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Genomic analyses of behavior traits in laying hen lines divergently selected for feather pecking

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Hanna Iffland

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Dean of the Faculty:	Prof. Dr. Ralf Vögele

Examination Committee:

Chair:	Prof. Dr. Martin Hasselmann
Supervisor and Reviewer:	Prof. Dr. Jörn Bennewitz
Second Reviewer:	Prof. Dr. Jens Tetens
Additional Examiner:	Prof. Dr. Volker Stefanski

Dedicated to the Animals,
who gave their Lives for Science

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SUMMARY

Feather pecking is a longstanding problem in commercial layer flocks and has been a topic of intensive research for decades. It often causes injured birds and as a consequence even cannibalism. As a result, economical losses as well as animal welfare issues occur. In the past, hens were beak trimmed to reduce feather pecking. Nevertheless, this procedure is already prohibited in some EU countries and others will presumably follow. Hence, a solution to this problem is urgently needed. Feather pecking is influenced by environmental and genetic factors and selection experiments illustrated the possibility to select for high and low feather pecking. The experimental populations analyzed in this thesis were formed by hens based on a founder line of a White Leghorn layer strain which were divergently selected for high and low feather pecking since 1995. The first experimental population of this thesis analyzed in chapter one was an F_2 cross of about 900 hens which was established of the 10^{th} generation of the pure selection lines. The second population which was analyzed in chapters two to four consisted of about 500 hens of the 15^{th} generation of these two lines. Based on the findings of former studies, the aim of this thesis was to gain further knowledge of the genetic background of feather pecking and its relation to additional behavior traits and the gut microbiome. This was done by phenotypic and genomic analyses and a focus on the extreme occurrence of feather pecking.

In **chapter one**, a novel model to detect extreme feather pecking hens was developed. Therefore, a mixture of two negative binomial distributions was fitted to feather pecking data of the F_2 cross. With the estimated parameters, the trait *posterior probability of a hen to belong to the extreme feather pecking subgroup* (pEFP) was calculated. The fear tests tonic immobility and emerge box were conducted at juvenile and adult age of the hens to relate fearfulness to pEFP. After dichotomization, all traits were analyzed in a multivariate threshold model and subsequent genomewide association studies (GWAS) were performed. The fit revealed that extreme feather peckers made up a proportion of about one third of the hens. The new trait pEFP has a medium heritability of 0.35 and is positively correlated with the fear traits. Breeding for this new trait could be an option to reduce the proportion of extreme feather peckers. An index of fear related traits might serve as a proxy to breed indirectly against pEFP. GWAS revealed that all traits are typical quantitative traits.

In **chapter two**, the model to detect extreme feather pecking hens was applied to the pure selection lines. After calculation of the trait pEFP, GWAS with a subsequent post GWAS analysis were performed. Additionally, to find genomic regions influencing feather pecking, selection signatures were mapped by applying the intra-population iHS and the inter-population

F_{ST} approach. Mapping of selection signatures revealed no clear regions under selection. GWAS revealed a region on chromosome one, where the existence of a quantitative trait locus (QTL) influencing feather pecking is likely. The candidate genes found in this region are a part of the GABAergic system, which has already been linked to feather pecking in previous studies. Despite the polygenic nature of feather pecking, selection on these candidate genes may reduce the extreme occurrence of it.

In **chapter three**, the relation between agonistic behavior and feather pecking was analyzed. Therefore, the active parts of the traits (delivery of feather pecking, aggressive pecking or threatening) as well as the passive parts (reception of the traits) were considered. These groups of traits were additionally summarized by means of an index formation which led to the two additional traits Activity and Passivity, because all these behaviors are undesired in their excessive manifestations. Moreover, Indices were built by subtracting the passive traits from the respective active traits to obtain the feather pecking index, the aggression index and the threat index. Phenotypic correlations were estimated between all traits which were followed by heritability estimations and GWAS. Feather pecking is significantly positively correlated with the agonistic traits in both lines. The average amount of feather pecks received in both lines is nearly the same. The active traits and the feather pecking index show medium heritabilities. Hence, selection on high feather pecking leads to an increase of agonistic behavior whereas the correlation probably depends on the phase of establishing the social hierarchy and might disappear, after a stable ranking is established. GWAS revealed that the heritable traits in this study seem to be typical quantitative traits with some QTL which have slightly greater effects on these traits.

Chapter four provides the analyses of the gut microbial composition of the two feather pecking lines, followed by the estimation of microbiabilities for feather pecking and the two agonistic behavior traits, to study the influence of the gut microbiome on behavior. Microbiota samples from digesta and mucosa were taken from ileum and caecum. The microbial communities were determined by using 16S RNA gene sequencing techniques. Although both lines differ significantly in some fractions of their gut microbial composition, the microbial animal effects were mostly negligibly small. Thus, the calculated microbiabilities were close to zero and not significant in both lines and for all traits investigated. Hence, trait variations were not affected by the gut microbial composition in both feather pecking lines.

The thesis ends with a general discussion where additional results of a meta-analysis of pEFP and breeding strategies against feather pecking are considered.

SUMMARY (GERMAN)

Das Federpicken ist ein seit langem bestehendes Problem in kommerziellen Legehennenherden und seit Jahrzehnten Gegenstand intensiver Forschung. Es führt häufig zu Verletzungen bei den Tieren und als Folge davon sogar zu Kannibalismus. Infolgedessen treten sowohl wirtschaftliche Verluste als auch Tierwohlprobleme auf. In der Vergangenheit wurden die Schnäbel von Hennen gekürzt, um das Federpicken zu reduzieren. Dieses Verfahren ist in einigen EU-Ländern jedoch bereits verboten und weitere werden vermutlich folgen. Daher ist eine Lösung für dieses Problem dringend erforderlich. Federpicken wird durch Umweltfaktoren und genetische Faktoren beeinflusst und Selektionsexperimente zeigten die Möglichkeit, auf hohes und niedriges Federpicken zu selektieren. Die in dieser Dissertation analysierten Versuchspopulationen wurden von Hennen gebildet, die auf einer Gründerlinie einer Weißen Leghorn Legerasse basierten und seit 1995 divergent für hohes und niedriges Federpicken selektiert wurden. Die erste Versuchspopulation dieser Dissertation, die in Kapitel eins analysiert wurde, war eine F_2-Kreuzung von etwa 900 Hennen, die aus der 10. Generation der reinen Selektionslinien gebildet wurde. Die zweite Population, die in den Kapiteln zwei bis vier analysiert wurde, bestand aus etwa 500 Hennen der 15. Generation dieser beiden Linien. Basierend auf den Ergebnissen früherer Studien war es das Ziel dieser Dissertation, weitere Erkenntnisse über den genetischen Hintergrund des Federpickens und dessen Beziehung zu weiteren Verhaltensmerkmalen sowie dem Darmmikrobiom zu gewinnen. Dies geschah durch phänotypische und genomische Analysen sowie den Fokus auf das extreme Vorkommen des Federpickens.

Im **ersten Kapitel** wurde ein neuartiges Modell zum Nachweis extremen Federpickens bei Hennen ausgearbeitet. Dazu wurde eine Mischung aus zwei negativen Binomialverteilungen an die Federpickdaten der F_2-Kreuzung angepasst. Mit den geschätzten Parametern wurde das Merkmal *die a posteriori Wahrscheinlichkeit einer Henne, zur Untergruppe der extremen Federpicker zu gehören* (pEFP), berechnet. Die Furchttests tonische Immobilität und Emerge Box wurden in juvenilem und adultem Alter der Hennen durchgeführt, um die Furcht mit pEFP in Beziehung zu setzen. Nach der Dichotomisierung wurden alle Merkmale in einem multivariaten Schwellenwertmodell analysiert und anschließend genomweite Assoziationsstudien (GWAS) durchgeführt. Die Anpassung der Verteilung ergab, dass extreme Federpicker einen Anteil von etwa einem Drittel der Hennen ausmachten. Das neue Merkmal pEFP hat eine mittlere Heritabilität von 0,35 und ist positiv mit den Furchtmerkmalen korreliert. Die Züchtung dieses neuen Merkmals könnte eine Option sein, um den Anteil extremer Federpicker zu reduzieren. Ein Index der furchtbezogenen Merkmale könnte als Hilfsmerkmal

dienen, um indirekt gegen pEFP zu züchten. Die GWAS ergab, dass es sich bei allen Merkmalen um typische quantitative Merkmale handelt.

Im **zweiten Kapitel** wurde das Modell zum Nachweis extremer Federpicker auf die zwei reinen Selektionslinien angewandt. Nach der Berechnung des Merkmals pEFP wurden GWAS mit einer anschließenden post GWAS Analyse durchgeführt. Um zusätzlich genomische Regionen zu detektieren die das Federpicken beeinflussen, wurden Selektionssignaturen durch Anwendung des intra-Population-iHS-Ansatzes und des inter-Population-F_{ST}-Ansatzes kartiert. Die Kartierung der Selektionssignaturen ergab keine eindeutigen Regionen, an denen Selektion stattgefunden hat. Die GWAS zeigte eine Region auf Chromosom eins, in der die Existenz eines quantitative trait locus (QTL), welcher Federpicken beeinflusst, wahrscheinlich ist. Die in dieser Region gefundenen Kandidatengene sind ein Teil des GABA-Systems, das bereits in früheren Studien mit Federpicken in Verbindung gebracht wurde. Trotz der polygenen Natur des Merkmals Federpicken könnte die Selektion auf diese Kandidatengene das extreme Auftreten des Federpickens reduzieren.

In **Kapitel drei** wurde die Beziehung zwischen agonistischem Verhalten und Federpicken analysiert. Dabei wurden sowohl die aktiven Anteile der Merkmale (Ausübung des Federpickens, aggressiven Pickens oder Drohens) als auch die passiven Teile (Empfang der Merkmale) betrachtet. Diese Merkmalsgruppen wurden zusätzlich mittels einer Indexbildung zusammengefasst, die zu den beiden zusätzlichen Merkmalen Aktivität und Passivität führte, da all diese Verhaltensweisen in ihrer exzessiven Ausprägung unerwünscht sind. Darüber hinaus wurden Indizes gebildet, indem die passiven Merkmale von den jeweiligen aktiven Merkmalen subtrahiert wurden, um den Federpick-Index, den Aggressionsindex und den Bedrohungsindex zu erhalten. Zwischen allen Merkmalen wurden phänotypische Korrelationen geschätzt, gefolgt von Heritabilitätsschätzungen und GWAS. Federpicken ist signifikant positiv mit den agonistischen Merkmalen in beiden Linien korreliert. Die durchschnittliche Menge an Federpicken, die in beiden Linien empfangen wurde, ist nahezu gleich. Die aktiven Merkmale und der Federpick-Index zeigen mittlere Heritabilitäten. Daher führt die Selektion auf hohes Federpicken zu einer Zunahme des agonistischen Verhaltens, wobei die Korrelation wahrscheinlich von der Phase der Etablierung der sozialen Hierarchie abhängt und sich auflösen könnte, nachdem eine stabile Rangordnung etabliert ist. Die GWAS ergab, dass es sich bei den vererbbaren Merkmalen in dieser Studie um typische quantitative Merkmale zu handeln scheint mit einigen QTL, die etwas größere Effekte auf diese Merkmale haben.

Kapitel vier enthält die Analysen der Zusammensetzung der Darmmikrobiota der beiden Federpicklinien, gefolgt von der Schätzung der Microbiabilities für Federpicken und den beiden agonistischen Verhaltensmerkmalen, um den Einfluss des Darmmikrobioms auf das Verhalten zu untersuchen. Mikrobiotaproben aus der Digesta und Schleimhaut wurden aus dem Ileum und Caecum entnommen. Die Mikrobengemeinschaften wurden mit Hilfe von 16S-RNA Gen-Sequenzierungstechniken bestimmt. Obwohl sich beide Linien in einigen Fraktionen ihrer mikrobiellen Zusammensetzung im Darm signifikant unterscheiden, waren die mikrobiellen Tiereffekte meist vernachlässigbar gering. Somit waren die berechneten Microbiabilities nahe Null und in beiden Linien sowie für alle untersuchten Merkmale nicht signifikant. Dies bedeutet, dass die Merkmalsvariationen nicht durch die Zusammensetzung des Darmmikrobioms in den Federpicklinien beeinflusst wurde.

Die Dissertation endet mit einer allgemeinen Diskussion, in der zusätzliche Ergebnisse einer Meta-Analyse von pEFP sowie Zuchtstrategien gegen Federpicken berücksichtigt werden.

GENERAL INTRODUCTION

Feather pecking is a long known and still existing problem in commercial layer flocks. It can be defined as severe non-aggressive pecks or pulls directed to the plumage of conspecifics and often leads to an injured integument and in consequence to cannibalism. As a result, farmers are facing two major problems, economic losses as well as severe animal welfare issues. To reduce feather pecking, the beaks of the hens were trimmed in the past, but this is already prohibited in some EU countries and others will presumably follow. Hence, a solution to this problem is urgently needed. Feather pecking has been a topic of intensive research for years and many studies revealed environmental factors influencing the undesired behavior like stocking density, nutrition, litter or light intensity (for reviews see e.g. Nicol et al. (2013) or Rodenburg et al. (2013)). Besides these environmental factors, it is already known that feather pecking is also influenced by a genetic component. Feather pecking is heritable with heritabilities estimated in a low to medium range (Wysocki et al., 2010; Bennewitz et al., 2014). Quantitative trait loci (QTL) influencing feather pecking could as well already be revealed on several chromosomes (Buitenhuis et al., 2003; Lutz et al., 2017) although the quantitative and complex nature of the trait was highlighted.

Feather pecking is not homogenous within groups of laying hens and it has already been shown that there exists a subgroup of hens which peck feathers considerably more often than their group members (Labouriau et al., 2009; Piepho et al., 2017). Furthermore, a relation between feather pecking and fearfulness could be shown (e.g. Veestergaard et al. (1993)). Aggressive pecking and threatening are clearly distinguishable from feather pecking (Savory, 1995; Kjaer et al., 2001) and can be assigned to agonistic behavior (Guhl, 1968). Nevertheless, like feather pecking, aggressive pecking often leads to damaged plumage and injured birds and thus also represents an animal welfare problem. QTL influencing aggressive pecking have been found by Lutz et al. (2017).

There was evidence that the gut microbiome of hens plays a role in the development of feather pecking behavior. For example, a relation between feather pecking and feather eating (e.g. Harlander-Matauschek and Bessei (2005)) as well as differences in the gut microbiota composition of hens showing different amounts of feather pecking (e.g. Meyer et al. (2013)) have already been shown. In addition, connections between the gut microbiome and behavior of farm animals have been illustrated and reviewed in Kraimi et al. (2019).

The experimental populations analyzed in this thesis were formed by hens based on a founder line of a White Leghorn layer strain in 1970 (Liljedahl et al., 1979). In 1995, divergent selection for high and low feather pecking started (Kjaer et al., 2001). The first experimental population

of this thesis analyzed in chapter one was an F_2 cross of about 900 hens which was established of the 10th generation of the pure selection lines (Bennewitz et al., 2014). The second population which was analyzed in chapters two to four consisted of about 500 hens of the 15th generation of these two lines.

Based on former findings by Lutz (2016) who studied feather pecking and related behavior traits in the F_2 cross, the aim of this thesis was to gain further knowledge of the genetic background of feather pecking, its relation to additional behavior traits and the gut microbiome by means of the analysis of the high and low feather pecking pure selection lines. This was done by conducting phenotypic and genomic analyses and with an additional focus on extreme feather pecking.

In **chapter one**, a novel model to detect extreme feather pecking hens was developed. A mixture of two negative binomial distributions was fitted to feather pecking data of the F_2 cross. With the estimated parameters, the trait *posterior probability of a hen to belong to the extreme feather pecking subgroup* (pEFP) was calculated. This new trait was dichotomized and analyzed together with the binary fear traits tonic immobility and emerge box, each juvenile and adult, in a multivariate threshold model. Additionally, genomewide association studies (GWAS) were conducted for the five traits.

In **chapter two**, the model to detect extreme feather pecking hens was applied to the feather pecking data of the pure selection lines. After calculation of the new trait pEFP, GWAS with a subsequent post GWAS analysis was performed for putative QTL for pEFP and feather pecking. Additionally, to find genomic regions influencing feather pecking, selection signatures were mapped by applying the intra-population iHS and the inter-population F_{ST} approach.

In **chapter three**, the relation between agonistic behavior and feather pecking was analyzed. Therefore, the active parts of the traits (delivery of feather pecking, aggressive pecking or threatening) as well as the passive parts (reception of the traits) were considered. These groups of traits were additionally each summarized by means of an index formation which led to the two additional traits Activity and Passivity. Moreover, Indices were built by subtracting the passive traits from the respective active traits to obtain the feather pecking index, the aggression index and the threat index. Phenotypic correlations were estimated between all traits which was followed by the estimation of heritabilities and GWAS.

Chapter four provides the analyses of the gut microbial composition of the two feather pecking lines followed by estimation of microbiabilities for feather pecking and the two agonistic behavior traits to study the influence of the microbiome on behavior.

At the end of this thesis, a general discussion is given where potential breeding strategies are discussed on how to reduce feather pecking in commercial layer flocks based on the results of this thesis. Furthermore, a meta-analysis of the GWAS for pEFP of both experimental populations is provided.

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CHAPTER ONE

A Novel Model to Explain Extreme Feather Pecking Behavior in Laying Hens

H. Iffland[1] · R. Wellmann[1] · S. Preuß[1] · J. Tetens[2] · W. Bessei[1] · H.-P. Piepho[3] · J. Bennewitz[1,*]

[1]Institute of Animal Science, University of Hohenheim, Garbenstr. 17, 70593 Stuttgart, Germany

[2]Department of Animal Science, University of Göttingen, Burckhardtweg 2, 37077 Göttingen, Germany

[3]Institute of Crop Science, University of Hohenheim, Fruwirthstr. 23, 70593 Stuttgart, Germany

*correspondence: j.bennewitz@uni-hohenheim.de

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Abstract

Feather pecking (FP) is a serious economic and welfare problem in the domestic fowl. It has recently been shown that the distribution of FP bouts within groups is heterogeneous and contains a sub-population of extreme feather peckers (EFP). The present study proposed a novel model to detect EFP hens. A mixture of two negative binomial distributions was fitted to FP data of a F_2 cross of about 960 hens, and, based on the results, a calculation of the posterior probability for each hen belonging to the EFP subgroup (pEFP) was done. The fit of the mixture distribution revealed that the EFP subgroup made up a proportion of one third of the F_2 cross. The EFP birds came more frequently into pecking mood and showed higher pecking intensities compared to the remaining birds. Tonic immobility and emerge box tests were conducted at juvenile and adult age of the hens to relate fearfulness to EFP. After dichotomization, all traits were analyzed in a multivariate threshold model and a genomewide association study was performed. The new trait pEFP has a medium heritability of 0.35 and is positively correlated with the fear traits. Breeding for this new trait could be an interesting option to reduce the proportion of extreme feather peckers. An index of fear related traits might serve as a proxy to breed indirectly for pEFP. GWAS revealed that all traits are typical quantitative traits with many genes and small effects contributing to the genetic variance.

Keywords Laying hen · Extreme feather pecking · Fearfulness · Genetic architecture · Mixture distributions

Introduction

Feather pecking is a serious economic and welfare problem in laying hens. In the past, damages caused by feather pecking and cannibalism were controlled by beak trimming. This treatment, however, is or will be prohibited in several European countries for welfare reasons. In order to find alternative solutions for the problem more detailed understanding of this abnormal behavior pattern is required. Causation of feather pecking and cannibalism is multifactorial determined and several environmental-associated factors like housing and feeding as well as endogenous factors are discussed (Rodenburg et al. 2013). A small to medium heritability for this trait was frequently reported (Kjaer and Sørensen 1997; Rodenburg et al. 2003; Bennewitz et al. 2014), and indicates that breeding for this trait is possible. This was proven by selection experiments (Kjaer et al. 2001; Grams et al. 2015a). Quantitative Trait Loci (QTL) mapping studies and genomewide association analyses (GWAS) confirmed the assumption that this trait is polygenic determined with some trait-associated chromosomal regions (Buitenhuis et al. 2003; Lutz et al. 2017).

Feather pecking shows some complex interrelationship with other behavior traits. Among them, fearfulness was frequently reported to be associated with feather pecking, with high feather pecking hens showing a higher level of fear than low feather pecking hens (Jensen et al. 2005; Jones et al. 1995; Veestergaard et al. 1993). Rodenburg et al. (2004) showed that there is a positive genetic correlation between fear response of juvenile birds in an open field test and feather pecking in adult hens in feather pecking selection lines. In contrast, van der Eijk et al. (2018) found a lower level of fearfulness associated with higher level of feather pecking. In a recent experiment we set up an F_2 design with almost 900 individuals derived from two founder lines divergently selected for feather pecking (Bennewitz et al. 2014). A number of behavior tests including open field test, tonic immobility test and emergence test were conducted at juvenile as well as in adult age of the hens. Only small positive genetic correlations between these traits measured in juvenile status and feather pecking in adults was observed (Grams et al. 2015b).

Modelling feather pecking in genetic analysis is notoriously difficult, because of the deviation of the data from normality. In a recent study we used generalized linear mixed models and a Poisson distribution for the estimation of genetic parameters (Bennewitz et al. 2014). The use of the Poisson distribution seems natural, because the observations are count data. However, depending on the length of the observation period in which the feather pecks of the hens are recorded, it might be that there is an excess in zero counts (Grams et al. 2014). This might favor the use of zero-inflated Poisson distributions, which is however usually not supported by standard software applications. Moreover, it seems that there exists a subgroup of individuals that peck notably more often than their group members. Labouriau et al. (2009) identified non peckers, low peckers and high peckers in a high feather pecking line by using a mixture of Poisson distributions and suggested, that there is a major allele causing hyperactive feather pecking. Piepho et al. (2017) analyzed data from lines selected for high and low feather pecking and their F_2-cross and found consistently a subgroup of so called extreme feather peckers across several generations by fitting a mixture of two Poisson distributions. A detailed investigation of this extreme feather pecking phenomenon has not been done so far. Thus it is unclear what the underlying drivers for this phenomenon are. There is also no information on its heritability and genetic architecture. In addition, the relationship of extreme feather pecking and other behavior traits, like fearfulness has still to be investigated.

In this study data from the F_2 cross of Bennewitz et al. (2014) and Grams et al. (2015b) were used for the following three aims. Firstly, we present a novel theory and statistical method for the analysis of the extreme feather pecking phenomenon. The model is based on the fit of

mixtures of negative binomial distributions. Secondly, we define the dichotomized posterior probabilities of the hens belonging to the extreme feather pecking group as a novel trait and analyze it with threshold and appropriate GWAS models. Finally, we analyze the interrelationship of this new trait with fearfulness, measured by tonic immobility and emergence tests in juvenile and adult birds.

Materials and methods

Experimental design, data collection and editing

The experimental design is described in detail in Bennewitz et al. (2014) and Grams et al. (2015b) and is therefore only briefly described in the following. Individuals of a White Leghorn layer strain divergently selected for high and low feather pecking for 10 generations were used as founder animals for the establishment of an F_2 cross with about 960 individuals. The fearfulness of the hens was measured by the emerge box test (EB) and the tonic immobility test (TI). Both are well established tests for this purpose (Jones and Mills 1983; Forkman et al. 2007).

The EB and TI tests were performed at the seven and nine days (EB_j and TI_j) as well as 40 weeks of age (EB_a and TI_a). For the EB test, the animals were placed in a box measuring 23 × 23 × 20 cm (length × breadth × height) in complete darkness. After 60 s, a trapdoor was opened and the latency of appearance of the head and the whole body was recorded. The test was finished after 3 min. A latency of 180 s was assigned to birds which did not emerge within the time of the test. For TI test the animals were turned on their back in a cradle and the latency until the birds raised themselves was recorded. Maximum amount of time of the test was 180 s as well. The latencies of EB and TI in juvenile as well as in adult birds (Fig. 1) represent truncated data sets. Therefore, data for both traits was dichotomized: The animals which raised themselves in the TI test or emerged from the box in the EB test within 180 s received a ‘1’ and the remaining a ‘0’.

Feather pecking observations started at the age of 27 weeks. It was defined as non-aggressive, severe pecks and pulls delivered to the plumage of group members. Few single feather pecks delivered in a short sequence without the hen changing its position were recorded as a single occurrence and called bouts per bird. The observation period was in total 420 min, recorded on three consecutive days. A histogram of the observed number of bouts per bird is shown in Fig. 2. Minimum and maximum number of bouts per bird were 0 and 198, respectively, with a mean of 14.1 bouts per bird. Blood samples for genotyping with the Illumina 60 K chicken Infinium iSelect chip were collected from 817 hens. A number of 29022 SNPs were used in this

study after data filtering. SNPs located on the sex chromosomes W and Z as well as SNPs that were not allocated to a specific chromosome based on positional information according to the chicken genome assembly GRCg6a were excluded. Furthermore, SNPs with a call frequency lower than 0.95 and a minor allele frequency lower than 0.03 were filtered out. Sporadic missing genotypes were imputed using Beagle 5.0 (Browning and Browning 2007).

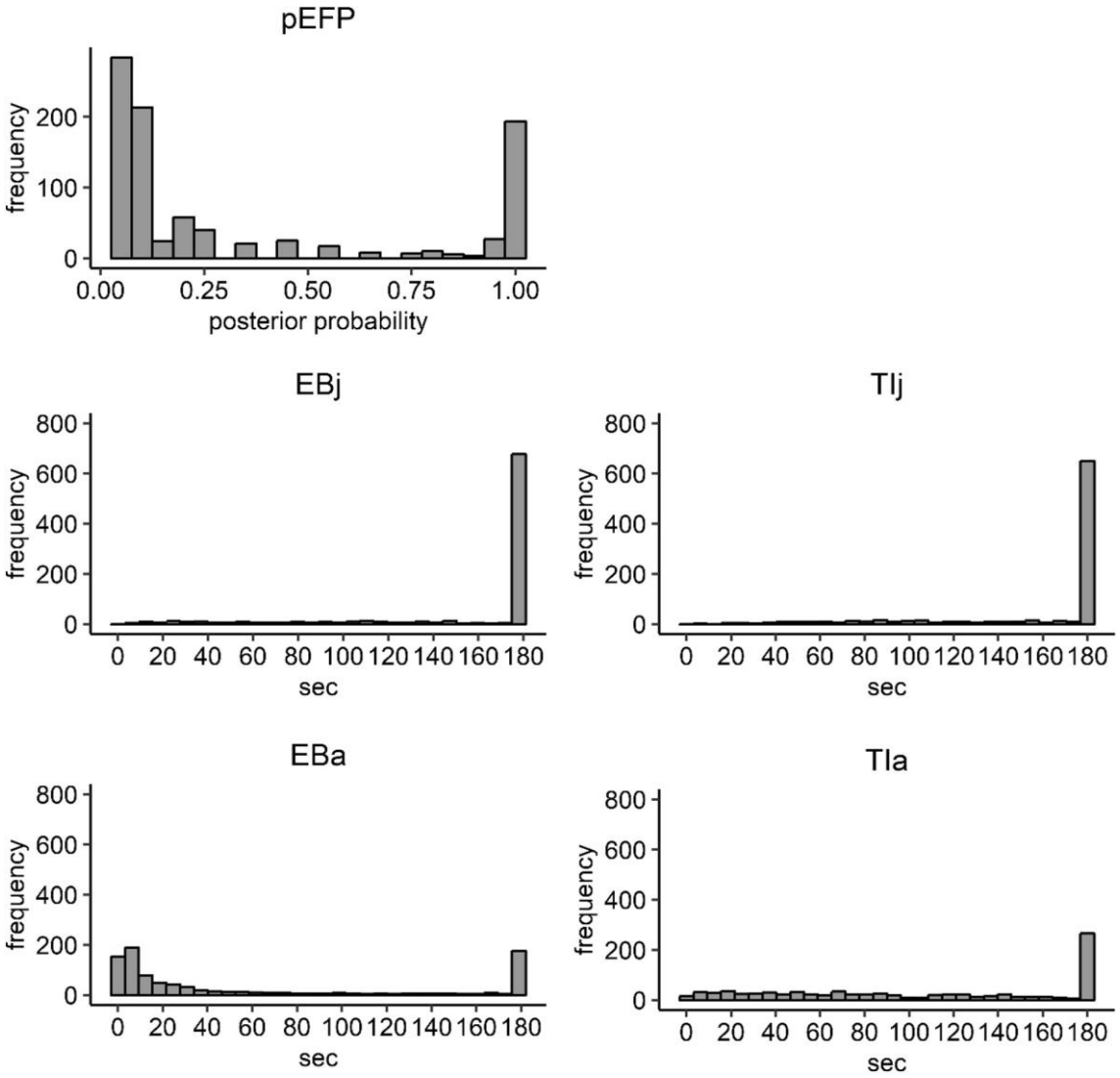

Fig. 1 Histogram of posterior probability of extreme feather pecking (pEFP, n=937), emerge box test at juvenile age (EB_j, n=891), tonic immobility test at juvenile age (TI_j, n=893), emerge box test at adult age (EB_a, n=893) and tonic immobility test at adult age (TI_a, n=867). The bar at 180 s represents the proportion of animals that did not move their head out of the box (EB) or turned around (TI) within the data collection period

Statistical analysis

A novel method to detect extreme feather peckers

The putative existence of an extreme feather pecking group (EFP) can be deduced from the visual inspection of the histogram (Fig. 2). Piepho et al. (2017) used mixtures of Poisson distributions to model this pattern. In this study we modelled the data as follows. Take *N* to be the number of times, a hen gets into feather pecking mood. We assume that these events are independent and occur at a constant rate during observation. Hence, *N* is Poisson distributed. Each time, a hen gets into pecking mood, it pecks one or several times. We assume that the frequency of pecks X_{ik} when hen *i* gets into pecking mood for the *k*-th time, follows a logarithmic distribution. Hence, the number of bouts per bird for hen *i*

$$N_{bpb}(i) = \sum_{k=1}^{N} X_{ik}$$

has a compound Poisson distribution, which in combination with the logarithmic distribution equals the negative binomial distribution (Johnson et al. 1993, Chap. 5). The parameters of the negative binomial distribution may be different for different hens.

