with the following GLMM. For the short period the linear predictor was:

$$\eta = Xb + Z_{pen}pen + Z_{pe}pe + Z_{de}de + Z_a a$$

where *b* is a vector of fixed effects (short period: observer, test-day and observer-by-test-day interaction; medium period: test-day), *pen* is a vector with random pen effects, *pe* is a vector with random permanent environment effects of the hens, *de* is a vector of random test-day-by-hen effects (only for the short period), *a* is a vector with the random additive-genetic effects, and X, Z_{pen}, Z_{pe}, Z_{de}, and Z_a are known design matrices. The covariance structure of the random effects were $\text{var}(pen) = I * \sigma^2_{pen}$ $\text{var}(pe) = I * \sigma^2_{pe}$, $\text{var}(de) = I * \sigma^2_{de}$, and $\text{var}(a) = A * \sigma^2_a$, where σ^2_{pen}, σ^2_{pe}, σ^2_{de}, and σ^2_a are pen variance, permanent environmental variance, test-day-by-hen variance, and additive genetic variance respectively, and A (I) is the numerator relationship (identity) matrix. For the medium period the same linear predictor was used, but without the *de* effect. The expectations of the observations were $\lambda = E(y \mid pen, a, pe, de) = g^{-1}(\eta)$ (short period) and $\lambda = E(y \mid pen, a, pe) = g^{-1}(\eta)$ (medium period), where g is the link function, in this case a log link, i.e. $g = \log_e(\eta)$. As we detected over dispersion relative to the Poisson model, a dispersion parameter (ϕ) was added to the models on the observed scale by assuming the variance function $\text{var}(y \mid pen, a, pe, de) = \lambda\phi$ (short period) and $\text{var}(y \mid pen, a, pe) = \lambda\phi$ (medium period). Unlike in linear mixed models, calculating the repeatability and heritability from the variance components is not straightforward for GLMMs. This is because it is not obvious how the residual variance can be obtained under a Poisson model. Based on the Delta method we approximated the residual variance as $\phi\lambda^{-1}$, which is an extension of the approach of Foulley et al. (1987) towards accounting for $\phi \neq 1$ (see Bennewitz et al. (2014)). Following this, the heritability on the η scale for the short period was computed according to:

$$h^2 = \frac{\sigma^2_a}{\sigma^2_{pen} + \sigma^2_a + \sigma^2_{pe} + \sigma^2_{de} + \phi\lambda^{-1}}.$$

The Poisson parameter λ was estimated for each subject and then averaged over all subjects. The repeatability was $t = (\sigma^2_a + \sigma^2_{pe})/(\sigma^2_a + \sigma^2_{pen} + \sigma^2_{pe} + \sigma^2_{de} + \phi\lambda^{-1})$. For the medium period the same computations were performed, but without σ^2_{de}.

Data analysis using linear mixed models

For this analysis the observations were accumulated over the entire observation period of 420 min (long period), resulting in one observation per hen. The following linear model was used:

$$y = Xb + Z_{pen}pen + Z_a a + e\,,$$

where the fixed effect included the hatch, *e* denotes for the residual term and the remaining terms are as defined above. This analysis was conducted, because it is a standard analysis of feather pecking data. It is noted, that an analysis of this data set by a Poisson model led to convergence problems and hence was not possible. All models were fitted for each trait separately using ASReml 3.0 (Gilmour et al. (2009)).

Results and Discussion

The histograms of the traits revealed that they are not normally distributed and that there are large proportions of zero counts, especially for the short period (not shown). The results of the GLMM analysis are shown in Table 1. The variance components were remarkably on a similar level for both observation periods. However, the residual variance was substantially smaller for the medium period, leading to a higher heritability and repeatability for this period. For the short period the heritability is in between 0.04 (APR) and 0.11 (FPD). For the medium period it varied between 0.14 (APR) and 0.24 (APD). The higher residual variance for the short period is due to a small average Poisson parameter λ, which over-compensated the effect of the smaller dispersion parameter ϕ for this period (Table 1). This possibly yielded downward biased heritability estimates for the short period. Maybe a distribution which is able to model the excess of zero counts explicitly would be more appropriate for the short period, e.g. a zero-inflated Poisson distribution.

Table 1. Estimated additive genetic variance ($\hat{\sigma}_a^2$), permanent environment variance ($\hat{\sigma}_{pe}^2$), test-day-by-hen variance ($\hat{\sigma}_{de}^2$), pen variance ($\hat{\sigma}_{pen}^2$), dispersion parameter ($\hat{\phi}$), residual variance ($\lambda^{-1}\hat{\phi}$), heritability ($\hat{h}^2$), and repeatability ($\hat{t}$) for the behaviour traits (standard errors are shown in parenthesis), results from the GLMMs

	Feather pecks delivered, FPD		Aggressive pecks delivered, APD		Aggressive pecks received, APR	
Item	Short period	Medium period	Short period	Medium period	Short period	Medium period
$\hat{\sigma}_a^2$	0.46 (0.18)	0.49 (0.19)	0.42 (0.13)	0.41 (0.13)	0.17 (0.06)	0.18 (0.06)
$\hat{\sigma}_{pe}^2$	1.40 (0.15)	1.33 (0.14)	0.53 (0.09)	0.52 (0.09)	0.30 (0.05)	0.34 (0.05)
$\hat{\sigma}_{de}^2$	0.62 (0.03)	-	0.35 (0.03)	-	0.25 (0.03)	-
$\hat{\sigma}_{pen}^2$	<0.001	<0.001	0.03 (0.02)	0.02 (0.02)	0.05 (0.02)	0.03 (0.02)
$\hat{\phi}$	1.13 (0.01)	3.27 (0.08)	0.79 (0.01)	1.37 (0.04)	0.85 (0.01)	1.29 (0.04)
$\lambda^{-1}\hat{\phi}$	1.85	0.80	3.04	0.76	3.40	0.75
$\hat{h}^2$	0.11	0.19	0.09	0.24	0.04	0.14
$\hat{t}$	0.43	0.56	0.22	0.54	0.12	0.40

A remarkable result is the high permanent environment effect for the traits, especially for FPD and APD, which was not reported so far. This indicates that if hens delivered feather pecks or aggressive pecks once, they might continue in doing this. But also for the trait APR there is a substantial permanent environment effect. This implies that once hens were identified as 'victims' they stayed within this category. On the other hand, there are hens that successfully avoid receiving pecks.

The results of the linear model and the long period are shown in Table 2. The heritability on the observed scale is 0.16, 0.26 and 0.27 for FPD, APD and APR, respectively. These figures are higher compared to the heritabilities obtained from the GLMM for the short period, but note that a formal comparison is not valid. The disadvantage of accumulating the bouts across the entire observation period, as done for the long period, is that it is not possible to separate random additive animal effects from permanent environment effects. The Pearson correlation coefficients between the random animal effects obtained from the three models are shown in Table 3. These are high, especially for the effects obtained from the two GLMMs. The correlations suggest that re-ranking of individuals might be an issue only if predictions of the linear model and the GLMMs are compared.

Table 2. Estimated additive genetic variance ($\hat{\sigma}_a^2$), residual variance ($\hat{\sigma}_e^2$), pen variance ($\hat{\sigma}_{pen}^2$), and heritability ($\hat{h}^2$) for the behavior traits (standard errors are shown in parenthesis), results from the linear model

Item	FPD, long period	APD, long period	APR, long period
$\hat{\sigma}_a^2$	93.93 (40.84)	15.26 (4.81)	7.30 (2.43)
$\hat{\sigma}_e^2$	573.89 (38.12)	40.81 (3.52)	19.39 (1.74)
$\hat{\sigma}_{pen}^2$	<0.001	0.50 (0.53)	1.06 (0.78)
$\hat{h}^2$	0.14 (0.06)	0.27 (0.08)	0.27 (0.08)

Behavior traits depend on the interactions among individuals. Models were developed that include interaction or associated effects (see Bijma (2013) and references therein). As suggested by Bijma (2013), we chose the simplest form to capture shared environment effects and associated effects by fitting a random pen effect to the GLMMs. The pen variances were small (Table 1 and 2), but interpreting the magnitude of associated effects is not trivial (Bijma (2013)).

Table 3. Pearson correlation coefficients between animal effects obtained from different models[§]

Trait	short - medium period	short - long period	medium - long period
FPD	0.99	0.92	0.93
APR	0.99	0.96	0.96
APD	0.99	0.93	0.94

[§] Animal effects of the short period and medium period were obtained from the GLMMs and of the long period from the linear model

Conclusions

The estimated variance components showed a relatively small standard error. This is due to the thorough observations of the traits using a standardized protocol. In addition it implies that the data set was of sufficient size to obtain accurate estimates. The permanent environment effects were substantial for all traits. Accounting for over-dispersion in the Poisson models was important. The heritability of the traits was low to medium, depending on the period considered and on the models used. Modelling the data as repeated observations (short and medium period) and analysing them with a dispersed Poisson model is a suitable option to separate the important permanent environment effects from the additive animal effects and to account for the non-normal distribution of the data.

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CHAPTER THREE

Quantitative Genetic Analysis of causal relationships between Feather Pecking, Feather Eating and General Locomotor Activity in Laying Hens using Structural Equation Models

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Quantitative Genetic Analysis of Causal Relationships between Feather Pecking, Feather Eating and General Locomotor Activity in Laying Hens using Structural Equation Models

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Abstract

The objective of this research was to analyze the relationship between feather pecking (FP) and feather eating (FE) as well as general locomotor activity (GLA) using structural equation models, which allow that one trait can be treated as an explanatory variable of another trait. This provides an opportunity to infer putative causal links between the traits. For the analysis, 897 F2-hens set up from two lines divergently selected for high and low FP were available. The FP observations were Box-Cox transformed, and FE and GLA observations were log- and square root transformed, respectively. The estimated heritabilities of FE, GLA and FP were 0.36, 0.29 and 0.20, respectively. The genetic correlation between FP and FE (GLA) was 0.17 (0.04). A high genetic correlation of 0.47 was estimated between FE and GLA. The recursive effect from FE to FP was $\hat{\lambda}_{FP,FE}$= 0.258, and from GLA to FP $\hat{\lambda}_{FP,GLA}$= 0.046. These results imply that an increase of FE leads to an increased FP behavior and that an increase in GLA results in a higher FP value. Furthermore, the study showed that the genetic correlation between the traits are mainly caused by indirect effects.

Introduction

Feather pecking (FP) is a widespread problem in laying hens. It leads to severe feather damages and feather losses. Poor feather cover usually increase feed consumption and reduce conversion rate of feed to eggs. In addition, FP is a risk factor for cannibalism (Kjaer and Sørensen, 2002), which is an important welfare issue. The importance of FP and cannibalism on the economics and welfare in layer flocks is increasing as several EU countries announced a ban of beak trimming. Despite intensive research on FP, the underlying mechanism of this behavior is unclear. It has been shown that many factors affect FP behavior (reviewed by Wysocki et al., 2010). The most widespread theory on the origin of FP is based on the assumption that it is a misdirected feeding and foraging behavior, which develops in the absence of suitable foraging and occupation material (Blokhuis, 1986, 1989; Huber-Eicher and Wechsler, 1997, 1998; Blokhuis and Wiepkema, 1998; El-Lethey et al., 2000).

However, foraging and occupation opportunities attenuate but do not prevent FP. Previous studies have shown that FP is closely related to feather eating (FE) (McKeegan and Savory, 1999, 2001; Harlander-Matauschek and Bessei, 2005; Harlander-Matauschek and Häusler, 2008). Harlander-Matauschek and Bessei (2005) demonstrated that hens selected for high and low FP significantly differ in their preference to feather eating. High feather peckers (HFP) ate significantly more feathers than low feather peckers (LFP), and when given the choice between feathers and fiber, HFP preferred feathers (McKeegan and Savory, 2001; Harlander-Matauschek and Bessei, 2005; Harlander-Matauschek and Häusler, 2008; Bögelein et al., 2010). Based on these results, Bessei and Kjaer (2015) suggested FE as a primary motivation for FP. Kjaer and Bessei (2013) assumed that nutrient deficiencies or imbalances caused FP. Although McCasland and Richardson (1966) proved that feathers have almost no nutritive value, ingested feathers increase feed passage time in birds (Harlander-Matauschek et al., 2006). Meyer et al. (2013) showed that a difference in intestinal microbial metabolites in HFP and LFP hens exist. Though feathers may not contribute to the essential nutrient supply, they influence the microbiota composition and various metabolites produced in the digestive tract of hens (Meyer et al., 2012), and these metabolites following absorption from the digestive tract might affect behavioral traits.

Kjaer (2009) presented a theoretical model where FP is caused by a hyperactivity disorder. It has been shown that chickens from the HFP line have a significant higher locomotor activity than chickens from the LFP line. The potential link between FP and locomotor activity is the malfunction of the dopamine system (Kjaer, 2009). Flisikowski et al. (2009) suggested that

DEAF1 (Deformed Epidermal Autoregulatory Factor 1), a neighboring gene of the dopamine receptor D4, which is associated with temperament and behavior, as a candidate gene for FP. The knockout of dopamine D1, D2, D4 in mice has resulted in decreased locomotor activity, due to decreased motivation (Viggiano et al., 2003). Nistico and Stevenson (1979) showed that apomorphine, a dopamine receptor agonist, increased the locomotor activity in chickens. Van Hierden et al. (2005) reported differences in apomorphine sensitivity between a high and low FP lines of laying hens. Furthermore, it has been shown that dopamine D1/D2 receptor agonists influence stereotypic pecking in pigeons (Goodman et al., 1983) and domestic fowl (Nistico and Stevenson, 1979) and FP in laying hens (Kjaer et al., 2004).

Linear mixed models have been used to quantify the relationship between FP and FE (Bennewitz et al., 2014) or between FP and activity (Rodenburg et al., 2004). A positive genetic correlation between FP and FE was found in a large F2 population, set up from lines selected divergent on FP (Bennewitz et al., 2014). For the same F2-population, the activity was recorded in an Open Field Test and had a low genetic correlation with FP (Grams et al., 2015a). Rodenburg et al. (2004) found a positive genetic correlation between the open field activity in young birds and FP behavior in adult hens. However, these studies did not answer the question if FE or activity affects FP.

Gianola and Sorensen (2004) proposed structural equation models (SEM) as an extension of standard multitrait models (MTM) to handle situations in which recursive or simultaneous effects among phenotypes can be considered in a multivariate system. SEMs allow that one trait can be treated as a predictor of another trait, which provide a causal link between the traits (Valente et al., 2010). If there is a possible causal link between traits, genes affecting directly one trait may affect also the second trait indirectly. The use of SEMs for modelling causal relationships among traits leads to a better understanding of biological pathways underlying complex traits (Rosa et al., 2011).

In the present study the traits FE, general locomotor activity (GLA) and FP collected in the F2 experiment described in Bennewitz et al. (2014) and Grams et al. (2015a) were analyzed and genetic parameters as well as structural coefficients were estimated using SEM. The causal relationships were formulated based on the following hypotheses. FE and GLA trigger FP and FE affects GLA. The aim of these analyses was to infer the magnitude of such putative causal effects among these three traits, and to discuss the genetic parameters underlying such causal models.

Material and Methods

Population and housing

Starting from a White Leghorn founder stock, two lines were divergently selected for low and for high FP, resulting in a LFP line and in a HFP line. Selection took place for five generations at the Danish Institute of Animal Science (Kjaer et al., 2001) and then for five additional generations at the Institute of Animal Husbandry and Breeding, University Hohenheim, Germany (Grams et al., 2015b). From these two selected lines, a large F2 cross was established as described in detail in Bennewitz et al. (2014). Four hatches were generated with a distance of two weeks. The total number of chickens with trait records varied between 897 and 937. The day-old chicks were neck banded for individual identification. Feeding, lighting program and management were carried out under conventional pullet rearing conditions. The research protocol was approved by the German Ethical Commission of Animal Welfare of the Provincial Government of Baden-Wuerttemberg, Germany.

Data collection

General Locomotor Activity (GLA). At the age of 18 weeks the GLA was recorded. The hens were tested for GLA in groups of 185 to 275 birds according to the respective hatches. For testing, each individual bird was fitted with a leg band carrying an electronic transponder weighing approx. 0.5 g and measuring 2 mm in diameter and 10 mm in length. Each transponder emitted a unique number when close to an antenna. Nine sets of antennas were used. Each set consisted of 10 circular antennas built together in a frame of plastic measuring 76 cm by 30 cm (length x width) and 2.7 cm in height. The 9 sets were placed under the litter in a 3 * 3 antenna grid in the central part (4.6 m * 3.1 m) of a 60.2 m^2 large pen (10.2 m length by 5.9 m width). The precise antenna area with active signal reception covered approximately 10% (5.7 m^2) of the total pen area. The recording system (Gantner Pigeon Systems GmbH, Schruns, Austria) scanned each antenna several times per second. When a transponder came within ca. 15 cm of an antenna, information for bird identity, antenna location, date and time of day was recorded. Multiple recordings (several birds recorded at the same time and place) were possible. Data were stored automatically in Ascii-files for each day. The recording covered all the 12 hours lighting period for 9 days, in total 108 hours of recording. The birds were left alone and were without behavioral restrictions of any kind during the recording period except for the daily feeding and control routines, which lasted about 10 min per day. In this way the recordings could be used to estimate a general level of movement around the central pen area whereas more specific behavior like velocity

or distance travelled was not possible to estimate using this technique. GLA was expressed as the sum of recordings at any (new) antenna during the 9 days (108 hours) observation period.

Feather eating (FE). At the age of about 19 weeks, the hens were transferred to individual cages for the feather eating test. Over a period of 10 consecutive days, a transparent plastic sheet containing 10 feathers of about 4 cm length was fixed next to the feeder. The number of feathers eaten was counted once a day and missing feathers were replaced. The maximum consumption of feathers was 100 per hen over the testing period. The number of feathers eaten over the entire period was used as a trait in the present study.

Feather pecking (FP). At the age of 26 weeks, the pullets were moved into deep-litter pens in group sizes between 36 and 42 individuals. The birds were marked with a plastic tag on the back. The behavior data collection started at the age of 27 weeks and was done by direct observation by seven experienced observers. FP was defined as non-aggressive allo pecking directed to the feathers of other hens and introducing visible movement of a feather, sometimes with feathers being pulled out and/or eaten. This definition follows the definition used in an earlier study on these lines (Bessei et al., 2013) and is comparable to the definition of 'feather pulling leading to feather loss' given in Savory (1995). In some cases more than a single FP was delivered by a certain hen within a short period of time (some seconds), but this was recorded as a single incidence of FP, but the term 'pecks' are used in the present report. It would, however, in other reports be referred to as bouts of FP, see for example Kjaer et al. (2001) for a more detailed description and discussion. Each pen was visually observed for 20 minutes per observer for three consecutive days. Due to low levels of pecking in hatch three and four, these hatches were observed twice for three consecutive days.

Statistical analyses

For the statistical analysis, birds without a record in one trait were excluded. In total 897 chickens with records in all three traits remained in the data set. The number of recorded pecks for FP was standardized to an observation period of 420 min. A Box-Cox transformation was applied to the FP records as follows:

$$y_{ti} = \frac{(y_i^{-0.2} - 1)}{-0.2},$$

where y_i is the number of FP bouts of each hen i and y_{ti} is the transformed observation. The power parameter was −0.2, which was found to give the best fit of the model applied by Su et al. (2005) using FP data. A one was added to the recordings of FE and these were subsequently log-transformed. The trait GLA was square root transformed. These

transformations were performed in order to reduce the deviation of the distribution from a normal distribution.

For the SEM as described in Gianola and Sorensen (2004), the causalities have to be defined a priori. Based on the outlines given in the introduction section, we assumed the following three recursive causalities: FE affects FP and GLA, and GLA affects FP. A graphical description of this model is given in Figure 1. The structural coefficients $\lambda_{FP,FE}$ and $\lambda_{GLA,FE}$ describe the rates of change of FP and GLA with respect to FE and $\lambda_{FP,GLA}$ that of FP with respect to GLA. The following trivariate recursive mixed linear animal model was applied, which is given in the notation as used by Valente et al. (2010) and Rosa et al. (2011):

$$y = (\Lambda \otimes I_n)y + X\beta + Zu + e$$

where *y* is the vector with the phenotypic records FP, FE and GLA, respectively, for the individuals; β is a vector of fixed hatch effects, u and e are vectors of the additive direct genetic effects and the model residuals, respectively; and *X* and *Z* are incidence matrices. *Λ* is a matrix of structural coefficients describing the causal structure $\Lambda = \begin{bmatrix} 0 & 0 & 0 \\ \lambda_{GLA,FE} & 0 & 0 \\ \lambda_{FP,FE} & \lambda_{FP,GLA} & 0 \end{bmatrix}$; *I* is an identity matrix and $\otimes$ indicates the Kronecker product. The distribution of the vectors *u* and *e* is:

$$\begin{bmatrix} u \\ e \end{bmatrix} \sim N\left\{\begin{bmatrix} 0 \\ 0 \end{bmatrix}, \begin{bmatrix} G \otimes A & 0 \\ 0 & R \otimes I \end{bmatrix}\right\},$$

where G and R are the additive genetic and residual covariance matrices, respectively, and A is the additive relationship matrix. In order to ensure parameter identifiability, it was assumed that the system residuals were uncorrelated, which is a standard method to ensure identifiability (Wu et al., 2010; Rosa et al., 2011). The ASReml software package was used for the analysis (Gilmour et al., 2006). According to Wu et al. (2010), the estimated parameters of the SEM pertain to 'system parameters' and need to be transformed in order to be comparable to their equivalents from a standard mixed model. In order to this, the following matrices were computed (Gianola and Sorensen 2004; Rosa et al. 2011).

$$G^* = (I - \Lambda)^{-1} G (I - \Lambda)'^{-1}$$

$$R^* = (I - \Lambda)^{-1} R (I - \Lambda)'^{-1}$$

$$P^* = G^* + R^*$$

From these matrices trait heritabilities as well as phenotypic and genetic correlations were estimated using standard notations. Standard errors were approximated by the use of the Delta

method as outlined in detail in Beck et al. (2016). For comparison purpose, the data were also analyzed with a standard MTM.

Results and Discussion

The histograms of the recorded and transformed traits FP, FE and GLA are presented in Figure 2. A summary statistic of the recorded traits is given in Table I. The maximum number of FP during an observation period of 420 min was 198. The average number of FP was low and with a substantially standard deviation. On average, the hens ate 62 feathers during a period of 10 consecutive days. The average activity was 49 antenna signals and the maximum was 210 during a period of 9 consecutive days.

Table I Traits, trait abbreviations (Abbr.), number of animals (N), mean, standard deviation (SD), minimum (min) and maximum (max) of the recorded traits

Trait	Abbr.	N	Mean	SD	Min	Max
Feather pecking	FP	897	13.82	26.13	0	198.33
Feather eating	FE	897	62.32	34.06	0	100.00
General locomotor activity	GLA	897	49.27	31.20	0	210.25

Structural coefficients

The recursive effects are given in the legend of Figure 1. The recursive effect from FE to FP ($\hat{\lambda}_{FP,FE}$= 0.258) is high and implies that an increase of FE leads to an increased FP behavior. The recursive effect from GLA to FP ($\hat{\lambda}_{FP,GLA}$= 0.046) indicates that an increase in GLA results in a higher FP value. The standard errors of these two recursive effects are small. A negligible recursive effect with a large standard error from FE to GLA ($\hat{\lambda}_{GLA,FE}$= 0.03) was estimated, which implies that an increase of FE does not result in an increased activity. Hence, this postulated causality is not supported by our data.

The structure shown in Figure 1 was chosen based on hypotheses that were previously formulated in the literature. Since no competing models were compared, we cannot rule out the possibility that the assumptions were incomplete. Methods for identifying causal structures among a wide range of possible structures were developed by Valente et al. (2010; 2011). Their methods are implemented in a Bayesian framework and further work is needed to adapt their method for a REML approach, as used in this study.

The trait GLA was recorded at week 18 week and FE at about 19 week of life. It might be argued that the model assumption about the effect of FE on GLA is questionable, because GLA was recorded before FE. The trait recording had to be organised this way, because the hens were moved from group housing to single cages in order to measure FE. However, we observed, that hens with a high level of FE during the data collection period in week 19 ate also many feathers during the group housing the week before (not shown). Therefore, we assumed that only the measurement of the traits took place with roughly one week in between, but both traits were expressed in the same period.

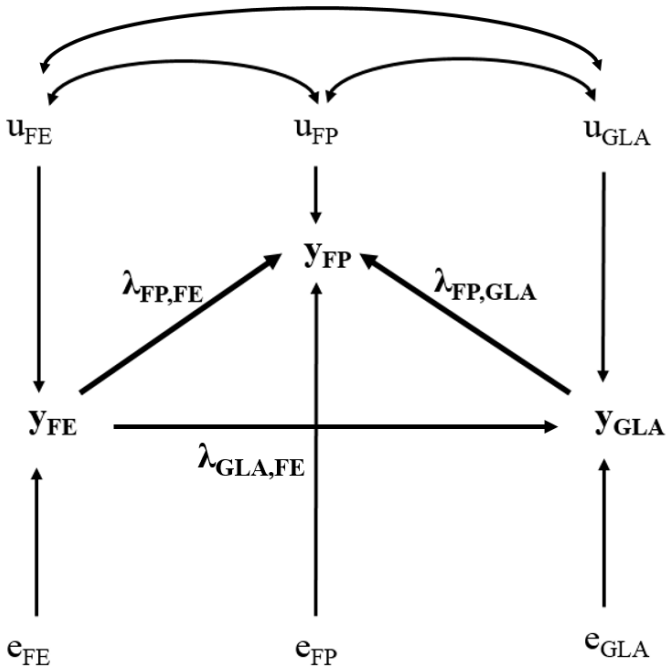

Figure 1 Recursive model for three traits feather pecking (FP), feather eating (FE) and general locomotor activity (GLA). Y denotes the phenotypic values and λ_{ij} denotes the structural coefficient, with trait *j* affecting trait *i*. The estimates are (standard errors in parenthesis) $\hat{\boldsymbol{\lambda}}_{\boldsymbol{FP,FE}}$= 0.258 (0.02), $\hat{\boldsymbol{\lambda}}_{\boldsymbol{FP,GLA}}$= 0.046 (0.01), and $\hat{\boldsymbol{\lambda}}_{\boldsymbol{GLA,FE}}$= 0.03 (0.06).

Genetic parameters

The genetic correlations and trait heritabilities estimated with the SEM are shown in Table II. The standard errors are given in this table as well. As expected, the genetic parameters obtained from the classical MTM model are the same (not shown). Trait FP showed a heritability of 0.20, which is in agreement with estimates of previous studies (Kjaer and Sørensen, 1997) and with studies partly using the same data set (Grams et al., 2015a; Bennewitz et al., 2014). For trait FE and GLA moderate to high heritabilities (0.36 and 0.29, respectively) were estimated. Kjaer (2013) developed divergent lines differing significantly in locomotor activity recorded in the home pen after 4 generations of selection. Heritability was estimated to be between 0.20 and 0.30 (J.B. Kjaer, unpublished).

Table II Phenotypic (below the diagonal), and genetic (above the diagonal) correlations, and trait heritability (on the diagonal) with standard errors (in parentheses), results from the structural equation model (SEM).

Trait[1]	FE	GLA	FP
FE	0.36 (0.09)	0.47 (0.19)	0.17 (0.24)
GLA	0.16 (0.04)	0.29 (0.10)	0.04 (0.25)
FP	0.28 (0.04)	0.09 (0.06)	0.20 (0.07)

[1]For trait abbreviation see Table I.

The standard errors of the genetic correlation estimates are large, indicating that these have to be interpreted with some cautions. In addition, the SEM applied assumes a normality of the trait distributions, which seems to be approximately given for FP and GLA due to the data transformation, but not for FE (Fig 1). This also points out the need for a careful interpretation of the results. A moderate phenotypic (0.28) and a low genetic (0.17) correlation was found between FP and FE (Table II), which are also substantially lower compared to correlations estimated in the study of Bennewitz et al. (2014). The traits FP and GLA showed a low phenotypic and genetic correlation (0.09 and 0.04, respectively, Table II). Similarly, Grams et al. (2015a) estimated a low genetic and phenotypic correlation between activity recorded in an Open Field Test and FP for the same F2-population. A high genetic correlation (0.47) was estimated between FE and GLA, although they are low phenotypically correlated (0.16) and no causality exists between these two traits (the recursive effect is close to zero with a large standard error, see Figure 1).

Table III shows the estimates of the additive-genetic and residual (co)variances of the system (i.e. the elements of the G, and R matrices obtained from the SEM). The elements of the matrices G^*, and R^* obtained from the SEM are shown in Table IV. The standard errors are given in these tables as well.

Table III Direct additive genetic and residual (co)variance of the 'system' together with standard error (in parentheses), results from the structural equation model (SEM)

(Co) variance	Trait[1]	FE	GLA	FP
Additive genetic ($\hat{G}$)	FE	0.553 (0.17)	0.378 (0.14)	-0.110 (0.06)
	GLA		1.234 (0.38)	-0.138 (0.09)
	FP			0.178 (0.06)
Residual ($\hat{R}$)	FE	0.976 (0.11)	0	0
	GLA		3.037 (0.27)	0
	FP			0.541 (0.04)

[1]For trait abbreviation see Table I.

The genetic correlation coefficients obtained from standard MTMs consider direct as well as indirect genetic effects, the latter being caused by the recursive effects (Figure 1). SEMs are capable of separating direct from indirect genetic effects (Wu et al., 2010). Hence, the 'system' genetic effects in Table III are solely due to direct effects and free from genetic effects mediated by other phenotypic traits that may also have causal influence on it (Gianola and Sorensen, 2004). Consequently, the genetic covariances between these effects are due to genes directly affecting the two traits or due to linkage disequilibrium between genes affecting the traits (Valente et al., 2013). Table IV shows the genetic covariances considering both, direct and indirect genetic effects (i.e. the elements of *G** and *R**). The covariance of the direct effects between FP and FE as well as between FP and GLA is negative (Table III), but positive when direct and indirect effects are considered simultaneously (Table IV). The indirect effects from FE to FP or GLA to FP (see Figure 1) have an opposite effect compared to the direct effects affecting both traits. This implies that indirect effects contribute substantially to the genetic correlation between the traits. Indirect effects may be related to FE changing retention or microbial activity in the digestive tract. It has been shown that including 5 % ground feathers (non-hydrolyzed) in the diet increased the abundance of keratinolytic bacteria in the ileum and cecae of hens, increased ammonia concentration in the cecae, and also a changed pattern of short-chain fatty acids produced in the cecae (Meyer et al. 2012). This points towards a partial hydrolysis of eaten feathers in the intestine. While digestibility was not determined by Meyer et al. (2012), ammonia and short-chain fatty acids can be absorbed and may affect behavioral traits. There is an increasing body of evidence that, in humans, variations in the composition of gut microbes may be associated with changes in the normal functioning of the nervous system (Forsythe et al., 2010).

Table IV Additive genetic and residual (co)variance together with standard error (in parentheses), results from the structural equation model (SEM)

(Co) variance	Trait[1]	FE	GLA	FP
Additive genetic ($\hat{G}^*$)	FE	0.554 (0.18)	0.392 (0.19)	0.051 (0.07)
	GLA		1.252 (0.38)	0.018 (0.11)
	FP			0.157 (0.06)
Residual ($\hat{R}^*$)	FE	0.977 (0.11)	0.024 (0.13)	0.253 (0.05)
	GLA		3.038 (0.27)	0.146 (0.08)
	FP			0.613 (0.05)

[1]For trait abbreviation see Table I.

Conclusion

In conclusion, the application of the SEM shed some light into the relationship between FP and FE as well as between GLA and FP. The recursive effect from FE to FP and from GLA to FP is relatively high, implying that FP is partly affected by FE and also by GLA. Heritability estimates of FE and GLA were moderate and higher than for FP.

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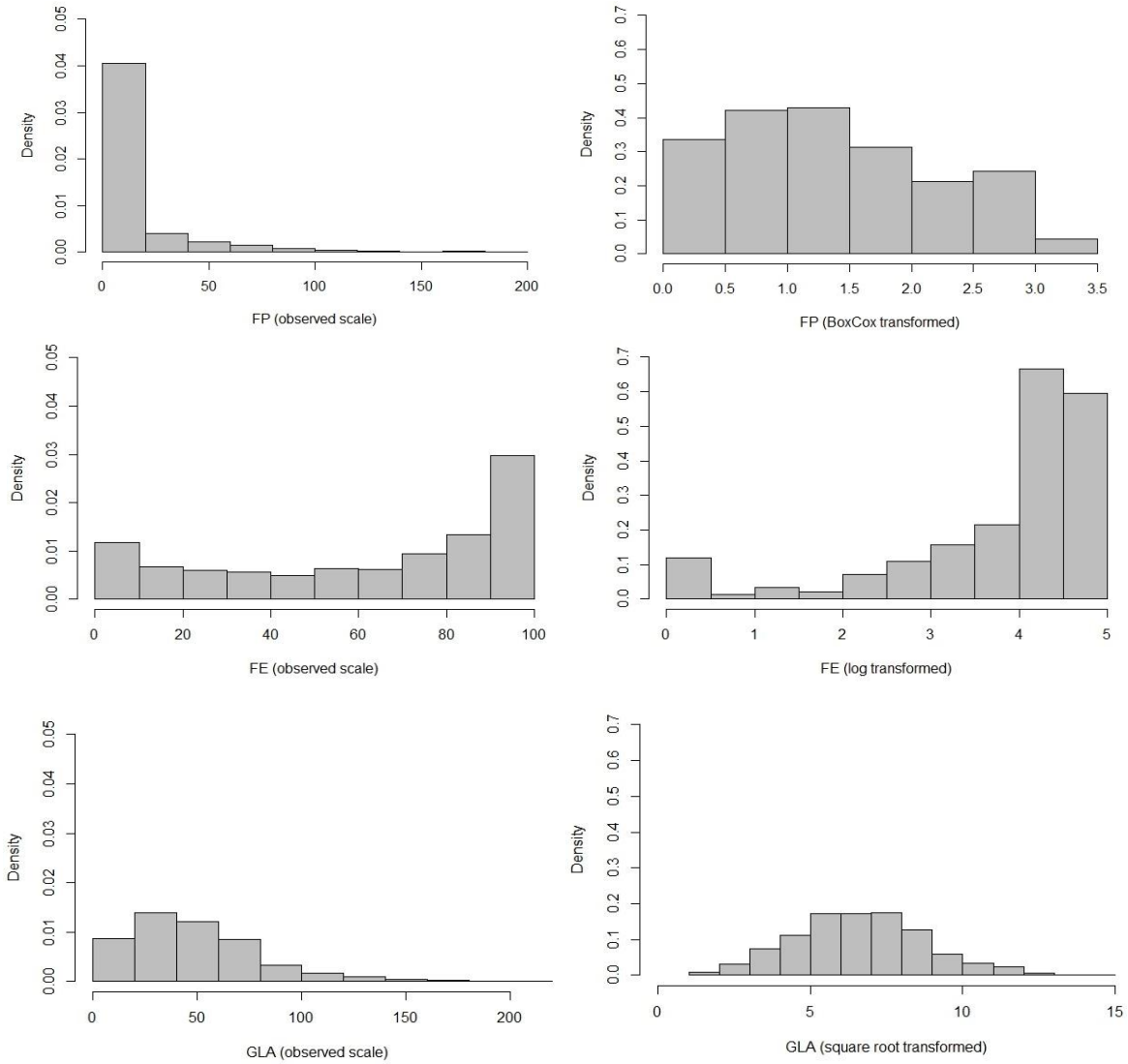

Figure 2. Histogram of the observed data (left panel) and transformed data (right panel) for feather pecking (FP) during the entire observation period of 420 min, for feather eating (FE) with a maximum consumption of 100 feathers per hen and for general locomotor activity (GLA) during the recording period of 12 hours for 9 days.