We assume that the hens can be assigned to two subgroups *j*, the feather peckers (FP) and the extreme feather peckers (EFP). Following this, the number of bouts per bird (N_{bpb}) is derived from a mixture of two negative binomial distributions:

$$N_{bpb} \sim \pi f_{EFP}(\mu_j, \sigma_j) + (1-\pi) f_{FP}(\mu_j, \sigma_j)$$

where π is the proportion of EFP in the population. The density of the negative binomial distribution is

$$f_{NB}(\mu_j, \sigma_j; \gamma) = \binom{\gamma + \frac{\mu_j^2}{\sigma_j^2 - \mu_j} - 1}{\gamma} \left(\frac{\sigma_j^2 - \mu_j}{\sigma_j^2}\right)^{\gamma} \left(\frac{\mu_j}{\sigma_j^2}\right)^{\frac{\mu_j^2}{(\sigma_j^2 - \mu_j)}}$$

where γ is the observed number of bouts per bird, μ_j the mean of bouts per bird of subgroup *j* and σ_j the standard deviation of bouts per bird of subgroup *j*. There are several other parametrizations available (Johnson et al. 1993). We used this mean–variance parametrization, because these were our parameters of interest. The model was fitted to the F_2 cross feather pecking data to detect the EFP subgroup using a combination of a Newton-type method and an EM algorithm in the R package 'mixdist' (Macdonald and Du 2018; R Core Team 2017).

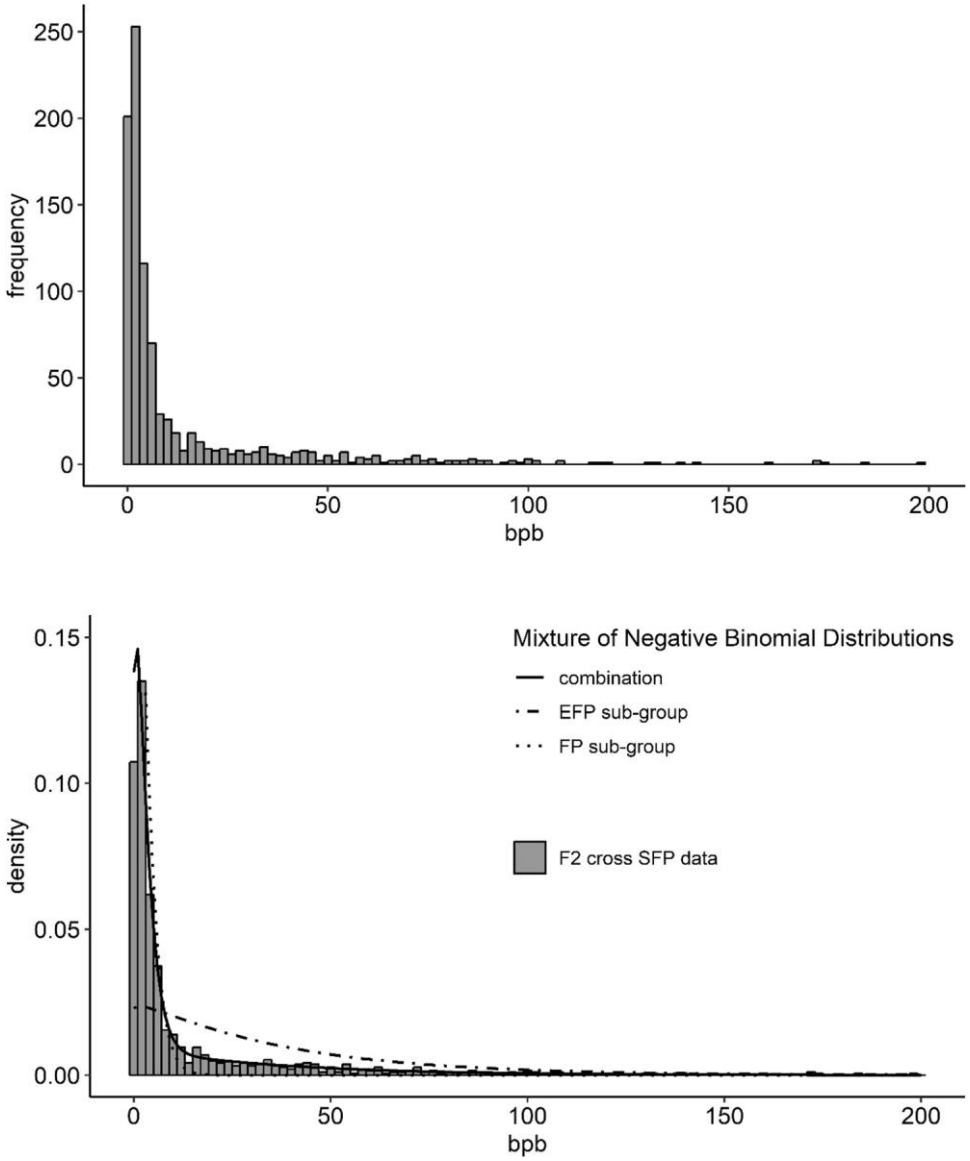

Fig. 2 Histogram of the F_2 cross feather pecking data in bouts per bird (bpb) (top graphic) and mixture of negative binomial distributions (black lines) fitted to the F_2 cross FP data (grey bars) (bottom graphic). The dotted line represents the fit of the FP subgroup, the dot-dashed line represents the EFP subgroup and the solid line represents the combination of both and shows the goodness of the fit of the mixture distributions

The number N_j of times a hen comes into pecking mood in subgroup j has mean

$$E(N_j) = \lambda_j = -\frac{\mu_j^2}{\sigma_j^2 - \mu_j} \ln\left(\frac{\mu_j}{\sigma_j^2}\right)$$

in which case the hen pecks on average $E(X_{ikj}) = \frac{\mu_j}{\lambda_j}$ times.

After fitting the mixture of negative binomial distributions to the data and thus estimating the parameters of the distributions, we calculated the posterior probability for each hen belonging to the EFP subgroup (pEFP) as follows:

$$pEFP = P(z = 1|\gamma) = \frac{\pi f_{EFP}(\gamma)}{(1-\pi)f_{FP}(\gamma) + \pi f_{EFP}(\gamma)}$$

where $P(z = 1|\gamma)$ is the posterior probability for observation γ to belong to the EFP subgroup while z denoting the latent variable for the subgroup, $f_{FP}(\gamma)$ the density function of the FP distribution and $f_{EFP}(\gamma)$ the density function of the EFP distribution. The new trait pEFP (Fig. 1) was dichotomized at a threshold of 0.5 for further analysis.

Pedigree-based estimation of genetic parameters using a threshold model

The five binary traits pEFP, EB_j, TI_j, EB_a and TI_a were analyzed simultaneously in a multivariate Bayesian animal threshold model as follows

$$\lambda = Xb + Za + e$$

where λ is the vector of liabilities of pEFP, EB_j, TI_j, EB_a and TI_a, b is the vector of fixed hatch effects, a is the vector with random additive-genetic effects, X and Z are known design matrices and e denotes for the residual term. The variance–covariance of the random animal effect was $var(a) = C \otimes A$, with C being a five times five matrix containing the additive genetic variances of the diagonals and the covariances on the offdiagonals and A being the numerator relationship matrix. The variance of the random residuals was $var(e) = I * E$, with E being a five times five matrix containing the residual variances on the diagonals and zero elsewhere and I being the identity matrix. The threshold model was run using the MCMCglmm package (Hadfield 2010) in R with flat priors. The length of the MCMC chain was set to 1,000,000 iterations with a burn-in of 100,000 and a thinning interval of 10. The residual variances were set to 1 in order to ensure indefinability and the heritability as well as the genetic and phenotypic correlations were estimated from the variance components using standard notations.

Genomewide association study

A single-marker GWAS was carried out to study the genetic architecture of the traits and to infer putative common chromosomal regions affecting the traits. In order to account for the 0/1 character of the data, we used the liability threshold linear model implemented in the LTSoft package (version 4.0) (Hayeck et al. 2015, 2017). We used the LT-Fam software, because we had family structures in our data. The heritabilities given to LT-Fam were those obtained from the previous pedigree-based analysis. The threshold given to the software was calculated using

the function *qnorm* in the 'stats' package in *R* (R Core Team 2017) while the prevalence needed for calculating the threshold was taken from the data. Conditioned by prevalence as well as the genomic relationship matrix and 0/1 status of all animals, LTSoft estimated the posterior mean liabilities using Gibbs sampling, and calculated a χ^2 association score statistic to infer the association between the candidate SNP and the posterior mean liabilities. During computation of the association score statistic, the chromosome with the candidate SNP is excluded from calculating the genomic relationship matrix. After computation, from the χ^2 values the corresponding p-values with one degree of freedom were calculated using the function *pchisq* in the 'stats' package in *R* (R Core Team 2017). Genomewide significance threshold levels were obtained by using the Bonferroni correction. Because it is known that Bonferroni correction is very conservative especially if the tests are not independent (as it is in our data set) we used an additional nominal significance criterion at $p \leq 5*10^{-5}$ to detect weak associated SNPs.

Results

The fit of two components was clearly superior compared to the fit of only one component. This was assessed using the Akaike information criterion (6286 for two component mixture compared to 6519 for one component, not shown elsewhere) and clearly confirms the existence of an EFP group in the data. The estimated mean of the number of bouts per bird was $\hat{\mu}_1 = 2.7$ (SE 0.18) for the FP group and $\hat{\mu}_2 = 36.7$ bouts per bird (SE 4.47) for the EFP group. The mixing proportion was $\hat{\pi} = 0.34$ (SE 0.04). During the observation hens from the FP group came on average $\lambda_1 = 1.63$ times into pecking mood, in which case they pecked on average $E(X_{ik1}) = 1.65$ times. In contrast, hens from the EFP group came on average $\lambda_2 = 3.77$ times into pecking mood and pecked on average $E(X_{ik2}) = 9.72$ times. The fit of the two components as well as the mixture distributions is shown in Fig. 2.

Results of the Bayesian threshold model analysis and thus heritability as well as phenotypic and genetic correlation estimates are shown in Table 1. pEFP, TI_j, EB_a and TI_a show medium and EB_j a low heritability. The phenotypic correlations are low between all of the five traits as well as the genetic correlation between pEFP and TI_j. The genetic correlation between pEFP and the fear traits are positive. It is higher between pEFP and the two EB traits than the corresponding correlations with TI_j and TI_a traits. The highest genetic correlations with 0.51 were found between EB_j and EB_a as well as TI_j and TI_a. Figure 3 shows the posterior distribution plots of the heritabilities, from which the convergence of the Gibbs sampler can be inferred.

The GWAS results are shown as Manhattan plots of the $-\log_{10}$ p-values in Fig. 4. No genomewide significant SNPs could be found for any trait. For pEFP, three nominal significant ($p \leq 5*10^{-5}$) SNPs (Gga_rs15622328, Gga_rs14027234 and GGaluGA079200) were found on chromosome 11 and one (GGaluGA093070) on chromosome 13. Furthermore, for EB_j, three nominal significant SNPs (GGaluGA259905, Gga_rs14471634 and GGaluGA259965) on chromosome 4 could be identified. Additionally, one nominal significant SNP (GGaluGA058092) was found for TI_a on chromosome 1. These SNPs are listed with their *p*- and χ^2-values in Table 2.

Table 1 Phenotypic (above the diagonal) and genetic (below the diagonal) correlation between traits and heritabilities (on the diagonal) as well as the standard deviations (in parenthesis), results from Bayesian multivariate threshold analyses

Traits[a]	pEFP	EB_j	TI_j	EB_a	TI_a
pEFP	**0.35 (0.15)**	0.05 (0.05)	0.01 (0.06)	0.15 (0.08)	0.05 (0.06)
EB_j	0.34 (0.29)	**0.10 (0.07)**	0.03 (0.04)	0.08 (0.05)	0.02 (0.04)
TI_j	0.04 (0.22)	0.25 (0.30)	**0.27 (0.12)**	0.09 (0.06)	0.10 (0.05)
EB_a	0.49 (0.19)	0.51 (0.27)	0.36 (0.23)	**0.29 (0.11)**	0.10 (0.06)
TI_a	0.21 (0.23)	0.18 (0.31)	0.51 (0.23)	0.43 (0.22)	**0.19 (0.08)**

[a] *pEFP* posterior probability of extreme feather pecking, EB_j emerge box juvenile, TI_j tonic immobility juvenile, EB_a emerge box adult, TI_a tonic immobility adult

Discussion

Extreme feather pecking

The results clearly showed the existence of an EFP subgroup, which was detected earlier by Piepho et al. (2017) using mixtures of Poisson distribution. The advantage of the novel method is, that it allows for more detailed information about the underlying mechanisms for this phenomenon. The method separated the bird`s frequency to get into the pecking mood and the frequency of pecking bouts within the pecking mood, which represents the intensity of pecking. EFP hens differed clearly in both criteria. They came on average more than twice as often in the mood for feather pecking ($\hat{\lambda}_1 = 1.63$ vs. $\hat{\lambda}_2 = 3.77$), and showed an about nine times higher pecking frequency within these periods.

This bi-partite distribution indicates that at least two different mechanisms are involved in expressing feather pecking. There might be a basic motivation to get into a pecking mood, which is subjected to a regulatory mechanism. In EFP birds the regulating mechanisms may lead to overstimulation, or prevent down regulation. It was repeatedly shown that the interface of the serotonergic and dopaminergic systems can influence feather pecking, as reviewed by de Haas and van der Eijk (2018). It can be speculated that low levels of serotonin stimulate the development of feather pecking in the juvenile phase of chickens, which might facilitate the basic motivation to come into the pecking mood. The dopaminergic mechanism might contribute to the impulsive/compulsive component, which prevents the birds from ceasing the sequence of feather pecking once it has started. This component is expressed in the elevated pecking intensity when the birds are in pecking mood. Research is needed to unravel how the serotonergic and dopaminergic systems affects the extreme feather pecking phenomenon observed in this study.

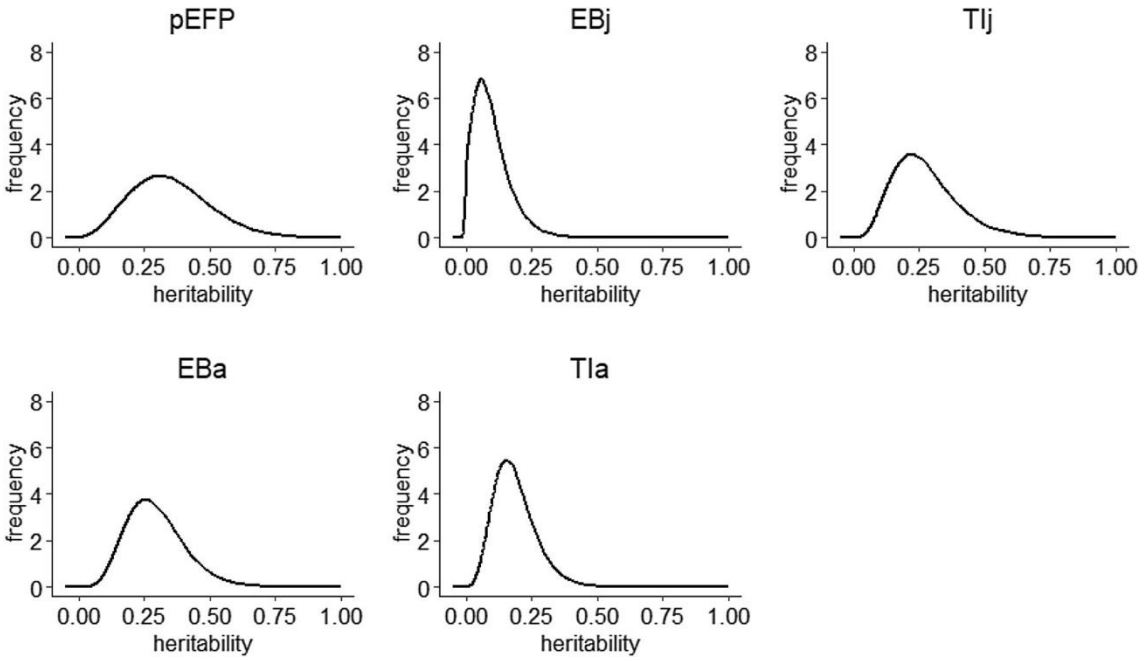

Fig. 3 Posterior distribution of heritabilities estimated in the multivariate Bayesian threshold model for pEFP, EB_j, TI_j, EB_a and TI_a (for abbreviations see Fig. 1)

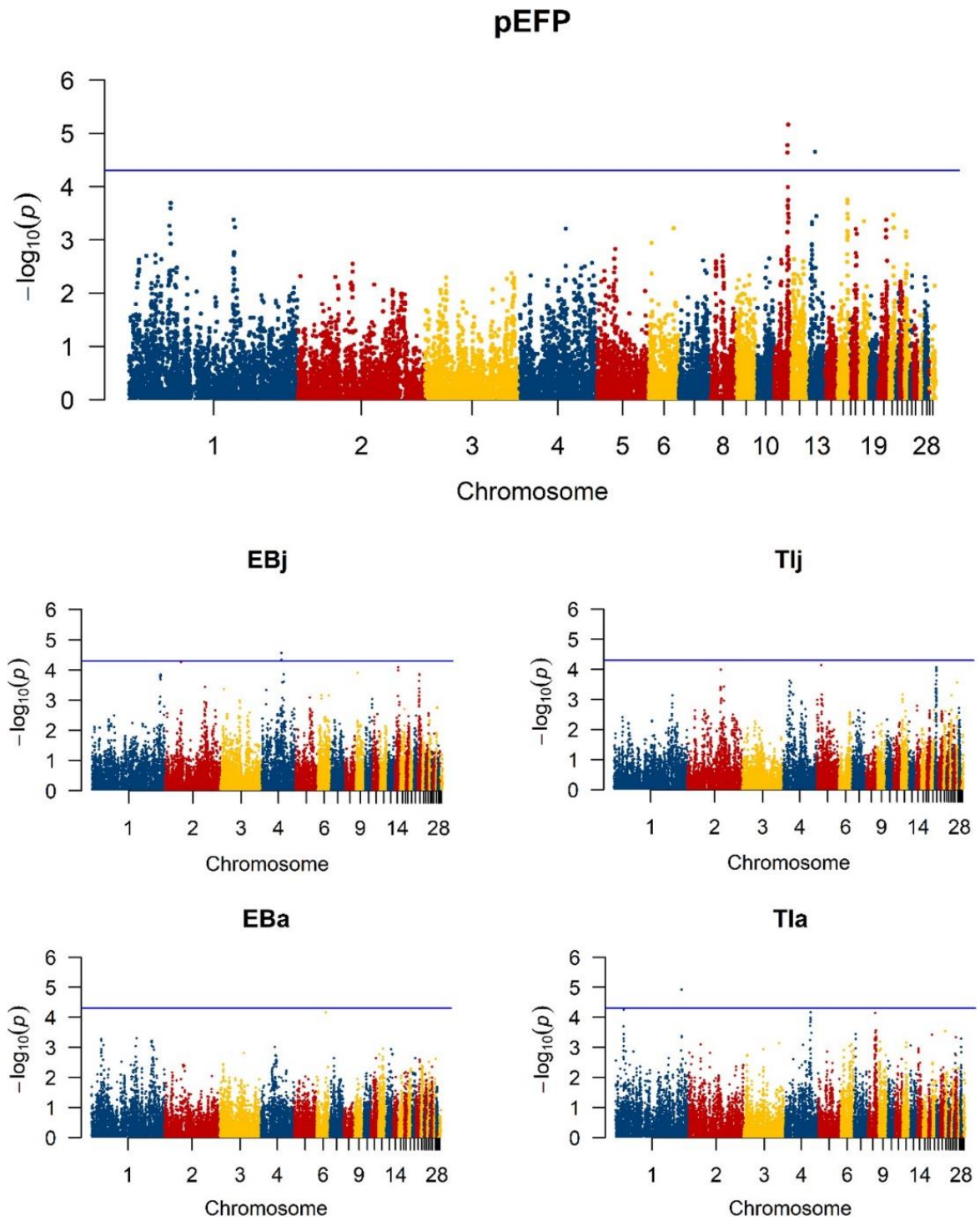

Fig. 4 Manhattan Plots of the $-\log_{10}$ p-values for association of SNPs with pEFP, EB_j, TI_j, EB_a and TI_a (for abbreviations see Fig. 1). The line indicates the nominal level of significance $p \leq 5*10^{-5}$

Table 2 List of the nominal significant SNPs for the five traits pEFP, EB_j, TI_j, EB_a, TI_a with $p \leq 5*10^{-5}$

Traits[a]	SNP	Chr[b]	Position[c]	$-\log_{10}(p)$[d]	χ^2-value[e]
pEFP	Gga_rs15622328	11	15910351	4.78	18.5
	Gga_rs14027234	11	15934863	4.64	17.9
	GGaluGA079200	11	16878999	5.16	20.2
	GGaluGA093070	13	8072026	4.65	18.0
EB_j	GGaluGA259905	4	53559697	4.34	16.6
	Gga_rs14471634	4	53653503	4.31	16.5
	GGaluGA259965	4	53659313	4.57	17.6
TI_a	GGaluGA058092	1	178590498	4.93	19.2

[a]For trait abbreviations see Table 1

[b]Chromosome number

[c]Position in bp

[d]p-value calculated from the χ^2-value from the LT-Fam

[e]χ^2-value from the LT-Fam statistic

To be effective, genetic strategies to reduce feather pecking should focus on extreme feather pecking birds. Separate recording and statistical treatment of feather pecking episodes and the frequency of feather pecks within these events will be helpful for identifying extreme feather peckers. The posterior probabilities belonging to the EFP group were U-shaped with many probabilities close to zero or one, respectively (Fig. 1). This shows that the method clearly separated FP and EFP hens. Further, the dichotomization could be done without any substantial loss in information. The pEFP heritability is in the medium range and is substantially above the estimates for bouts per bird in this data set (Bennewitz et al. 2014; Grams et al. 2015b). This is partly due to the different models used for analyzing the data, but it also underlines that breeding for the new trait pEFP is possible and would result in selection response, if the pEFP heritability in breeding populations is on a similar level as in this experimental data set. EFP hens can be seen as drivers for the development of feather pecking episodes in a flock of hens by stimulating pecking behavior of pen mates. Thus reducing the proportion of EFP hens is desired in order to reduce the problem, and the results of this study revealed that it could be done effectively by a breeding effort. The novel method needs feather pecking count data, which was done manually in this study and this is an extremely hard to measure process in a routine application. New phenotyping strategies are needed in order to establish an automatized counting process (e.g.

video or sensory techniques, Rodenburg et al. 2017). Alternatively, genetic correlated proxy traits have to be identified that are easier to measure.

Even though the GWAS revealed four significant SNPs for pEFP, it seems that it is a typical quantitative trait with many genes and small effects contributing to the genetic variance (Fig. 4). The four SNPs are only nominal significant. Hence, the assumption of a major gene causing extreme feather pecking (Labouriau et al. 2009) cannot be confirmed. Nevertheless, Buitenhuis et al. (2003) also identified QTL on chromosome 2 for severe FP. Lutz et al. (2017) detected some genomewide significant SNPs on chromosomes 8 and 9 for FP bouts in a meta-analysis with the same F_2 cross involved as in the present study. These SNPs were however not significant for pEFP, which might in part be due to the lower power in the present study and also by the statistical treatment of the observed feather pecking data. Mapping trait associated SNPs for EFP was done using a two-step approach, i.e. first the estimation of pEFP using the novel method and then using pEFP as observations in a GWAS approach. This is convenient, because it allows for the use of standard methods for GWAS (or indeed also quantitative-genetic analysis, done in this study). However, combining the two steps is desired in order to circumvent the problem of error propagation. Further research is needed in this direction.

Fear related traits

pEFP seems to be genetically positive correlated with fearfulness, especially with EB (Table 1), which was also reported for FP bout data by Rodenburg et al. (2004) and Grams et al. (2015b). Since fearfulness of a hen can be more easily assessed using standardized tests as FP activity measured manually by counting FP bouts, a combination of fear test trait results indeed might be suitable as proxy traits to breed for reduced pEFP, as suggested for FP bouts by Rodenburg et al. (2004). Because the standard deviations of the genetic correlations are high (Table 1), the results have to be confirmed in a larger study. This is also needed to decide which fear tests conducted at which age should be considered to be included in a fear index, which might be used to breed indirectly for reduced pEFP.

The GWAS results (Fig. 4) showed that the fear traits are also highly polygenic traits with no genes of large effects. QTL for fearfulness measured by TI type tests were found on chromosome 1 by Schütz et al. (2004), on chromosomes 1, 10, and 20 by Mignon-Grasteau et al. (2017) and on chromosome 5 in Japanese Quails by Recoquillay et al. (2015). No test statistic peak could be observed in our data in the corresponding chromosomal regions (Fig. 4), except for TI_a on chromosome 1.

Experimental design

Data from 960 and 817 individuals from an F_2 cross were used for quantitative-genetic analysis and GWAS, respectively. This number can be considered as substantial for an experimental F_2 design and indeed, results in some power to map QTL (Schmid et al. 2018). The lack of genomewide significant SNPs for the traits considered in this study point towards a highly polygenic nature of these behavior traits. Thus, mapping genes underlying the genetic variation needs much larger experiments or the combination of GWAS results from different studies using meta-analysis. Larger datasets would enable us also to study not only correlations between the behavior traits, but also causalities among them. This is essential if the results are to be used for understanding the phenomenon of FP and EFP and also if intervention strategies are to be developed. Causalities among quantitative traits can be detected e.g. by structural equation models (Gianola and Sorensen 2004) or Mendelian randomization (Evans and Smith 2015). It is hoped that new phenotyping strategies will provide us data sets that are large enough for this purpose.

Conclusions

The novel method showed that a subgroup of extreme feather peckers does exist and makes up about one third of the animals. The EFP birds came more frequently into pecking mood and showed higher pecking intensities compared to the remaining birds. The new trait pEFP has a medium heritability and is a typical quantitative trait with many genes and small gene effects. Breeding for this new trait could be an interesting option to reduce the proportion of extreme feather peckers. An index of fear related traits might serve as a proxy to breed indirectly for pEFP. Large datasets are needed to estimate genetic parameters precisely and to infer which fear tests should eventually be combined in such an index. Large datasets are also needed in order to disentangle correlations from causalities, which is needed to understand the phenomenon of extreme feather pecking.

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Compliance with Ethical Standards

Conflict of interest H. Iffland, R. Wellmann, S. Preuß, J. Tetens, W. Bessei, H.-P. Piepho, J. Bennewitz declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent All institutional and national guidelines for the care and use of laboratory animals were followed.

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CHAPTER TWO

Genomewide Mapping of Selection Signatures and Genes for Extreme Feather Pecking in Two Divergently Selected Laying Hen Lines

Hanna Iffland [1,*], Robin Wellmann [1], Markus Schmid [1], Siegfried Preuß [1], Jens Tetens [2], Werner Bessei [1] and Jörn Bennewitz [1]

[1] Institute of Animal Science, University of Hohenheim, Garbenstr. 17, 70593 Stuttgart, Germany;

[2] Department of Animal Science, University of Göttingen, Burckhardtweg 2, 37077 Göttingen, Germany;

*Correspondence: hanna.iffland@uni-hohenheim.de

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Simple Summary: Feather pecking is a behavior frequently occurring in commercial layer flocks. It often leads to skin injuries and cannibalism. Besides economic losses, severe animal welfare problems cannot be ignored. Previous research has shown that the trait is heritable. Thus breeding against feather pecking is possible, but phenotyping in a commercial environment is economically unfeasible at the moment because of the lack of proper techniques. Therefore, understanding the genetic background of the trait is mandatory to establish a genomic breeding program. This would require genotypic information of the hens, which is feasible under practical conditions. In the present study, we used different methods to identify regions in the genome that influence feather pecking and extreme feather pecking. We found one trait associated with the genomic region. The use of genotypic information from this region in terms of selection against the undesired behavior may help to improve animal welfare in layer flocks.

Abstract: Feather pecking (FP) is a longstanding serious problem in commercial flocks of laying hens. It is a highly polygenic trait and the genetic background is still not completely understood. In order to find genomic regions influencing FP, selection signatures between laying hen lines divergently selected for high and low feather pecking were mapped using the intra-population iHS and the inter-population F_{ST} approach. In addition, the existence of an extreme subgroup of FP hens (EFP) across both selected lines has been demonstrated by fitting a mixture of negative binomial distributions to the data and calculating the posterior probability of belonging to the extreme subgroup (pEFP) for each hen. A genomewide association study (GWAS) was performed for the traits pEFP and FP delivered (FPD) with a subsequent post GWAS analysis. Mapping of selection signatures revealed no clear regions under selection. GWAS revealed a region on Chromosome 1, where the existence of a QTL influencing FP is likely. The candidate genes found in this region are a part of the GABAergic system, which has already been linked to FP in previous studies. Despite the polygenic nature of FP, selection on these candidate genes may reduce FP.

Keywords: laying hens; selection signatures; extreme feather pecking; divergent selection; QTL

1. Introduction

Feather pecking is a long known and still existing problem in commercial flocks of laying hens. It causes feather damages and skin lesions often resulting in cannibalism, which is an animal welfare issue and leads to economic losses. For years it has been a topic of intensive research and many aspects influencing feather pecking have already been revealed like stocking density,

light intensity, nutrition, or litter [1]. Besides these environmental factors, genetic factors were shown to influence the occurrence of feather pecking as well. Low to medium heritabilities were frequently found [2,3] indicating the possibility to breed for this trait which has also been proven in several selection experiments [4]. A number of mapping studies revealed quantitative trait loci (QTL) and trait-associated genome-regions, but also highlighted the quantitative and complex nature of this trait [5,6], because the few significant QTL by far did not explain the total genetic variance.

One approach to identify candidate regions in the genome is to map selection signatures in selected lines. As selection increases the frequency of an advantageous allele, nearby variants in linkage disequilibrium (LD) with the superior allele will also increase in frequency, termed as “hitch-hiking” alleles. At the beginning of selection, this results in long haplotype segments that are surrounding the advantageous alleles. Their increase in frequency in the population is called a “selective sweep” which leads to a reduced genetic diversity in the vicinity of advantageous alleles [7,8]. Hence, selection pressure leads to a specific formation of selection signatures which can be detected with several inter- and intra-population methods. In this study, two lines selected for high (HFP) and low (LFP) feather pecking for 15 generations were used to map selection signatures using the intra-population haplotype-based integrated haplotype score (iHS) [9] and the inter-population SNP-based F_{ST} index [10,11]. Chromosomal regions with significant selection signatures point to the presence of QTL within these regions.

Feather pecking is not homogenous within groups of laying hens and several studies reported the presence of a subgroup of extreme feather peckers (EFP), i.e., showing an exceptional high severe feather pecking activity compared to group mates [12–15]. In a previous study [15], we detected a subgroup of extreme feather pecking hens within an F2-cross of the HFP and LFP lines mentioned above by fitting a mixture of two negative binomial distributions to feather pecking data. A proportion of 33% extreme feather pecking hens was found. We also showed that extreme feather pecking hens came more than twice as often into the motivation period for feather pecking and pecked about five times more feathers than the other hens when they were in the motivation period. This led to the conclusion that genetic strategies should focus on extreme feather pecking. Consequently, it is important to detect the individual extreme feather peckers and to analyze their genetic background. Using the results from the fit of the mixture of negative binomial distributions, the posterior probability of belonging to the distribution representing the extreme feather pecking subgroup was calculated for each hen. We used this probability (pEFP) as a new trait for extreme feather pecking and a genomewide association study revealed several significant SNPs for pEFP [15].

The aim of the present study was to map selection signatures in lines that were divergently selected for feather pecking. In a second step, we identified extreme feather pecking hens by fitting a mixture of two negative binomial distributions to feather pecking data and calculated the new trait pEFP. Finally, genomic regions associated with feather pecking were identified by conducting a GWAS for the traits feather pecks delivered (FPD) and pEFP. Those regions were further analyzed to reveal the putative candidate genes.

2. Materials and Methods

2.1. Experimental Design, Data Collection and Editing

Based on a founder line of a White Leghorn layer strain established in 1970 as a control population in the Scandinavian selection and cross-breeding experiment of Liljedahl et al. [16], divergent selection for HFP and LFP started in 1996 at the Danish Institute of Agricultural Sciences [17]. Breeding values for feather pecking behavior were estimated and used for selection in the HFP line and in the LFP line. After five generations, fertilized eggs were transferred to the experimental farm of the University of Hohenheim in Germany where the selection for HFP and LFP continued. Current data was generated by phenotyping hens of the HFP and LFP lines of the 15th generation, hatched in 2017. Rearing and husbandry conditions have not been changed since then and are briefly described in Bennewitz et al. [3].

Three hatches were produced in two week intervals. The hens of the first two hatches took part in the first experimental run at the age of 33 and 31 weeks, respectively and hatch three in the second run at the age of 32 weeks. One week before behavioral observations, the hens were marked with numbered plastic tags on their back for individual identification and then transferred to observation pens in a ratio of 1:1 of LFP to HFP hens and around 42 individuals per pen in at total seven pens in the first run.

Because of less total hens and a lower number of LFP in the second run, six groups each of about 40 hens were used with a ratio of 1:2 LFP to HFP. A 14 hours' light program was provided by incandescent bulbs from 3 am to 5 pm. There was additional natural light through transparent plastic material at the upper part of the side walls. Depending on the fluctuation of the natural light, light intensity increased occasionally from 20 up to 2500 lux.

Observations were done in two sessions each day, starting at 10 am on four consecutive days. In order to ensure a balanced observation scheme, the number of observers corresponded with the number of observation pens (7 in the first and 6 in the second run). Each pen was observed by each observer on each day in 20 min sessions. The observers changed the compartments in

a rotational system. This resulted in at total 560 min of observation time in the first run and 480 min in the second. To gain compatibility with data from an F2 design of these lines, observation time was standardized to 420 min [18]. FPD as well as feather pecks received were recorded in the morning sessions whereas aggression was recorded in the afternoon (not further analyzed in this study).

FPD was recorded as non-aggressive severe pecks or pulls directed to the plumage of group members with sometimes resulting in pulled out feathers and a recipient which tolerates or moves away [18,19]. A series of pecks delivered in a short sequence without the hen changing its behavior were recorded as a single occurrence and called a bout per bird (bpb). A number of 492 hens (270 HFP and 222 LFP) were phenotyped sufficiently.

For the GWAS, FPD was Box-Cox transformed to reduce the deviation of the distribution from a normal distribution. After adding 1 to the FPD recordings the following transformation was applied: $y_{ti} = \frac{(y_i^{-0.2}-1)}{-0.2}$, where y_i is the number of bpb of each hen i and y_{ti} is the transformed observation. The power parameter −0.2 was used according to Lutz et al. [20] and Su et al. [21] showing the best fit for feather pecking data.

Blood was collected from the hens to extract the DNA and to perform genotyping with the Illumina 60 K chicken Infinium iSelect chip. SNPs with a call frequency lower than 0.95 and a minor allele frequency of zero were filtered out. Additionally, SNPs located on the sex chromosomes as well as SNPs that were not allocated to a specific chromosome according to positional information of the chicken genome assembly GRCg6a were excluded. This filtering resulted in 29,020 SNPs and 494 hens (270 HFP and 219 LFP) with sufficient genotype information. Sporadic missing genotypes were imputed and the genotypes were phased with Beagle 5.0 [22]. The total amount of hens with sufficient phenotypic as well as genotypic data was 489.

The research protocol was approved by the German Ethical Commission of Animal Welfare of the Provincial Government of Baden-Wuerttemberg, Germany (code: HOH 35/15 PG, date of approval: April 25, 2017).

2.2. Statistical Analysis

2.2.1. Multidimensional Scaling

In order to visualize the genetic distance between the divergently selected feather pecking lines, a multidimensional scaling was performed. In the first step, using R package “optiSel” [23], the

segment-based kinship $f_{SEG}\,(i,j)$ between all pairs of individuals i,j was calculated. Each segment comprised at least 20 markers and was at least 3.3 cM long.

The 20 markers per segment are considered to be enough to ensure that two segments with identical marker alleles are not identical by chance [24]. In accordance with Browning [25], the minimum length of a segment was chosen as 3.3 = $\frac{100}{2g}$ cM, where g denotes the number of generations after the base population has been established, which is 15 in our case. It needs to be ensured that the number of SNPs that remained in the dataset after filtering is enough to deliver segments which are sufficiently long. This can be seen as follows. Under the assumption of equally spaced SNPs, $0.167 = \frac{100}{40\,g}$ cM is the maximum allowed marker distance between adjacent SNPs if segments cover at least 20 markers [26]. This is more than four times larger than the actual average marker distance, which is $\frac{10.4 \cdot 100}{29020} = 0.035$ cM, where 10.4 M is the length of the chicken genome. The shortest detectable segment is thus $20 \cdot 0.035 = 0.7$ cM long, which is considerably smaller than the minimum segment length, which was set as 3.3 cM. Even under the knowledge that the SNPs are not equally spaced, more than four times as many seem to be a sufficient number of SNPs.

The kinship between individuals i,j was calculated as

$$f(i,j) = 0.02 + 0.98 f_{SEG}(i,j), \quad (1)$$

where $f_{SEG}(i,j)$ is the segment-based kinship between individuals i and j, and 0.02 is the ancestral kinship that is assumed to be not covered by the markers. The kinship, which is a value between 0 and 1, was then mapped to the positive real numbers and converted into a dissimilarity measure with function

$$Dissimilarity(i,j) = \log\,(f(i,j))^2. \quad (2)$$

The individuals were arranged on a two-dimensional plane such that their distances correspond to their genetic dissimilarities as good as possible by performing multidimensional scaling with R package “smacof” [27].

2.2.2. Mapping Selection Signatures

Selection signatures within each line were mapped with the iHS statistic of R package “rehh” [28,29]. The test for a single population compares the average lengths of haplotype segments around a focal SNP s that carry the ancestral allele A with those that carry the derived allele D. For each SNP, the allele with the highest average frequency over both populations was set as

ancestral and the other as derived. A selection signature is detected if both average segment lengths differ significantly from each other. The average length of haplotype segments that carry a given allele $a \in \{A, D\}$ at position s is estimated by the integrated extended haplotype homozygosity IHH_s^a as follows. Two haplotypes are assumed to be chosen at random without replacement from the population from all haplotypes that carry the a-allele, and the proportion $EHH_{s,t}^a$ of cases is computed in which they are identical between positions s and t. This value decreases from 1 to 0 when SNP t moves away from the focal SNP s. The average haplotype segment length IHH_s^a is then estimated as the integral of $EHH_{s,t}^a$ over all SNP t with $EHH_{s,t}^a > 0.05$. The test statistic is a monotone function of

$$\text{unstandardized } iHS_{\text{pop}i}(s) = \ln\left(\frac{IHH_s^A}{IHH_s^D}\right). \quad (3)$$

If the value is smaller than 0, then haplotypes that carry the derived allele are on average longer than the haplotypes that carry the ancestral allele, so it might be expected that the derived allele has swept up in frequency. A large positive value, however, can also indicate a selective sweep. This is the case when ancestral alleles hitchhike with the selected site. Therefore, a two-sided test was carried out. The standardized iHS value was used as the test statistic. The standardization was done conditionally on the allele frequency, which removed the effect of the allele frequency on the distribution of the test statistic. The "rehh" package assumes that the standardized iHS value has a normal distribution [9]. For this test, only SNPs with a minor allele frequency (MAF) larger than 0.01 within each line were considered, which resulted in 22,425 SNPs for the HFP and 23,084 SNPs for the LFP line. Both lines had 16,766 of these SNPs in common.

In order to increase the power of the iHS test, the test was also carried out for both populations simultaneously. First, the standardized iHS values were calculated separately for the two divergently selected lines. The standardized difference of both iHS values was then used as the test statistic, i.e.,

$$\text{combined } iHS(s) = \frac{iHS_{\text{pop1}}(s) - iHS_{\text{pop2}}(s)}{mad\left(iHS_{\text{pop1}} - iHS_{\text{pop2}}\right)}. \quad (4)$$

Thereby, the mad is the scaled median absolute deviation, which is a robust estimate for the standard deviation of a normal distribution. The test statistic has therefore a standard normal distribution under H_0. The rationale behind this approach is that signatures from selective sweeps in both lines that result from selection in the same direction tend to cancel each other,

whereas signatures from selective sweeps that result from divergent selection increase in magnitude. Since the lines are divergently selected for feather pecking, it is likely that the resulting selection signatures result from selection on feather pecking. As large negative and large positive values both indicate a selective sweep, the p-values for the combined iHS test were calculated as

$$p_{iHS} = 2\Phi(-|\text{combined } iHS(s)|), \quad (5)$$

where Φ() is the distribution function of the standard normal distribution.

Selection signatures between the HFP and LFP lines were mapped using the genetic differentiation index F_{ST}. Similar allele frequencies in the two subpopulations are represented by small F_{ST} values whereas different allele frequencies lead to large F_{ST} values and thus indicate regions under selection [10]. The F_{ST} index was already calculated in Grams et al. [30] for data of hens of the 11th generation of the two feather pecking lines. The same approach is used in the current study and hence in the following only described briefly. F_{ST} indices were computed using Equation (8) of Weir and Cockerham [11]:

$$F_{ST} = \frac{\sigma_p^2}{\bar{p}(1-\bar{p})}, \quad . \quad (6)$$

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

To account for differences in allele frequencies because of genetic drift, a statistical test was developed by Grams et al. [30]. The distribution of the F_{ST}-values under the null-hypothesis that an allele is neutral was obtained by simulation. Each allele was given a starting allele frequency of 0.5 in the base population. The allele frequencies for the two populations were simulated for 15 generations. For the first 11 generations, the effective population size (Ne) was the same as in Grams et al. [30]. From generation 12 to 15, the Ne was 40 because of a wider selection of breeding animals in these generations. The simulation was repeated 100,000 times resulting in 100,000 F_{ST} values. This revealed the distribution of F_{ST} indices under the null hypothesis of genetic drift but without selection. Finally, the *p*-value for each real SNP ($p_{nominal}$) was computed as the proportion of simulated SNPs that showed a greater F_{ST} index than the SNP under consideration. F_{ST} computations were performed using an own written R script.

The correction for multiple testing was done by applying a Bonferroni correction as $p_{genomewide} = 1 - (1 - p_{nominal})^{\#SNP}$, where the number of SNPs was 29,020 and the significance level $p_{genomewide}$ was set to ≤ 0.05. The other two additional levels of significance were set to $p_{nominal} \leq 5 \times 10^{-4}$ and $p_{nominal} \leq 5 \times 10^{-5}$ as well due to the very conservative approach of the Bonferroni correction. False discovery rates (FDR) for every $p_{nominal}$ value were calculated with the R package "qvalue" [31] to estimate the number of false positives among the significant SNPs.

In a final step, a meta-analysis was performed by combining the *p*-values of the combined iHS test and the F_{ST} test. This was done as follows using Fisher's combined probability test [32]:

$$\chi^2_{2k} \sim -2 \sum_{i=1}^{k} \ln(p_i), \tag{7}$$

where k is the number of studies being combined ($k = 2$ in this study) and p_i is the *p*-value for the i^{th} hypothesis test. The significance level that was used for the meta-analysis was the same as for the iHS statistics.

2.2.3. Detecting Extreme Feather Peckers

Iffland et al. [15] introduced a novel method to detect extreme feather peckers in a group of laying hens, which was applied to the two lines jointly as described briefly in the following. It is assumed that each hen belongs to one of two subgroups, which are the extreme feather pecking (EFP) and the normal feather pecking (FP) subgroup. Hence, the density of the number of bouts per bird N_{bpb} is

$$f(N_{bpb}) = \pi f_{EFP}(N_{bpb}) + (1 - \pi) f_{FP}(N_{bpb}), \tag{8}$$

where π is the proportion of EFP hens in the population. Here, $f_{EFP}(N_{bpb})$ is the density of N_{bpb} in the EFP subgroup, and $f_{FP}(N_{bpb})$ is the density of N_{bpb} in the FP subgroup. It is assumed that N_{bpb} has a negative binomial distribution within each subgroup. The distributions have parameters $\mu_{\text{EFP}}, \sigma_{\text{EFP}}$ and $\mu_{\text{FP}}, \sigma_{\text{FP}}$, respectively, where μ_j is the mean of N_{bpb} for subgroup *j* (*j* = EFP or FP) and σ_j is the standard deviation. The parameters were estimated with R package "mixdist" [33].

If a hen belongs to subgroup *j*, then its number of bouts can be written as $N_{bpb} = \sum_{k=1}^{N_j} X_{kj}$,

where N_j is the number of times the hen came into the pecking motivation period (i.e., time period with motivation for feather pecking), and X_{kj} is the number of pecks when the hen came into the pecking motivation period for the *k*-th time. These values can be estimated from the results of the fit of the mixture of negative binomial distributions as follows [15]

$$E(N_j) = \lambda_j = -\frac{\mu_j^2}{\sigma_j^2-\mu_j}\ln\left(\frac{\mu_j}{\sigma_j^2}\right), \text{ and } E(X_{kj}) = \frac{\mu_j}{\lambda_j}. \quad (9)$$

Hence, the separation into pecking motivation periods and number of pecks within pecking motivation periods was not part of the applied ethogram, but is our interpretation of the parameters of the mixture distributions. In a second step, using the estimated parameters of the fit of the mixture distributions, the posterior probability for each hen to belong to the EFP subgroup (pEFP) is calculated as follows:

$$pEFP = P(j = \text{EFP} \mid N_{bpb}) = \frac{\pi f_{EFP}(N_{bpb})}{f(N_{bpb})}, \quad (10)$$

where $P(j = EFP|N_{bpb})$ is the posterior probability for a hen with N_{bpb} bpb to belong to the EFP subgroup. Finally, the new trait pEFP was dichotomized at a threshold of 0.5 for further analysis.

2.2.4. GWAS and Estimations of Genetic Parameters

To analyze the genetic background of pEFP and FPD, a single-marker GWAS was carried out using the software GCTA [34]. The chromosome with the candidate SNP was excluded from calculating the genetic relationship matrix *G*. For each of the two traits, the following model was applied:

$$y = Xb + Wu + g + e, \quad (11)$$

where *y* is a vector of observations of the corresponding trait, *b* is a vector containing the fixed effect of the line as well as a fixed combinational effect consisting of the experimental run and pen. *X* is the corresponding design matrix, *u* is a vector of the fixed SNP effects to be tested, and *W* is the standardized genotype matrix. The random vector *g* contains the additive animal effects with $g \sim N(0, \sigma_g^2 G)$, where *G* is the genomic relationship matrix. The vector *e* residual effects has distribution $e \sim N(0, \sigma_e^2 I)$ with *I* being the identity matrix. For GWAS computation, the minor allele frequency (MAF) was set to 0.01, which resulted in 28,525 remaining SNPs.

The variance components were also estimated with GCTA [34], where the model was reduced to: $y = Xb + g + e$.

A prevalence of 0.24 was specified for pEFP to transform the heritability estimated on the observed scale within a case-control threshold model on the liability scale. The prevalence was estimated from the data.

2.2.5. Post GWAS Analysis

In regions of the genome where QTL are likely according to the GWAS results of pEFP and FPD, screening for positional candidate genes was done going from significant Peak-SNP 1.5 Mb up- and downstream using the NCBI (National Center for Biotechnology Information) genome data viewer. The positional candidate genes were then analyzed with the database for annotation, visualization and integrated discovery (DAVID) version 6.8 [35,36] to perform a functional annotation clustering and thus to identify enriched annotation terms. For clustering, a similarity threshold of 0.85, an EASE score of 0.1 and an enrichment score of ≥1.3 ($p \leq 0.05$) were used as stated out in Huang et al. [36].

3. Results

3.1. Multidimensional Scaling

The multidimensional scaling plot can be seen in Figure 1. HFP and LFP lines are clearly separated from each other with both having two crescent-shaped substructures in their clusters.

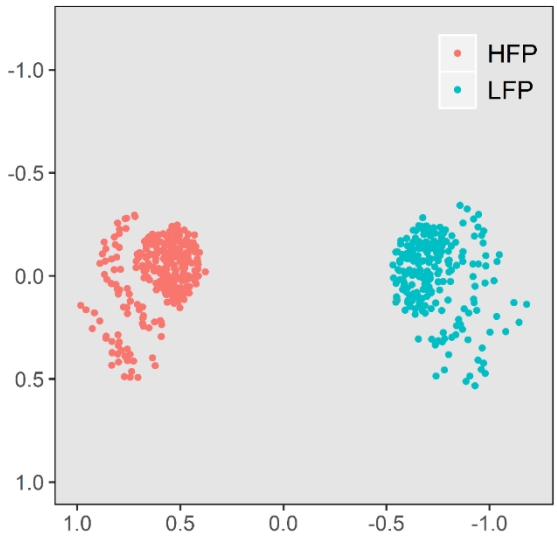


Figure 1. Multidimensional scaling of the high (HFP, n = 272) and low (LFP, n = 222) feather pecking lines. The distance to each other visualize their genetic distance.

Figure 2 shows the histograms of bpb of each of the two lines. Hens of the HFP line are pecking considerably more often, compared to the hens of the LFP line.

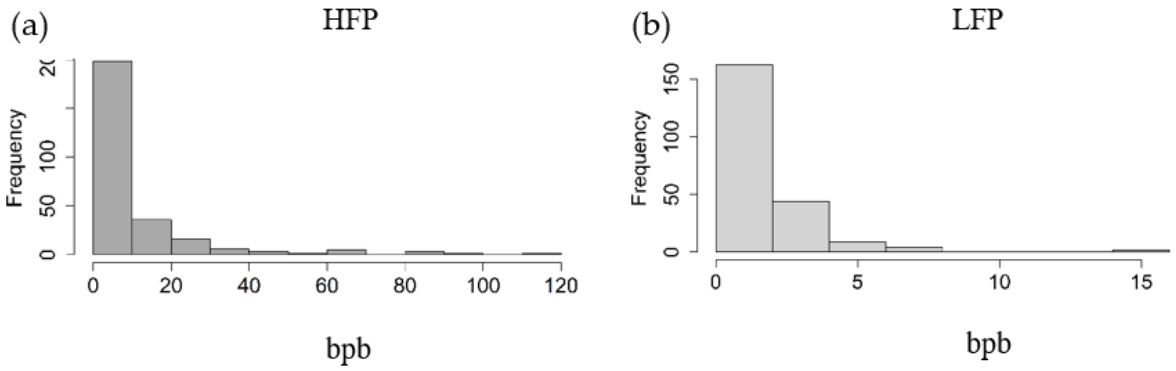


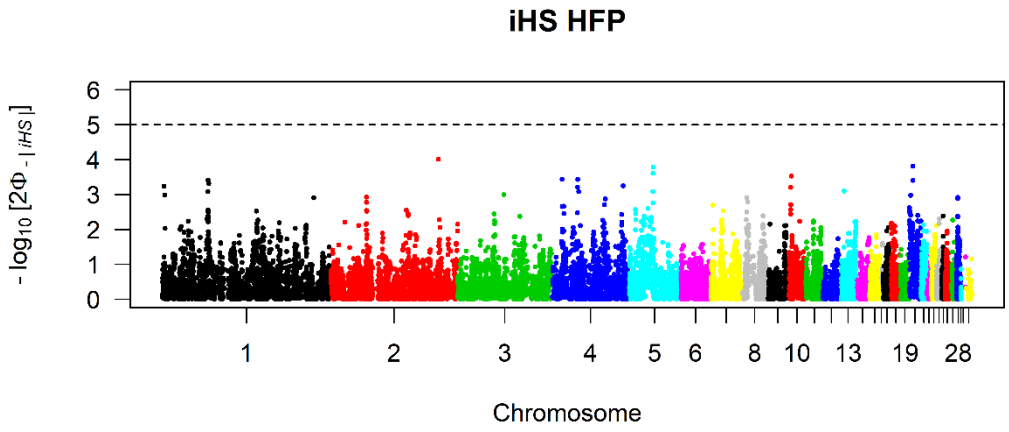
Figure 2. Histogram of feather pecks delivered in bouts per bird (bpb) for (**a**) the high (HFP, n = 270) and (**b**) low (LFP, n = 222) feather pecking lines. Note the different scales used for clarity.

3.2. Selection Signatures

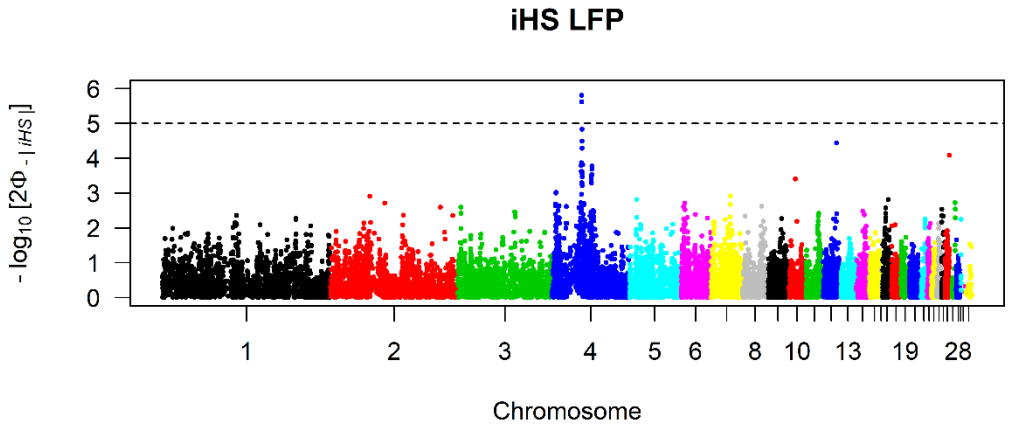
For LFP, the iHS approach revealed two nominal significant ($p \leq 10^{-5}$) SNPs on Chromosome 4 (Figure 3). No significant SNPs and thus no selection signatures could be found for HFP or for the combination of both lines (Figure 3).

Results of F_{ST} statistics are shown in Figure 4. A number of 57 SNPs on 13 chromosomes reached significance ($p_{nominal} \leq 5 \times 10^{-5}$) with an FDR of 0.021. With the relaxed level of significance ($p_{nominal} \leq 5 \times 10^{-4}$), another 92 SNPs on in total 22 chromosomes reached significance (FDR 0.056). The most significant SNPs ($p_{nominal} \leq 5 \times 10^{-4}$) were found (in descending order) on Chromosomes 2, 3, 1, 8 and 11. No genomewide significant SNPs could be revealed because SNPs did not reach genomewide significance.

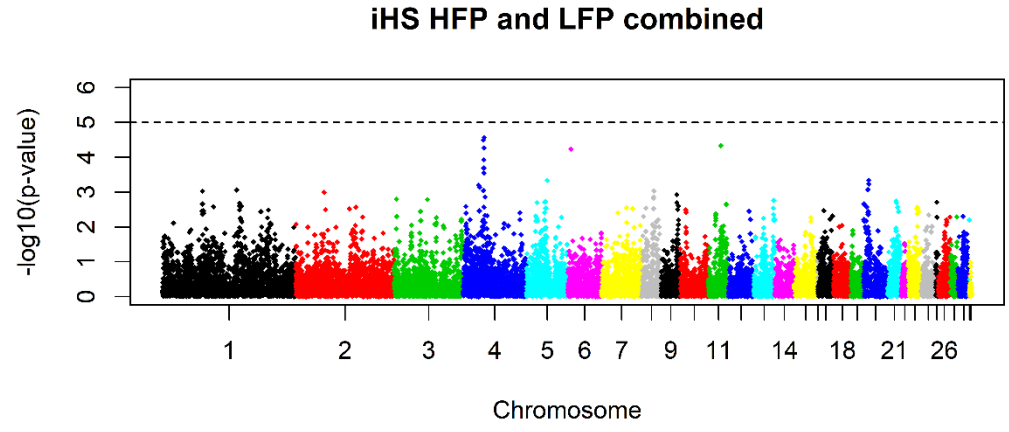
The meta-analysis of the combination of the *p*-values of the combined iHS statistic and the F_{ST} indices is plotted in Figure 4. No SNPs reached significance.