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CHAPTER FOUR

Genetic parameters and signatures of selection in two divergent laying hen lines selected for feather pecking behaviour

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Genetic parameters and signatures of selection in two divergent laying hen lines selected for feather pecking behaviour

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Abstract

Feather pecking (FP) in laying hens is a well-known and multi-factorial behaviour with a genetic background. In a selection experiment, two lines were developed for 11 generations for high (HFP) and low (LFP) feather pecking, respectively. Starting with the second generation of selection, there was a constant difference in mean number of FP bouts between both. We used the data from this experiment to perform a quantitative genetic analysis and to map selection signatures. Pedigree and phenotypic data were available for the last six generations of both lines. Univariate quantitative genetic analyses were conducted using mixed linear and generalized mixed linear models assuming a Poisson distribution. Selection signatures were mapped using 33 228 single nucleotide polymorphisms (SNPs) genotyped on 41 HFP and 34 LFP individuals of generation 11. For each SNP, we estimated Wright's fixation index (F_{ST}). We tested the null hypothesis that F_{ST} is driven purely by genetic drift against the alternative hypothesis that it is driven by genetic drift and selection. The mixed linear model failed to analyze the LFP data because of the large number of 0s in the observation vector. The Poisson model fitted the data well and revealed a small but continuous genetic trend in both lines. Most of the 17 genome-wide significant SNPs were located on chromosomes 3 and 4. Thirteen clusters with at least two significant SNPs within an interval of 3 Mb maximum were identified. Two clusters were mapped on chromosomes 3, 4, 8 and 19. Of the 17 genome-wide significant SNPs, 12 were located within the identified clusters. This indicates a non-random distribution of significant SNPs and points to the presence of selection sweeps. Data on FP should be analysed using generalised linear mixed models assuming a Poisson distribution, especially if the number of FP bouts is small and the distribution is heavily peaked at 0. The F_{ST}-based approach was suitable to map selection signatures that need to be confirmed by linkage or association mapping.

Background

Feather pecking (FP) in laying hens is a well-known, but yet unsolved problem. This abnormal behaviour is characterized by non-aggressive pecks directed towards the plumage of other hens [1]. It causes economic losses due to increases in feeding costs when large parts of the body are denuded and in mortality rates when FP leads to cannibalism. In addition to a number of environmental conditions, physiological, nutritional as well as genetic and epigenetic factors are known to influence FP (see [2-6]). Quantitative genetic analyses have reported heritability estimates in the range of 0.1 to 0.4, depending on the trait definition, design of the study, age of the hens, statistical model applied, and data collection period [1, 7-9]. FP shows a complex genetic relationship with other traits such as feather eating and number of laid eggs [10-11], traits related to aggressiveness and fear response [12-14], and general activity and explorative behaviour [15].

Kjaer et al. [1] carried out a selection experiment to develop a high feather pecking line (HFP) and a low feather pecking line (LFP), starting from a common base population. After two rounds of selection, FP was significantly more pronounced in the HFP line than the LFP line. Su et al. [2] used the data from the first five generations of these lines to estimate variance components and heritabilities. Heritability ranged from 0.11 to 0.17. Five additional rounds of selection were then conducted. Our aim was to perform a quantitative genetic analysis of FP on animals from these additional rounds of selection in order to discuss the data obtained with those reported by Su et al. [2], and to determine the best approach for analyzing such data.

As indicated by Wysocki et al. [5], performing a genome-wide study to map QTL (quantitative trait loci) or genes that underlie genetic variation of FP would help to better understand this abnormal behaviour and its complex relationships with other traits. QTL linkage and association mapping rely on genotypes and phenotypes that are preferably collected from a large-scale study. However, since FP is not recorded in routine breeding programs, such large-scale designs cannot rely on existing datasets and need to be established, which is a time-consuming and costly effort, because observing and recording FP is labour intensive.

Based on Qanbari and Simianer [16], selection signatures are defined as regions of the genome that harbour functionally important sequence variants and have changed under selection. It is well known that strong selection leads to reduced nucleotide diversity around the loci under selection. Not only is the diversity of the target loci reduced, but also that of loci in high linkage disequilibrium (LD) with the target loci. This is known as genetic hitch-

hiking [17] and results in selection signatures in the genome. Mapping selection signatures has been a matter of intense research during the last years; see [16, 18] and references in these two papers. A genome scan to map selection signatures requires a dense genetic map in order to exploit LD. In chicken, a 60 K SNP (single nucleotide polymorphism) Illumina iSelect chip was developed by the USDA Chicken GWMAS Consortium. Kranis et al. [19] reported the development of a 600 K Affymetrix HD genotyping chicken array. A large range of methods is available for the detection of selection signatures, which can be classified according to whether intra- or inter-population information is used. To analyze inter-population information, Wright's fixation index, F_{ST}, is widely used, for which several estimators are described [20, 21].

Given the availability of dense SNP chicken arrays, an alternative to using linkage or association mapping to detect QTL for FP, is to search for selection signatures using data from the last generation of the HFP and LFP lines. This approach could lead to the identification of chromosomal regions that contain genes having responded to divergent selection, and hence, contribute to the genetic variation of FP. Therefore, our second aim was to conduct a genome scan to map selection signatures based on data from the last generation of the selection experiment described above by applying the F_{ST} statistic.

Methods

Animals, data collection and selection

Chickens of a White Leghorn layer line were divergently selected for high and low FP for 11 generations. The selection started in the Danish Institute of Animal Sciences, Foulum, Denmark, for the first six generations (0-5) [1]. Thereafter, five rounds of selection took place at the Institute of Animal Science, University of Hohenheim, Germany. The common base population of both lines was established in 1995 and derived from a foundation stock, which was created in 1970 as a control population in the Scandinavian selection and cross-breeding experiment of Liljedahl et al. [22], see also Kjaer et al. [1] and Su et al. [2]. In the base population (generation 0), FP was recorded on 123 hens at the age of 67 weeks. This information was used to estimate breeding values and, then, 30 females and 10 males with the highest and lowest estimated breeding value for FP were selected as the founder animals of the HFP and LFP lines, respectively.

This selection procedure was repeated in the subsequent generations. Up to generation 5, at about 30 weeks of age, groups of 20 hens (10 HFP and 10 LFP) per pen were transferred into

observation pens (size 2m x 4 m). The observation period started 7 to 12 days after the hens were transferred to the observation pens. Feather pecking behaviour was recorded by video camera during three hours and the number of FP bouts was counted for each hen. An FP bout was defined as a series of continuous pecks directed to the same part of the body of a recipient hen. At each generation, 10 males and 30 females per line were selected based on their breeding value for the number of FP bouts. For a detailed description of the experiment and the results of the statistical analysis, see Kjaer et al. [1] and Su et al. [2].

Behaviour testing and the selection procedure from generation 6 to 11 were carried out at the experimental farm of the University of Hohenheim. At about 30 (25 to 37) weeks of age, groups of 40 hens (20 HFP and 20 LFP) per pen were transferred into floor pens measuring 16 m^2. For individual identification, a plastic tag was attached to the back of each bird. The observation period started one week after the birds were transferred to the floor pens and the number of FP bouts (defined as above) was counted for each hen. Each pen was observed by each observer (one observer per one pen at a time) during sessions of 20 min over three consecutive days. Each hen was observed during a total of three hours. Selection was based on the number of FP bouts. At each generation and for each line, 60 females and about 10 males were selected based on their estimated breeding value that was calculated using an animal model. In this study, observation records and pedigree data were available only from generation 6 onwards. In total, 1526 hens were phenotyped for FP behaviour from generation 6 to 11. The research project was approved by the University of Hohenheim Committee of Animal Care and the Provincial Government of Baden-Wuerttemberg, under the authorisation number HOH 35/15PG.

Estimation of variance components

Statistical analyses of the data recorded during the last six generations were performed using an animal model and the ASREML software package [23]. Two different models were used, i.e. a generalized linear mixed model and a linear mixed model. In both models, HFP and LFP lines were analyzed separately. The vector containing the linear predictors of the observations ($\boldsymbol{\eta} = \{\eta_i\}$) was:

$$\boldsymbol{\eta} = \mathbf{1}\boldsymbol{\mu} + \mathbf{Z}_{\mathbf{gen}}\mathbf{gen} + \mathbf{Z}_{\mathbf{a}}\mathbf{a}, \qquad (1)$$

where μ is the intercept, **gen** is a vector with random generation effects, **a** is a vector with the random additive-genetic effects, and $\mathbf{Z}_{gen}$ and $\mathbf{Z}_a$ are known design matrices. Covariance structures of random effects were $\mathrm{var}(\mathbf{gen}) = \mathbf{I} * \sigma^2_{gen}$ and $\mathrm{var}(\mathbf{a}) = \mathbf{A} * \sigma^2_a$, where σ^2_{gen} and

σ_a^2 are generation variance and additive genetic variance, respectively, and **A** and **I** are the numerator relationship and identity matrices, respectively. An observation was equal to the number of FP bouts recorded over the entire observation period and stored in vector **y**. Expectations of the observations were as follows:

$$\boldsymbol{\lambda} = \mathrm{E}(\mathbf{y}|\mathbf{gen}, \mathbf{a}) = \mathrm{g}^{-1}(\boldsymbol{\eta}),$$

where $\boldsymbol{\lambda} = \{\lambda_i\}$ is a vector containing the Poisson parameters of the observations and g is the link function, in this case log link.

In the Poisson model, the residual variance is not an explicit part of the model. If estimating the heritability is of interest, the residual variance has to be modelled entirely on the link scale. Formulas to do this are given in Foulley et al. [24] and Bennewitz et al. [10].

For the analysis of the data using the linear mixed model, the observations were Box-Cox transformed as follows:

$$y_{ti} = \frac{(y_i^{-0.2} - 1)}{-0.2},$$

where y_i is the number of FP bouts for each hen i summed up over the entire observation period and y_{ti} is the transformed observation. The power parameter was −0.2, which was found to give the best fit of the model applied by Su et al. [2] using data from the first six generations of the same selection experiment. The following mixed model was used:

$$\mathbf{y_t} = \mathbf{1}\boldsymbol{\mu} + \mathbf{Z_{gen}}\mathbf{gen} + \mathbf{Z_a}\mathbf{a} + \mathbf{e}, \qquad (2)$$

where $\mathbf{y_t}$ is the vector of transformed observations, e denotes the random residual and the remaining terms are as defined in model (1). HFP and LFP lines were analyzed separately, because pedigree information was not available up to the common base population and trait means differed constantly between the two lines across generations (Figure 1). A generation effect was included to capture the large fluctuations in the means for each generation that were observed from generation 6 onwards. This effect also captures at least part of the putative genetic progress across generations, a point that will be discussed later. In this model, heritability was estimated using standard procedures.

Genotyping

Genotyping was performed on 41 HFP and 34 LFP hens from generation 11 using the

Illumina 60K chicken Infinium iSelect chip. A total of 57 636 SNPs were detected and after control checks, 33 228 remained for the statistical analyses described below. SNPs that were located on one of the sex chromosomes W or Z or on linkage groups LGE22C19W28_E50C23 or LGE64, respectively, and SNPs that were not allocated to a specific chromosome or linkage group were excluded. In addition monomorphic SNPs (minor allelic frequency (MAF) = 0.0) and SNPs with a call frequency less than 0.95 were filtered out. The remaining SNPs were checked for correct clustering by Illumina's GenomeStudio software. For this purpose, SNPs were sorted consecutively by using different metrics (heterozygote excess, cluster separation, parent-parent-child errors) and those that showed extreme values were checked visually for correct clustering and, where appropriate, were manually re-clustered. SNPs for which a manual re-clustering was not possible were excluded from analyses.

Estimation of F_{ST} index and mapping of selection signatures

To identify regions under selection, we used the population differentiation index F_{ST}. In general, F_{ST} provides a measure to quantify levels of differentiation between subpopulations [25, 20]. A small F_{ST} (e.g. < 0.05) indicates that allele frequencies in both subpopulations are similar, whereas an F_{ST} greater than 0.05 indicates that allele frequencies are different. We used the F_{ST} computation of Weir and Cockerham (Equation 8 in [25]), which is for a single SNP:

$$F_{ST} = \frac{\sigma_p^2}{\bar{p}(1-\bar{p})}, \qquad (3)$$

where $\bar{p}$ is the mean allele frequency for the two lines and σ_p^2 is the variance of the allele frequency across the two lines. σ_p^2 is estimated as $\sigma_p^2 = \left(\overline{p^2}\right) - \left(\overline{p}^2\right)$, where $\overline{p^2}$ is the mean of the squared allele frequencies in the two lines.

Single F_{ST} values can vary greatly. In addition, selection sweeps will affect the F_{ST} of consecutive SNPs due to the LD between them. Therefore, we also calculated F_{ST} for sliding windows that each consisted of 25 SNPs and moved in steps of one SNP forward. The computation was done using the following formula, which is a multi-marker extension of (3):

$$F_{ST} = \frac{\sum_i [(\overline{p_i^2}) - (\bar{p}_i^2)]}{\sum_i \bar{p}_i(1-\bar{p}_i)},$$

where index *i* denotes the *i*th SNP in the sliding window.

In our experiment, the differences in allele frequencies between the two lines could be driven by genetic drift and selection. To unravel these two processes, a statistical test was developed, which is based on the assumption, that genetic drift affects the whole genome, while selection affects only SNPs that are in LD with causal genes. F_{ST} values were used as test statistics. For each SNP, we tested the null hypothesis that F_{ST} was driven purely by genetic drift against the alternative hypothesis that it was driven by genetic drift and selection. To derive a null distribution of the test statistic, we simulated the effect of genetic drift stochastically. We were able to do this, because, as described above, the breeding history of each line starting from the common base population was known. In the first five rounds of selection, 10 males and 30 females were selected in each line [2], which resulted in an effective population size (Ne) of 30. In the next five rounds of selection, the number of females was increased to about 60, resulting in an Ne of approximately 35. Since genetic drift is largest for intermediate allele frequencies, the allele frequency in the base population was assumed to be equal to 0.5. Two populations with one SNP were simulated from the common base population and were bred for 11 generations independently by assuming an Ne of 30 for the first five rounds of selection and 35 for the next five rounds of selection. At generation 11, F_{ST} for these two populations at the SNP was computed using formula (2). This was repeated 100 000 times and resulted in a distribution of F_{ST} values under the null hypothesis of no selection.

The error probability for each real SNP ($p_{nominal}$) was computed as the proportion of simulated SNPs that had a greater F_{ST} than the real SNP under consideration. To correct for multiple testing, we applied the Bonferroni correction as $p_{genomewide} = 1 - (1 - p_{nominal})^{\#SNP}$, where the number of SNPs was equal to 33 228. The genome-wide significance level was set at $p_{genomewide} \leq 0.05$. Because the Bonferroni correction is very conservative due to the assumption of independence of tests (which is not the case in our study due to the LD structure of consecutive SNPs and due to selection), we considered two additional levels of significance, i.e. $p_{nominal} \leq 5\text{x}10^{-5}$, and $p_{nominal} \leq 5\text{x}10^{-4}$. In order to estimate the number of false positives among the significant SNPs, we calculated false discovery rates (FDR).

Clustering

As denoted above, it is likely that selection led to increased F_{ST} indexes for a series of consecutive SNPs. Therefore, we identified clusters of SNPs, which provided stronger evidence of selection sweeps, compared to F_{ST} indexes for single SNPs. A cluster contained a minimum of two significant SNPs ($p_{nominal} \leq 5\text{x}10^{-5}$) with a maximum distance of 3 Mb

between them.

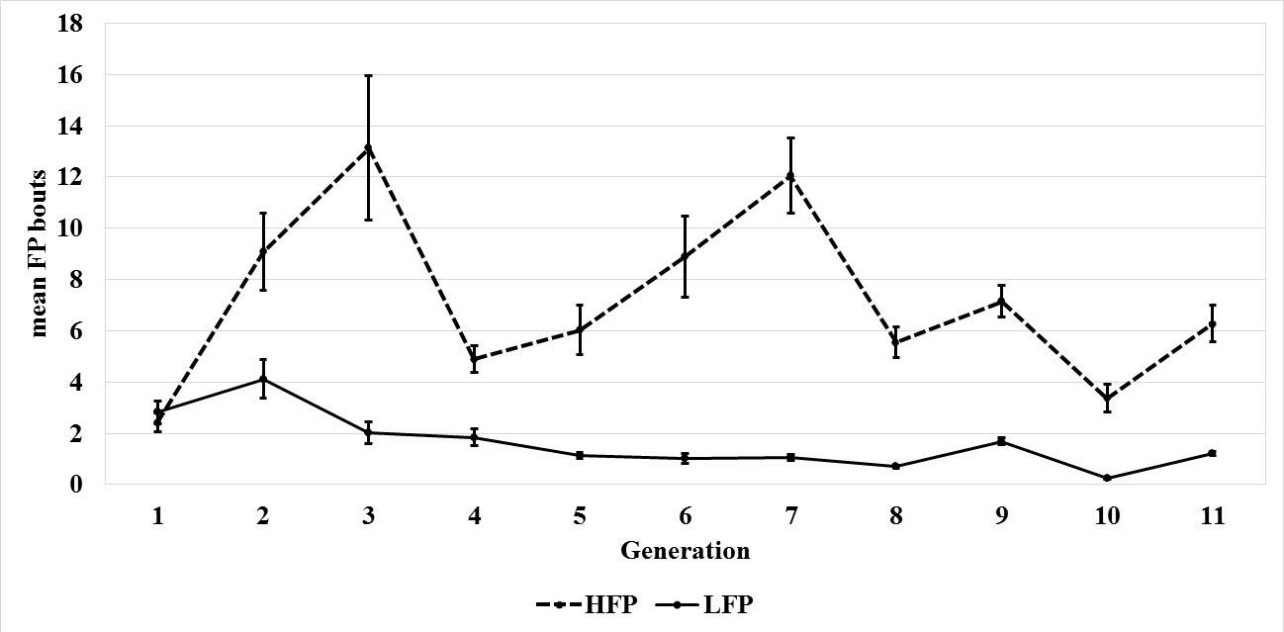

Figure 1 Phenotypic trend over 11 generations. The interrupted and continuous lines show the average number of feather pecking bouts per hen during the observation period of 180 minutes with respective standard errors for the high and low feather pecking line.