(**a**)

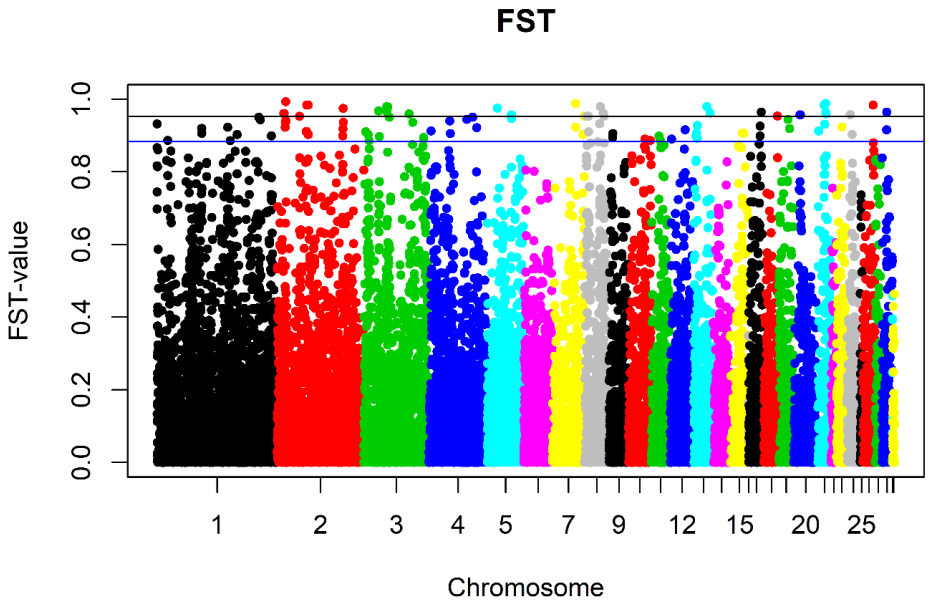


(**b**)

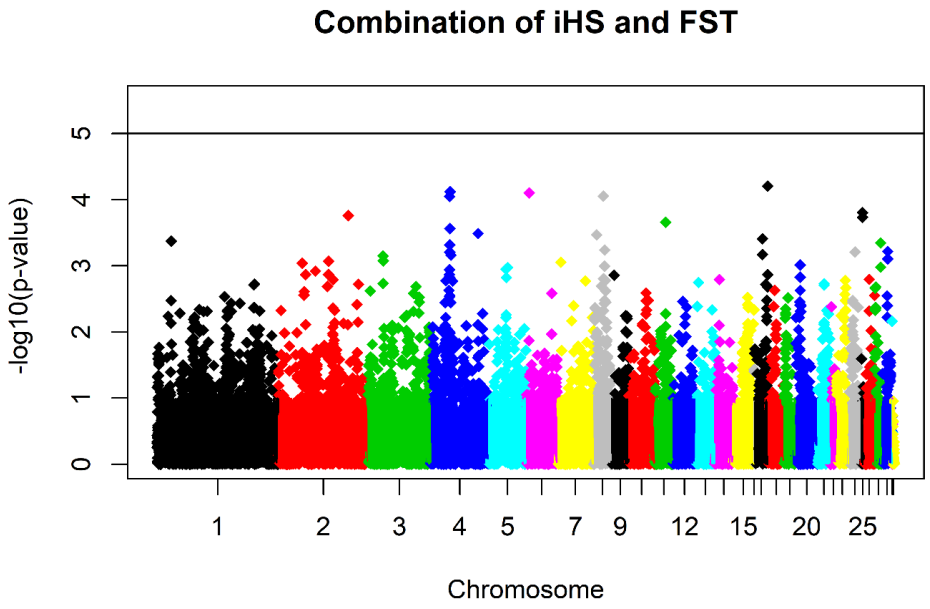


(**c**)

Figure 3. *P*-values of the integrated haplotype score (iHS) for (**a**) the high feather pecking line (HFP, n_{SNP} = 22,425), (**b**) the low feather pecking line (LFP, n_{SNP} = 23,084) and (**c**) their combination (n_{SNP} = 16,766). The dashed lines indicate the nominal level of significance $p \leq 10^{-5}$.



(**a**)



(**b**)

Figure 4. (**a**) Manhattan plot of F_{ST}-indices (n_{SNP} = 29,020). The top line indicates the nominal level of significance $p_{nominal} \leq 5 \times 10^{-5}$ and the bottom line the nominal level of significance $p_{nominal} \leq 5 \times 10^{-4}$. (**b**) Manhattan plot of the $-\log_{10}$ *p*-values of the combination of the combined iHS *p*-values and the F_{ST} p-values (n_{SNP} = 16,766). The line indicates the nominal level of significance $p \leq 10^{-5}$.

3.3. Extreme Feather Peckers

In Figure 5, the combination of the feather pecking data of both lines is shown with the mixture of two negative binomial distributions fitted to it to reveal the FP and EFP subgroups. Hens in the FP subgroup pecked feathers on average $\hat{\mu}_{FP} = 2.26$ (SE 0.33) times during observation and made up $1 - \hat{\pi} = 63\%$ of the whole experimental population. Additionally, they came on average $\lambda_{FP} = 1.62$ times into pecking motivation period, in which case they pecked feathers on average $E(X_{ikFP}) = 1.65$ times. In contrast, hens from the EFP subgroup pecked feathers on average $\hat{\mu}_{EFP} = 13.8$ (SE 4.25) times during observation and made up $\hat{\pi} = 37\%$ of the experimental population. They came on average $\lambda_{EFP} = 1.88$ times into pecking motivation period in which case they pecked feathers on average $E(X_{ikEFP}) = 7.34$ times.

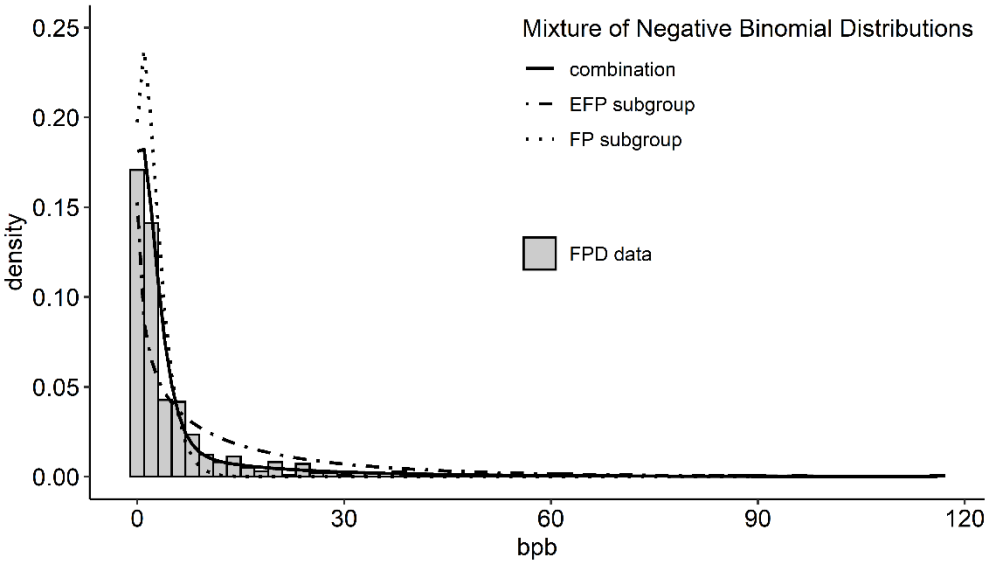
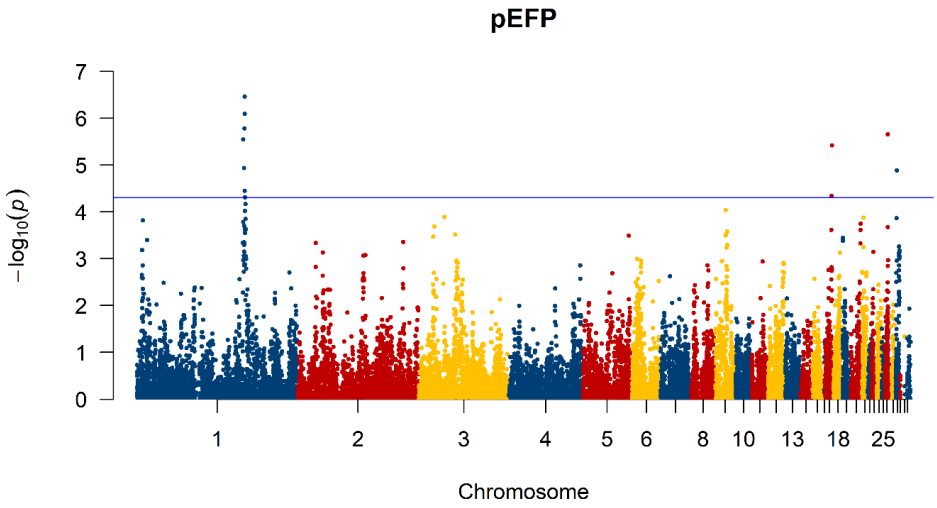


Figure 5. Mixture of negative binomial distribution (black lines) fitted to the feather pecking data (grey bars) in bouts per bird (bpb) of the high and low feather pecking hens (n = 492). The dotted line represents the fit of the feather pecking (FP) subgroup, the dot-dashed line represents the extreme feather pecking (EFP) subgroup and the solid line represents the combination of both and visualizes the good of fit of the mixture distributions.

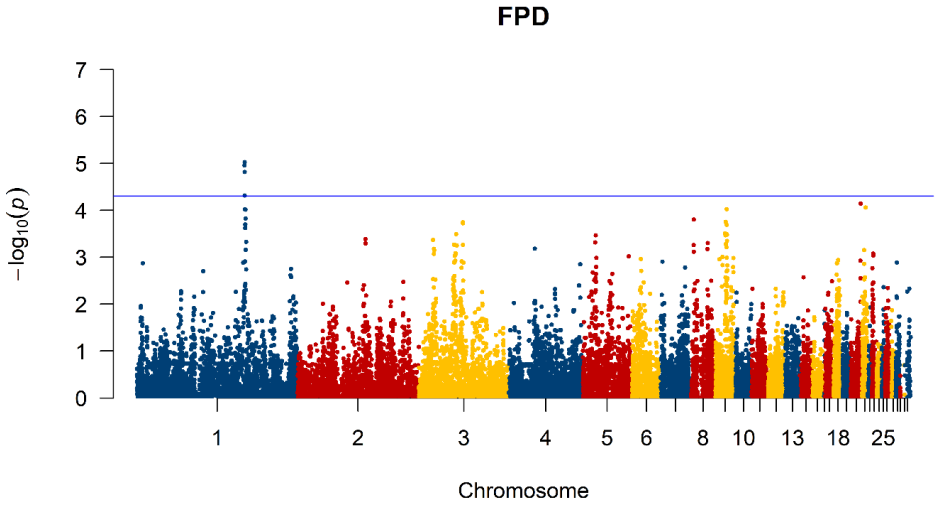
3.4. GWAS and Estimations of Genetic Parameters

GWAS revealed four nominal significant ($p \leq 5 \times 10^{-5}$) SNPs on Chromosome 1 for FPD (Figure 6). For pEFP, seven significant SNPs on Chromosome 1 were found including the same four mentioned above for FPD, two on Chromosome 17 and one on Chromosome 26 and 28 (Figure 6), respectively. The full list of nominal significant SNPs can be seen in Table 1.

The estimated variance components and heritabilities for the two traits FPD and pEFP are shown in Table 2. For both traits, the heritabilities are in a medium range with 0.20 (SE 0.08) for FPD on the observed scale and 0.26 (SE 0.14) for the binary trait pEFP on the liability scale.



(**a**)



(**b**)

Figure 6. Manhattan plots of the $-\log_{10}$ *p*-values for association of SNPs (n_{SNP} = 28,525) for (**a**) the posterior probability of extreme feather pecking (pEFP) and (**b**) feather pecks delivered (FPD). The line indicates the nominal level of significance $p \leq 5 \times 10^{-5}$.

Table 1. Significant SNPs with $p \leq 5 \times 10^{-5}$ from GWAS.

[1] Trait	[2] Chr	SNP	Position (bp)	$-\text{Log}_{10}$ (p)
FPD	1	**GGaluGA044500**	132,686,520	4.96
	1	**Gga_rs14888608**	132,789,468	5.03
	1	**GGaluGA044531**	132,792,863	4.82
	1	**Gga_rs13940234**	132,960,547	4.32
pEFP	1	Gga_rs13938103	131,055,669	5.55
	1	Gga_rs14887858	132,015,352	4.94
	1	**GGaluGA044500**	132,686,520	5.78
	1	**Gga_rs14888608**	132,789,468	6.46
	1	**GGaluGA044531**	132,792,863	6.09
	1	**Gga_rs13940234**	132,960,547	4.45
	1	Gga_rs13624646	133,345,452	4.31
	17	Gga_rs15792349	8,366,984	4.34
	17	Gga_rs14098115	8,458,039	4.34
	17	Gga_rs14097650	8,891,679	5.42
	26	Gga_rs16203090	3,684,301	5.66
	28	Gga_rs15249217	1,623,905	4.89

[1] FPD = feather pecks delivered; pEFP = posterior probability of extreme feather pecking; [2] Chromosome number; SNPs printed bold are significant in both traits.

Table 2. Results of the variance component analyzes.

[1] Trait	Prevalence	[2] V_P ([3]SE)	[4] V_A (SE)	[5] $H^2_{obs.}$ (SE)	[6] $H^2_{liab.}$ (SE)
FPD	–	0.41 (0.03)	0.08 (0.04)	0.20 (0.08)	–
pEFP	0.24	0.15 (0.01)	0.02 (0.01)	0.14 (0.07)	0.26 (0.14)

[1] For trait abbreviations see Table 1; [2] V_p = phenotypic variance; [3] SE = standard error; [4] V_A = additive genetic variance; [5] $h^2_{obs.}$ = heritability on the observed scale; [6] $h^2_{liab.}$ = heritability on the underlying liability scale.

3.5. Post GWAS Analysis

Genes were screened 1.5 Mb up- and downstream from the significant peak SNP Gga_rs14888608 of the GWAS analyses for pEFP and FPD on Chromosome 1 (Table 1). This resulted in the specific window of 131,289,468 bp-134,289,468 bp. Fifty-two positional candidate genes were found in this window and input to DAVID which itself identified 44 of

these genes and used them for functional annotation clustering. One significant cluster was identified and revealed 15 significant enriched terms (Table 3). The terms are all related to the same three genes: GABRA5, GABRB3, and GABRG3. In three terms, two additional genes are involved, i.e., CNGA3 and RP2.

Table 3. Top enriched annotation cluster determined with DAVID from genes in significant GWAS region of [1] pEFP as well as [1] FPD on Chromosome 1.

Category	Term	[2] p	Genes	[3] B
Annotation Cluster 1 Enrichment Score 2.22				
REACTOME_PATHWAY	R-GGA-977441	4.8×10^{-4}	GABRA5, GABRB3, GABRG3	1.3×10^{-2}
GOTERM_CC_DIRECT	GABA-A receptor complex (GO:1902711)	8.5×10^{-4}	GABRA5, GABRB3, GABRG3	4.1×10^{-2}
GOTERM_MF_DIRECT	GABA-A receptor activity (GO:0004890)	9.2×10^{-4}	GABRA5, GABRB3, GABRG3	3.7×10^{-2}
INTERPRO	Gamma-aminobutyric acid A receptor (IPR006028)	1.6×10^{-3}	GABRA5, GABRB3, GABRG3	1.3×10^{-1}
REACTOME_PATHWAY	R-GGA-975298	1.6×10^{-3}	GABRA5, GABRB3, GABRG3	2.2×10^{-2}
INTERPRO	Neurotransmitter-gated ion-channel transmembrane domain (IPR006029)	5.1×10^{-3}	GABRA5, GABRB3, GABRG3	2.0×10^{-1}
INTERPRO	Neurotransmitter-gated ion-channel, conserved site (IPR018000)	5.1×10^{-3}	GABRA5, GABRB3, GABRG3	2.0×10^{-1}
INTERPRO	Neurotransmitter-gated ion-channel (IPR006201)	5.4×10^{-3}	GABRA5, GABRB3, GABRG3	1.5×10^{-1}
INTERPRO	Neurotransmitter-gated ion-channel ligand-binding (IPR006202)	5.4×10^{-3}	GABRA5, GABRB3, GABRG3	1.5×10^{-1}
UP_KEYWORDS	Ion channel	6.7×10^{-3}	CNGA3, GABRA5, GABRB3, GABRG3	3.1×10^{-1}

UP_KEYWORDS	Synapse	1.9×10^{-2}	GABRA5, GABRB3, GABRG3	4.1×10^{-1}
UP_KEYWORDS	Ion transport	2.1×10^{-2}	CNGA3, GABRA5, GABRB3, GABRG3	3.2×10^{-1}
GOTERM_CC_DIRECT	cell junction (GO:0030054)	4.5×10^{-2}	GABRA5, GABRB3, GABRG3	6.7×10^{-1}
UP_KEYWORDS	Cell junction	5.5×10^{-2}	GABRA5, GABRB3, GABRG3	5.3×10^{-1}
UP_KEYWORDS	Cell membrane	9.7×10^{-2}	GABRA5, GABRB3, GABRG3, RP2	6.7×10^{-1}

[1] For trait abbreviations see Table 1; [2] p = *p*-value; [3] B = Benjamini test to correct for multiple testing.

4. Discussion

As shown in Figure 1, two crescent-shaped structures are notable in both feather pecking lines which are based on genetic similarities within the lines. An explanation leading to the subgroups might be the fact that for some generations, the hens were mated in 10 half-sib families within each line. Nevertheless, two clear groups of HFP and LFP hens are notable visualizing the genetic difference between the two lines after 15 generations of separate breeding. The phenotypic differences between the two lines in the 15th generation can be seen in Figure 2. In Piepho et al. [14], feather pecking data of the 5th to the 11th generation of both lines is shown with the same phenotypic difference over these seven generations. Hence, the two feather pecking subpopulations are both phenotypically and genotypically distinguishable, but there was little selection response in the last generations.

Despite of the lack of recent selection response, one might expect to find selection signatures in both lines. Mapping of selection signatures in each line using the haplotype-based iHS approach revealed only two significant SNPs on Chromosome 4 for the LFP line and no significant SNPs for the HFP line. After combination of both statistics to increase the power of the test, there were no significant SNPs anymore. One explanation might be that the selection response in the first generations did not lead to detectable selective sweeps because of the low effective sizes the lines have had in the subsequent generations.

The results of the SNP-based F_{ST} statistic did not reveal any genomewide significant SNPs because the lines were separated for many generations and had a moderately low effective size, so SNPs could be divergently fixed by chance. The SNPs with very high F_{ST} values were equally distributed over the whole genome. The mean F_{ST} was 0.16. According to Akey et al. [37], this specific pattern of equally distributed significant SNPs is a sign of genetic drift and not selection. Selection, irrespective whether natural or artificial, would be more locus specific and thus lead to single significant peaks. The effective population size in each of the two feather pecking lines over the last 15 generations was on average only 35. A huge impact of drift is thus likely leading to differentiation between and uniformity within the subpopulations which in turn leads to huge allele frequency differences and thus great F_{ST} indices.

Nevertheless, Grams et al. [30] mapped selection signatures in the 11th generation of these lines using the F_{ST} index as well and found 13 clusters harboring significant SNPs on eight chromosomes with a concentration of significant SNPs (in descending order) on Chromosomes 4 and 3 using a sliding window approach. In the current study as well, Chromosome 3 harbored the second most significant SNPs. The mean F_{ST} index in Grams et al. [30] over all SNPs with 0.15 is slightly smaller showing that the allele frequency differences have increased further over the last four generations. The FDR for significant SNPs were also low. In contrast to Grams et al. [30], in the current study no genomewide significant SNPs were found and the number of significant SNPs in total was also lower with 342 in Grams et al. [30] versus 206 in this study. This might be due to the six times higher number of animals used in the current study.

After combining the p-values of the F_{ST} indices with the p-values of the combined iHS statistics in the meta-analysis, no more selection signatures reaching nominal significance could be found. A reason why no selection signatures could be detected might be that feather pecking is a highly polygenic trait influenced by many genes with small effects. During selection, selection pressure is distributed on those many genes leading to a slow accumulation of low to medium allele frequencies. Hence, there might be divergent selection but it is not detectable for us with the current study design (i.e., amount of animals, SNP density, applied methods).

Another reason for the lack of selection signatures might be as follows. At the beginning of the divergent selection, breeding was successful and the two lines differed recognizably from each other. Over the past generations, the lines remained divergent in the trait because it was still the basis of selection but no more breeding progress could be gained and thus only the status quo was maintained [30] (Figure 1). A threshold exists for both directions of the trait feather

pecking. Low feather pecking cannot go below zero and extremely high feather pecking hens cannot be kept in their groups anymore due to their harmful behavior.

The fit of the mixture distributions revealed the existence of FP and EFP subgroups. The results are confirmed by a previous study where the mixture distributions were fitted to F2 cross FPD data [15]. The proportion of EFPs in the population of the F2 cross was 34% and thus slightly smaller than in the current study but they pecked feathers on average 2.7 times more often. Hens in the F2 cross came also more often in the pecking motivation period where they pecked more feathers as hens of the EFP subgroup in this study. The results of the FP subgroup are nearly the same in both studies.

Significantly associated SNPs for both traits with highly significant *p*-values for pEFP were found in one region on Chromosome 1. This indicates the presence of a QTL. Hence, screening of positional candidate genes was done in this window. The terms in the significant cluster of the functional annotation clustering are linked to neurotransmitter-gated ion-channels, particularly gamma-aminobutyric acid receptor A ($GABA_A$). These receptors are made up of five subunits with several isoforms, which can be divided into the following classes: α (1–6), β (1–4), γ (1–3), δ, ε, θ, π, and ρ (1–3) [38]. The three candidate genes found in this study, which are related to all terms, are encoding for the subunits α5 (GABRA5), β3 (GABRB3), and γ3 (GABRBG3). $GABA_A$ receptors are mainly located in the central nervous system. They act as inhibitory ion channels representing an important antagonist to excitatory forces regarding the transmission of axon potentials and thus neuronal activity in the brain [39]. These facts lead to the hypothesis that a mutation in the candidate genes cause a malfunction of the $GABA_A$ receptors resulting in a loss of inhibitory processes for feather pecking. Further research is needed to get more detailed information on the role of $GABA_A$ in this regard, for example via eQTL studies. Iffland et al. [15] already assumed that EFP hens might miss a regulatory factor preventing downregulation of extreme feather pecking. In a study by Poshivalov [40] it has been shown that mice that were kept isolated over 12 weeks became aggressive and antisocial. After application of a GABA agonist or an irreversible inhibitor of a GABA degrading enzyme aggressiveness decreased and sociability increased. This exemplifies the regulatory effect of GABA and the $GABA_A$ receptors on behavior patterns. Bennewitz et al. [3] reported a positive genetic correlation between FPD and aggression in an F2 cross of the lines selected for high and low feather pecking. This leads to the suggestion that GABA may also influence social behavior in chickens. Brinker et al. [41] found a GABAergic system related candidate gene (GABBR2, Chromosome 2) for direct genetic effects for survival time which is linked to cannibalism in crossbred laying hens. Lutz et al. [6] found a candidate gene (SLC12A9) on

Chromosome 9 for FPD in a study of a large F2 cross of the HFP and LFP lines. The SCL12 gene family plays a role in the GABAergic system as well. They also linked the serotonergic system to their findings because serotonin mediated by 5-HT_2 receptors inhibits $GABA_A$ receptor currents [42]. As reviewed by de Haas and van der Eijk [43] it was repeatedly shown that the serotonergic system can be genetically linked to feather pecking. Flisikowski et al. [44] identified serotonergic related genes (DRD4 and DEAF1) on Chromosome 5 to influence feather pecking behavior.

Direct observations by several observers for measuring FP behavior, as was used in this study, leads to some limitations. Although our observers were trained to differentiate between severe FP, gentle FP and aggressive pecking, a bias due to subjectivity cannot be completely excluded. In order to minimize this, we used as many observers as we had pens and hence all groups were observed by all observers at the same day in a rotational scheme. Thus, an inter-observer reliability was not calculated. The calculation of the intra-observer reliability requires video-records in order to provide the same feather pecking pattern repeatedly to the same observer. This technique, however, was not applicable in our study because of the relatively large groups with about 40 individually tagged hens. As reviewed by Ellen et al. [45], it could already be shown in the PhenoLab project that ultra-wideband as well as video tracking of hens of another HFP and LFP line explored differences in activity of both lines with an accuracy of up to 85% compared to the human observer [46]. It was also possible to detect individual FP hens due to their increased activity levels compared to the victims [46]. The use of this technology in the future is promising and would lead to more objectivity in measuring FP behavior.

5. Conclusions

Mapping of selection signatures in lines of laying hens divergently selected for feather pecking behavior revealed no clear regions under selection indicating that they are either not detectable with the current study design because of the polygenic nature of the trait or that there are no selection signatures because of the lack of stringent selection response over the last generations. A GWAS for the traits feather pecks delivered and the posterior probability of a hen belonging to the extreme feather peckers revealed a region on Chromosome 1 where the existence of QTL influencing the feather pecking phenomenon is likely. The candidate genes found in this region are a part of the GABAergic system which is related to the serotonergic system. Both systems were frequently linked to feather pecking which is confirmed by the current study as well. The candidate genes found in the present study may play an important role in the occurrence of the

phenomenon. However, feather pecking is a quantitative trait influenced by many genes with more or less small effects.

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CHAPTER THREE

Phenotypic and genomic analyses of agonistic interactions in laying hen lines divergently selected for feather pecking

Hanna Iffland [a,*], Markus Schmid [a], Siegfried Preuß [a], Werner Bessei [a], Jens Tetens [b,c], Jörn Bennewitz [a]

[a]Institute of Animal Science, University of Hohenheim, Garbenstraße 17, 70593 Stuttgart, Germany

[b]Department of Animal Sciences, University of Göttingen, Burckhardtweg 2, 37077 Göttingen, Germany

[c]Center for Integrated Breeding Research, University of Göttingen, Albrecht-Thaer-Weg 3, 37075 Göttingen, Germany

*Corresponding author: hanna.iffland@uni-hohenheim.de

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Abstract

In poultry, aggressive pecking and threatening are normal agonistic behavior patterns which serve to establish a social hierarchy. As agonistic behavior is a stressor for animals, its excessive occurrence is undesired in layer flocks. Feather pecking is a longstanding serious problem in layer flocks and its relationship to agonistic behavior is still not clear. Therefore, phenotypic and genomic analyses of the agonistic and feather pecking behavior of two laying hen lines divergently selected for high and low feather pecking were conducted. The hens were phenotyped for the active traits aggressive pecks delivered (APD)[1], threats delivered (TD)[2], feather pecks delivered (FPD)[3] and the passive traits aggressive pecks received (APR)[4], threats received (TR)[5] and feather pecks received (FPR)[6]. Indices were built by subtracting the passive traits from the respective active traits to obtain the aggression index, the threat index, and the feather pecking index. As all three behavior patterns in their excessive manifestations are undesired, the index-traits Activity and Passivity were also defined by combining each the active and passive traits. The results showed that FPD is significantly positive correlated with APD and TD in both lines, but with higher coefficients in the high feather pecking line. The average amount of FPR in both lines is nearly the same and no correlation was found between FPR and FPD, APD or TD in any of the lines. The active traits and the feather pecking index showed medium heritabilities, whereas the heritability was negligibly small for the other traits. GWAS revealed four nominal significant ($p \leq 5*10^{-5}$) SNPs for APD on chromosome 6, the same four and three additional SNPs on chromosome 8 for Activity and three SNPs on chromosome 1 for the feather pecking index. It is concluded, that selection on high feather pecking leads to an increase of agonistic behavior. The correlation probably depends on the phase of establishing the social hierarchy in which the hens in a newly formed group are at the time of observation, and might disappear, after a stable ranking is established. The reception of feather pecking is similar in both lines. GWAS revealed that TD, APD, Activity and the feather pecking index seem to be typical quantitative traits with associated regions for the latter three which have slightly greater effects on these traits than other regions in the genome.

Keywords: Poultry; Aggressive pecking; Threatening; Heritability; GWAS

[1] Aggressive pecks delivered (APD)
[2] Threats delivered (TD)
[3] Feather pecks delivered (FPD)
[4] Aggressive pecks received (APR)
[5] Threats received (TR)
[6] Feather pecks received (FPR)

1. Introduction

Agonistic behavior is a normal social behavior between conspecifics which is shown for example to get access to food, mating or establish and maintain social hierarchies (Young, 2019). In poultry, threatening and aggressive pecking are part of the agonistic behaviors (Guhl, 1968). Aggressive pecking is defined as pecks delivered in an upright body posture against the head of the recipient conspecific, but due to withdrawing of the recipient, also other parts of its body can be pecked (Bilcik and Keeling, 2000; Kjaer et al., 2001). Threatening is performed in the same upright body posture directed towards the recipient bird, but no pecks are delivered (Bessei et al., 2013a). In newly formed flocks, agonistic interactions decrease after a few weeks when the social hierarchy is established (Siegel and Hurst, 1962; Guhl, 1968). Moreover, it has been shown that aggression in laying hens occurs more frequently in smaller groups and decreases in larger groups (Nicol et al., 1999). In contrast, the occurrence of feather pecking in layer flocks tends to be exactly the opposite. The larger the flock size, the more frequently feather pecking appears (Nicol et al., 1999; Bilcik and Keeling, 2000). Feather pecking is considered as an abnormal behavior frequently occurring in commercial layer flocks and is clearly distinguishable from aggressive pecking. Hens are severely pecking against and pulling at feathers of conspecifics which leads to destruction of the vane and rachis and sometimes results in pulled out feathers (Savory, 1995; Bessei et al., 2013a). There are two different forms of feather pecking, i.e. gentle and severe feather pecking. Since gentle feather pecking causes minor or no damages (Savory, 1995), severe feather pecking was considered in the present study. In addition to feather pecking, recurring aggressive encounters are stressful (Gross and Siegel, 1985) and can also lead to injured birds and thus to welfare as well as economic problems.