Results

The phenotypic trend for feather pecking during the 11 generations of selection is shown in Figure 1. From the first round of selection onwards, lines HFP and LFP differed in mean numbers of FP bouts. Selection response on the phenotypic scale was greatest during the first two rounds of selection, then, the mean number of FP bouts decreased sharply from generation 3 to 4 for the HFP line. The explanation is that HFP males were killed by accident in generation 3 and had to be replaced by males from a control line to produce the next generation. After generation 4, variability in pecking behaviour is most likely caused by environmental effects. For line LFP, the level of pecking behaviour was constantly low during the 11 generations of selection and showed only a small and almost undetectable decrease in the mean number of FP bouts over generations.

Table 1 Estimated additive genetic variance (σ_a^2), generation variance (σ_{gen}^2), residual variance (σ_e^2), heritability (h^2) and standard error (in parenthesis) for trait feather pecking bouts using a Poisson model and a linear model

Model	**Line**	σ_a^2	σ_{gen}^2	σ_e^2	$\mathbf{h^2}$
Poisson	HFP	2.760 (0.24)	0.27 (0.23)	-	-
	LFP	1.430 (0.15)	0.35 (0.24)	-	-
Linear	HFP	0.090 (0.04)	0.08 (0.05)	0.55 (0.04)	0.15 (0.07)
	LFP	0.001 (0.01)	0.03 (0.02)	0.26 (0.02)	0.01 (0.03)

Estimated variance components are shown in Table 1. With the Poisson model (model 1), additive genetic variance for line HFP is almost twice as large as that for line LFP. However, even in line LFP, this variance is significantly different from zero since it has a small standard error. Generation variance is small compared to additive genetic variance and its value is similar in both lines. In contrast, the generation variance estimated with the linear mixed model (model 2) is substantially larger, compared to the additive genetic variance. The heritability of FP for the HFP line was equal to 0.15 and was at the lower bound of the range of values reported in the literature. For line LFP, the additive genetic variance and hence the heritability were close to zero.

Table 2 Number of significant SNPs, F_{ST}-indexes and FDR of significant SNPs for three levels of significance

Significance level		**Number of SNPs**	**F_{ST}-index**	**FDR**
$p_{genome\ wide}$	< 0.05	17	1.000	< 0.001
$p_{nominal}$	$\leq 5x10^{-5}$	49	0.901	≤ 0.015
$p_{nominal}$	$\leq 5x10^{-4}$	276	0.730	≤ 0.034

An overall F_{ST} index of 0.15 was estimated for the whole set of SNPs. The number of significant F_{ST} values is shown in Table 2. FDR for the significant SNPs were low, even at the relaxed significance level. The 17 genome-wide significant SNPs had an F_{ST} value of 1, i.e. alleles were divergently fixed in the two lines. A full list of significant SNPs is in Table S1 [See Additional file 1 Table S1]. Manhattan plots of the F_{ST} values are in Figure 2. Most of the genome-wide significant SNPs are located on chromosome 4, followed by chromosome 3. The results of the sliding window approach (Figure 3) revealed five distinct peaks, i.e. two on chromosome 3, and one on each of chromosomes 4, 8, and 19. Thirteen clusters with at least two significant SNPs were identified (Table 3). Based on Figure 3, two clusters were observed on chromosomes 3, 4 and 19. These clusters harboured several genome-wide

significant SNPs, especially the cluster on chromosome 4. The size of the clusters is small, except for those on chromosomes 3 and 4. Among the 17 genome-wide significant SNPs, 12 are located within the identified clusters.

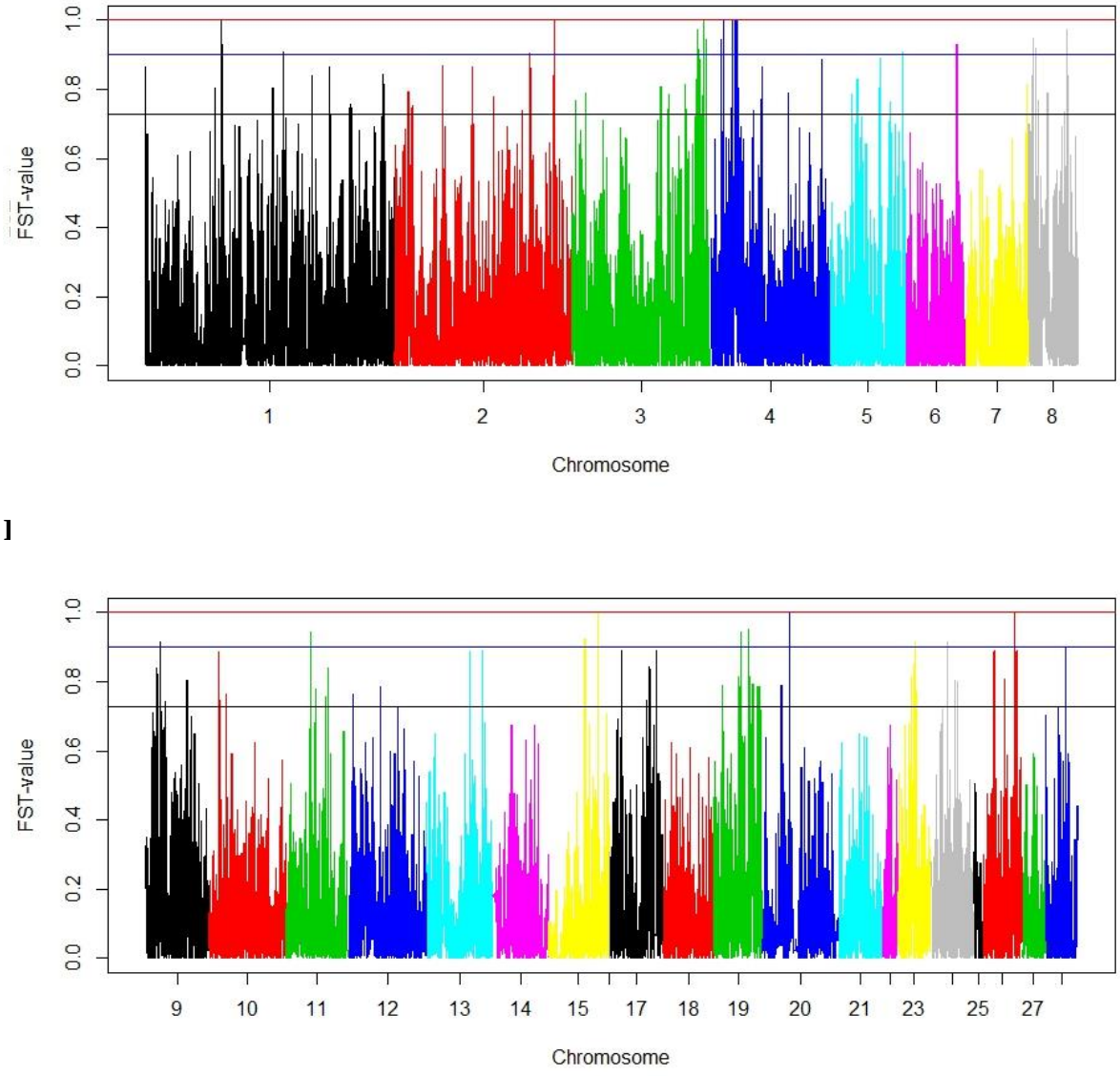

Figure 2 Manhattan plots of F_{ST}-indexes. The top panel shows the F_{ST}-values of each marker from chromosomes 1 to 8 and the bottom panel for chromosomes 9 to 28. The top threshold value indicates the genome-wide significance level $p_{genome\ wide} < 0.05$; the middle and bottom threshold values are the nominal significance levels $p_{nominal} \leq 5x10^{-5}$ and $p_{nominal} \leq 5x10^{-4}$, respectively.

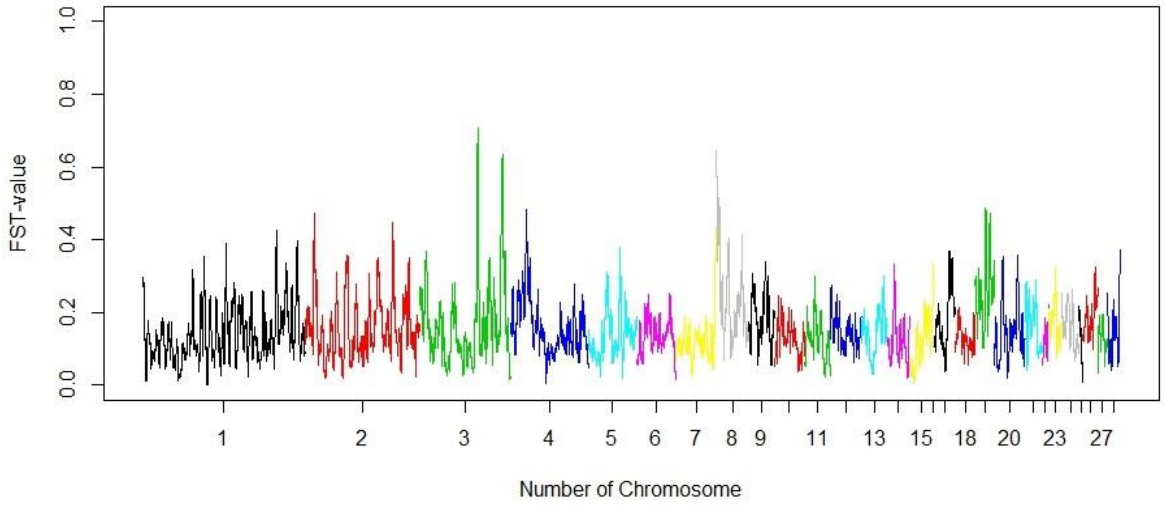

Figure 3 Manhattan plots of FST-indexes in a sliding window of 25 consecutive SNPs.

Discussion

One of the main reasons for establishing short-term selection experiments is to demonstrate that selection results in a selection response and thus, that it is feasible to breed for the trait under consideration. With regard to this, the selection experiment described in this study was moderately successful since selection response became immediately visible and the mean trait values of the two divergent selection lines differed for all generations, with the mean for line HFP always greater than that for line LFP (Figure 1). Although not formally tested, it can be reasonably assumed that the consistent difference in the means of the number of FP bouts for both lines represents a true difference rather than a sampling effect, which was also stated by Kjaer et al. [1] and Su et al. [2]. This is also supported by the small standard errors estimated for the means (Figure 1). The initial selection response in line HFP could not be maintained in subsequent generations. The reason is that it was often not possible to retain the animals with the highest estimated breeding value as parents to breed the next generation because of handling problems and increased mortality rates with these birds. This limited the selection intensity and hence genetic progress.

The data were analyzed with two very simple models, because no information was available on the observer, the pen, or other effects known to influence FP behaviour. The generation effect captured part of these effects. However, inclusion of this effect was a compromise since it probably captured a least part of the genetic progress. To some extent, the two models

produced different results. A formal model comparison would be possible by assessing model predictive ability using cross-validation, but this was beyond the aim of our study. The dataset was too small for cross-validation, especially with the generation structure in the data.

Table 3 Number of clusters, chromosomes and chromosomal position in bp, length in Mb and number of significant SNPs (significant level $p_{nominal} \leq 5x10^{-5}$ and $p_{gemome\ wide} \leq 0.05$) in each cluster

Cluster number	**Chr.**	**start/end position in bp**	**Length in Mb**	**Number of SNPs $p_{nominal} \leq 5x10^{-5}$**	**Number of SNPs $p_{gemone\ wide} \leq 0.05$**
1	1	58.108.441-58.537.760	0.43	4	1
2	3	103.609.224-105.597.337	1.40	5	0
3	3	108.252.363-109.945.836	1.69	2	1
4	4	10.364.490-10.575.112	0.21	3	3
5	4	18.580.845-21.323.065	2.74	7	7
7	6	31.974.670-32.086.164	0.11	2	0
8	8	4.002.499-4.211.591	0.21	2	0
9	8	23.892.743-23.911.149	0.02	2	0
10	11	11.015.338-11.139.271	0.12	2	0
11	15	7.826.821-7.879.094	0.05	2	0
12	19	5.204.468-5.273.813	0.07	2	0
13	19	6.883.105-6.896.487	0.01	2	0

It seems that the linear model attributed more variance to the generation effect, while in the Poisson model the additive genetic variance was greater (Table 1). In addition, the linear model estimated an additive genetic variance close to 0 in line LFP, while the Poisson model did not. Hence, although Figure 1 suggests that line LFP is close to reaching a selection limit, the trend of the animal effects estimated with the Poisson model across generations still revealed a small selection response (Figure 4). This response is not detected based on the trend of the mean additive effects estimated with the mixed linear model, as expected given the low additive genetic variance. The estimated heritability for line HFP was at the lower bound of the range of values reported in the literature (see Background section). For line HFP,

the Poisson model revealed a continuous selection response (Figure 4), which was not detectable with the mixed linear model. It seems that, in the mixed linear model, the generation effect completely captured the small genetic progress that was gained over generations, which was not the case in the Poisson model. The larger amount of variance

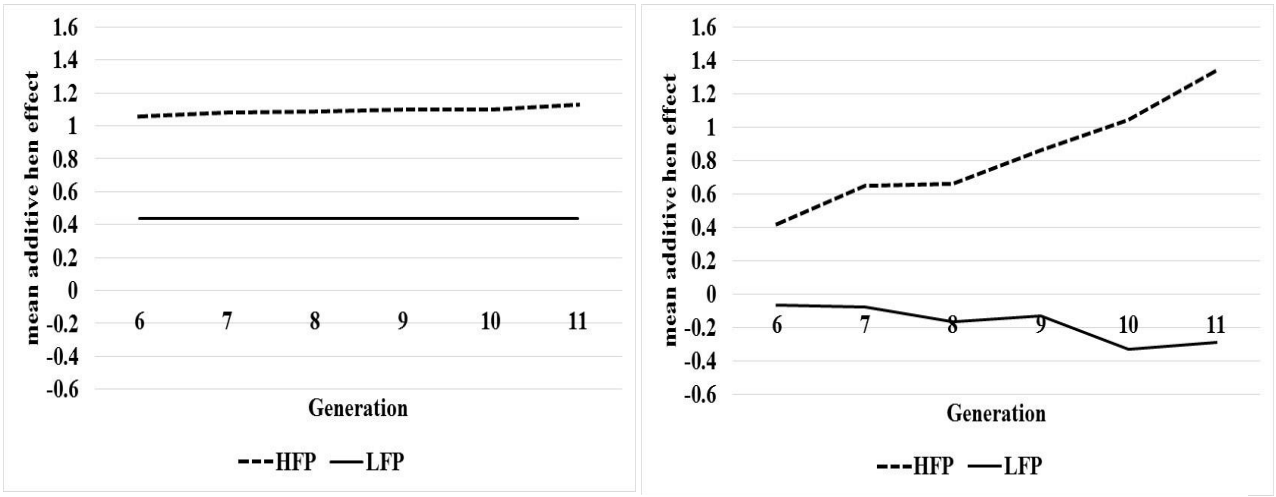

Figure 4 Selection response in the high (HFP) and low (LFP) feather pecking lines across generations. The mean additive hen effects were estimated with a linear mixed model (top panel) and with a Poisson model (bottom panel). The estimated intercept was added to the additive hen effects.

explained by the generation in the mixed linear model compared to the Poisson model (Table 1) supports this explanation. The rank correlations between additive genetic effects of animals estimated with the mixed linear and Poisson models were equal to 0.74 (HFP line) and 0.68 (LFP line).

In analyses for the detection of selection sweeps, one of the main challenges is to separate drift and selection effects. In our study, due to the complete knowledge of the demographic history of the two selected lines since their common base population, we were able to model drift effect stochastically. This led to the detection of significant SNPs and selection signatures. The histogram of real and simulated (pure drift model) F_{ST} values is in Figure S1 [See Additional file 2 Figure S1]. Compared to the histogram of simulated F_{ST}, a thick tail is observed for the histogram of real F_{ST} values, which is likely due to the effect of selection. The applied test statistic is somewhat conservative, because the simulated gene frequency in the base population was set to 0.5, for which genetic drift is highest.

Based on the assumption that selection affects several consecutive SNPs, criteria to build a cluster of SNPs were defined, and based on these criteria, 13 clusters were identified. Most clusters were small and included only few significant SNPs (Table 3). The extent of LD for the SNPs included in this study for the two lines is not known. However, the drift that is

operating during the selection experiment is expected to create a greater long-range LD within the lines compared to within the base population. In addition, selection results in LD around the functional gene. Given the relatively short selection period, the clusters that point to selection sweeps can be expected to be large. This might hold true for selection sweeps that are present only within one line, which, however, cannot be detected with the F_{ST} approach applied. Two interesting clusters were slightly larger than 2 Mb and included multiple significant SNPs on chromosomes 3 and 4 (Table 3). In addition, the 17 genome-wide significant SNPs were not randomly distributed across the genome, but, in most cases, located within the clusters, which supports the presence of selection sweeps around these clusters.

In contrast to quantitative genetic studies related to FP behaviour, to our knowledge, only a few QTL mapping experiments have been conducted and were mostly based on microsatellite linkage analysis (e.g. [26, 27, 13]). Buitenhuis et al. [26] reported a QTL for FP on chromosome 1, two on chromosome 2 and one on chromosome 10. We also identified clusters with selection sweeps on these chromosomes (Table 3), but a fine comparison of the QTL positions on these chromosomes is limited by the wide confidence intervals in QTL linkage studies. Biscarini et al. [28] performed an across-line SNP association study for genetic effects on feather damage in nine genetic lines and reported that the gene *HTR2C* (*5-hydroxytryptamine (serotonin) receptor 2C, G protein-coupled*) is associated with FP behaviour.

Molecular analyses suggested putative candidate genes for feather pecking behaviour [29-31]. According to Keeling et al. [29], the *PMEL 17* (*premelanosome protein*) gene affects plumage melanisation and the amount of feather pecking received. Two other candidate genes, *dopamine receptor D4* (*DRD4*) and *DEAF1 transcription factor* (*DEAF1*), have been shown to be associated with FP behaviour [30]. Gene expression analyses with brain tissues collected from individuals of the same HFP and LFP lines as those used here have led to the identification of six candidate genes, namely *HTR1B* (*5-hydroxytryptamine (serotonin) receptor 1B, G protein-coupled*), *SIP1* (Smad interacting protein 1), *PSEN1* (*presenilin-1*), *GLUL* (*glutamate-ammonia ligase*), *TSPO* (*translocator protein*) and *MAOA (monoamine oxidase A*), which may be involved in FP behaviour [31]. However, none of these candidate genes were located in the 13 cluster regions identified in our study.

Conclusions

In conclusion, the use of a Poisson model is advantageous to analyze data on FP behaviour, because the assumptions made by the linear model are too heavily violated. This is especially

the case if the number of FP bouts is small and the distribution is heavily peaked at 0, as is the case for line LFP.

The F_{ST}-based approach that we applied to the genotypic data from individuals of the last generation of lines HFP and LFP was suitable to map selection signatures. Only a few individuals had to be genotyped and it was not necessary to perform individual phenotyping in addition to routine phenotyping. The non-random distribution of genome-wide significant SNPs indicates the presence of selection sweeps. A more detailed analysis of e.g. putative gene effects or the explained variance can only be done by using linkage or association mapping experiments. We have set up a large F2 design from lines HFP and LFP. The individuals of this experimental cross were phenotyped for a number of behaviour traits [10, 14] and are being genotyped using a high-density SNP chip. In the near future, we shall carry out QTL mapping using this F2 population and we shall combine the results with those obtained here as reported in Schwarzenbacher et al. [32] in order to detect and confirm QTL that affect FP behaviour.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MG, WB, and JK conducted the selection experiment; SP performed the genotyping and quality checks of the genotypic data; RW and JB developed the statistical models; VG did the statistical analysis; VG and JB wrote the paper. All authors read and approved the final manuscript.

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ADDITIONAL FILES

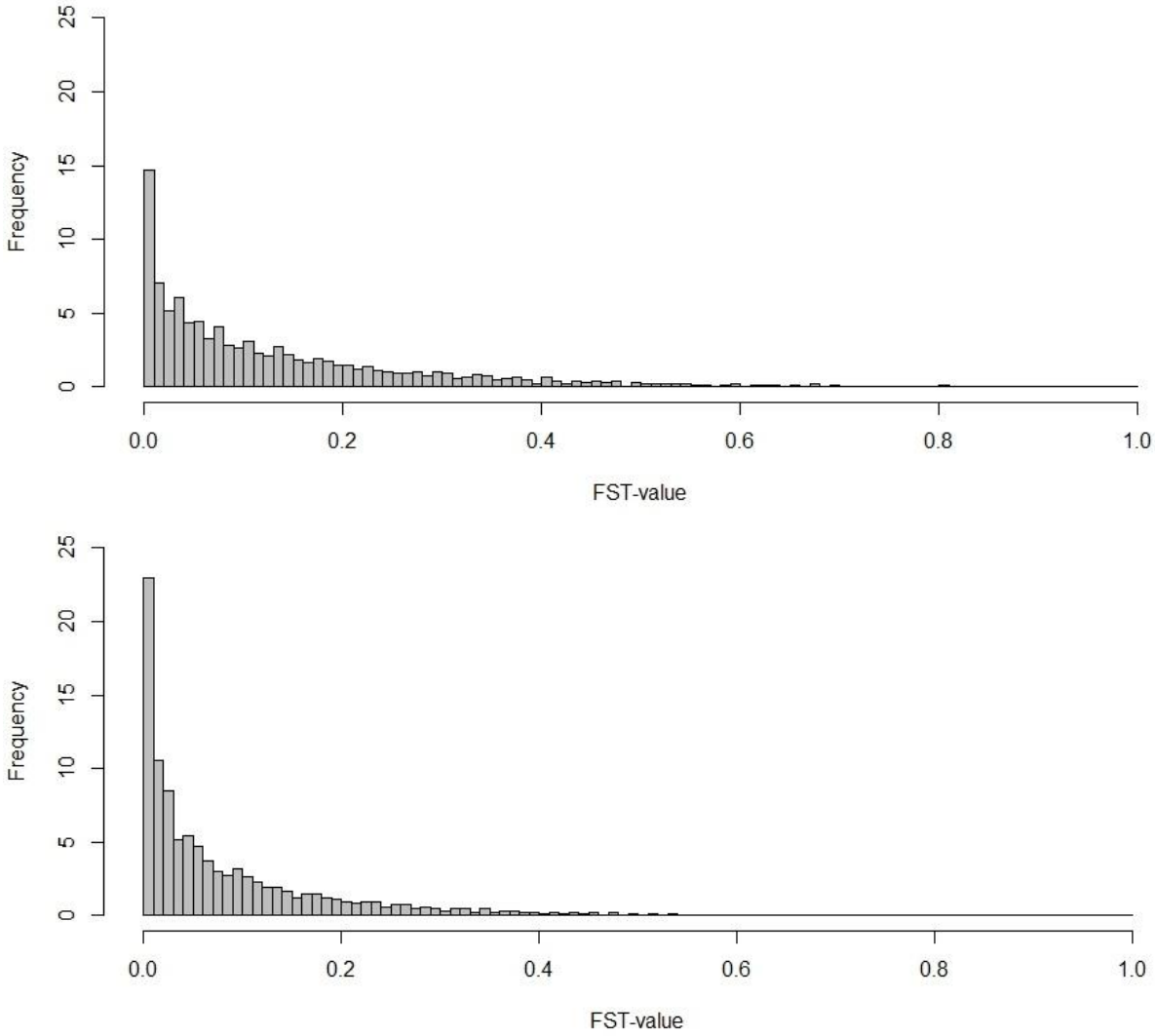

Figure S1 Histogram of real (top panel) and simulated (bottom panel) F_{ST}-indexes. Figure S1 shows the histogram of the real and the simulated F_{ST}-indexes.

Table S1 SNPs with a genome wide significance level $p_{genome\ wide} < 0.05$ and their position (bp) on chromosome, F_{ST}-value and cluster number from Table 3 of the main text.

SNP	Chromosome	Position (bp)	F_{ST}-value	Cluster from table 3
GGaluGA019372	01	58108441	1.00	1
Gga_rs16139375	02	138867071	1.00	-
GGaluGA238408	03	108252363	1.00	3
Gga_rs13643399	04	10364490	1.00	4
Gga_rs13643409	04	10387283	1.00	4
GGaluGA245350	04	10575112	1.00	4
GGaluGA248019	04	16462888	1.00	-
Gga_rs16371032	04	18580845	1.00	5
Gga_rs10725958	04	18594711	1.00	5
Gga_rs15508371	04	18762763	1.00	5
GGaluGA248971	04	21004836	1.00	5
Gga_rs16371453	04	21226700	1.00	5
Gga_rs14436318	04	21308984	1.00	5
GGaluGA49012	04	21323065	1.00	5
Gga_rs15024957	15	10460502	1.00	-
Gga_rs10726111	20	5064598	1.00	-
Gga_rs14300656	26	4035940	1.00	-

CHAPTER FIVE

A genome-wide association study in a large F2-cross of laying hens reveals novel genomic regions associated with feather pecking and aggressive pecking behavior

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Abstract

Background

Feather pecking and aggressive pecking in laying hens are serious economic and welfare issues. In spite of extensive research on feather pecking during the last decades, the motivation for this behavior is still not clear. A small to moderate heritability has frequently been reported for these traits. Recently, we identified several single-nucleotide polymorphisms (SNPs) associated with feather pecking by mapping selection signatures in two divergent feather pecking lines. Here, we performed a genome-wide association analysis (GWAS) for feather pecking and aggressive pecking behavior, then combined the results with those from the recent selection signature experiment, and linked them to those obtained from a differential gene expression study.

Methods

A large F2 cross of 960 F2 hens was generated using the divergent lines as founders. Hens were phenotyped for feather pecks delivered (FPD), aggressive pecks delivered (APD), and aggressive pecks received (APR). Individuals were genotyped with the Illumina 60K chicken Infinium iSelect chip. After data filtering, 29,376 SNPs remained for analyses. Single-marker GWAS was performed using a Poisson model. The results were combined with those from the selection signature experiment using Fisher's combined probability test.

Results

Numerous significant SNPs were identified for all traits but with low false discovery rates. Nearly all significant SNPs were located in clusters that spanned a maximum of 3 Mb and included at least two significant SNPs. For FPD, four clusters were identified, which increased to 13 based on the meta-analysis (FPDmeta). Seven clusters were identified for APD and three for APR. Eight genes (of the 750 investigated genes located in the FPDmeta clusters) were significantly differentially-expressed in the brain of hens from both lines. One gene, *SLC12A9*, and the positional candidate gene for APD, *GNG2*, may be linked to the monomanine signaling pathway, which is involved in feather pecking and aggressive behavior.

Conclusions

Combining the results from the GWAS with those of the selection signature experiment substantially increased the statistical power. The behavioral traits were controlled by many genes with small effects and no single SNP had effects large enough to justify its use in marker-assisted selection.

Background

Feather pecking in laying hens is a serious economic and welfare issue that can be observed in commercial and non-commercial chicken flocks. In spite of extensive research on feather pecking during the last decades, the motivation for this behavior is still unclear. The most widespread theory on the origin of feather pecking is that it is a redirected feeding and foraging behavior [1]. Some authors reported that feather pecking is related to dust-bathing [2]. Environmental factors such as light intensity [3], stocking density [4], and food form [5] can influence feather pecking. Feather pecking behavior has also been associated with fear [6-9]. Other studies suggested that the underlying motivation for feather pecking is feather eating [10-13] or that it is the consequence of a general hyperactivity disorder [14]. Feather pecking is often confounded with aggressive pecking but these two behaviors are clearly distinguishable, both in terms of form and motivation; aggressive pecks are delivered in an upright body posture, are mainly directed to the head of the other birds and aim at establishing and maintaining social hierarchy [15], while feather pecking is performed in a non-aggressive posture. Reports on the relationship between aggressive pecking and feather pecking show no consistent trend. While some authors found no correlation between the two behaviors, positive genetic and phenotypic correlations have been reported in lines selected for high and low feather pecking and their F2-crosses [16, 17]. Depending on the definition of the trait, study design, age of hens, statistical model applied, and data collection period, heritability estimates for feather pecking are low to moderate and range from 0.1 to 0.4, while heritability estimates for aggressive pecking range from 0.04 and 0.14 [17-20].

In a previous study, we analyzed two divergent lines that were selected for 11 generations for high (HFP) and low (LFP) feather pecking, respectively [20, 21]. We estimated genetic parameters within the lines and the phenotypic trend across generations. From the first round of selection onwards, the two lines differed in their means for feather pecking bouts. The highest selection response on the phenotypic scale was obtained during the first rounds of selection and thereafter, no clear trend was observed in the HFP line. The LFP line showed a constant low level of pecking behavior across the 11 generations of selection. Heritabilities of feather pecking estimated based on linear mixed models were equal to 0.15 and 0.01 in the HFP and LFP lines, respectively. The distribution of feather pecking bouts within each line and for each round of selection are discussed in detail in [21].

In addition, we performed a genome scan to map selection signatures in these two divergent HFP and LFP lines using an FST-based approach [20]. The analysis provided 17 genome-

wide significant single-nucleotide polymorphisms (SNPs), most of which were located in clusters, which supports the presence of selection signatures.

These HFP and LFP lines formed the base population of the F2-population used in the current study, in which a genome-wide association analysis (GWAS) for feather pecking and aggressive pecking behavior was performed. The results obtained were combined with those from the previous selection experiment [20] in a meta-analysis, and then linked to those obtained from a differential gene expression study.

Methods

Experimental population

Chickens from a White Leghorn line were divergently selected for low and high feather pecking for 11 generations, resulting in a LFP and a HFP line. Selection took place for five generations at the Danish Institute of Animal Science [18] and then for five additional generations at the Institute of Animal Science, University Hohenheim, Germany [20]. From these two lines, a large F2 cross was established. Five sires and ten dams from generation 11 of each line were used to generate 10 F1 families. Each HFP sire was crossed with two LFP full-sib dams and vice versa. Then, 10 F1 sires were used to generate the F2 families. Each sire was mated with eight F1 hens four times by artificial insemination. A total of 960 F2 offspring were produced in four hatches, with an interval of three weeks between hatches.

Phenotypes

At 27 weeks of age, feather pecks delivered (FPD) and aggressive behavior [aggressive pecks delivered (APD) and aggressive pecks received (APR)] were recorded in groups of 36 to 42 hens. The applied ethogram was according to Savory [22] and Bessei et al. [16] and was as follows. Feather pecking was defined as a non-aggressive behavior and included forceful pecks, sometimes with feathers being pulled out and the recipient hen either tolerating this action or moving away. Aggressive pecking was defined as fast pecks towards the head and body of conspecifics. Usually, the hen that was attacked moved away but may have incurred tissue damage. For the behavioral observations, the hens were marked with numbered plastic batches on their backs. Seven observers visually recorded feather pecking and aggressive pecking within each pen during 20-min sessions for three consecutive days during daytime. Hatches 3 and 4 were observed twice for three consecutive days. The total number of observers varied between five and seven persons per observation day. The numbers of FPD,

APD, and APR were summed over the entire observation period and standardized to an observation period of 420 minutes. Heritabilities of FPD (APD, and APR), estimated with a linear mixed model in this F2 cross, were equal to 0.12 (0.27, and 0.27) [23]. Genetic and phenotypic correlations of 0.2 and 0.09, respectively, were obtained between FPD and APD [9]. Correlations of estimated breeding values between FPD and APR and between APD and APR were 0.18 and -0.23, respectively [17].

Genotypes

A total of 817 F2 hens were genotyped with the Illumina 60K chicken Infinium iSelect chip. For the remaining hens no samples were collected. A total of 57,636 SNPs were genotyped and after data filtering, 29,376 SNPs remained in the dataset. Based on positional information according to the chicken genome assembly galGal2.1, SNPs that were located on the sex chromosomes W or Z or in the linkage groups LGE22C19W28_E50C23 or LGE64, and SNPs that were not allocated to a specific chromosome or linkage group were excluded. In addition, SNPs with minor allele frequencies (MAF) lower than 0.03 and SNPs with a call frequency lower than 0.95 were filtered out.

Statistical analysis

In order to investigate the mapping resolution of the design, the linkage disequilibrium (LD) structure was investigated for the first nine chromosomes i.e. GGA1 to GGA9 (GGA for *Gallus gallus* chromosome). The Beagle Genetic Software Analysis [24, 25], which is included in the synbreed R package [26], was used to phase haplotypes and then the common LD measure r^2 was estimated using PLINK [27] for pairs of SNPs that were less than 5 Mb apart across the autosomes.

GWAS are frequently conducted using mixed linear models (e.g., [28]). In its simplest form, such models include a general mean, a fixed SNP effect and a random family effect. The latter is important to capture population stratification effects and, hence, to prevent inflation of type I errors (e.g., [29]). Previous studies showed that FPD, APD and APR are not normally distributed and that Poisson models should be used for the statistical analyses [17, 20]. Poisson models with fixed and random effects belong to a class of generalized linear mixed models (GLMM). Due to the lack of a closed form of expression of the likelihood for these models, approximate likelihood techniques are often used, as for example in the software ASReml [30]. However, for hypothesis testing, the behavior of these techniques has not been sufficiently well investigated, and Collins [31] recommended that GLMM should not be used

for this purpose. Therefore, we used the following generalized linear model based on the Poisson distribution and no random effects for single-marker association analysis:

$$\eta_{ijm} = H_j + S_i + D_i + b_m x_{im}, \quad (1)$$

where η_{ijm} is the linear predictor for hen i and SNP m, H_j is the fixed hatch effect, S_i and D_i are the fixed sire and dam effects, respectively, x_{im} denotes the number of copies of the minor allele of SNP m (x = 0, 1, or 2), and b_m is the regression coefficient for SNP m. Thus, instead of fitting a random family effect, we included fixed sire and dam effects in the model to account for population stratification effects.

In a previous study, we detected substantial permanent environmental effects for FPD, APD and APR [17], which could also be caused by dominant gene effects. Because dominance and additive gene effects tend to be correlated such that larger dominance deviations are observed for genes with larger additive effects [32], we tested only genome-wide significant SNPs from Model (1) or from the meta-analysis (described below) for dominance effects using the following Poisson model:

$$\eta_{ijm} = H_j + S_i + D_i + b_m x_{im} + \tilde{b}_m z_{im} \quad (2)$$

where z_{im} is an indicator variable, which is 1(0) if the individual is heterozygous (homozygous) at SNP m and $\tilde{b}_m$ is a fixed regression coefficient, which is a dominance estimate. The other terms are defined as in Model (1).

To correct for multiple-testing, we applied a Bonferroni-type correction as:

$$p_{genome-wide} = 1 - (1 - p)^{\#\mathrm{SNP}},$$

where the number (#) of SNPs was equal to 29,376. The genome-wide significance level was set at $p_{genome-wide} \leq 0.05$. Because Bonferroni's correction is very conservative, we considered an additional nominal significant level; i.e. $p \leq 5.10^{-5}$. To estimate the number of false positives among the significant SNPs, we calculated a false discovery rate (FDR) q-value for each association test by using the software QVALUE [33]. The FDR q-value of the significant SNP with the largest p-value provided an estimate of the proportion of false positives among the significant SNPs.

A meta-analysis was performed using the data from the selection experiment and the F2-cross experiment. We combined the p-values from both studies using the inverse chi-square method

of Fisher [34], known as Fisher's combined probability test, as follows:

$$\chi^2_{2k} \sim -2\sum_{i=1}^{k} ln(p_i),$$

where p_i is the p-value for the i^{th} hypothesis test and k is the number of studies being combined (i.e., k = 2 in our study). The significance levels were used for the p-value obtained from the meta-analysis were the same as those for the GWAS (Model 1).