The relationship between agonistic behavior and feather pecking is still not quite clear. While some authors found no or very low correlations between aggressive pecking and feather pecking (Blokhuis and Arkes, 1984; Kjaer et al., 2001; Bessei et al., 2013a; Grams et al., 2015), others reported medium correlation coefficients (Bessei et al., 2013b; Bennewitz et al., 2014). Bessei et al. (2013a) found no phenotypic correlation between both traits in laying hen lines selected for high (**HFP**) and low (**LFP**) feather pecking for nine generations. However, in a subsequent study working with an F2 cross of these lines, the authors found a nonlinear interdependence between both traits (Bessei et al., 2013b).

Bessei et al. (2013a) calculated an aggression index for each hen based on the differences of aggressive pecks delivered and aggressive pecks received. The aggression index of the HFP

line had a clearly wider range than that of the LFP line. Thus, hens of the HFP line not only delivered more aggressive pecks than hens of the LFP line, but also received significantly more aggressive pecks. According to the authors, LFP hens seemed to be able to avoid aggressive acts.

Heritabilities for aggressive pecks delivered were estimated in several studies and vary from almost zero to 0.27 (Rodenburg et al., 2003; Bennewitz et al., 2014; Grams et al., 2015). A number of 45 SNPs associated with aggressive pecking have been found by Lutz et al. (2017) on several chromosomes in the above mentioned F2 cross of the HFP and LFP lines. Buitenhuis et al. (2009) detected 40 differently expressed genes between two groups of an HFP line which was divided into an aggressive pecking receiver and aggressive pecking deliverer group.

The aim of the present study was to analyze agonistic behavior and its relationship to feather pecking within and between the 15th generation of two laying hen lines divergently selected for feather pecking. This was supplemented by a genomewide association study (GWAS) to shed light on the underlying genetic mechanisms of agonistic behaviors in laying hens.

2. Material and methods

The research protocol was approved by the German Ethical Commission of Animal Welfare of the State Government of Baden-Wuerttemberg, Germany.

The experimental population bases on animals of a White Leghorn layer strain which were divergently selected for HFP and LFP. The origin of the strain and the experiment are described in detail in Iffland et al. (2020). Hens of the 15th selection generation of the lines were kept in mixed HFP and LFP groups of about 42 hens in the first and 40 hens in the second experimental run for observation. The entire flock was divided into two experimental runs, because only a limited number of observation pens and experienced observers was available. In the first experimental run, the hens were distributed to seven pens at the age of 33 and 31 weeks (two hatches). Only six pens were needed for observation in the second run because of a lower number of hens of the third hatch at the age of 32 weeks. As there were several families per line, it was tried to distribute hens of each family equally in all of the pens. Within the families, hens were selected randomly.

The dimensions of the pens were 4 × 4 m which resulted in an average stocking density of 2.6 hens / m². Two-third of the pens were equipped with perforated plastic floor and one-third was a 1-tier litter area. The pens contained nipple drinkers, round feeders, perches and nests. Feeding and management proceeded as under conventional conditions. A light program of

14 h was carried out with light switched on from 03:00 to 17:00 h and additional daylight through windows at the upper part of the side wall.

For individual identification, the hens were marked with numbered plastic tags on their back while grouping. These tags did not allow the observer to distinguish between the feather pecking lines. Behavioral observations started one week after housing in the hens. Feather pecks delivered (**FPD**) and feather pecks received (**FPR**) were recorded in morning sessions from 10:00 to 12:30 h. Aggressive pecks delivered (**APD**) and aggressive pecks received (**APR**) as well as threats delivered (**TD**) and threats received (**TR**) were recorded in afternoon sessions from 13:30 to 16:00 h. A behavior sampling was performed where each occurrence of the considered behavior, i.e. the deliverer and receiver hen, was recorded. Each group was observed directly in the pen for 20 min by each observer on four consecutive days resulting in 560 min observation time in the first run and 480 min in the second. The observation time was standardized to 420 min to gain compatibility with data from another study. No observer reliability had to be calculated as all pens were observed by each observer at each observation day in a rotational scheme (discussed in Iffland et al. (2020)).

The unit of recording was bouts per bird (**bpb**), which is defined as the respective behavioral action without a changed behavior of the hen. This means e.g. for FPD, that a series of pecks was recorded as one bpb. The ethogram (Table 1) provides a detailed description of the traits.

At the end of each experimental run, most of the hens were slaughtered (CO_2 stunned with subsequent ventral neck cut) for taking tissue samples and blood whereas part of the hens (randomly chosen from each family) were left alive for line preserving. From these hens, blood was taken from the wing vein. Body weight was measured before slaughter or blood sampling, respectively and was on average 1.55 kg across all hens. The dataset contained a number of 490 hens (n_{HFP} = 269, n_{LFP} = 221).

For genotyping with the Illumina 60 K chicken Infinium iSelect chip, DNA was extracted from the blood samples. DNA was isolated from EDTA stabilized blood collected during slaughtering. 200 µl were transferred into a cartridge of Promega's Maxwell 16 Blood DNA Purification Kit. For the fully automated extraction, a Maxwell 16 MDx AS3000 was used. Concentration of extracted DNA was measured on a Nanodrop 2000 and adjusted to 50 ng/µl. Imputation of sporadic missing genotypes and phasing was done using Beagle 5.0 (Browning and Browning, 2007). SNPs with a minor allele frequency < 0.01, a call frequency < 0.95, a location on the sex chromosomes and with a missing allocation to a specific chromosome

according to positional information of the chicken genome assembly GRCg6a were discarded. After data filtering, 28,525 SNPs remained in the dataset.

Table 1

Ethogram of the recorded traits and calculations of the corresponding indices.

Trait	Abbreviation	Definition
Aggressive pecks delivered	APD	Pecks delivered in an upright body posture against (mainly) the head and other parts of the body of the recipient.
Aggressive pecks received	APR	Reception of APD
Aggression index	AI	$AI = APD - APR$
Threats delivered	TD	Visual fixation towards the recipient in an upright body posture followed by avoidance or withdrawal behavior of the recipient.
Threats received	TR	Reception of TD
Threat index	TI	$TI = TD - TR$
Feather pecks delivered	FPD	Non-aggressive severe pecks or pulls directed to the plumage of conspecifics with sometimes resulting in pulled out feathers and a recipient, which tolerates or moves away. Thereby, the deliverer does not adopt any special body posture.
Feather pecks received	FPR	Reception of FPD
Feather pecking index	FI	$FI = FPD - FPR$
Activity	Activity	$Activity = \sum_{i=1}^{n} \frac{P_i - \mu_{p(i)}}{\sigma_{p(i)}}$, with $i = APD, TD,$ and FPD
Passivity	Passivity	$Passivity = \sum_{i=1}^{n} \frac{P_i - \mu_{p(i)}}{\sigma_{p(i)}}$, with $i = APR, TR,$ and FPR

According to Bessei et al. (2013a) and Lee et al. (1982), indices were formed by subtracting the APR, TR and FPR recordings for each feather pecking line separately from the corresponding APD, TD and FPD recordings. This resulted in three new traits, the aggression index (**AI**), the threat index (**TI**) and the feather pecking index (**FI**) which are described in Table 1. This leads to an index value of zero for hens which delivered the same amount of bpb as they received and which are referred to as ‘neutrals’ in the current study. Animals with index values greater zero

representing hens which delivered more bpb than they received and which are referred to as ‘offenders’. Hens with index values less than zero are those who received more bpb than they delivered and are thus referred to as ‘victims’. Thus, by building the indices, the extreme behavior in both directions were made visible. Although the typically used terminology dominant, subdominant and subordinate to describe dominance hierarchies would be appropriate for AI and TI, the trait feather pecking is not a classical trait to depict social structures. To be consistent, these terms were not used.

As all three behavior patterns (i.e. aggression, threatening and feather pecking) in their excessive manifestations are undesired, the active parts of the corresponding traits (APD, TD and FPD) and the passive parts (APR, TR and FPR) were combined to obtain the overall traits **Activity** and **Passivity**. Therefore, an index *I* for each of the two new traits within each feather pecking line was constructed as follows:

$$I = \sum_{i=1}^{n} \frac{P_i - \mu_{p(i)}}{\sigma_{p(i)}},$$

where P_i is the vector of the phenotypes for trait *i*, $\mu_{p(i)}$ is the mean of the phenotypes for trait *i* and $\sigma_{p(i)}$ is the standard deviation of the phenotypes for trait *i*. In case of Activity (Passivity), *i = APD, TD,* and *FPD* (*i = APR, TR,* and *FPR,* respectively). Hens with positive (negative) values represent animals that show more (less) activity than the population mean.

Within the feather pecking lines, correlations between the traits were calculated using Spearman’s rank correlation coefficient and tested for significance. The correlations as well as descriptive statistics were calculated using functions of R (R Core Team, 2019).

To reduce the deviation from a normal distribution of the data for the following analysis, the records of the traits APD, TD, and FPD as well as APR, TR, and FPR were Box-Cox transformed by using the following formula $y_{ti} = \frac{((y_i+1)^{-\lambda_i}-1)}{-\lambda_i}$, where y_i is the vector of phenotypes of trait *i*, λ_i is the power parameter for trait *i* and y_{ti} is the transformed vector of phenotypes for trait *i*. The specific power parameter λ_i for each trait was chosen manually going in 0.05 steps up- and downwards from zero and comparing the quantile-quantile plots visually of the transformed trait to achieve the best fit. This resulted in a λ of +0.2 for APD, TD and FPR, −0.05 for APR, −0.25 for TR and −0.2 for FPD. Because the index traits AI, TI and FI as well as Activity and Passivity contain negative values as well, they were transformed with the

Johnsons family of distributions to reduce their deviation from a normal distribution by using the R package 'jtrans' (Wang, 2015).

Single-marker GWAS were performed with the software GCTA (Yang et al., 2011) with the following model applied:

$$y = Xb + g + e,$$

where *y* is a vector of observations of the corresponding trait, *b* is a vector containing the fixed effect of the line, a fixed combinational effect consisting of the experimental run and pen and the effect of the tested SNP. *X* is the corresponding design matrix. The SNP genotypes are coded in *X* as the number of copies of the 1-allele (i.e. 0, 1, 2 copies). The random vector *g* contains the additive animal effects with $g \sim N(0, \sigma_g^2 G)$, where *G* is the genomic relationship matrix. For GWAS, the chromosome with the candidate SNP was excluded from calculating *G*. The vector *e* contains the residual effects and has a distribution $e \sim N(0, \sigma_e^2 I)$ with *I* being the identity matrix. Correction for multiple testing was done using the Bonferroni correction as follows: $p_{corrected} = 1 - (1 - p)^{28,525}$ with the $p_{genomewide}$ significance level set to $p_{corrected} \leq 0.05$. This resulted in a $-log_{10}(p_{genomewide})$ significance level of 5.75. Because of the very conservative approach of this type of correction, an additional level of significance, i.e. $p_{nominal} \leq 5 * 10^{-5}$, was applied. Estimation of variance components and heritabilities were also done using GCTA. Therefore, the model was applied without any fixed SNP effect.

3. Results

3.1. Phenotypic analyses (behavior traits, indices and correlations)

In Fig. 1A, the histograms and means of the recorded phenotypes APD, TD and FPD for both feather pecking lines are shown. Within the feather pecking lines, the pattern of the APD and TD histograms are very similar. HFP hens delivered 2.9 times more aggressive pecks and 2.5 times more threats than LFP hens. Additionally, hens of the HFP line pecked 6.8 times more feathers than hens of the LFP line. Fig. 1B shows the histograms and means for the traits APR, TR and FPR for both lines. Hens of the HFP line received 1.4 times more threats than LFP hens whereas LFP hens received 1.2 times more aggressive pecks. The reception of feather pecks is nearly the same for both lines.

The calculation of the overall trait Activity resulted in a standard deviation of 2.34 bpb for the HFP line and a smaller one of 2.03 bpb for the LFP line. For Passivity, the standard deviation is slightly larger in the LFP line with 2.06 bpb in contrast to the HFP line with 2.03 bpb.

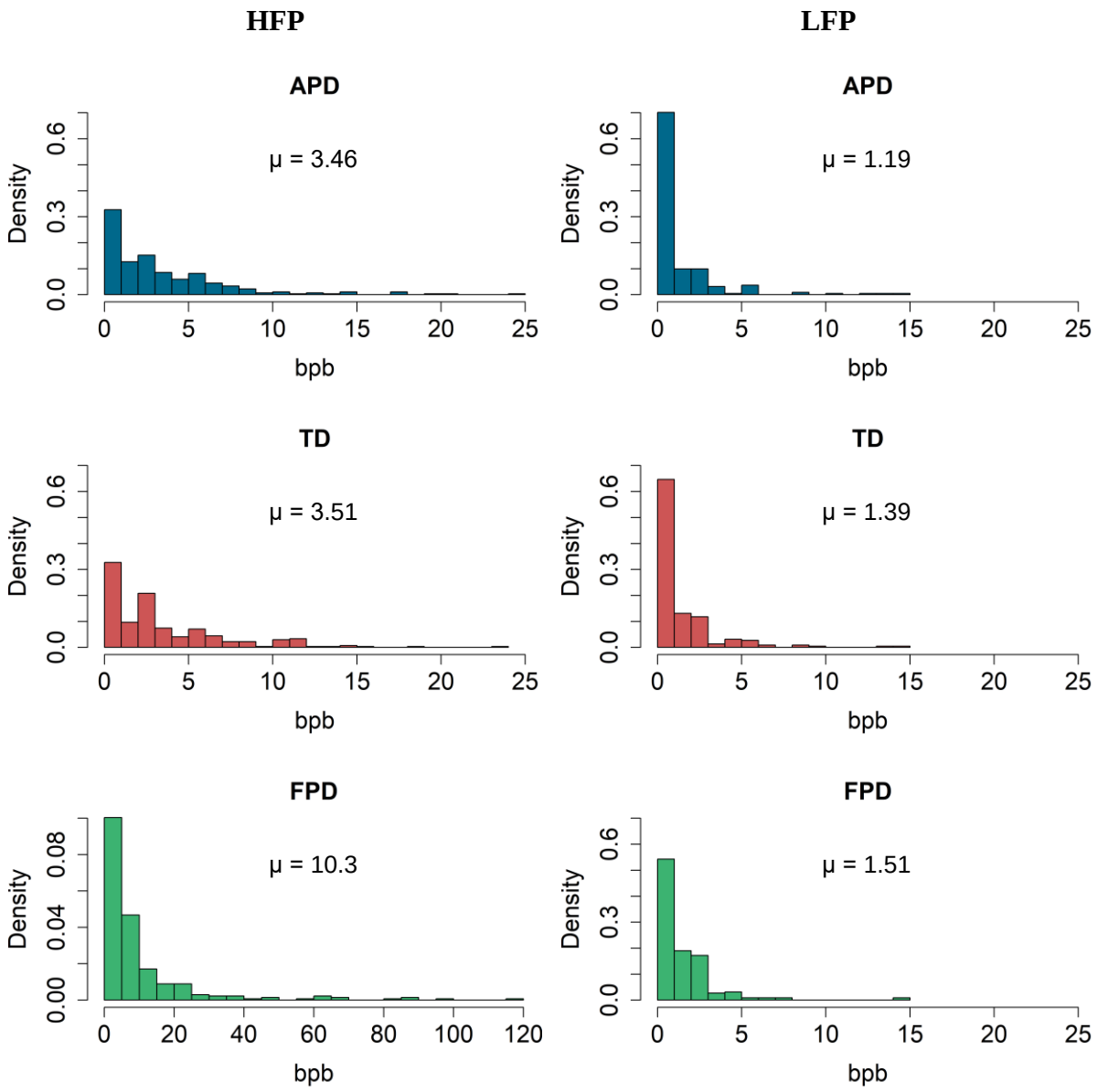

Fig. 1. A Histograms and means of the traits aggressive pecks delivered (**APD**), threats delivered (**TD**) and feather pecks delivered (**FPD**)[1] in bouts per bird (bpb) for the high (HFP, n = 269) and low (LFP, n = 221) feather pecking lines. Note the different axis scale for FPD in the HFP line for clarity.

[1]The Histograms of this trait for both lines were already shown in Iffland et al. (2020). Here they are depicted for the sake of completeness and a better understanding of the recorded traits. **B** Histograms and means of the traits aggressive pecks received (**APR**), threats received (**TR**) and feather pecks received (**FPR**) in bouts per bird (bpb) for the high (HFP, n = 269) and low (LFP, n = 221) feather pecking lines.

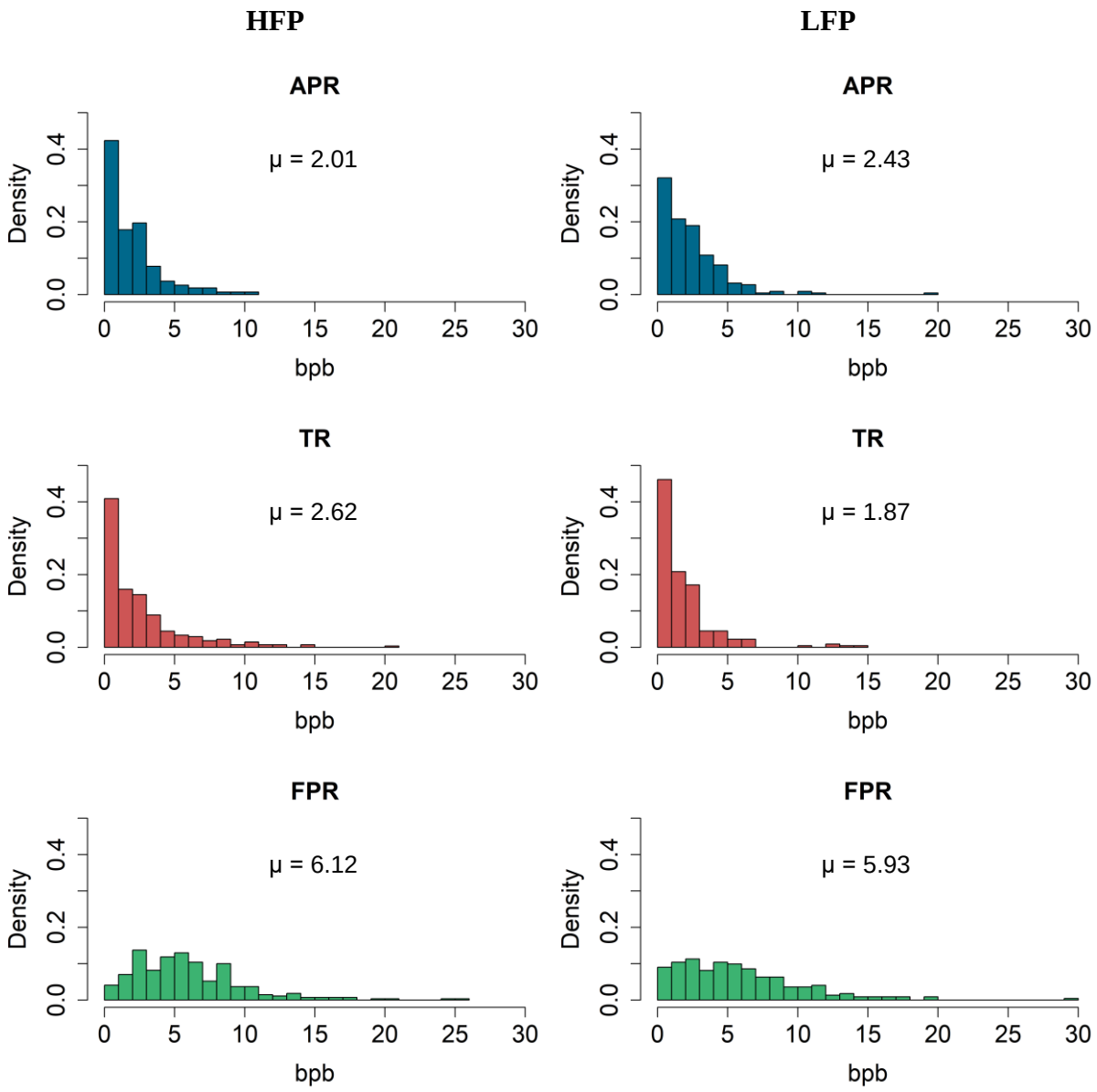

Fig. 1. (continued).

The three indices for each of the two feather pecking lines with the respective percentages of offenders and victims are shown in Fig. 2. For each trait, the HFP line contained a larger proportion of offenders than the LFP line.

Phenotypic correlations between all traits including the indices within the HFP and LFP line are shown in Table 2. FPR showed no significant correlation with any of the other traits except with the FI and Passivity in both lines, where it is itself a part of. In general, significant medium to high correlations can be found between the indices and the traits which were included in the respective indices. The active traits APD, TD and FPD are positively correlated with the indices

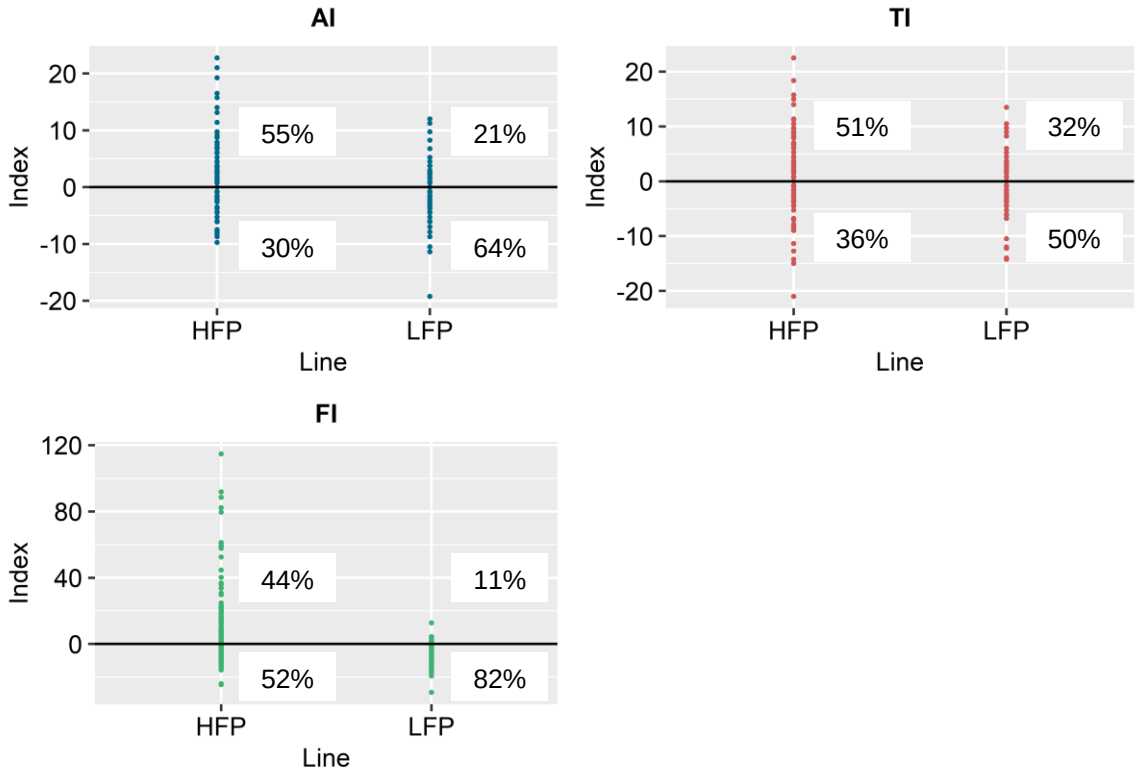

Fig. 2. Plots of the aggression index (**AI**), threat index (**TI**) and feather pecking index (**FI**) for the high (HFP, n = 269) and low (LFP, n = 221) feather pecking lines, respectively. Each dot represents the difference between delivered and received for a hen. The number above the zero line shows the percentage of offenders and the number below the line the percentage of victims per feather pecking line. Percentages of neutrals result from the subtraction of the respective offender and victim values from 100%.

whereas the passive traits APR, TR and FPR are negatively correlated. The traits APD and TD as well as APR and TR are significantly medium to high correlated in both lines. In both lines, FPD is significantly correlated with TD and APD, with higher correlation coefficients found in the HFP line.

3.2. Genomic analyses (heritabilities and GWAS)

SNP-based heritabilities are shown on the diagonal in Table 2. Heritabilities in a medium range were estimated for Activity, TD, FPD, APD and FI. For the other traits, low heritabilities with high standard errors were estimated.

As FPD was already analyzed in Iffland et al. (2020), in the current study GWAS was done for the heritable traits APD, TD, FI and Activity. Manhattan plots of the results are shown in

Fig. 3. For APD, four SNPs on chromosome 6 reached nominal significance ($p \leq 5 \times 10^{-5}$). The same SNPs were associated also with Activity, together with three additional SNPs on chromosome 18. For FI, three SNPs on chromosome 1 were found to be significantly associated with the trait. The full list of nominal significant SNPs can be seen in Table 3. None of the SNPs reached genomewide significance and no significant SNPs were found for TD.

Table 2

Spearman's rank correlation coefficients between the traits threats delivered (**TD**), threats received (**TR**), aggressive pecks delivered (**APD**), aggressive pecks received (**APR**), feather pecks delivered (**FPD**), feather pecks received (**FPR**), **Activity, Passivity** and their indices threat index (**TI**), aggression index (**AI**) and feather pecking index (**FI**). Above the diagonal are the correlations of the high feather pecking line and below the diagonal the correlations of the low feather pecking line. SNP-based heritabilities[2] are on the diagonal with standard errors in parenthesis.

	TD	TR	TI	APD	APR	AI	FPD	FPR	FI	Activity	Passivity
TD	0.22 (0.09)	-0.07	0.78 ***	0.62 ***	0.02	0.52 ***	0.28 ***	-0.03	0.27 ***	0.84 ***	-0.04
TR	-0.11	0.03 (0.05)	-0.60 ***	-0.09	0.46 ***	-0.34 ***	0.11	0.01	0.07	-0.01	0.66 ***
TI	0.69 ***	-0.72 ***	0.03 (0.05)	0.53 ***	-0.26 ***	0.61 ***	0.14 *	-0.05	0.16 **	0.63 ***	-0.43 ***
APD	0.53 ***	-0.07	0.44 ***	0.17 (0.08)	0.07	0.80 ***	0.33 ***	-0.07	0.32 ***	0.86 ***	-0.05
APR	-0.16 *	0.38 ***	-0.36 ***	-0.06	0.02 (0.05)	-0.45 ***	0.08	0.03	0.04	0.07	0.69 ***
AI	0.45 ***	-0.32 ***	0.57 ***	0.61 ***	-0.77 ***	0.08 (0.07)	0.22 ***	-0.07	0.24 ***	0.69 ***	-0.41 ***
FPD	0.19 **	-0.04	0.15 *	0.19 **	-0.16 *	0.22 ***	0.20 (0.08)	0.01	0.80 ***	0.56 ***	0.10
FPR	0.03	0.03	-0.01	-0.07	0.06	-0.09	-0.03	0.06 (0.06)	-0.49 ***	-0.04	0.56 ***
FI	0.03	-0.05	0.07	0.12	-0.11	0.15 *	0.36 ***	-0.91 ***	0.16 (0.08)	0.50 ***	-0.21 ***
Activity	0.73 ***	-0.13	0.58 ***	0.69 ***	-0.19 **	0.56 ***	0.65 ***	-0.06	0.29 ***	0.26 (0.09)	0.01
Passivity	-0.15 *	0.61 ***	-0.52 ***	-0.14 *	0.67 ***	-0.58 ***	-0.12	0.60 ***	-0.58 ***	-0.22 **	0.04 (0.05)

[1]Levels of significance: $p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; [2]The heritability for FPD was already shown in Iffland et al. (2020).