Cluster identification

We assumed that a causative mutation is in LD with several SNPs, and thus built clusters of SNPs, which provided strong evidence for trait-associated chromosomal regions compared to single significant SNPs, although of course it cannot be guaranteed that the mutation is within these clusters. A cluster contained at least two significant SNPs ($\leq 5.10^{-5}$), with a maximum distance of 3 Mb between them. The bounds of each cluster were identified using the LD structure as well as the p-values of SNPs with lower statistical support, as follows. Starting from the midpoint of the cluster of significant SNPs ($p \leq 5.10^{-5}$) and moving in both directions up to 1.5 Mb on each side, we searched for weakly significant SNPs. The weakly significant SNPs ($p \leq 5.10^{-4}$) at a maximum distance of 1.5 Mb from the cluster midpoint in both directions were used as the cluster bounds.

Differential gene expression analysis

Within each FDP_{meta} cluster, genes were investigated for differential expression. Expression data were generated in an earlier study [35]. In brief, the brains of nine hens each from the HFP and LFP line were collected after slaughter. RNA was extracted from the whole brain, reverse-transcribed into cDNA and then converted into labeled cRNA by *in vitro* transcription. Following this procedure, 1.65 µg of each single cRNA sample was hybridized on the Chicken Gene Expression Microarray (4 x 44 K format, Agilent Technologies) and fluorescent signal intensities were detected. The quantile-normalized and log2-transformed data were averaged across the hens within each line. A total of 1083 transcripts included in the microarray gene expression chip were located within the FDP_{meta} clusters. The average expression levels of these genes only were compared between the two lines using a standard Welch t-test. Correction for multiple-testing was performed using Bonferroni's test, assuming 1083 independent tests. Sequences of probes with no assigned gene or only a LOC number were subjected to BLAST analysis against the most recent genome database galGal 5.0

(assembly GCA_000002315.3) to identify the corresponding gene. Results of the expression analysis were subsequently compared to the candidate genes that were identified within the associated clusters. Clusters that contained differentially-expressed transcripts were checked for potential enrichment of those transcripts, because this indicates the presence of cis-acting QTL. The corrected p-values obtained in the original study [35] were used to separate transcripts into three categories of significance i.e. $p \leq 0.1$, $p \leq 0.05$, and $p \leq 0.01$, respectively. For each of these categories, the proportions of significantly differentially-expressed genes within clusters were compared to genome- and chromosome-wide proportions.

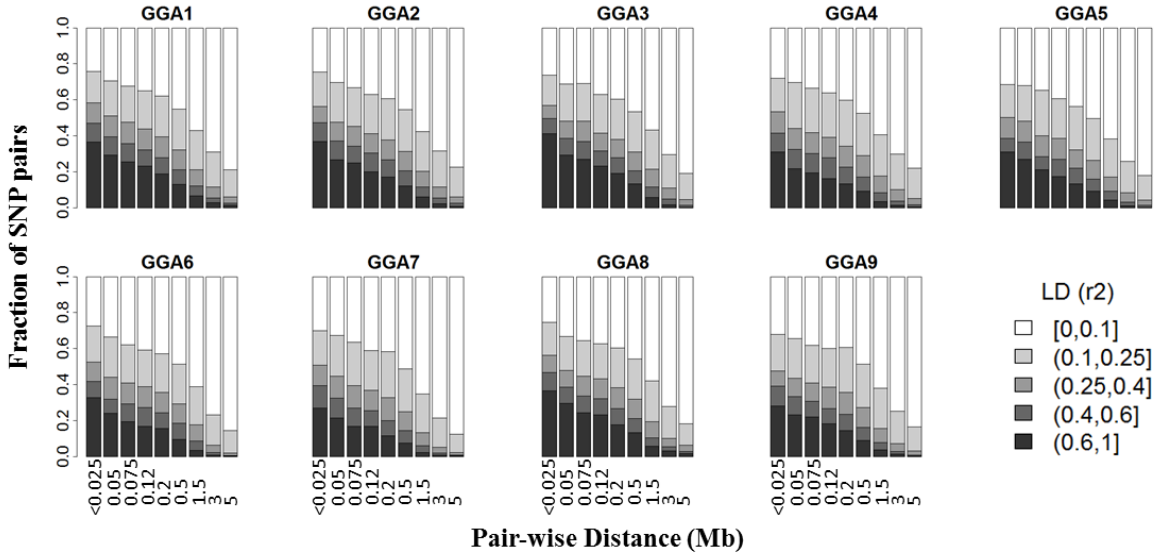

Figure 1 Linkage disequilibrium patterns. Level of linkage disequilibrium decay according to inter-SNP distance up to 5 Mb for the first nine chicken chromosomes (GGA1 to GGA9). The proportion of SNP pairs with different levels of linkage disequilibrium is shown for different distances between SNPs (in Mb) for the following bins (0, 0.025), (0.025, 0.05), (0.05, 0.075), (0.075, 0.12), (0.12, 0.2), (0.2, 0.5), (0.5, 1.5), (1.5, 3), (3, 5).

Results

Results of the LD analysis are in Fig. 1 and illustrated as a plot of the LD against the physical distance of the loci up to 5 Mb. Figure 1 shows that for small distances, the level of LD was high and decreased as distance increases, especially for distances larger than 1.5 Mb. This holds true for all nine investigated chromosomes.

Table 1 List of genome-wide significant SNPs for the traits APD and FDP_{meta}.

Trait	SNP	Chr	Position	-log10(p)	Gene frequency	F_{ST}
APD	Gga_rs14552589	5	57353834	6.8	0.13	-
	GGaluGA290503	5	57401911	6.4	0.13	-
	Gga_rs13923655	1	116041775	6.0	0.44	-
	Gga_rs15388609	1	116062599	5.8	0.44	-
FDP_{meta}	GGaluGA341482	9	17128657	7.4	0.45	0.76
	Gga_rs14676055	9	16629471	6.4	0.44	0.80
	GGaluGA341217	9	16764865	6.4	0.44	0.80
	Gga_rs13766455	9	5961337	6.0	0.46	0.82
	Gga_rs16519883	5	59368007	5.9	0.44	0.91
	Gga_rs14667686	9	6739756	5.9	0.48	0.92
	Gga_rs14652254	8	23911149	5.8	0.48	0.97
	Gga_rs15930799	8	23892743	5.8	0.48	0.97
	Gga_rs14652966	8	24679820	5.8	0.41	0.84

Chr chromosome number
Position in bp
Gene frequency in the F2-design
p-value obtained from Model (1)
FST-value obtained from the previously conducted selection signature experiment

The GWAS (Model 1) revealed 45 (20, 19, and 58) significant SNPs at $p < 5.10^{-5}$ for ADP (APR, FDP, and FDP_{meta}). The FDR for the significant SNPs associated with ADP, APR, FDP, and FDP_{meta} were less than 0.025, 0.07, 0.05, and 0.01, respectively. Lists of these significant SNPs are in Tables S1 and S2 [See Additional file 1 Table S1 and Additional file 2 Table S2]. Plots of the test statistics for the GWAS (i.e., -log10p-values) are in Fig. 2. For APD, four genome-wide significant SNPs were identified; i.e., two on GGA1 and two on GGA5 (Table 1). The latter two SNPs also showed a significant dominance effect ($p = 0.01$, results from Model 2, not shown). For FDP_{meta}, nine genome-wide significant SNPs were identified (Table 1) with none showing a significant dominance effect.

Results from the cluster analyses are in Tables 2 and 3. For FDP, four clusters were identified, and for FDP_{meta} 13 clusters were identified. Only the cluster on GGA8 overlapped between the two traits. Seven of the nine genome-wide significant FDP_{meta} SNPs were located within clusters on GGA8 and 9. For APD, seven clusters were identified and the four genome-wide significant SNPs were located within two clusters on GGA1 and 5. For APR, three clusters were identified on GGA7 and almost all the significant SNPs were located in clusters [See Additional file 1 Table S1 and Additional file 2 Table S2].

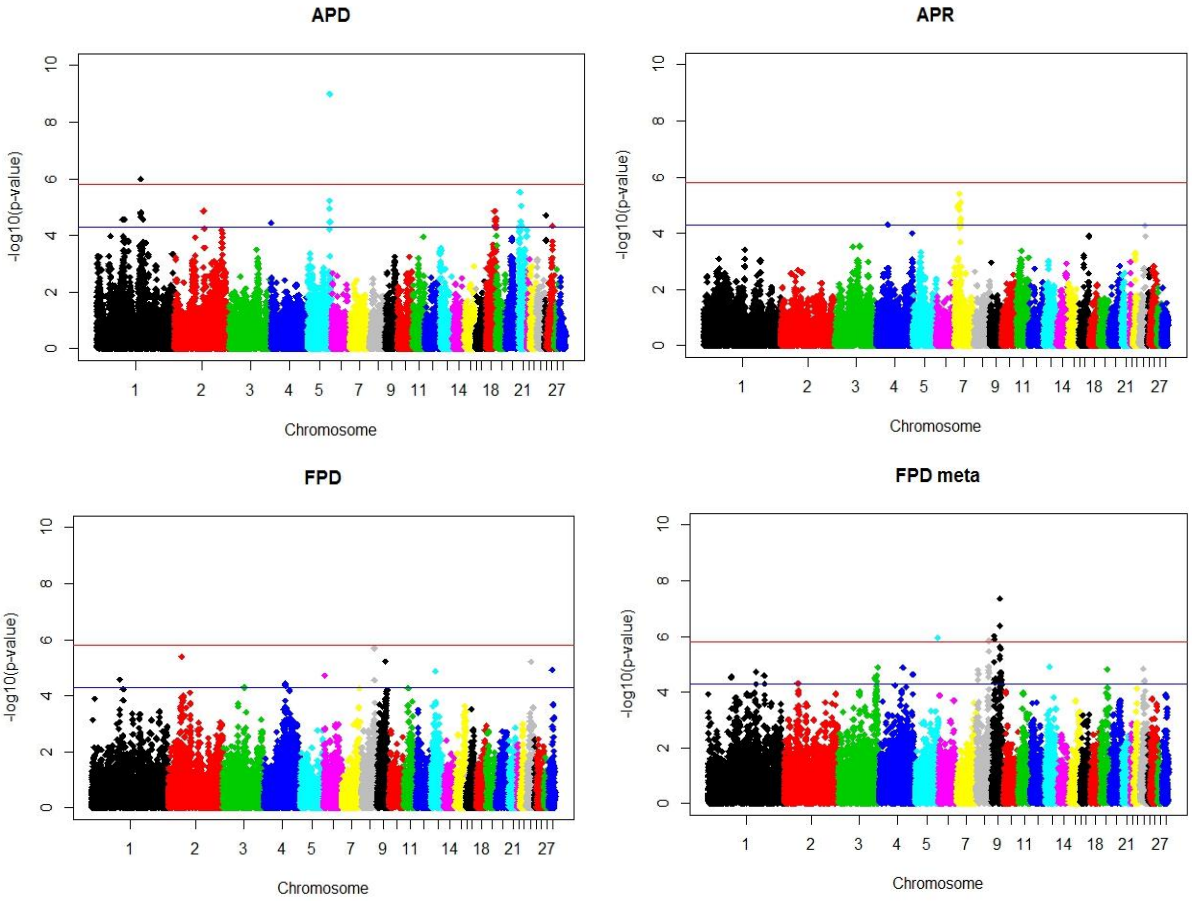

Figure 2 Manhattan plots. Manhattan plots of the -$\log_{10}$ p-values for association of SNPs with APD, APR and FPD, and the meta-analysis (FDP_{meta}). The top horizontal line indicates the genome-wide significance level $p_{genome-wide} \leq 0.05$, and the bottom line indicates the nominal level of significance $p \leq 5.10^{-5}$.

Results from the gene expression analysis are in Table 4. Nine of the 26 probe sets that showed significant results (nominal $p-$value ≤ 0.0001) were assigned to a LOC number or were not assigned to any gene. BLAST analysis identified the corresponding gene for only one of these. The 26 probes represented 22 different genes (Table 4). Sixteen of the 1083 probes showed a significant differential expression level, among which seven had a fold difference greater than 2, and one a fold difference of 7.8. Six of the Bonferroni's test-corrected significant transcripts were located within the same cluster, i.e. cluster number 9. The largest number of differentially-expressed transcripts was observed on GGA9, among which eight were experiment-wide significant and four were significant probes that mapped to clusters 9 and 10.

Table 2 Numbers of clusters, chromosomal positions, and numbers of significant SNPs for the traits FDP and FDP_{meta}

Trait	Cluster number	Chr	Start/end position in bp 3 Mbp interval	Length in Mb	Number of SNPs $p \leq 5.10^{-5}$	Number of SNPs $p_{genome-wide} \leq 0.05$
FPD	1	3	58,834,628 – 59,725,450	0.89	3	0
	2	4	53,335,653 – 53,945,398	0.61	6	0
	3	6	3059,760 – 3075 330	0.02	2	0
	4	8	25,309,634 – 25,399,547	0.09	2	0
FPD_{meta}	1	1	58,412,953 – 58,831,069	0.42	3	0
	2	1	149,753,999 – 150,465,791	0.71	2	0
	3	2	37,372,218 – 39,828,657	2.46	2	0
	4	3	102,969,523 – 105,470,402	2.50	2	0
	5	3	107,262,448 – 109,945,836	2.68	3	0
	6	4	87,030,671 – 87,082,448	0.05	2	0
	7	8	3612,454– 5410,229	1.80	3	0
	8	8	23,799,410 – 26,002,938	2.20	9	3
	9	9	5650,341– 7645,421	2.00	5	2
	10	9	16,342,044 – 18,770,002	2.43	13	3
	11	9	18,726,350 – 20,815,056	2.09	4	0
	12	19	6883,105 – 8064,270	1.18	2	0
	13	24	2480,724 – 3900,089	1.42	3	0

Chr Chromosome
significance level $p \leq 5.10^{-5}$ and $p_{genome-wide} \leq 0.05$

Table 3 Numbers of clusters, chromosomal positions, and numbers of significant SNPs for the traits APD and APR

Trait	Cluster number	Chr	Start/end position in bp 3 Mbp interval	Length in Mb	Number of SNPs $p \leq 5.10^{-5}$	Number of SNPs $p_{genome-wide} \leq 0.05$
APD	1	1	64,103,417 – 67,037,983	2.93	3	0
	2	1	116,041,775 – 117,435,846	1.39	6	2
	3	2	83,445,347 – 86,114,050	2.67	2	0
	4	4	33,821 – 552,165	0.52	7	0
	5	5	56,835,282 – 58,214,037	1.38	6	2
	6	18	8135,718 – 101,911,44	2.06	11	0
	7	21	504,778 – 3009,557	2.50	7	0
APR	1	7	6241,588 – 6327,771	0.09	3	0
	2	7	9746,560 – 12,631,641	2.89	10	0
	3	7	13,378,513– 14,679,901	1.30	5	0

Chr Chromosome
significance level $p \leq 5.10^{-5}$ and $p_{genome-wide} \leq 0.05$

In the previous expression study [35], 16.5, 9.7, and 2.3% of the annotated probe sets were significantly differentially-expressed with corrected *p*-values less than 0.1, 0.05 and 0.01, respectively. For the individual chromosomes tested in this study, marked deviations from these fractions were found for GGA8 and GGA19 (Fig. 3). Among the seven FDP_{meta} clusters that harbored differentially-expressed transcripts, substantial enrichment was found for FPD_{meta} cluster 4 and a moderate enrichment for FPD_{meta} cluster 9 (Fig. 3). FDP_{meta} cluster 10 showed a slight enrichment only for *p*-values less than 0.01 (Fig. 3).

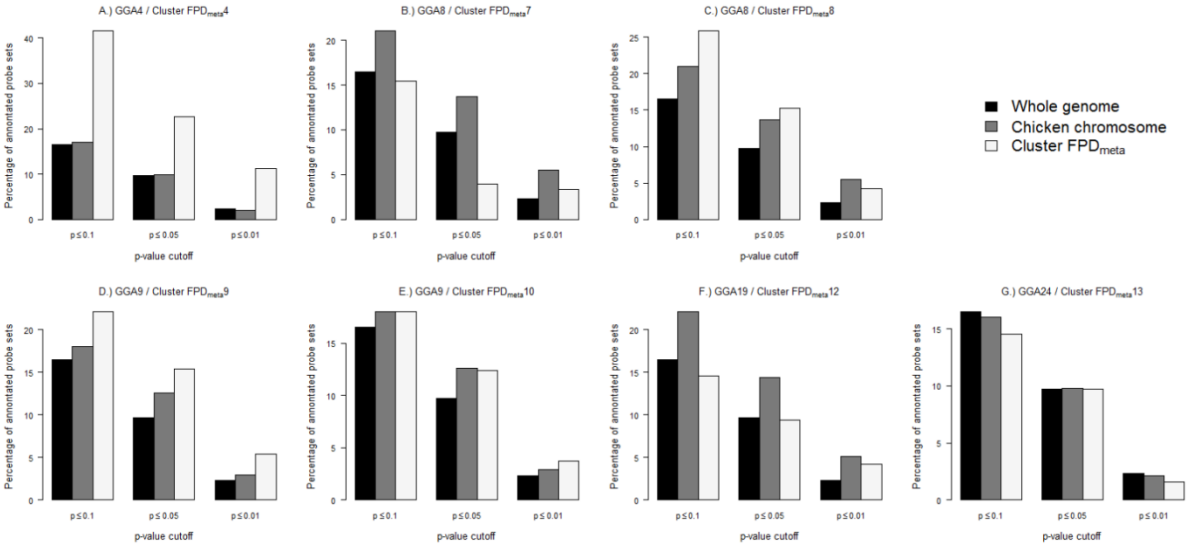

Figure 3 Enrichment of differentially-expressed transcripts in association clusters. Bars depict the fractions of differentially-expressed transcripts at different *p*-value thresholds at the genome- (left bar) and chromosome-wide (middle bar) level, as well as for individual clusters (right bar) that harbor differentially-expressed transcripts.