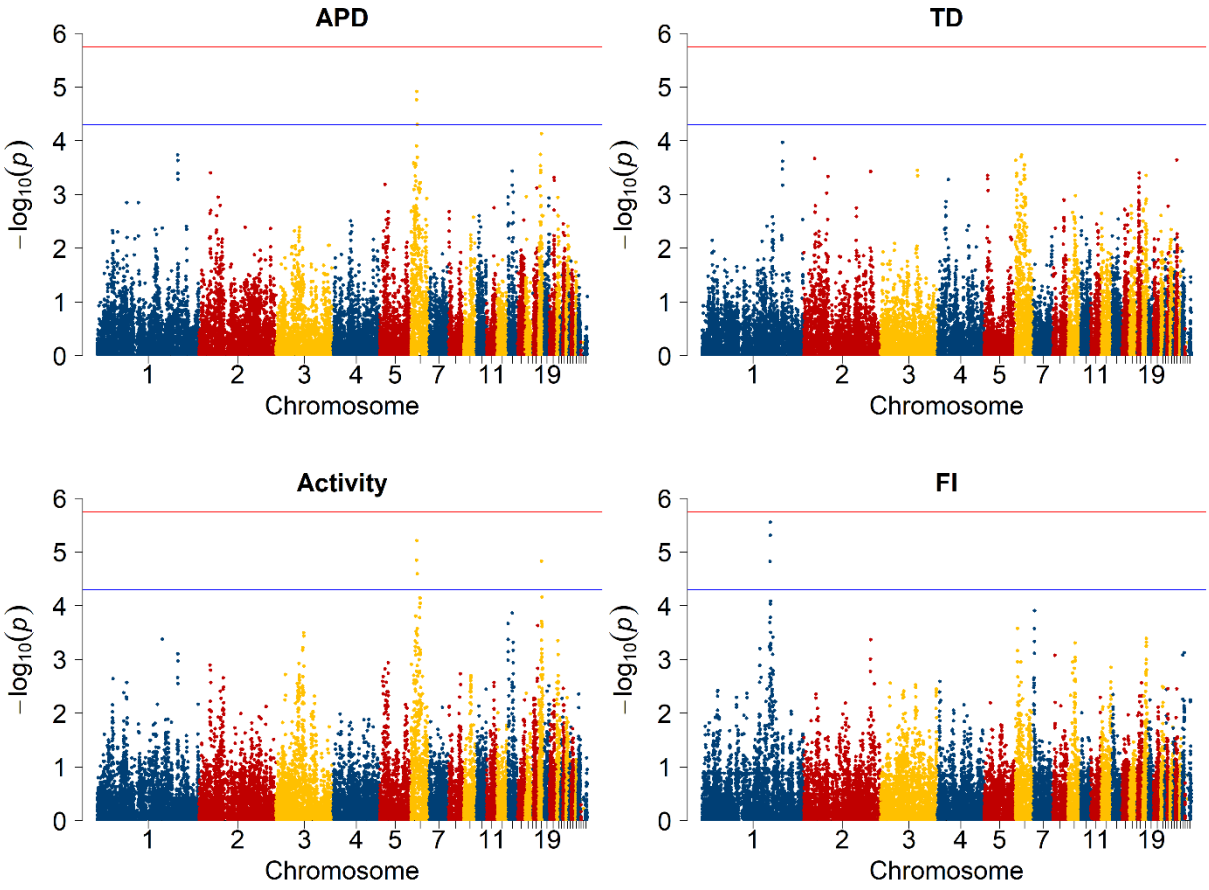

Fig. 3. Manhattan plots of the -$\log_{10}$ p-values for association of SNPs for aggressive pecks delivered (**APD**), threats delivered (**TD**), **Activity** and the feather pecking index (**FI**). The bottom line indicates the nominal level of significance $p_{nominal} \leq 5 * 10^{-5}$ and the top line indicates the genomewide level of significance $p_{genomewide} \leq 0.05$.

4. Discussion

The current study revealed that hens of the HFP line showed more FPD, APD and TD than hens of the LFP line. Additionally, FPD is significantly positive correlated with the agonistic traits in both lines. The average amount of FPR in both lines is nearly the same and no correlation was found between FPR and FPD, APD or TD in any of the lines. On the contrary, HFP hens received more threats than LFP hens whereas LFP hens received more aggressive pecks. Only for the active traits (APD, TD, FPD, and Activity) and FI, medium heritabilities were found. The GWAS for these traits (results for FPD are shown in Iffland et al. (2020)) revealed some nominal significant SNPs for APD on chromosome 6, for Activity on chromosomes 6 and 18 and for FI on chromosome 1.

Table 3

Significant SNPs with $p \leq 5 * 10^{-5}$ from GWAS for **APD**, **Activity** and **FI**.

Trait	Chromosome	SNP	Position (bp)	$-\text{Log}_{10}$ (p)
aggressive pecks delivered (APD)	6	Gga_rs14573845	11719048	4.8
	6	Gga_rs14573872	11737928	4.8
	6	Gga_rs16543029	12349545	4.9
	6	GGaluGA298494	13250515	4.3
Activity	6	Gga_rs14573845	11719048	4.8
	6	Gga_rs14573872	11737928	4.8
	6	Gga_rs16543029	12349545	5.2
	6	GGaluGA298494	13250515	4.6
	18	Gga_rs15823953	5701606	4.8
	18	Gga_rs15824153	5759679	4.8
	18	GGaluGA120826	5771844	4.8
feather pecking index (FI)	1	GGaluGA044500	132686520	4.8
	1	Gga_rs14888608	132789468	5.6
	1	GGaluGA044531	132792863	5.3

4.1. Phenotypic analyses (behavior traits, indices and correlations)

Divergent selection for feather pecking behavior has also led to differences in agonistic behaviors, i.e. APD and TD. First of all, hens of the HFP line delivered notably more feather pecks than hens of the LFP line. Even after building the FI, still 44 % of the HFP hens belonged to the offenders versus only 11 % of the LFP hens (Fig. 2). However, it appears that the lines also differed in APD and TD, namely that hens from the HFP line showed on average more aggressive pecking and threatening where the difference to the LFP line was not as great as with feather pecking. The positive correlation coefficients between FPD and the two agonistic traits in both lines showed as well that a higher performance of FPD came along with higher APD and TD. Again, for AI and TI the HFP line consisted of clearly more offenders than victims, in contrast to the LFP line. These findings are in line with Bessei et al. (2013b) and Bennewitz et al. (2014) who both analyzed the behaviors in the F2 cross of the 10th generation of the feather pecking lines. Other authors reported no or weak correlations between feather pecking and aggressive pecking (Hughes and Duncan, 1972; Blokhuis and Arkes, 1984; Kjaer et al., 2001; Buitenhuis and Kjaer, 2008; Bessei et al., 2013a; Grams et al., 2015). This might indicate that the relation found between APD and FPD is a phenomenon, which only exists in these specific HFP and LFP lines. But of course, different hen strains were used and different statistical analyses were applied in the studies which makes a comparison difficult.

Furthermore, it should be noted that in the current experiment, the social hierarchy was still being established, because the groups were assembled 7 days before the observation started. Hence, APD in the present study reflected spontaneous aggressive acts as a result of the genetic predisposition. After the social order is installed, the occurrence of agonistic behavior will usually be reduced (Siegel and Hurst, 1962) and thus the correlation between FPD and the agonistic traits may no longer exist. Controversial results in the relationship between APD and FPD may also be caused by environmental factors like flock size, which influence the occurrence of agonistic as well as feather pecking behavior (Nicol et al., 1999).

The mean number of bpb for FPR is nearly the same for both lines and no significant correlations could be found between FPR and FPD or the agonistic traits in any of the lines. Thus, hens of the HFP line did neither distinguish at whom they peck feathers at nor was there a relation between the agonistic traits and FPR. This is supported by the findings of Bessei et al. (2013a), except that they found a positive correlation between FPR and APD in the HFP line.

Hens of the HFP line received 1.4 times more threats than LFP hens whereas LFP hens received 1.2 times more aggressive pecks than HFP hens. Hence, the total amount of agonistic behaviors received was quite similar for both lines but reversed for both traits. This is in contrast to observations of Bessei et al. (2013a) where hens of the HFP line delivered and received more threats and aggressive pecks. The authors suggested a mechanism to avoid aggressive encounters. The divergent results may be explained by the particular social conditions in both experiments. While the social hierarchy was not fully established in the newly assembled groups in the present experiment, the groups of Bessei et al. (2013a) had been given sufficient time (9 weeks) to develop a stable rank order. In newly assembled flocks, the birds do not recognize the social rank of their pen mates. Consequently, LFP birds may try to avoid the feather pecking approach of HFP hens through threats. Since LFP hens have a lower social rank, they will not be in a position to perform aggressive pecks towards HFP hens. But their threats will elicit aggressive attacks by the dominant HFP hens.

According to the non-significant correlation coefficients, a higher amount of Activity has no influence on the reception of agonistic behavior or feather pecking for hens of the HFP line. For hens of the LFP line, the rate of receiving aggressive pecks decreased with a higher amount of Activity.

4.2. Genomic analyses (heritabilities and GWAS)

For APD, a medium heritability was estimated in the current study. Rodenburg et al. (2003) found no significant heritability for aggressive pecks in hens of an F2 cross of two commercial lines which differ in production traits as well as in feather pecking behavior. Bennewitz et al. (2014) reported a low heritability for hens of an F2 cross of the HFP and LFP lines of generation 10. Grams et al. (2015) who worked with the same F2 cross as Bennewitz et al. (2014), detected a heritability of 0.27 for APD and Recoquillay et al. (2013) found a heritability of 0.42 for aggressive pecks in Japanese quail. Comparisons between these studies are difficult because of the different methodology used in trait recordings and statistical analyses. Nevertheless, the heritability estimated for APD in this study fits the literature reports. The heritability of 0.20 for FPD is also supported by former studies which found low to medium heritabilities for this trait (Kjaer and Sørensen, 1997; Kjaer et al., 2001; Bennewitz et al., 2014; Grams et al., 2015). No heritabilities estimations could be found in literature for the other three heritable traits (TD, FI and Activity). However, the medium heritability estimations for these traits are in a realistic range for typical behavior traits. The remaining traits appear to be not heritable.

The four nominally significant SNPs found for APD in the current study were not reported in literature so far. Lutz et al. (2017) found 45 nominally significant SNPs on chromosomes 1, 2, 4, 5, 18, 21, 25 and 26 to be associated with APD in the F2 cross. Li et al. (2016) analyzed aggressive behavior subdivided in four different aggression traits in male broiler chickens. They detected in total 40 SNPs that were significantly associated with aggression traits. Most of the SNPs were found on chromosome 4, followed by chromosomes 2 and 12. Recoquillay et al. (2015) revealed QTL on chromosome 1 and 2 in a QTL linkage study in Japanese quail for the trait aggressive pecks. In a microsatellite-based linkage study by Buitenhuis et al. (2003) in an F2 cross of HFP and LFP lines, a QTL on chromosome 12 was identified.

APD seems to have a large impact on the overall trait Activity as the four significant SNPs on chromosome 6 are the same for both traits. In a previous study (Iffland et al., 2020), we found significant SNPs on chromosome 1 for FPD and the posterior probability of extreme feather pecking (pEFP). Interestingly, even though FPD is a part of Activity, no significant trait associated chromosomal regions were found on chromosome 1. This is probably because the peak SNP in the aforementioned study was found for pEFP, which in turn is not part of the trait Activity. Thus, the association of FPD with the significant SNPs on chromosome 1 in Iffland et al. (2020) is not as strong as the association with pEFP. However, the three significant SNPs for FI as a new trait on chromosome 1 in the current study as well as the four significant SNPs

for FPD and the seven significant SNPs for pEFP from the previous study (Iffland et al., 2020) which partially match, support the findings that chromosome 1 harbors feather pecking associated chromosomal regions.

5. Conclusion

Selection on high feather pecking leads to an increase of agonistic behavior like aggressive pecking and threatening. The correlation probably depends on the phase of establishing the social hierarchy in which the hens in a newly formed group are at the time of observation and might disappear, after a stable ranking is established. Feather pecking hens do not distinguish at whom they peck feathers at. Additionally, the reception of feather pecking is independent from the occurrence of the other observed traits. Genomic analyses revealed that all traits investigated seem to be typical quantitative traits with low to moderate heritabilities. Some trait associated chromosomal regions for APD, Activity and FI were detected by applying GWAS. The results imply that besides optimizing environmental conditions, selection against feather pecking behavior reduces agonistic behavior and hence might improve animal welfare in commercial layer flocks. For the practical application, a validation in commercial laying hen lines is required as the lines investigated in this study were experimental populations.

Declaration of Competing Interest

None.

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CHAPTER FOUR

Gut Microbial Composition and Predicted Functions Are Not Associated with Feather Pecking and Antagonistic Behavior in Laying Hens

Daniel Borda-Molina [1,†], Hanna Iffland [1,†], Markus Schmid [1], Regina Müller [1], Svenja Schad [1], Jana Seifert [1], Jens Tetens [2,3], Werner Bessei [1], Jörn Bennewitz [1] and Amélia Camarinha-Silva [1,*]

[1] Institute of Animal Science, University of Hohenheim, 70599 Stuttgart, Germany;

[2] Department of Animal Sciences, University of Göttingen, 37073 Göttingen, Germany;

[3] Center for Integrated Breeding Research, University of Göttingen, 37075 Göttingen, Germany

* Correspondence: amelia.silva@uni-hohenheim.de

[†] Shared authorship.

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Abstract: Background: Feather pecking is a well-known problem in layer flocks that causes animal welfare restrictions and contributes to economic losses. Birds' gut microbiota has been linked to feather pecking. This study aims to characterize the microbial communities of two laying hen lines divergently selected for high (HFP) and low (LFP) feather pecking and investigates if the microbiota is associated with feather pecking or agonistic behavior. Methods: Besides phenotyping for the behavioral traits, microbial communities from the digesta and mucosa of the ileum and caeca were investigated using target amplicon sequencing and functional predictions. Microbiability was estimated with a microbial mixed linear model. Results: Ileum digesta showed an increase in the abundance of the genus *Lactobacillus* in LFP, while *Escherichia* was abundant in HFP hens. In the caeca digesta and mucosa of the LFP line were more abundant *Faecalibacterium* and *Blautia*. Tryptophan metabolism and lysine degradation were higher in both digesta and mucosa of the HFP hens. Linear models revealed that the two lines differ significantly in all behavior traits. Microbiabilities were close to zero and not significant in both lines and for all traits. Conclusions: Trait variation was not affected by the gut microbial composition in both selection lines.

Keywords: gut microbiota; feather pecking; microbiability; laying hen; agonistic behavior

1. Introduction

Feather pecking is a detrimental behavior pattern shown in layer flocks, leading to injured birds and, consequently to the welfare and economic problems. Research over the last few decades revealed the underlying mechanisms of feather pecking (for a review, see Rodenburg et al. [1]). Still, it remains an unsolved problem in the poultry industry worldwide.

It is well known that environmental and genetic factors determine feather pecking. Previous research led to the assumption that the gut microbial composition is also involved in developing the undesired behavior. In laying hen lines divergently selected for feather pecking, supplementation with the essential amino acid L-tryptophan significantly reduced feather pecking by increasing the serotonergic tone [2]. Tryptophan supplementation increases the abundance of non-pathogenic bacteria (Bifidobacteria and Enterococci) known to support gut integrity and health [3]. A higher amount of feather pecking comes with a higher amount of feather eating [4–7], although raw feathers do not have any nutritional value [8]. Lutz et al. [9] identified a causal effect of feather eating on feather pecking using structural equation models. Meyer et al. [10] found differences in the gut microbiota and their metabolites between laying hen strains fed with different amounts of feathers. Some studies revealed that laying hen lines

divergently selected for feather pecking also differed in some aspects of their gut microbial composition [11–13]. These findings suggested that the gut microbial composition might be associated with feather pecking and even might be one cause for it.

The ileum represents a major nutrient absorption site of the gastrointestinal (GI) tract in chickens and is dominated by *Lactobacillus* [14], *Streptococcus*, and *Escherichia coli* [15]. The caeca are colonized by a huge diversity of bacterial members, specifically Clostridiaceae, Bacteroidaceae, Lactobacillaceae, Proteobacteria, and butyrate-producing clusters as well as several uncultured bacteria [15,16]. The chicken caeca are an important fermentation site. They are responsible for the digestion of foods rich in cellulose, starch, and resistant polysaccharides, which impact the health and performance of the animals. Therefore, the contributing microbiota has been extensively examined [16–18]. Although it is known that the intestinal microbiota differs between mucosa and digesta samples, yet most studies characterized the digesta [19,20]. In all GI sections, mucosa samples showed higher microbial diversity than the digesta samples [15].

The term microbiability [21] describes the part of the phenotypic variance of a trait which is explained by the microbial composition. This parameter can be estimated with microbial mixed linear models. Microbiabilities in a medium-range were estimated for feed-related traits in pigs [22,23]. Verschuren et al. estimated high microbiabilities for the digestibility of several nutrients in fecal samples of pigs [24]. In a study on Japanese quails, medium microbiabilities for feed-related traits were identified [25]. Hence, the usefulness of microbiability to define the gut microbiome's effect on feed-related traits in pigs and poultry could be revealed successfully.

Research on humans, rodents, and livestock showed that the gut microbiota composition influences behavior, e.g., anxiety-related, social, or feeding behavior [26]. Germ-free quail chicks were selected for high emotional reactivity (measured with tonic immobility test) and received either feces of conventional adults of the same line or a line selected for low emotional reactivity [27]. Germ-free chicks that received gut microbiota of the fearless line showed significantly less emotional reactivity than chicks with the fearful line's microbiota. After two weeks, the gut microbial composition returned to its equilibrium, which was partially determined by the host genome [27]. Probiotic supplementation reduced fearfulness, improves memory, and reduces agonistic poultry behavior [26,28].

The present study aimed to characterize the gut microbial composition and its predicted functionality from two laying hen lines divergently selected for high (HFP) and low (LFP) feather pecking behavior. A possible influence of the gut microbiota composition toward

feather pecking and agonistic behavior was investigated by applying microbial mixed linear models.

2. Materials and Methods

2.1. Birds and Experimental Procedures

The experiment and the experimental population's establishments are described in Iffland et al. [29]. Briefly, hens of a White Leghorn layer strain were divergently selected for the severe form of feather pecking for 15 generations. Hens were reared together, regardless of the line, and were kept under the same conditions from hatching on. For behavioral observations at around 32 weeks of age, the hens were divided into smaller mixed HFP and LFP groups of about 40 animals and housed in deep litter pens. Observation, by experienced observers, began one week after group formation and took place during four consecutive days [30]. Due to a limited number of pens, two experimental runs were performed phenotyping a total of 492 hens (n_{HFP} = 270, n_{LFP} = 222). Besides others, three behavior traits were recorded, feather pecks delivered (FPD), aggressive pecks delivered (APD), and threats delivered (TD). The ethogram is displayed in Table 1.

Table 1. Ethograms of the recorded traits feather pecks delivered (FPD), aggressive pecks delivered (APD) and threats delivered (TD).

Trait	Definition
FPD	Non-aggressive severe pecks or pulls are directed to the plumage of conspecifics, sometimes resulting in pulled-out feathers and a recipient, which tolerates or moves away. Therefore, the deliverer does not adopt any special body posture.
APD	Pecks delivered in an upright body posture against (mainly) the head and other parts of the recipient's body.
TD	Visual fixation on the recipient in an upright body posture followed by the recipient's avoidance or withdrawal behavior.

All recorded traits were BoxCox transformed to reduce their deviation from a normal distribution. After the observation period of each experimental run, the hens were slaughtered at around 35 weeks of age. Both ileum and caeca were longitudinally opened, and digesta was collected with a sterile spoon. The mucosa was washed with a sterile phosphate-buffered saline solution and scraped with a sterile glass slide. Samples were stored in RNAlater at –80 °C until further analysis. The samples were divided into eight groups based on intestinal section (ileum or caecum), type of samples (digesta or mucosa), and line affiliation (HFP or LFP). The number

of phenotyped animals with samples is shown separately for the sections and sample types in Table 2.

Table 2. Number of animals of the high (HFP) and low (LFP) feather pecking line with samples for the respective gut section and type of samples used in the microbial linear mixed model.

Gut Section and Sample Type	HFP	LFP	∑
Ileum mucosa	96	73	169
Ileum digesta	95	82	177
Caecum mucosa	48	42	90
Caecum digesta	48	43	91

The German Ethical Commission of Animal Welfare of the State Government of Baden-Wuerttemberg, Germany approved the research protocol.

2.2. DNA Extraction Illumina Amplicon Sequencing and Bioinformatic Analysis

DNA was extracted from approximately 250 mg of each digesta and mucosa sample using FastDNA™ SPIN Kit for soil from MP Biomedicals (Solon, OH, USA) following the manufacturer instructions. The quality and concentration of DNA were assessed through NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and DNA was stored until use at −20 °C. The V1-2 region of the 16S rRNA gene was amplified to produce the Illumina sequencing library. The protocol followed the methodology of Kaewtapee et al [31]. Briefly, one microliter of DNA was used as a template in the first PCR, where the forward primer contains a six-nucleotide barcode, and both primers have sequences complementary to the Illumina adapters. Master mixes include the PrimeSTAR® HS DNA Polymerase kit (TaKaRa, Beijing, China). One microliter of the first PCR product was used in a second PCR following the same PCR conditions where both primers were complemented to the sequences of Illumina multiplexing and index primers. Amplicons were verified by agarose gel electrophoresis, purified, and normalized using SequalPrep Normalization Kit (Invitrogen Inc., Carlsbad, CA, USA). Samples and negative controls were sequenced using 250 bp paired-end sequencing chemistry on an Illumina MiSeq platform.

Raw sequence reads obtained from Illumina MiSeq system (Illumina, Inc., San Diego, CA, USA) were analyzed using QIIME v1.9.1 pipeline [32], following a subsampled open-reference operational taxonomic units (OTUs) calling approach [33]. Demultiplexing and trimming of sequencing reads were done using the pipeline's default parameters with a maximum sequence length of 360 bp [34]. The reads were merged into one FASTA-file and aligned using the

SILVA Database (Release 132) [35]. Chimeras were identified and removed using usearch [36]. Reads were clustered at 97% identity into OTUs. Only OTUs present on average abundance higher than 0.0001% and a sequence length of >250 bp were considered for further analysis. The closest representative was manually identified with the seqmatch function of the Ribosomal Database Project. An average of 44,240 reads were obtained per sample. Sequences were submitted to European Nucleotide Archive under the accession number PRJEB40535.

Prediction of functionality was carried out with the R package Tax4Fun2 [37], which relied on the SILVA database [38] and used the Kyoto Encyclopedia of Genes and Genomes (KEGG) hierarchy for the assignations, which comprise gene catalogs from sequenced genomes [39]. The biom table to assign this functionality was obtained from the QIIME pipeline. Genomes from 16S rRNA gene sequences identified in this study were downloaded from the National Center for Biotechnology Information (NCBI) database to produce a case-study-specific database for the ileum and caeca of laying hens.

2.3. Statistical Analysis

Linear discriminant analysis effect size (LEfSe) was applied to observe differences at the OTU level between the HFP and LFP line. The default cutoff was used, including q value < 0.1 and linear discriminant analysis score > 2.0 [40]. Random forest analysis overview was obtained at the OTU level to differentiate the impact of HFP and LFP on the prediction in microbiome data classification. Values by default were 500 trees, and the plots included the out-of-bag error [40].

Datasets were analyzed using PRIMER (version 7.0.9, PRIMER-E, Plymouth Marine Laboratory, Plymouth, UK) [41]. Data was standardized by total, and a similarity matrix was created using the Bray-Curtis coefficient [42]. PERMANOVA analysis, using a permutation method under a reduced model, was used to study the significant differences obtained when the dietary treatments were analyzed and considered significantly different if $p < 0.05$ [41]. The community similarity structure was depicted through non-metric multidimensional scaling plots. Similarity percentage analysis was used to identify the OTUs responsible for the groups' differences. Diversity indices (Shannon diversity and Pielou's evenness) were calculated based on abundance data with PRIMER software.

For estimation of the microbial variance components and the microbiability, the following microbial mixed linear model was applied using ASReml-R (Version 3.0) [43,44]. The model was applied separately for each trait and each gut section.

$$y = Xb + m + e \quad (1)$$

where y is the vector containing the trait records for the corresponding trait (i.e., FPD, APD, or TD). X is a design matrix for vector b, which contained the line's fixed effect, and a combination of experimental run and pen, if significant. Vector e denotes the random residual term. The residuals were modeled heterogeneously within the two feather pecking lines. The Vector m contains the random microbiota animal effects with distribution

$$m \sim N\ (\ 0, M\sigma_m^2\ ) \tag{2}$$

with *M* being the microbial relationship matrix and σ_m^2 denoting the microbial variance. *M* was calculated as

$$M = \frac{XX^T}{N} \tag{3}$$

with *N* being the number of OTUs, and *X* is a $n \times N$ matrix, where *n* is the number of animals. The standardized and log-transformed abundances of the OTUs are contained in *X* [22]. The microbiabilities m_l^2 for each trait and line *l* (*l* = HFP or LFP) were estimated as the fraction of the phenotypic variance in the lines explained by σ_m^2. A likelihood-ratio test on the random microbial animal effect was performed to test the significance of the microbiabilities. The test statistic was calculated as

$$D = 2[\log(L_2) - \log(L_1)] \tag{4}$$

with L_1 being the likelihood of the reduced model, i.e., model (1) without the random microbiota animal effect and L_2 the likelihood of the full model. The test statistic *D* under the null-hypothesis was chi-squared distributed with one degree of freedom. In addition, the two feather pecking lines were analyzed separately with the same model but without a fixed-line effect.

3. Results

3.1. Microbial Community

A significant (p = 0.003) difference in section and feather pecking line interaction was demonstrated by PERMANOVA (Table S1A). Samples of both ileum and caeca clustered by mucosa and digesta (Figure 1) and significant differences were obtained for the feather pecking lines and the type of samples (digesta or mucosa) (Table S1B,D,E). The Shannon diversity

index showed significant ($p \leq 0.05$) differences between ileum and caeca, being higher in the caeca but not between mucosa and digesta samples or the two lines of hens (Figure S1).

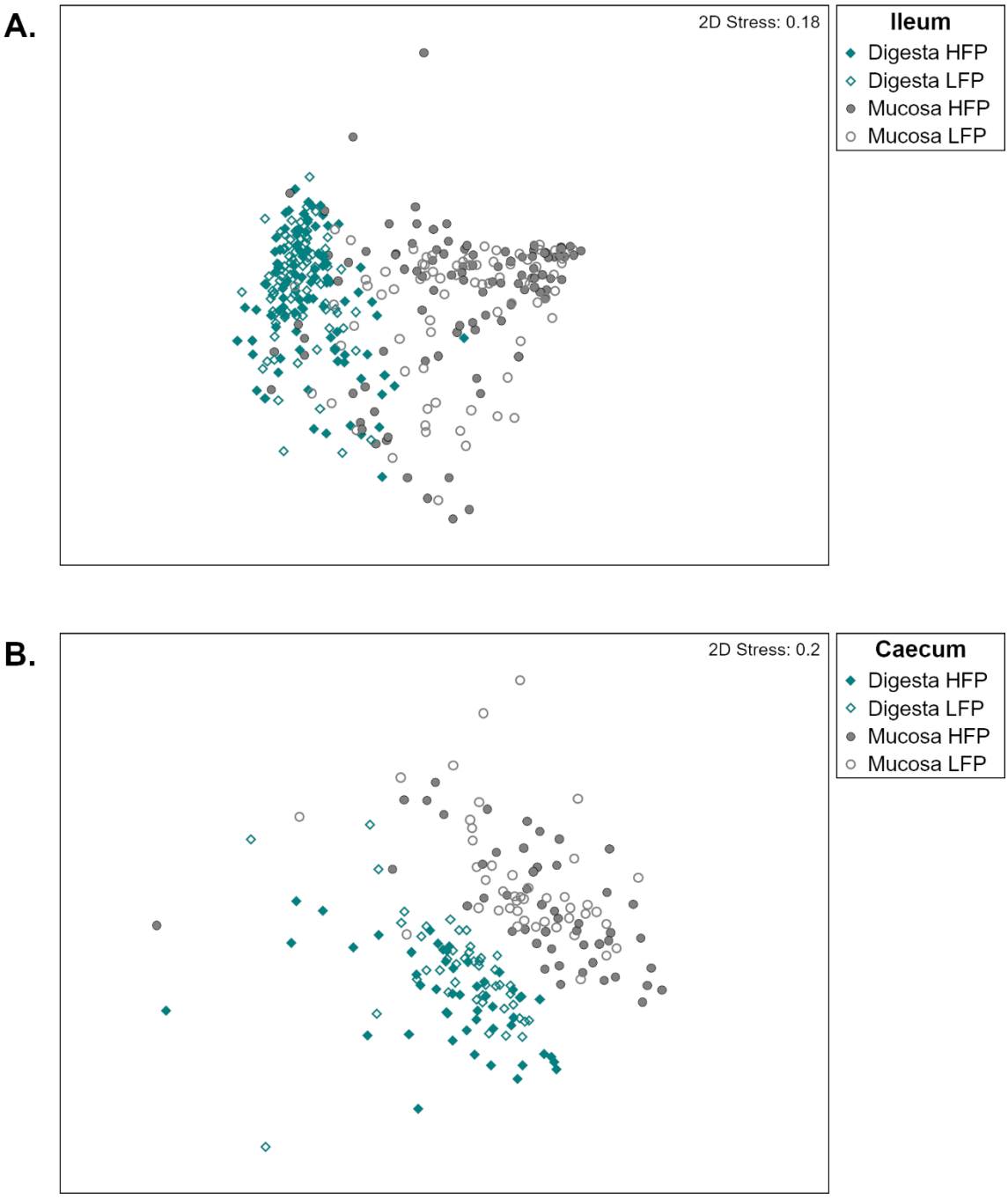

Figure 1. Non-metrical dimensional scaling plot showing the microbial community distribution for ileum (**A**) and caeca (**B**) samples of the high (HFP) and low (LFP) feather pecking line.