Table 4 Genes located in one of the FPD_{meta} clusters (Table 2) that were significantly differentially-expressed (nominal p-value ≤ 0.0001) in the HFP and LFP lines

ProbeSetID[a]	Chr[b]	Position [Mb][b]	FPD_{meta} cluster	-log10 p	Gene symbol	Gene name	Nfold	Reg
A_87_P022983	**3**	**104.30**	**4**	**4.58**	***WDR35***	**WD repeat domain 35**	**7.80**	**up**
A_87_P021624	**3**	**104.33**	**4**	**5.85**	***LAPTM4A***	**lysosomal protein transmembrane 4 alpha**	**1.28**	**down**
A_87_P018137	**3**	**104.80**	**4**	**5.23**	***HS1BP3***	**HCLS1 binding protein 3**	**2.53**	**up**
A_87_P254443	3	104.84	4	4.28	*LDAH*	lipid droplet associated hydrolase	1.34	up
A_87_P176188	3	105.39	4	4.16	*LOC769627*	Unknown[c]	1.94	down
A_87_P304288	**8**	**3.73**	**7**	**5.64**	***LOC101751271***	**1-phosphatidylinositol phosphodiesterase-like**	**1.88**	**down**
A_87_P052241	8	4.03	7	4.01	*MTA1*	metastasis associated 1	1.19	down
A_87_P079496	**8**	**25.66**	**8**	**4.58**	***GLIS1***	**GLIS family zinc finger 1**	**2.02**	**down**
A_87_P016336	8	26.00	8	4.10	*TTC4*	tetratricopeptide repeat domain 4	1.37	down
A_87_P022335	**8**	**26.00**	**8**	**4.35**	***PARS2***	**prolyl-tRNA synthetase 2, mitochondrial (putative)**	**1.36**	**up**
A_87_P139413	**9**	**5.67**	**9**	**4.17**	***AQP12***	**aquaporin 12**	**1.78**	**up**
A_87_P012759	**9**	**5.67**	**9**	**6.92**	***AQP12***	**aquaporin 12**	**1.67**	**up**
A_87_P077026	**9**	**5.68**	**9**	**4.09**	***PAK2***	**p21(RAC1)activated kinase 2**	**1.96**	**up**
A_87_P280878	**9**	**5.69**	**9**	**7.95**	***PAK2***	**p21(RAC1)activated kinase 2**	**1.81**	**up**
A_87_P285338	**9**	**5.76**	**9**	**5.38**	***RNF168***	**ring finger protein 168**	**1.27**	**down**
A_87_P017768	9	5.98	9	4.12	*PPP1R7*	protein phosphatase 1, regulatory (inhibitor) subunit 7	1.21	down
A_87_P223178	9	5.98	9	4.05	*PPP1R7*	protein phosphatase 1, regulatory (inhibitor) subunit 7	1.28	down
A_87_P023784	**9**	**6.18**	**9**	**6.00**	***ETV5***	**ets variant 5**	**1.40**	**down**
A_87_P077621	9	16.69	10	4.17	*SLC12A9*	solute carrier family 12 (potassium/chloride transporters), member 9	1.51	down
A_87_P005339	**9**	**16.78**	**10**	**6.92**	***CYP2J6L1***	**cytochrome P450 2J6-like 1**	**2.24**	**up**
A_87_P177293	9	16.78	10	4.09	*CYP2J6L1*	cytochrome P450 2J6-like 1	1.97	up
A_87_P077646	**9**	**16.79**	**10**	**7.95**	***CYP2J2L5***	**cytochrome P450 2J2-like 5**	**2.25**	**up**
A_87_P181713	19	6.94	12	4.07	*FAM101B*	family with sequence similarity 101 member B	2.17	down
A_87_P017169	**19**	**7.26**	**12**	**4.86**	***PTRH2***	**peptidyl-tRNA hydrolase 2**	**1.16**	**down**
A_87_P011731	**19**	**8.05**	**12**	**7.95**	***CA4***	**carbonic anhydrase IV**	**1.74**	**down**
A_87_P018194	24	25.84	13	4.19	*VPS26B*	VPS26 retromer complex component B	3.85	up

[a]Unique Agilent ID for the 60mer probe on the Agilent Chicken Gene Expression Microarrays
[b]Chromosomal assignment and position according to genome release galGal2.1
[c]Recording was discontinued and the probe set could not be assigned to any gene.
The experiment-wide significant genes (Bonferroni corrected, $p \leq 0.05$) are written in boldface

Discussion

Experimental design and statistical analysis

We used an experimental F2-design, which has frequently been analyzed using classical linkage analyses. However, we applied single-marker GWAS, which was justified by the high level of LD between adjacent SNPs (Fig. 1). In addition, the decay of LD for SNPs separated by more than 1.5 Mb shows that the mapping resolution for these distances was generally high. Intuitively, this might be surprising, because it is usually assumed that an F2-design results in very long range LD. However, a recent simulation study showed that this holds true only if the founder lines of the F2 cross are 'distantly' related. If they are 'closely' related, the mapping resolution is high (and sometimes even higher than in the founder lines) [36]. In the current study, the founder lines were separated by 11 generations, and thus they can be considered to be between closely and distantly related, which resulted in the high mapping resolution for distances greater than1.5 Mb.

Several significant trait-associated SNPs were identified for the traits included in this study and the FDR of these significant SNPs was low. In addition, nearly all significant SNPs were located within clusters. The power to map significant FDP-associated SNPs was substantially increased by combining the results from the association mapping study in the F2 cross and the selection signature results obtained in the earlier study, as can be deduced from the roughly three-fold larger number of significant SNPs for FDP_{meta} compared to FPD. This shift in power was also observed in an experiment on bovine data [37]. Intermediate gene frequencies and high F_{ST} values (only for FDP_{meta}) were obtained in the earlier selection signature experiment [20] for the genome-wide significant SNPs (see Table 1). This earlier study pointed to divergent gene frequencies in the HFP and LFP lines. Such a gene frequency pattern was expected for these genome-wide significant SNPs, because the variance contributed by an additive gene is maximized at these values. The assumption of the Fisher's combined probability test is that the p-values to be combined are independent. In our study, individuals from the same population were used; i.e., a sample of individuals from the HFP and LFP lines for selection signature mapping [20] and F2 individuals obtained from these lines for association mapping. However, a different type of information was used in each experiment, i.e. in the selection signature experiment differences in gene frequencies between the two lines were used, whereas in the association analysis SNP genotypes and trait phenotypes were used. A correlation of nearly 0 was found between the p-values obtained in

the selection signature and those in the association studies ($r = -0.003$), which provided further evidence for the independence of these studies.

Comparison of results with literature reports

Buitenhuis et al. [38] conducted a microsatellite-based linkage study to map QTL for feather pecking and identified QTL on GGA1 and 2. We also found significant clusters on these chromosomes, but a detailed comparison of the results was hampered by the wide confidence intervals obtained in the QTL linkage study. Recently, Recoquillay et al. [39] conducted a QTL linkage study for several behavior and production traits in Japanese quail. They did not detect a QTL for feather pecking but reported QTL for aggressive pecking on chromosomes 1 and 2. The corresponding position of the QTL on quail chromosome 1 on the chicken genome [39] was close to cluster number 1 for APD (Table 3), but the QTL on quail chromosome 2 could not be confirmed. Flisikowski et al. [40] suggested the genes *dopamine receptor D4* (*DRD4*) and *DEAF1 transcription factor* (*DEAF1*) as candidates for feather pecking and found significant trait associations in brain samples from the HFP and LFP lines. These lines were the same as used in Grams et al. [20] and in our study to create the F2 cross. *DRD4* and *DEAF1* are located on GGA5. We did identify one cluster for FDP_{meta} on GGA5, but it was not in the vicinity of these candidate genes. No single SNP in the chromosomal region that included these genes showed a nominal significant p-value. In addition, although two probes were located in *DRD4* and three in *DEAF1*, none of these showed significant differential expression in the HFP and LFP lines. Thus, based on results from the current study, the candidate status of these genes was not supported.

Comparison of our study with reports from the literature revealed few congruent results, which can be due to several reasons. First, it is very likely that different ethograms were used in these studies, resulting in different definitions of the traits. Second, in addition to differences in mapping procedures and in the genetic maps used, the size of the experimental populations also differed substantially between studies, with the largest size in the current study. Finally, it is also possible that significant associations were not confirmed simply because they do not segregate in other populations.

Candidate gene identification

The association clusters spanned more than 20 Mbp for all analyzed traits, i.e. a region comprising hundreds of genes, which makes the identification of candidate genes very speculative. However, inclusion of gene expression data can be used to classify positional

candidate genes on a functional basis, as was done in the current study, which was based on genome-wide expression data that were restricted to association clusters to reduce the multiple-testing burden. Differentially-expressed genes that are located within QTL regions can indicate the presence of a cis-acting regulatory mutation. However, hundreds of differentially-expressed transcripts were located within the association clusters, which made such an assumption very speculative. However, enrichment of such transcripts within clusters compared to the whole genome or individual chromosomes supports the hypothesis that differential expression can, at least partly, be explained by cis-acting regulatory mechanisms. In that case, it is expected that enrichment is stronger for more stringent p-value cutoffs. The most substantial enrichment in the current study was obtained for FDP_{meta} cluster 4 (Fig. 3). However, no functionally plausible candidate gene was identified within this region.

Positional candidate gene *SLC12A9* in FDP_{meta} cluster 10 on GGA9 exhibited experiment-wide significant differential expression between the HFP and LFP lines. However, for this cluster only a slight enrichment was observed for the most stringent p-value cutoff. Nevertheless, *SLC12A9* remains a functionally very plausible candidate gene for this QTL. It belongs to a family of nine genes that code for electroneutral cation-chloride-cotransporters [41]. Although the function of this gene is unclear, other *SLC12* transporters are known to be crucial in the control of the electrochemical chloride gradient that is required for hyperpolarizing the postsynaptic inhibition that is mediated by $GABA_A$ and glycine receptors [42]. This is remarkable, because reduction of postsynaptic $GABA_A$ receptor currents is also an effect of serotonin mediated by 5-HT_2 receptors [43]. There is a growing body of evidence that brain monoamines, such as serotonin and dopamine, are involved in the occurrence of feather pecking and aggressive pecking in hens [44-48] and in aggressive behavior in humans [49]. Kops et al. [43] showed that differences in dopamine turnover between a low mortality and a control hen line were largest, in particular, in the arcopallium region of the brain. Another purely positional candidate gene for feather pecking was located in FDP_{meta} cluster 9, i.e. *CLSTN2* (*calsyntenin 2*), which is also involved in postsynaptic signaling related to excitatory synaptic transmission [50].

For APD, the *GNG2* (*G protein subunit gamma 2*) gene was identified as a positional candidate gene in FDP_{meta} cluster 5 on GGA5 (Table 3). This gene is also involved in monoamine signaling, particularly in postsynaptic signaling at serotonergic (KEGG pathway ko04726) and dopaminergic (KEGG pathway ko04728) synapses.

Shared environment and associated effects

Behavior traits involve interactions between individuals. Statistical models that include interaction or associated effects have been developed, as reviewed by Bijma [51] and Ellen et al. [52], which have shown that these effects can substantially contribute to the heritable variation in survival of hens related to feather pecking and cannibalism [52]. Indeed, these interactions might also be another possible explanation for the low genetic trend in later generations in our selection experiment [20]. In a recent study, we chose the simplest form to capture shared environment effects and associated effects by fitting a random pen effect to the model [17]. Since pen variances were very small, they were not included in the current study. Moreover, the size of the pens used here was rather large for social interaction models.

Conclusions

Several significant trait-associated clusters of SNPs were identified, especially for the trait FPD_{meta} but also for aggressive pecking. However, behavioral traits, appeared to be controlled by many genes with small effects and no single SNP was promising for selection purposes. However, understanding the motivation for feather pecking is of interest in its own right. In-depth sequence-based association analyses of the clusters identified in this study and subsequent identification of candidate genes from a small list of putative positional genes will help to formulate and validate hypotheses for the expression of this abnormal behavior pattern. Clearly, for this purpose additional data need to be collected.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MG, WB, and VG conducted the field experiment; SP performed the genotyping and cleaned up the genotypic data; PS performed the LD structure analysis; VG performed the remaining statistical analysis; VG, SP, JT, and JB interpreted the results and wrote the paper; WB and JB Initiated and oversaw the project. All authors read and approved the final manuscript.

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Additional files

Table S1 List of significant SNPs with a p-value ≤ 5 x 10^{-5}, their chromosomal region and their *p*-value for trait feather pecks delivered (FPD) and the Metal-Analysis (FPD_{meta}).

Trait	SNP	Chr	Position	-log10(p)	Cluster
FPD	GGaluGA023840	1	69812429	4.569	-
	Gga_rs13669267	2	37372218	5.398	-
	GGaluGA224037	3	58834628	4.319	1
	GGaluGA224156	3	59186202	4.319	1
	GGaluGA224309	3	59725450	4.319	1
	Gga_rs13521814	4	53375709	4.444	2
	Gga_rs14469602	4	53397816	4.444	2
	Gga_rs14469696	4	53453267	4.420	2
	Gga_rs14469702	4	53461607	4.444	2
	Gga_rs15579522	4	53892204	4.357	2
	Gga_rs14470197	4	53945398	4.357	2
	Gga_rs16526327	6	3059760	4.721	3
	Gga_rs13561199	6	3075330	4.721	3
	Gga_rs14653727	8	25309634	4.553	4
	GGaluGA331049	8	25399547	5.699	4
	GGaluGA341482	9	17128657	5.222	-
	GGaluGA093070	13	7103987	4.886	-
	Gga_rs13605122	24	3315257	5.222	-
	Gga_rs13546091	28	2140227	4.921	-
FPD_{meta}	GGaluGA019519	1	58412953	4.548	1
	GGaluGA019545	1	58477922	4.516	1
	Gga_rs14828914	1	58537760	4.543	1
	GGaluGA042442	1	127308590	4.727	-
	Gga_rs13712580	1	149753999	4.590	2
	Gga_rs13712669	1	150001928	4.300	2
	Gga_rs13669267	2	37372218	4.327	3
	GGaluGA142420	2	39486006	4.325	3
	Gga_rs13702304	3	103609224	4.511	4
	Gga_rs13702581	3	104349320	4.437	4
	Gga_rs14408039	3	107262448	4.388	5
	GGaluGA238045	3	107786255	4.601	5
	Gga_rs13525874	3	109945836	4.890	5
	Gga_rs16419024	4	59770870	4.886	-
	Gga_rs14500076	4	87030671	4.650	6
	Gga_rs15637216	4	87076909	4.627	6
	Gga_rs16519883	5	59368007	5.948	-
	Gga_rs15900019	8	4002499	4.434	7
	Gga_rs10723790	8	4211591	4.788	7
	GGaluGA323765	8	5410229	4.641	7
	Gga_rs15930799	8	23892743	5.844	8

Gga_rs14652254	8	23911149	5.844	8
Gga_rs14652925	8	24646720	4.544	8
Gga_rs14652966	8	24679820	5.839	8
GGaluGA330724	8	24758580	5.460	8
Gga_rs15932003	8	24834266	4.928	8
GGaluGA330792	8	24921219	4.783	8
GGaluGA330826	8	24980300	4.888	8
GGaluGA331049	8	25399547	4.706	8
Gga_rs15962845	9	5754325	5.069	9
Gga_rs13766455	9	5961337	6.017	9
Gga_rs14667686	9	6739756	5.891	9
Gga_rs14667611	9	6812627	4.339	9
GGaluGA336891	9	7219744	4.453	9
Gga_rs14665188	9	9007799	4.502	-
Gga_rs13608664	9	16342044	4.388	10
Gga_rs14675951	9	16548816	5.197	10
Gga_rs15976485	9	16608755	5.307	10
Gga_rs14676055	9	16629471	6.392	10
GGaluGA341173	9	16688339	5.287	10
GGaluGA341217	9	16764865	6.376	10
GGaluGA341245	9	16810500	5.625	10
GGaluGA341277	9	16849902	4.677	10
GGaluGA341293	9	16877654	4.536	10
GGaluGA341482	9	17128657	7.368	10
GGaluGA341680	9	17579192	4.706	10
Gga_rs14677433	9	18297108	4.567	10
Gga_rs14677551	9	18425812	5.558	10
Gga_rs15979368	9	19293924	4.692	11
Gga_rs16006794	9	19411600	4.382	11
Gga_rs16742615	9	19427105	4.361	11
GGaluGA342417	9	20815056	4.426	11
GGaluGA093070	13	7103987	4.924	-
Gga_rs14122190	19	6883105	4.824	12
GGaluGA127801	19	6896487	4.819	12
GGaluGA191629	24	2533040	4.840	13
Gga_rs13605122	24	3315257	4.376	13
Gga_rs14295311	24	3419984	4.418	13

The genome-wide significant SNPs (Bonferroni corrected, $p \leq 0.05$) are written in boldface

Table S2 List of significant SNPs with a *p*-value ≤ 5 x 10^{-5}, their chromosomal region and their *p*-value for trait aggressive pecks delivered (APD) and aggressive pecks received (APR).