In the ileum digesta, the predominant phylum was Firmicutes with an average relative abundance of 93.5% for the LFP line, in comparison to 89.9% for the HFP line ($p \leq 0.05$) (Figure 2). Actinobacteria were detected in the HFP line in higher abundance than in the LFP line (8.0% vs. 5.6%) ($p \leq 0.05$). The percentage of Proteobacteria in the HFP line was also slightly higher (1.1%) than in the LFP line (0.8%). Ileum mucosa of LFP birds had more Firmicutes (88.6%) and Bacteroidetes (6.3%) than HFP birds (86.5% and 4.6%, respectively). Proteobacteria was more abundant in the HFP line (4.6%) than in the LFP line (2.2%) ($p \leq 0.05$). Fusobacteria ($p \leq 0.05$) and Actinobacteria were detected in higher relative abundance in HFP than LFP animals.

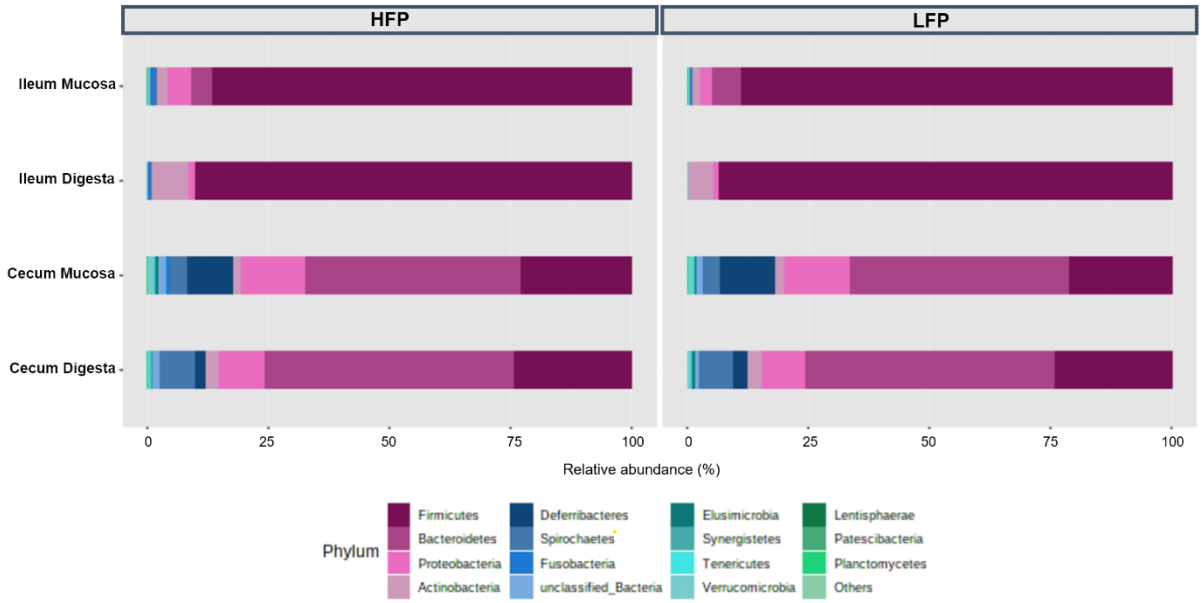

Figure 2. Percentage of relative abundance for phyla distribution in the ileum mucosa, ileum digesta, caeca mucosa, and caeca digesta in the high (HFP) and low (LFP) feather pecking line.

In caeca digesta samples, a significant ($p \leq 0.05$) difference was detected for Firmicutes relative abundance (24.2% in HFP compared to 23.9% in LFP). With a percentage lower than 3%, Deferribacteres and Tenericutes increased in HFP hens ($p \leq 0.05$) (Figure 2). A significant ($p \leq 0.05$) difference was shown in caeca mucosa for Firmicutes (HFP 22.7% compared to LFP 21.1%). In LFP, Elusimicrobia and Fusobacteria were present in less than 2.5% relative abundance, but both increased in HFP hens ($p \leq 0.05$) (Figure 2). Only Actinobacteria gave a higher value for the LFP birds ($p \leq 0.05$).

Random forest analysis was evaluated based on the global prediction error rate after 500 random forests [45]. After this classification, higher error rates for the microbial communities were

obtained in the LFP line for ileum and caeca, mucosa and digesta (Figure S2). This result could imply a more predictable microbial composition in the HFP line since the lowest accuracy was observed in the LFP line.

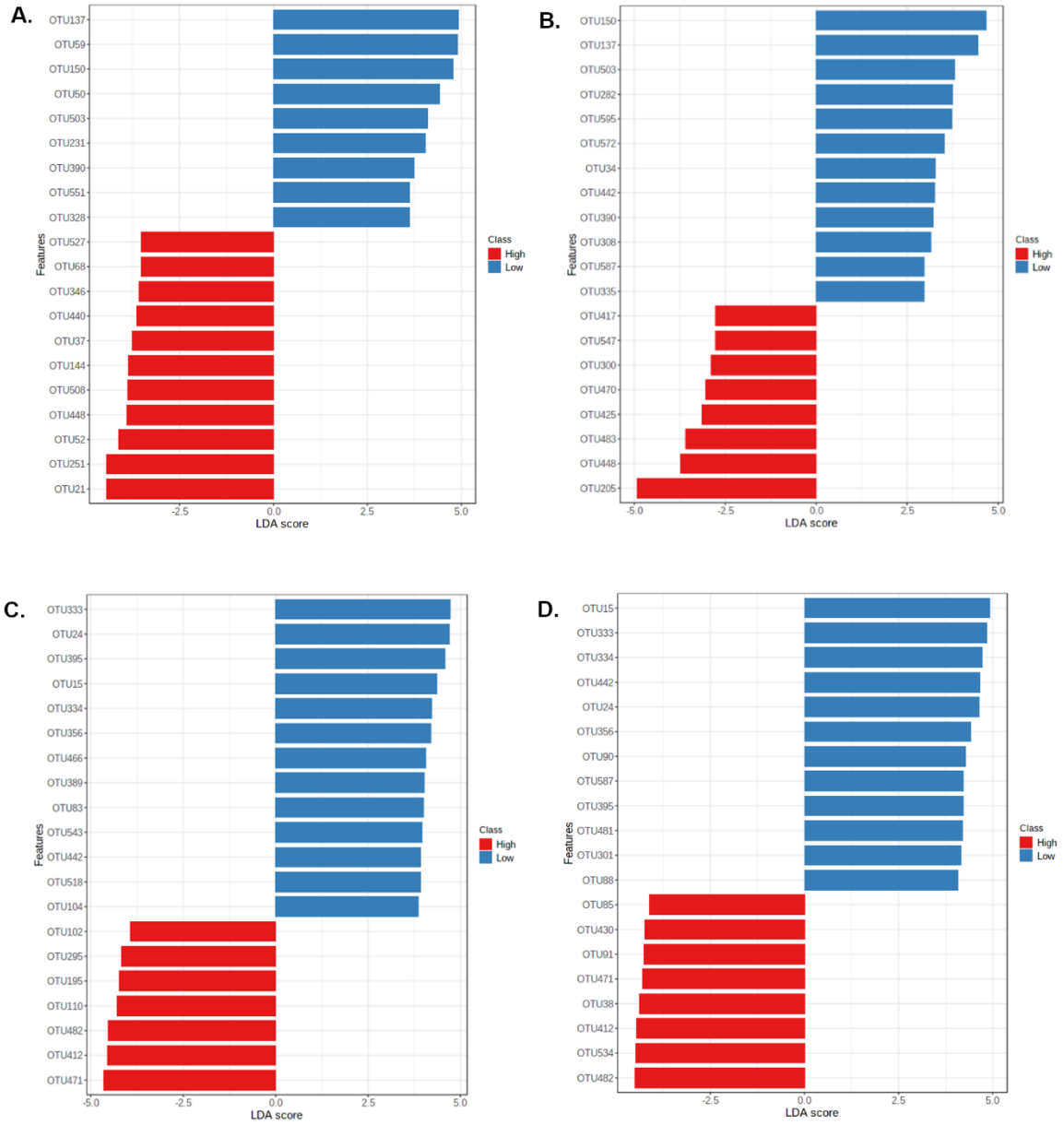

Figure 3. Linear discriminant analysis effect size (LEfSe) analysis for ileum digesta (**A**), ileum mucosa (**B**), caeca digesta (**C**), and caeca mucosa (**D**). The linear discriminant analysis (LDA) score is shown. The high feather pecking line is indicated by red and the low feather pecking line by blue.

LefSe analysis was consistent, showing differences for the same OTUs in the ileum and caeca of the two feather pecking lines (Figure 3). *Lactobacillus* species (OTUs: 50, 59, 137, 150, 231, 390, 503, 551) based on LefSe analysis only appeared in the ileum and the occurrence was

higher in the LFP line (Figure 3A,B). In the ileum digesta, the relative abundance of the OTUs 64 (Unclassified (Unc.) *Olsenella*), 67 (Unc. Clostridiaceae 1), and 251 (*Clostridium rectum*) were higher in the HFP line than in the LFP line (Figure 4). Microbial communities in the HFP hens for the ileum mucosa also included OTU37 (*Escherichia coli*) and OTU251 ($p \leq 0.05$) (Figure 4).

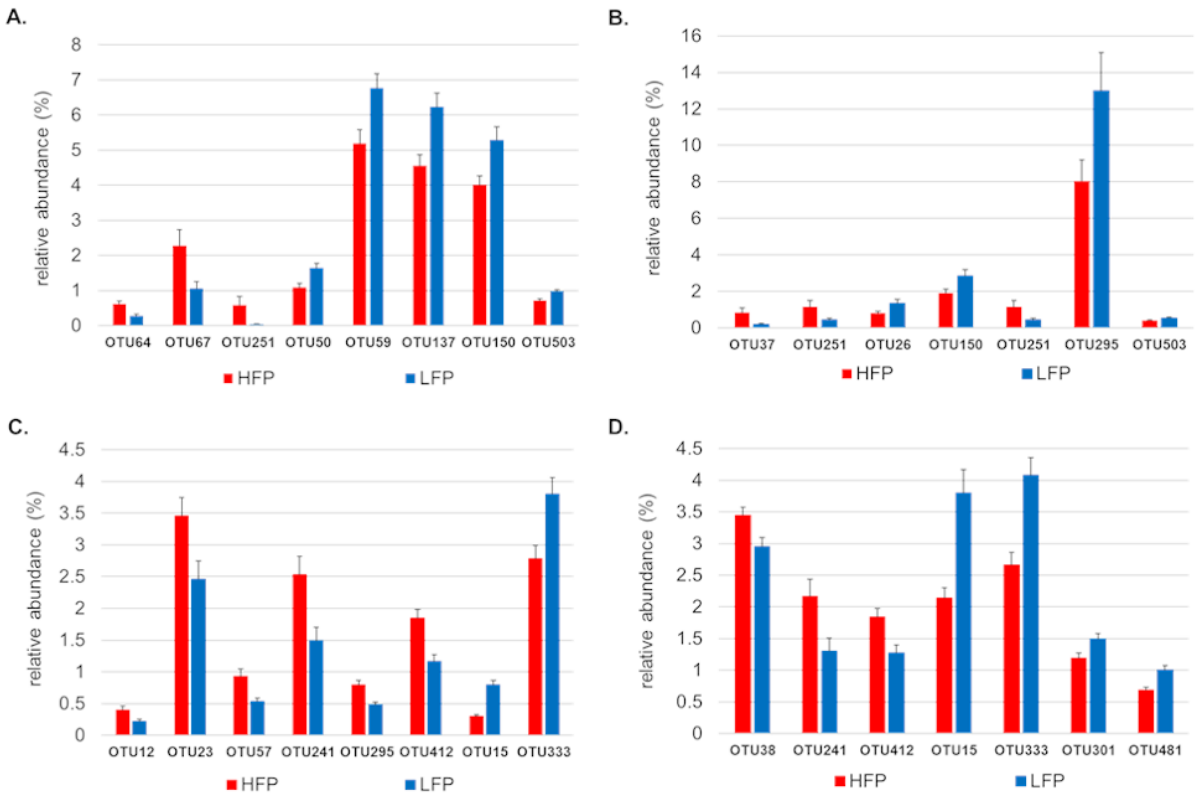

Figure 4. Relative abundance for the OTUs showing a significant difference for ileum digesta (**A**), ileum mucosa (**B**), caeca digesta (**C**), and caeca mucosa (**D**). The high (HFP) feather pecking line is indicated by red and the low (LFP) feather pecking line by blue.

The caeca were colonized by a greater number of bacterial species than the ileum as also represented by a higher diversity index (Figure S1). OTU12 (*Lactobacillus kitasatonis*), OTU23 (Unc. *Paraprevotella*), OTU57 (*Lactobacillus gallinarum*), OTU241 (Unc. Bacteroidales), OTU295 (Unc. *Romboutsia*), and OTU412 (Unc. Proteobacteria) ($p \leq 0.05$) (Figure 4) were detected in higher relative abundance in the HFP line. Less OTUs resulting in significant ($p \leq 0.05$) differences were observed for the LFP line; the OTU15 (Unc. *Mucispirillum*) and OTU333 (Unc. Bacteroidaceae) had higher abundances (Figure 4). In the caecum mucosa of HFP line, OTU38 (Unc. *Phascolarctobacterium*), OTU241 (Unc. Bacteroidales), and OTU412 (Unc. Proteobacteria) were more abundant ($p \leq 0.05$); while for the LFP line again OTU15,

OTU 333, and OTU301 (Unc. *Suterella*) and OTU481 (Unc. *Treponema*) ($p \leq 0.05$) were detected (Figure 4).

Functional prediction showed significant differences for the feather pecking lines in the caeca microbiota, but not in the ileum (Table S2B–E). In the category of amino acid metabolism, tryptophan metabolism, and lysine degradation appeared in both digesta and mucosa, and it was higher in the HFP line. In contrast, cysteine and methionine metabolism and lysine biosynthesis were only predicted in the digesta with increased values in the HFP line. Metabolic pathways of other amino acids were observed in increased abundance in the LFP line in both the digesta and mucosa samples (Figure S3).

In the category of carbohydrate metabolism, 10 out of 15 subcategories resulted in a significant ($p \leq 0.05$) difference between the digesta samples of both lines. At the same time, only five were found in the mucosa (Figure S4). In both sections, glycolysis/ gluconeogenesis and amino sugar and nucleotide sugar metabolism were higher in the HFP line. LFP hens had more functions related to glyoxylate and dicarboxylate metabolism and C5-branched dibasic acid metabolism. The category of energy metabolism showed enhanced numbers of nitrogen metabolism in LFP digesta and mucosa samples. Oxidative phosphorylation and carbon fixation pathways were only observed in the digesta and enhanced for the LFP line (Figure S5). Membrane transports had higher values for the bacterial secretion system subcategory in LFP birds. ATP-binding cassette (ABC) transporters and phosphotransferase system increased in the HFP birds (Figure S6). LFP birds (digesta and mucosa) showed major significant differences (Figure S7) regarding biosynthesis of secondary metabolites.

Lipid metabolism increased in both digesta and mucosa samples of HFP line for glycerolipid, arachidonic acid, and glycerophospholipid metabolism ($p \leq 0.05$) (Figure S8). Cell motility, specifically biofilm formation in *E. coli* and *Pseudomonas aeruginosa*, was predicted in the HFP samples (Figure S9).

3.2. Microbial Parameters

The linear models revealed that the lines differ significantly in the three behavior traits in all subsets of animals. The estimations of microbial parameters and microbiabilities for ileum mucosa in the HFP and LFP lines are shown in Table 3. For the agonistic traits APD and TD, low to medium microbial animal effects were estimated, which resulted in low to medium microbiabilities without significance. For FPD in ileum mucosa and all three traits in the other intestinal sections and samples, i.e., ileum digesta, caecum mucosa, and caecum digesta, the

microbial animal effect estimators were fixed at the boundary by the algorithm. Hence, the microbial animal effects and thus the microbiabilities were nearly zero and not significant.

The results of the separated analyzes of the two lines (not shown) revealed that none of the microbial animal effects in any of the lines and traits were significant.

Table 3. Estimated microbial parameters for the ileum mucosa microbial composition of 169 hens of the high (HFP) and low (LFP) feather pecking line for the three behavior traits feather pecks delivered (FPD), aggressive pecks delivered (APD) and threats delivered (TD).

	Ileum Mucosa					
	σ^2_m (SE)	σ^2_e(HFP) (SE)	σ^2_e(LFP) (SE)	m^2_{HFP}	m^2_{LFP}	*p*-Value
FPD	<0.001 (NA)	0.55 (0.08)	0.26 (0.05)	<0.001	<0.001	1
APD	0.08 (0.11)	1.04 (0.17)	0.52 (0.12)	0.07	0.13	0.54
TD	0.19 (0.12)	1.04 (0.17)	0.35 (0.10)	0.15	0.35	0.37

4. Discussion

4.1. Microbial Community

The characterization of the intestinal microbiota of both lines used in this study resulted in a similar microbial composition as previously described in laying hens [13] and chickens [15,16], including specific patterns such as the higher diversity in the caeca compared to the ileum. *Lactobacillus* species are known to be essential inhabitants of the GI tract of animals and are used as probiotic microorganisms due to their health-promoting properties [46,47]. *Lactobacillus* reduces the GI colonization of pathogens in broiler chickens such as *Campylobacter* [48], *Clostridium* [49], and *Salmonella* [50]. LEfSe analysis showed that in the ileum of LFP laying hens, mainly *Lactobacillus* species, such as *L. johnsonii* and *L. crispatus*, drove the community. La Ragione et al. [49] found that *L. johnsonii* significantly reduced *E. coli* colonization in chickens' small intestine. *L. crispatus* showed high amylase activity, positively affecting feed conversion and broiler performance [51]. *Lactobacillus* stimulated serotonin receptors [52] or increased serotonin and dopamine in the brain [53], influencing the locomotor activity or decreased anxiety and depression-related behavior [53–55].

The role of *Romboutsia* species in the small intestines is still unknown due to the limited availability of cultivated representatives [56]. Here, this genus was highly dominating the caeca digesta of HFP birds. The genus *Mucispirillum* was positively associated with mucus

production [57] and therefore related with a healthy intestine [58,59], in the present study it was detected in higher abundance in LFP than in HFP hens.

Random forest analysis is intended to classify and select the microbial data's main features [40]. It demonstrated that the HFP line comprises less out-of-bag error, which probably indicates a specific microbiota simpler to predict. In contrast, LFP promotes a host-microbiome with more differences leading to higher misclassification rates [45].

In the literature, it was shown that birds fed with feathers differed from control birds in the microbial metabolites and microbial composition. Feather fed birds showed higher numbers of enterobacteria in the ileum and caecum and higher numbers of clostridia in the caecum [10]. Thus, it is expected that feathers' consumption could change the microbial composition [13] and is assisted by the identified appearance of *E. coli* in LEfSe analysis in ileum digesta of the HFP hens.

A previous study demonstrated that gut microbes thrive the release of metabolites such as hydrogen sulfide and other sulfur-containing substances or biogenic amines, which are reactive and potentially influence behavior [10]. These findings were also observed in the predicted functions from this study. Another potential influence on behavior was the predicted promotion of biosynthesis of tryptophan in LFP hens. Tryptophan is the precursor of serotonin, and it was assumed that the alteration on the serotonergic system would impact the feather pecking behavior [60]. Indeed, feather pecking was reduced in diets with 2% of tryptophan compared to supplementation of 0.16% [2,60].

4.2. Microbial Parameters

For some of the traits, sample types, and gut sections as for FPD in Table 1, no variance components could be estimated. This was in line with the clustering of the microbial community distribution shown in Figure 1A. Except for ileum mucosa, no cluster separation was observed within and between the lines. For ileum mucosa, a tendency of separation of the two lines was noticeable implying a differentiation of the two lines' gut microbiota. The limited number of individuals in the present study might be the reason variance components could only be estimated in the ileum mucosa when both lines were analyzed together. No significant effect was determined in the estimated variance components and microbiabilities. Thus, for the behavior traits FPD, APD, and TD, no part of the phenotypic variance could be associated with the gut microbial composition. This means that even though the hens differed significantly in these behavior traits as well as in some fractions of the gut microbial composition, the gut microbiota composition was not associated with the behavior traits. The two feather pecking

lines of the 15th generation were genetically distinguishable from each other with huge allele frequency differences between the two lines. This resulted in a mean F_{ST} value of 0.16 [30], which was predominately due to drift and only to a minor extent due to selection [30,61]. Hence, these genetic differences might be the cause for the microbial differences as it is known that the microbiota is partially shaped by the host genome [62]. Another explanation might be that HFP hens picked and digested more feathers than LFP hens which altered the gut microbial composition [10].

Besides the idea to repeat the study with larger cohorts, one might apply a similar experimental setup as Kraimi et al. [27], where a microbiota transfer between divergently selected feather pecking lines was conducted, to finally rule out whether the microbiota is responsible for the differences in feather pecking behavior. This setup would also include gut microbiota, which cannot be identified or cultivated with the current techniques. Hence, if there is any influence of the microbiota on feather pecking, it could be revealed by this experiment.

5. Conclusions–Does the Microbial Composition in Ileum or Caecum Influences Feather Pecking Behavior?

No, as far as it is known from the recent results. Although significant differences in the gut microbial composition between the HFP and LFP line were found, it was impossible to show the microbiome's influence on the behavior traits FPD, APD, and TD.

Supplementary Materials: The following are available online at https://www.mdpi.com/2075-1729/11/3/235/s1, Figure S1. Shannon diversity index for the ileum and caeca in the mucosa and digesta samples coming from the high (HFP) and low feather pecking (LFP) laying hen lines. Figure S2. Random forest analysis based on the estimation for the out of the error bag (OOB) (y-axis) with a bootstrap of 500 created trees (x-axis), based on abundance information of operational taxonomic units at genus level data in ileum digesta (A), ileum mucosa (B), caeca digesta (C), and caeca mucosa (D). The table explained the classification performance for the high feather pecking line (green), the low feather pecking line (blue), and across both lines (red). Figure S3. Functional predictions for the caeca digesta and mucosa in the subcategory amino acid metabolism in the high and low feather pecking laying hen lines. Figure S4. Functional predictions for the caeca digesta and mucosa in the subcategory carbohydrate metabolism in the high and low feather pecking laying hen lines. Figure S5. Functional predictions for the caeca digesta and mucosa in the subcategory energy metabolism in the high and low feather pecking laying hen lines. Figure S6. Functional

predictions for the caeca digesta and mucosa in the subcategory membrane transport in the high and low feather pecking laying hen lines. Figure S7. Functional predictions for the caeca digesta and mucosa in the subcategory biosynthesis of other secondary metabolites in the high and low feather pecking laying hen lines. Figure S8. Functional predictions for the caeca digesta and mucosa in the subcategory lipid metabolism in the high and low feather pecking laying hen lines. Figure S9. Functional predictions for the caeca digesta and mucosa in the subcategory cell motility in the high and low feather pecking laying hen lines. Table S1A–E. Permanova test for the 16S rRNA gene identified bacterial species dataset obtained from the gut microbiome samples of the mucosa and digesta (type) taken either from the ileum or caeca (section) from the high and low feather pecking laying hen lines (line). Table S2A–E. Permanova test for the predicted functions based on 16S rRNA gene identified bacterial species obtained from the gut microbiome samples of the mucosa and digesta (type) taken either from the ileum or caeca (section) from the high and low feather pecking laying hen lines (line).

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Gut Microbial Composition and Predicted Functions are not Associated with Feather Pecking and Antagonistic Behavior in Laying Hens

Daniel Borda-Molina[1,†], Hanna Iffland[1,†], Markus Schmid[1], Regina Müller[1], Svenja Schad[1], Jana Seifert[1], Jens Tetens[2,3], Werner Bessei[1], Jörn Bennewitz[1] and Amélia Camarinha-Silva[1,*]

Supplementary Material

1 Supplementary Figures

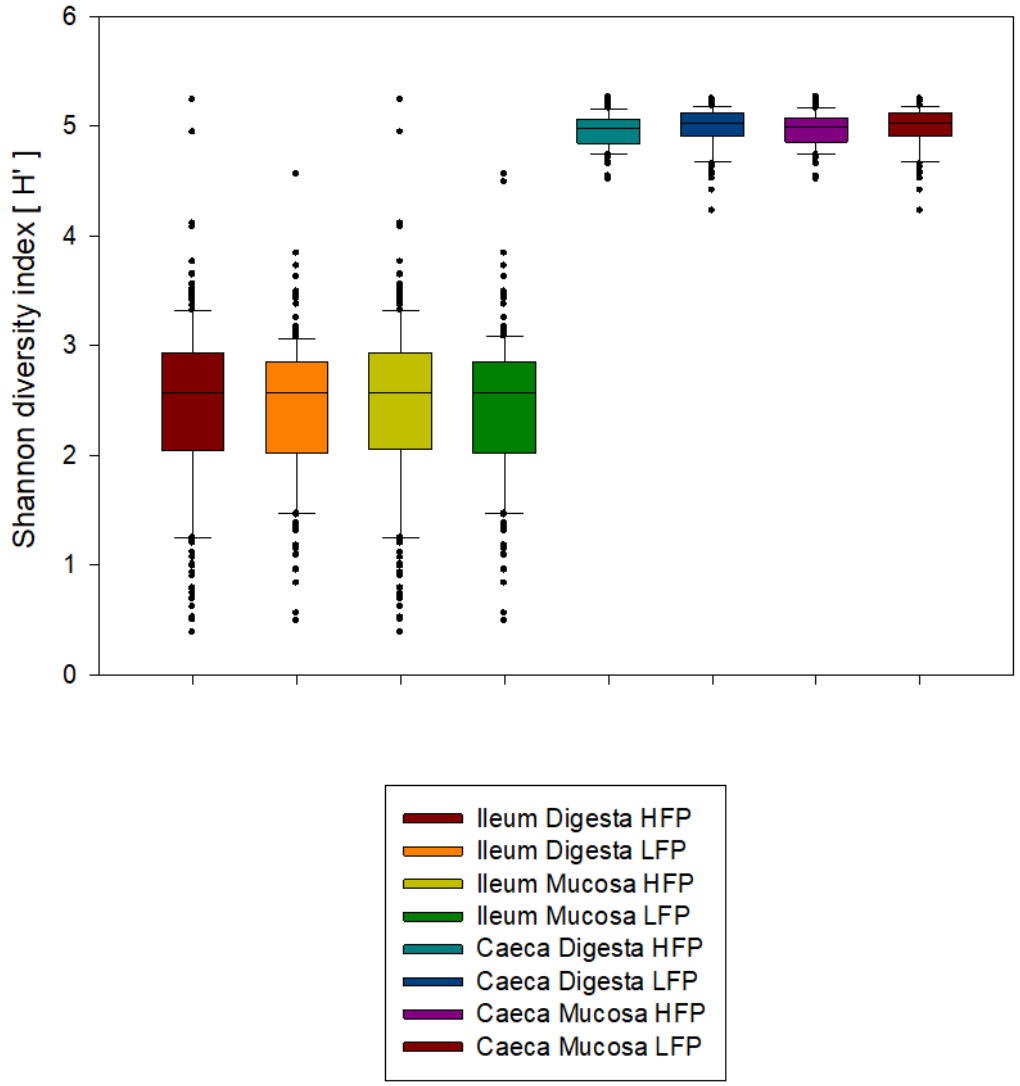

Figure 1. Shannon diversity index for the ileum and caeca in the mucosa and digesta samples coming from the high (HFP) and low feather pecking (LFP) laying hen lines.