Trait	SNP	Chr	Position	-log10(p)	Cluster
APD	Gga_rs14834942	1	64103417	4.548	1
	Gga_rs14837208	1	66726295	4.550	1
	Gga_rs15296857	1	67037983	4.561	1
	Gga_rs13923655	1	116041775	6.024	2
	Gga_rs15388609	1	116062599	5.838	2
	Gga_rs15388671	1	116098957	4.797	2
	Gga_rs13923933	1	116353270	4.632	2
	Gga_rs14875693	1	116457611	4.748	2
	GGaluGA038883	1	116538528	4.835	2
	Gga_rs16738272	1	121495752	4.568	-
	Gga_rs14210222	2	83445347	4.841	3
	Gga_rs13636444	2	86114050	4.886	3
	Gga_rs14418954	4	33821	4.456	4
	Gga_rs14418847	4	310336	4.456	4
	Gga_rs14418830	4	332468	4.456	4
	Gga_rs14418813	4	360213	4.456	4
	Gga_rs14418723	4	518571	4.456	4
	Gga_rs14418713	4	540435	4.456	4
	Gga_rs14418710	4	552165	4.456	4
	Gga_rs14552049	5	56835282	4.465	5
	Gga_rs14552339	5	57143305	5.259	5
	Gga_rs14552504	5	57246827	4.952	5
	Gga_rs14552589	5	57353834	6.829	5
	GGaluGA290503	5	57401911	6.400	5
	GGaluGA290917	5	58214037	4.485	5
	Gga_rs15832113	18	8658969	4.854	6
	GGaluGA122356	18	8928217	4.324	6
	GGaluGA122973	18	9699487	4.353	6
	Gga_rs15469578	18	9898566	4.590	6
	GGaluGA123114	18	9906133	4.512	6
	GGaluGA123129	18	9928246	4.514	6
	GGaluGA123133	18	9932567	4.514	6
	Gga_rs16347507	18	9945623	4.514	6
	Gga_rs14417028	18	9949736	4.514	6
	Gga_rs16347539	18	9983890	4.599	6
	Gga_rs16347624	18	10029434	4.599	6
	Gga_rs16177511	21	1095000	4.320	7
	Gga_rs16177666	21	1199251	5.475	7
	GGaluGA182481	21	1204493	5.475	7
	GGaluGA182516	21	1266391	5.501	7
	GGaluGA182786	21	1914059	4.494	7
	GGaluGA183181	21	2414911	5.067	7

	GGaluGA183305	21	2583575	4.349	7
	Gga_rs16072064	25	1438046	4.719	-
	Gga_rs16203090	26	3510820	4.338	-
APR	Gga_rs14443929	4	30737168	4.310	-
	GGaluGA310577	7	6241588	4.959	1
	Gga_rs14604136	7	6325307	4.959	1
	Gga_rs14604144	7	6327771	4.959	1
	Gga_rs15838552	7	9746560	4.959	2
	Gga_rs14605439	7	10476794	4.959	2
	Gga_rs15839283	7	10505869	4.959	2
	GGaluGA311212	7	10510834	4.959	2
	Gga_rs15839450	7	10582674	5.000	2
	Gga_rs15839686	7	10726089	5.000	2
	Gga_rs15840596	7	11199463	5.000	2
	Gga_rs13742807	7	12394545	5.398	2
	Gga_rs10729685	7	12514023	4.824	2
	GGaluGA311997	7	12631641	4.824	2
	Gga_rs14608583	7	13420198	4.347	3
	GGaluGA312585	7	13933219	4.367	3
	GGaluGA312831	7	14595204	4.509	3
	Gga_rs15846766	7	14652969	5.097	3
	GGaluGA312856	7	14679901	4.509	3

The genome-wide significant SNPs (Bonferroni corrected, $p \leq 0.05$) are written in boldface.

GENERAL DISCUSSION

The main objective of the present study was to detect the genetic foundation of the behaviour trait feather pecking and additionally to infer the ethological interrelationship between this behaviour and certain other traits. The data of two divergently selected lines for feather pecking behaviour was available, and a large F2-cross, set up from these divergently selected lines, was established to achieve this main objective. Chickens of a White Leghorn layer line were divergently selected for high and low feather pecking for 11 generations. The selection started in the Danish Institute of Animal Sciences, Foulum, Denmark, for the first six generations (0-5) (Kjaer et al., 2001). Thereafter, five rounds of selection took place at the Institute of Animal Science, University of Hohenheim, Germany. The large F2-cross was established from the 10^{th} selection generation, and a comprehensive data collection of behaviour and performance traits of 960 hens was performed. These two data sets were used for the following five research chapters and a general discussion. **Chapter one** included a quantitative genetic analysis of feather pecking and fear traits, using the data of F2-cross laying hens set up from two lines divergently selected for high and low feather pecking. In **chapter two** a comparison of genetic parameters resulting from a linear and a Poisson model by considering different observation periods was performed. In **chapter three,** structural equation models were used to assess the assumption that feather eating or general locomotor activity causes feather pecking. In **chapter four**, an F_{ST}-based approach was applied to detect selection signature in the two divergent laying hens lines selected for high and low feather pecking. A genome-wide association and a differential gene expression study was performed in **chapter five** using data from the F2-cross laying hens.

Some additional analyses about the interrelations of the behavior and other recorded traits

It was generally assumed that aggressive birds perform feather pecking behavior in this respect that a high social rank facilitates the approach of pen mates and, thus feather pecking (Hughes, 1982). This theory is supported by a low positive correlation between feather pecking and aggression (Hughes and Duncan, 1972; Wennrich, 1975). Nevertheless, it has been shown that feather pecking and aggressive pecking underlie different motivations. Aggressive pecks are used to establish and maintain a stable dominance hierarchy in poultry flocks (Guhl, 1968). Aggressive pecks are delivered in an upright body posture and are mainly directed towards the head of the recipient birds (Bilcik and Keeling, 1999; Kjaer et al.,

2001), whereas feather pecking is characterized by non-aggressive pecks directed towards the plumage of other hens (Kjaer et al., 2001). Subsequent investigations showed that the correlation between these two traits are closed to zero (Blokhuis and Arkes, 1984; McKeegan and Savory, 1999). Kjaer et al. (2001) found no difference in aggressive pecking behavior between two divergently selected lines for high and low feather pecking behavior for the first 3 generations of selection (Kjaer et al., 2001). Bessei et al. (2013a) analyzed the relationship between feather pecking and aggressive pecking of the same selection lines and showed that the aggression level was significantly higher in the high feather pecking line compared to the low feather pecking line in the 10th generation of selection. The correlations between aggressive pecking and feather pecking were positive but not significant in the high feather pecking line. The same correlations were close to zero in the low feather pecking line. In a subsequent study of Bessei et al. (2013b), F2-cross hens, set up from these divergent selected lines, were split in a high and low feather pecking group for analyzing the relationship between feather pecking and aggressive pecking. The high feather peckers delivered significantly more feather pecks and aggressive pecks than low feather peckers, and the results showed a close and nonlinear relationship between aggressive pecking and feather pecking. Bennewitz et al. (2014) analyzed the same F2-cross data with generalized linear mixed models and estimated a high approximated genetic correlation between feather pecking and aggressive behavior. In **chapter one** of this thesis, the results provided a genetic correlation of 0.2 between these two traits using a standard linear mixed model. Based on these results, we analyzed the possible causal relationship between feather pecking and aggressive pecking behavior with structural equation models. Such structural equation models can be used to study recursive causal relationships between traits in multivariate analyses, and hence, allow to separate causal effects of phenotypes from the genetic and environmental correlations among traits (Gianola und Sorensen, 2004; Valente et al., 2010, 2013). Consequently, we could estimate the recursive effect (indirect effect) from one trait to another. Hence, we analyzed the assumption whether the demand for feather pecking leads to aggressive pecking, or aggressive pecking affects feather pecking behavior. However, these postulated causalities could not supported by our data, because the estimated recursive effects between the traits showed large standard errors (results are not shown). Finally, we could say that selection for high and low feather pecking behavior has obviously produced a simultaneous divergence in aggressive behavior, which is supported by the estimated genetic correlation between these two traits (Bennewitz et al., 2014; Grams et al., 2015). However, we could not prove that feather pecking leads to aggressive pecking and vise verse. Hence,

there is an interrelationship between feather pecking and aggressive behavior, but the exact causal relationship is not clear so far.

Most studies suggested that fearfulness is a consequence of feather pecking (Hughes and Duncan, 1972; Vestergaard et al., 1993; Rodengurg et al., 2004; Rodenbrug et al., 2010). Studies found that hens with a higher pecking activity showed also a higher level of fear (Jones 1996; Vestergaard et al., 1993; Jensen et al., 2005). De Hass (2014) suggested fear as a major cause of feather pecking in layer lines, because investigations have been shown that groups differ in levels of fear and stress-sensitivity, and that fearfulness of young birds can lead to stress-sensitivity in adult hens, which create a risk for feather pecking. Rodenburg et al. (2004) suggested that open-field activity, a measure for fear, in young birds may be useful as a predictor of later pecking behaviour, and hence, the open-field test may be used to select against feather pecking. Based on these prevailing opinions, in **chapter one** we performed a quantitative genetic analysis of fear traits and feather pecking using the data of the large F2-cross, set up from the divergent selected lines for high and low feather pecking behavior. Fear was recorded by the tonic immobility test, the open field activity and the emerge box test at juvenile and adult age. The estimated genetic correlation could not confirm the assumption of Rodenbrug et al. (2004) that open field activity at juvenile can be used as a predictor for feather pecking at adult age (see, Table 3 of chapter one). The genetic correlation was close to zero in this study. The appreciable genetic correlation between fear traits and feather pecking was tonic immobility at juvenile age (r_g=0.27). Therefore, tonic immobility at juvenile age might be used as a predictor for feather pecking behaviour in adult hens and hence, for selection against feather pecking. However, in this case, the estimated selection response for feather pecking is higher by direct selection on it in comparison with the selection on TI_j and hence, selecting for TI_j is inefficient in reducing feather pecking. In addition, the recording of fear traits are also time-consuming. Furthermore, we estimated the causal relationship between fear traits and feather pecking, based on the assumption that fear triggers feather pecking behavior using structural equation models. In this respect, our data could not support the postulated assumption that fearfulness is a consequence of feather pecking (results not shown).

Several studies suggested an association between feather eating and feather pecking (Mc Keegan and Savory, 1999, 2001; Harlander-Matauschek and Bessei, 2005; Harlander-Matauschek and Häusler, 2008). Moreover, Kjaer (2009) presented a theoretical model where feather pecking is caused by a hyperactivity disorder, i.e. birds with a higher activity behavior

perform feather pecking. Hence, it is assumed that feather pecking is related to a general locomotor activity. These causal relationships between feather pecking and feather eating as well as general locomotor activity were analyzed in **chapter three** using structural equation models. We assumed that feather eating and general locomotor activity triggers feather pecking, and feather eating leads to a higher general locomotor activity. The estimated genetic correlation between general locomotor activity and feather pecking is close to zero. The recursive effect, however, indicated that an increase in general locomotor activity results in a higher feather pecking value. Previous analyses showed that higher feather pecking birds have a significant higher general locomotor activity than low feather peckers (Kjaer, 2009). Kjaer (2009) suggested that the dopamine system is the potential link between feather pecking and general locomotor activity.

The assumption that birds are more active, because they are searching for feathers, could not support by our data (chapter three). The recursive effect were small and had a high standard error. Nevertheless, we estimated a high genetic correlation between feather eating and general locomotor activity. Hence, there is a link between feather eating and general locomotor activity due to pleiotropy or linkage disequilibrium and not caused by indirect effects.

One major motivation for feather pecking seems to be feather eating. It has been demonstrated that hens selected for high and low feather pecking significant differ in their preference to feather eating (Harlander-Matauschek and Häusler, 2008). High feather peckers ate significantly more feathers than low feather peckers (Mc Keegan and Savory, 2001; Harlander-Matauschek and Bessei, 2005; Harlander-Matauschek and Häusler, 2008; Bögelein, 2010). Bennewitz et al. (2014) reported a high positive genetic correlation between feather pecking and feather eating. In **chapter three**, we could demonstrate that feather eating triggers feather pecking behavior. The applied structural equation model provided a high recursive effect with a small standard error from feather eating to feather pecking. Hence, if it would possible to prevent feather eating, it would also reduce feather pecking behavior. The reason for eating feathers is still unclear. McCasland and Richardson (1996) proved that feathers have no nutritive value and it is still assumed that dietary factors play a role in relation to the consumption of feathers (Harlander-Matauschek et al., 2006b; Harlander-Matauschek and Häusler, 2008). Analyses showed that feathers increased the speed of feed passage in the digestive passage of high feather pecking birds, which ate significant more feathers than low feather peckers (Harlander-Matauschek et al., 2006b). It is assumed that the

physical structure of the feathers are the reason for the rapid feed passage in high feather pecking lines (Harlander-Matauschek et al., 2006b). Fiber dietary has a positive effect on the anterior tract and gizzard function (Hetland et al., 2004). Different experiments have demonstrated that fiber in the diet reduced feather pecking behavior and cannibalism (Aerni et al., 2000; El Lethey et al., 2000; Hartini et al., 2002). Investigations showed that high feather peckers have a higher preference for fiber compared to low feather peckers (Kalmendal and Bessei, 2012). Choice experiments have even demonstrated that high feather peckers prefer feathers instead of wood shavings (Harlander-Matauschek et al., 2006a). Further experiments showed that feathers have an influence on the bacterial composition of the intestinal microbiota in chickens (Meyer et al., 2012). Including 5% ground feathers (non-hydrolyzed) in the diet lead to an increased abundance of keratinolytic bacteria in the ileum and cecae of hens and an increased ammonia concentration in the cecae, and also to a changed pattern of short-chain fatty acids produced in the cecae (Meyer et al., 2012). Further experiment has been shown that the ammonia concentration and short-chain fatty acids were higher in the cecae of high feather pecking birds compared to low feather pecking birds due to the difference in feather eating between the lines (Meyer et al., 2013). In humans, it has been shown that an interaction between gut microbiota and brain exist and hence, the intestinal microbiota have an impact on behavior and nervous system (Forsythe et al., 2010; Collins et al., 2012; Sommer and Bäckhed, 2013). In our case, it is still not obvious how intestinal microbiota metabolism affecting the behavior of hens. Furthermore, it is not clear whether the difference in microbiota between high and low feather peckers exist due to feather eating or it has a genetic basis.

To conclude, feather eating is one major motivation to perform feather pecking, but the reason for feather eating is still unclear and hence, further investigations are necessary.

Identification and further investigations of candidate genomic regions and genes

The identification of genomic regions and genes that might be have an influence on phenotypic variation can be performed in two different ways: i) from phenotype to genome, which is used in LD-based association mapping, so-called genome-wide association study, and, ii) from genome to phenotype, which involves the statistical evaluation of genomic data to localize footprints of past selection (Qanbari et al., 2010). The latter one is used to identify pattern of linkage disequilibrium in the genome or between populations, which are incompatible with the hypothesis of genetic neutrality, and these patterns are called selection signatures (Qanbari et al., 2010). A genome-wide association study is a trait-based analysis in

which an association between marker and the trait of interest are tested, i.e. the effects of every SNP on phenotypic variation will be estimated (Gianola and Simianer, 2014), and hence, the individuals have to be phenotyped. Whereas selection signature analyses have the target to identify chromosomal regions that have responded to selection. Such methods are independent from individual phenotyping. A further difference is that a genome-wide association study need a certain number of animals to obtain a sufficient power. The number of animals and hence, the power of a genome-wide association study depends on the heritability of the obtained trait and the proportion of the variance explained by the SNP (Goddard and Hayes, 2009). Because the most quantitative traits are controlled by many genes with small effect and few genes with a larger effect, a high number of genotyped individuals are necessary (Goddard and Hayes, 2009).

The selection signature analysis was perform in **chapter four** using the data of the divergently selected lines for high and low feather pecking behavior. The divergent selection was done for 11 generations and across all generations, the two lines showed a consistent difference in the mean trait value. We used an F_{ST}-based approach to maps selection signature. The F_{ST}-based approach of the divergently selected lines provided 17 genome-wide significant SNPs with an F_{ST}-value of 1, i.e. alleles were divergently fixed in these two lines, and a full list of significant SNPs at $p \leq 5x10^{-5}$. Based on the assumption that selection affects several consecutive SNPs we could identify 13 cluster on chromosome 1, 3, 4, 6, 8, 11, 15 and 19. The genome-wide association study (GWAS) for the traits feather pecking and aggressive pecking behavior was performed in **chapter five** using the data from the large F2-cross, set up from these divergently selected lines, and to combine the results of this GWAS with the results from the selection experiment (chapter for) using a meta-analysis. A number of significant SNPs at $p \leq 5x10^{-5}$ were mapped for feather pecking, aggressive pecking delivered and received. The meta analysis provided nine genome-wide significant SNPs and also a full list of significant SNPs for feather pecking. Based on the assumption that a causative mutation is in a linkage disquilibrium with several SNPs we could identify four cluster for feather pecking and 13 in the meta analysis on chromosome 1, 2, 3, 4, 5, 6, 7, 8, 9, 19 and 24. For aggressive pecking delivered we detected seven cluster on chromosome 1, 2, 4, 5, 18 and 21. However, there is no overlap with corresponding regions found in the literature (Buitenhuis et al., 2003; Recoquillay et al., 2015) and our data could not support the candidate status for feather pecking behaviour of the genes dopamine receptor D4 (DRD4) and DEAF1 transcription factor (DEAF1) on chromosome 5, which were suggested by Flisikowski et al. (2009). Numerous genes were located in the detected clusters of chapter five, however the

candidate status of these genes is not yet clear and could be supported by post-GWAS analyses.

In conclusion, it seems that behavior traits are controlled by many genes with small effects and no single polymorphism is promising for selection purpose. However, understanding the motivation for feather pecking is of interest in its own right. A sequence based association analyses in the clusters identified in this thesis and subsequently identification of candidate genes from a small list of putative positional genes will help to formulate hypotheses for the motivation to express this abnormal behavior pattern.

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der bedankt sich auch.
(Wilhelm Busch)

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CURRICULUM VITAE

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2013-2016	Promotion am Fachgebiet für Genetik und Züchtung landwirtschaftlicher Nutztiere über das Thema „Genetic Analyses of Feather Pecking and related Behavior Traits of Laying Hens“
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Juli-September 2011	Studentische Hilfskraft am Institut für Tierhaltung und Tierzucht, Universität Hohenheim
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Eidesstattliche Versicherung

gemäß § 8 Absatz 2 der Promotionsordnung der Universität Hohenheim zum Dr.sc.agr.

1. Bei der eingereichten Dissertation zum Thema „Genetic Analyses of Feather Pecking and related Behavior Traits of Laying Hens“ handelt es sich um meine eigenständig erbrachte Leistung.

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