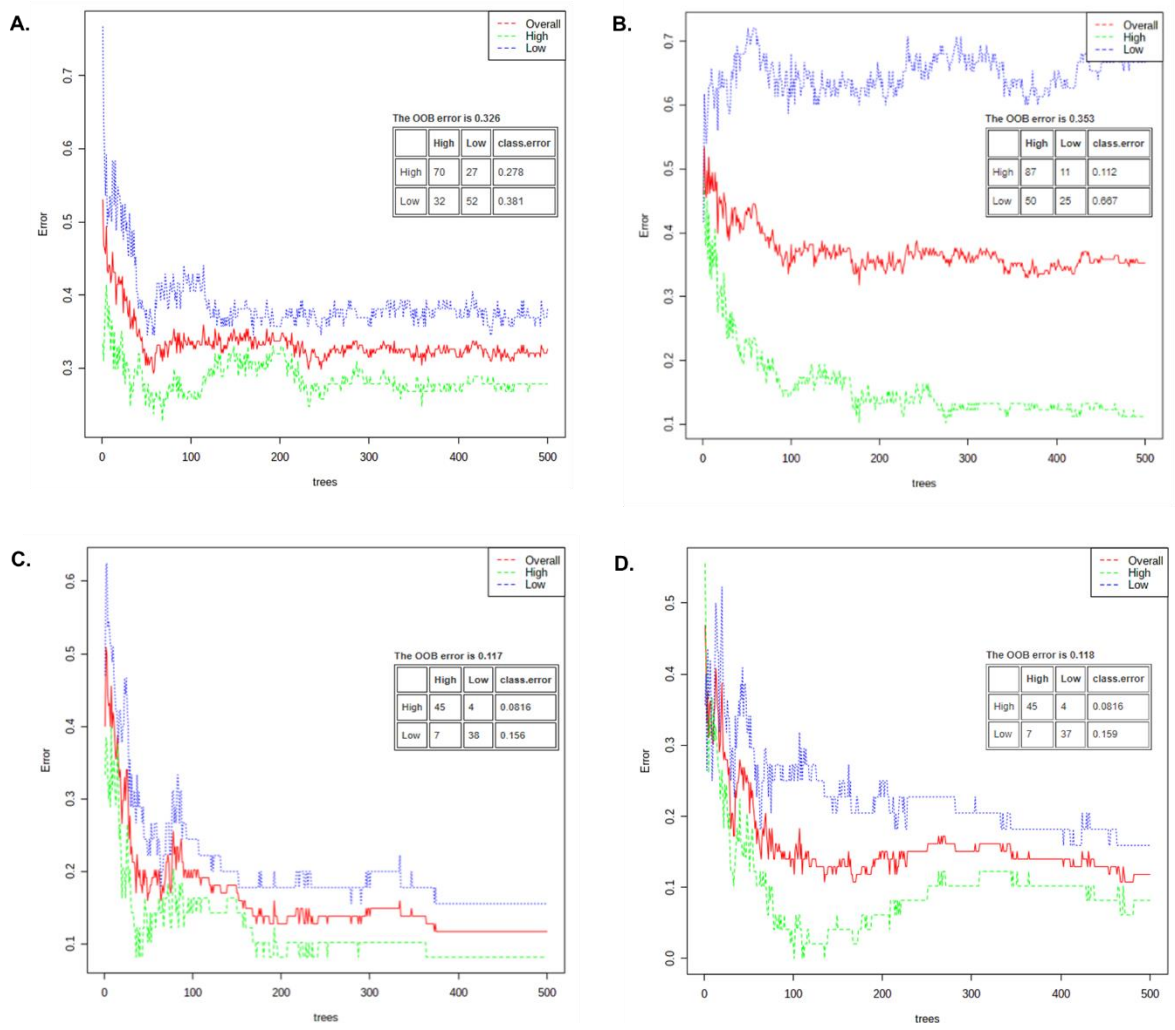

Figure S2. Random forest analysis based on the estimation for the out of the error bag (OOB) (y-*axis*) with a bootstrap of 500 created trees (x-*axis*), based on abundance information of operational taxonomic units at genus level data in ileum digesta (A), ileum mucosa (B), caeca digesta (C), and caeca mucosa (D). The table explained the classification performance for the high feather pecking line (green), the low feather pecking line (blue), and across both lines (red).

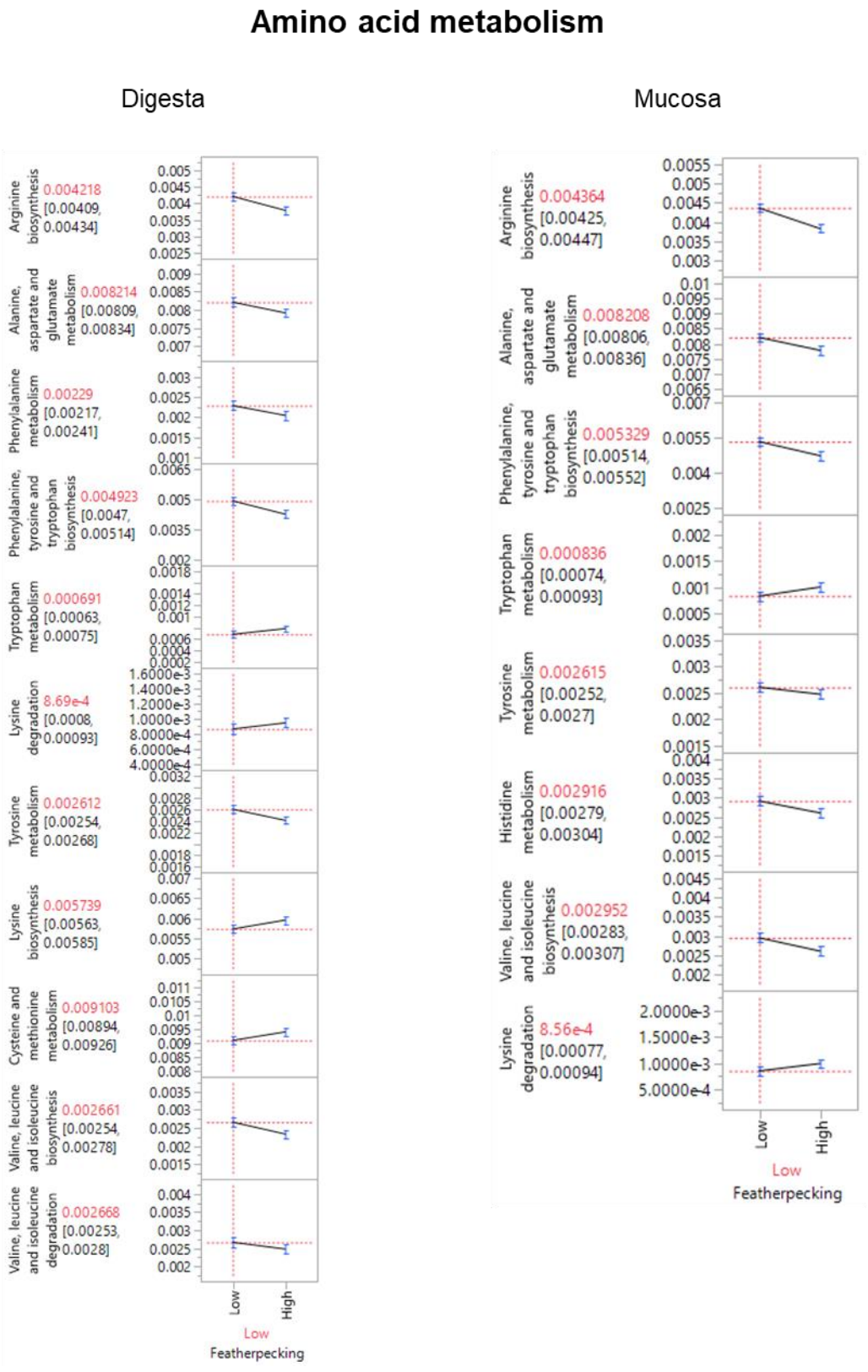

Figure S3. Functional predictions for the caeca digesta and mucosa in the subcategory amino acid metabolism in the high and low feather pecking laying hen lines. The mean values of the measures are highlighted in red. The range of the observed values is given in brackets.

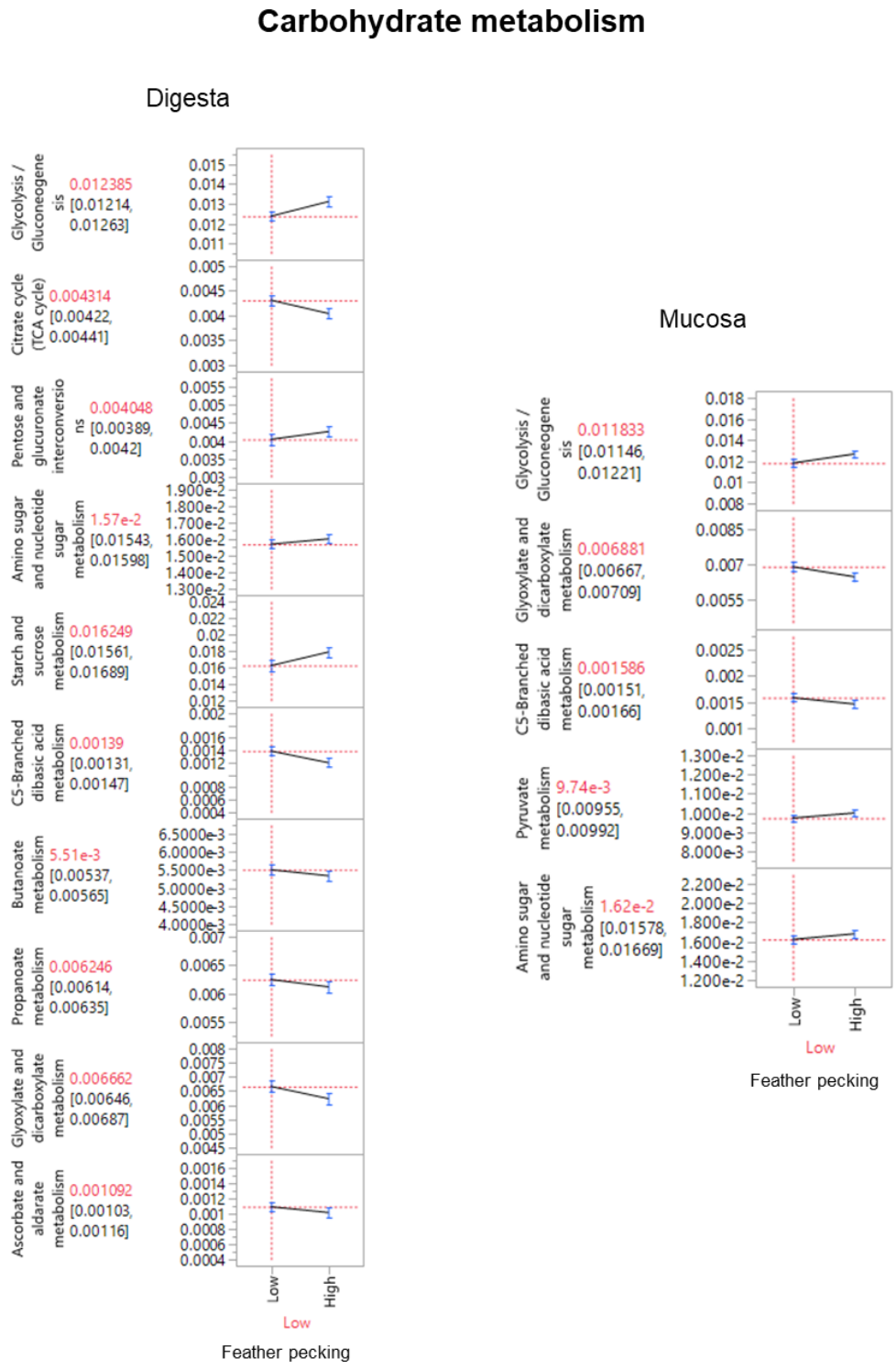

Figure S4. Functional predictions for the caeca digesta and mucosa in the subcategory carbohydrate metabolism in the high and low feather pecking laying hen lines. The mean values of the measures are highlighted in red. The range of the observed values is given in brackets.

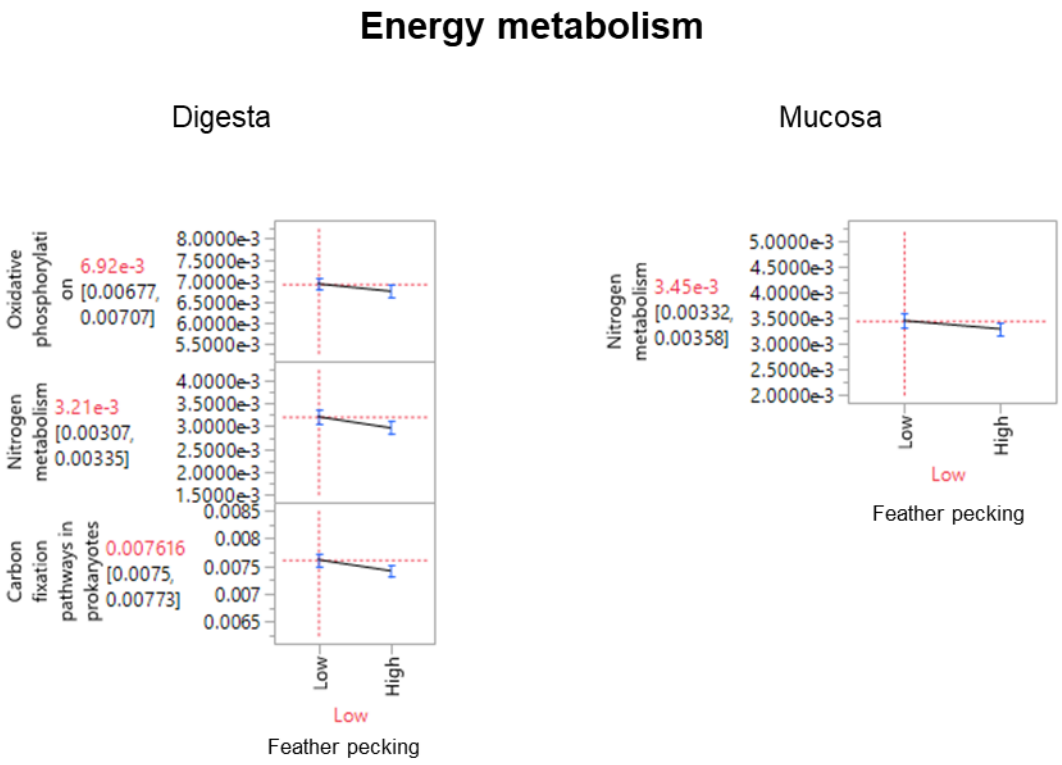

Figure S5. Functional predictions for the caeca digesta and mucosa in the subcategory energy metabolism in the high and low feather pecking laying hen lines. The mean values of the measures are highlighted in red. The range of the observed values is given in brackets.

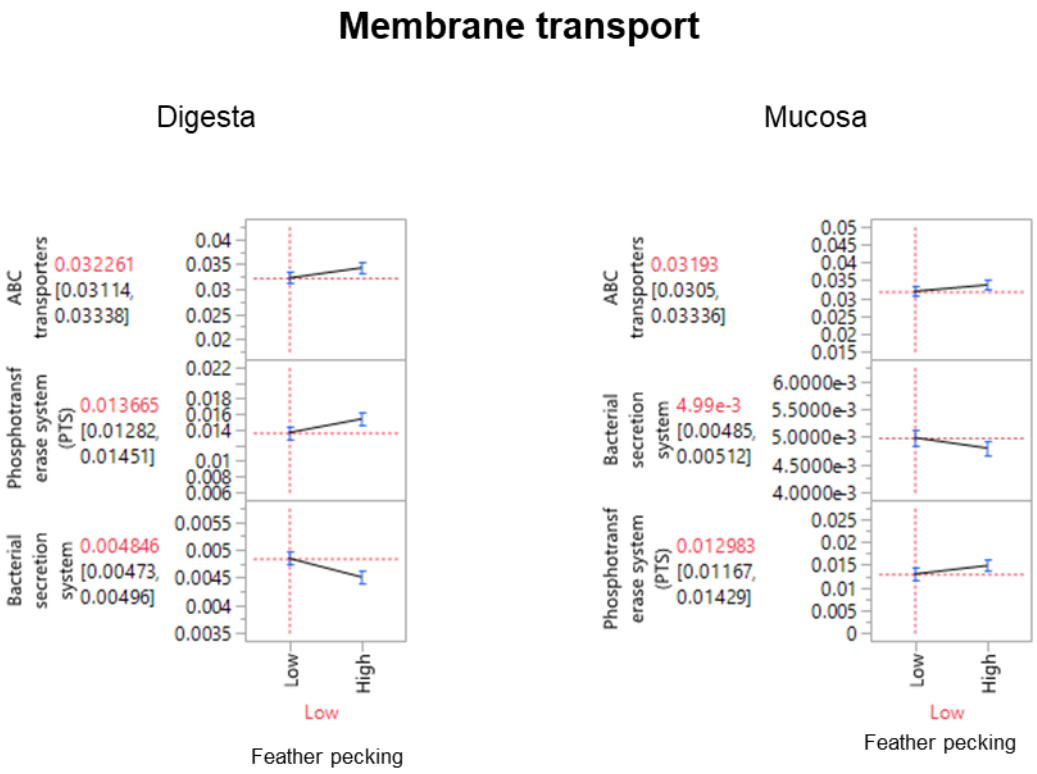

Figure S6. Functional predictions for the caeca digesta and mucosa in the subcategory membrane transport in the high and low feather pecking laying hen lines. The mean values of the measures are highlighted in red. The range of the observed values is given in brackets.

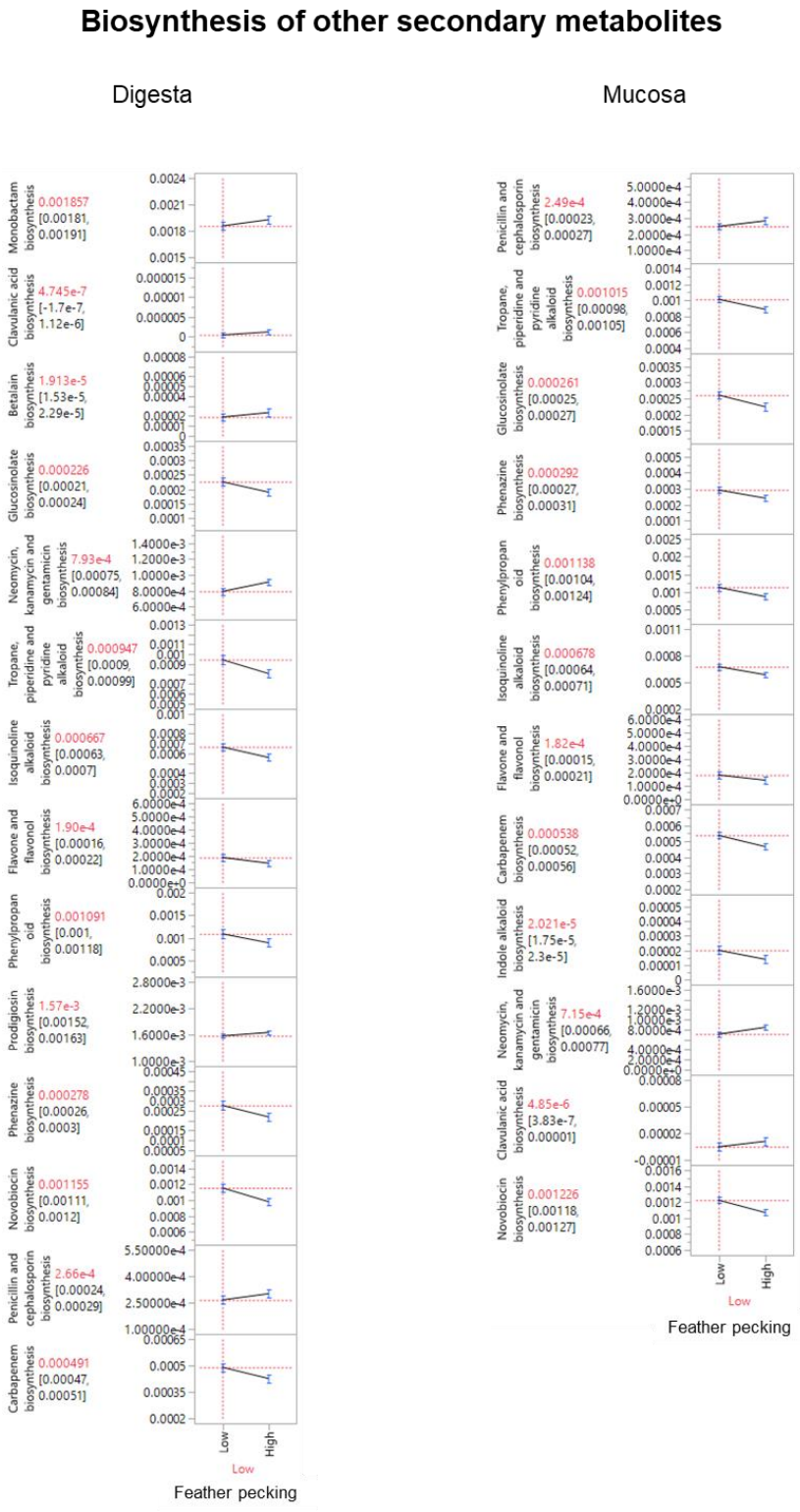

Figure S7. Functional predictions for the caeca digesta and mucosa in the subcategory biosynthesis of other secondary metabolites in the high and low feather pecking laying hen lines. The mean values of the measures are highlighted in red. The range of the observed values is given in brackets.

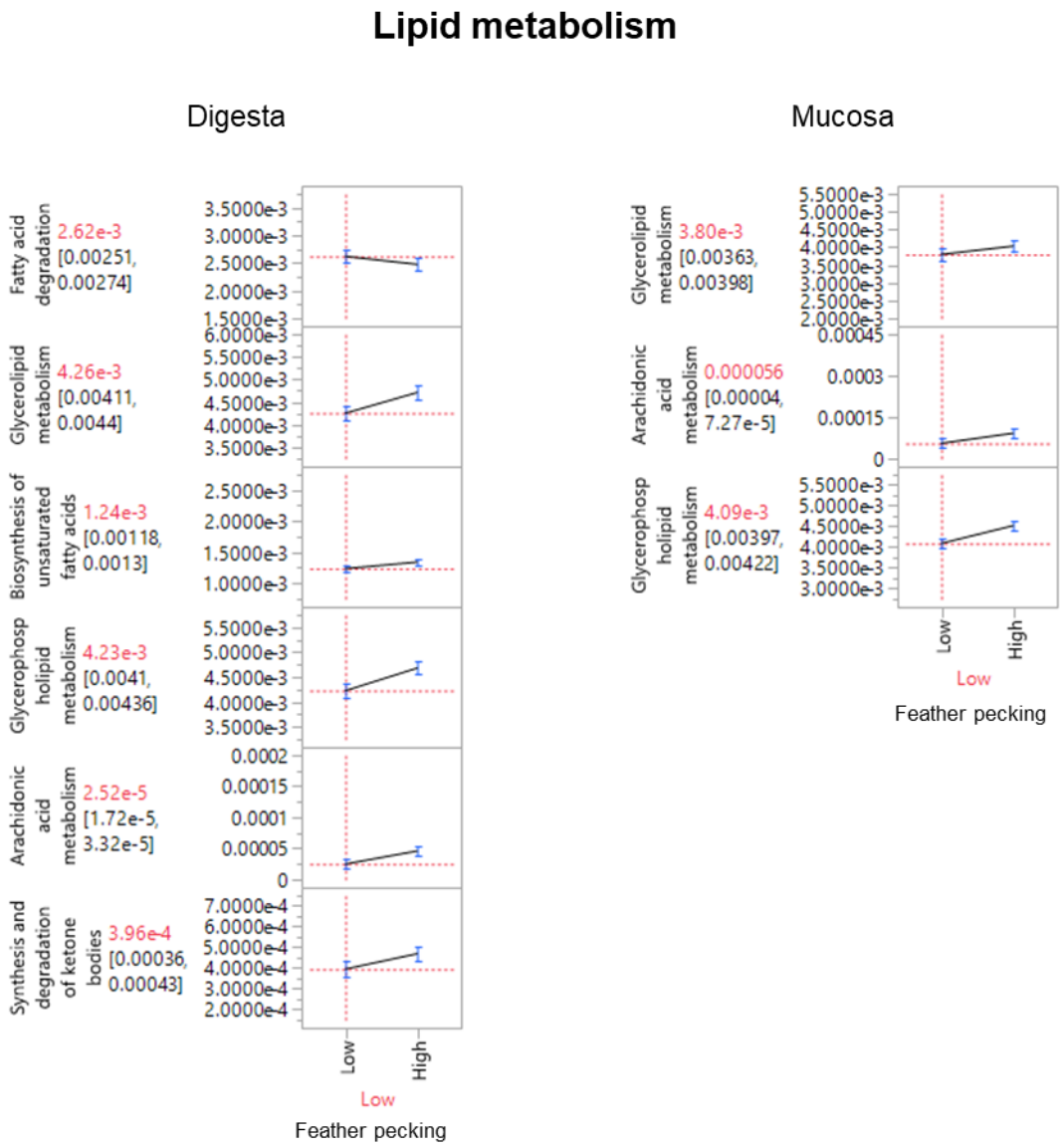

Figure S8. Functional predictions for the caeca digesta and mucosa in the subcategory lipid metabolism in the high and low feather pecking laying hen lines. The mean values of the measures are highlighted in red. The range of the observed values is given in brackets.

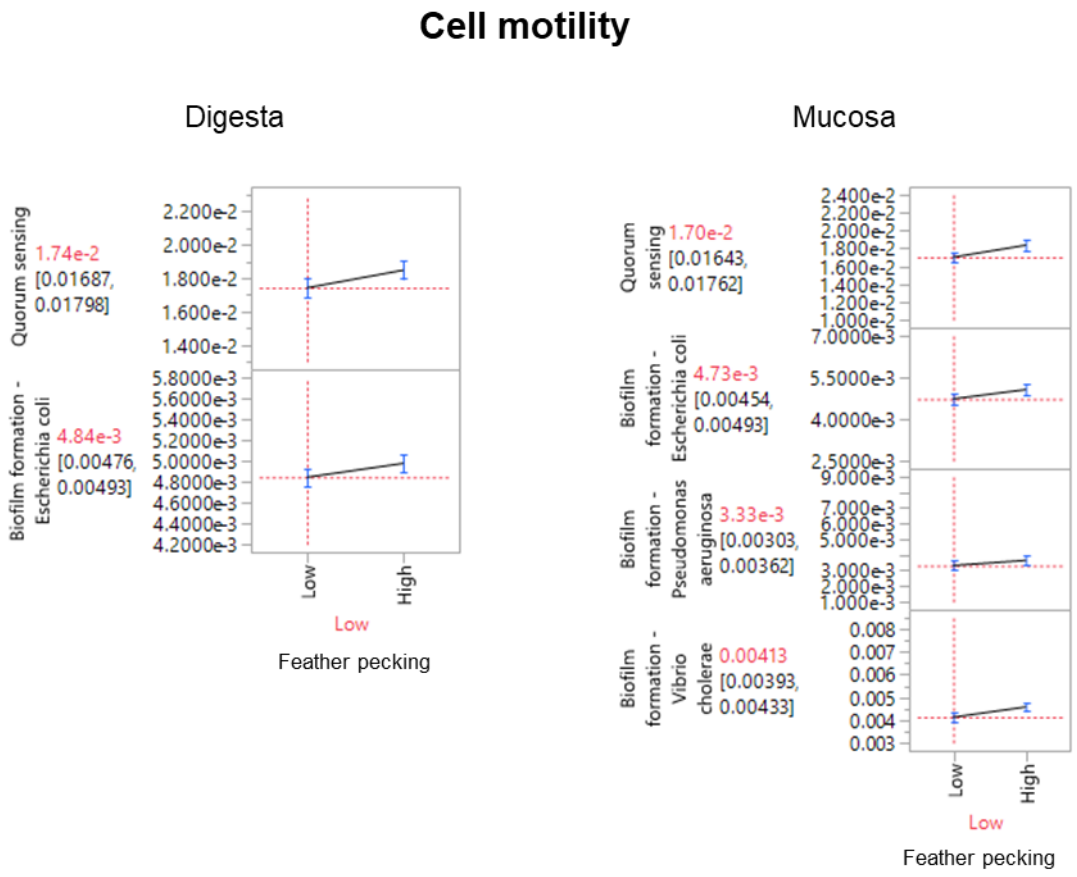

Figure S9. Functional predictions for the caeca digesta and mucosa in the subcategory cell motility in the high and low feather pecking laying hen lines. The mean values of the measures are highlighted in red. The range of the observed values is given in brackets.

2 Supplementary Tables

Table 1. A-E. Permanova test for the 16S rRNA gene identified bacterial species dataset obtained from the gut microbiome samples of the mucosa and digesta (type) taken either from the ileum or caeca (section) from the high and low feather pecking laying hen lines (line).

A. Overall test

PERMANOVA table of results

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Type	1	89289	89289	66.759	0.0001	9931
Section	1	6.95E+09	6.95E+09	519.66	0.0001	9936
Line	1	5954.3	5954.3	44.519	0.0033	9938
Type x Section	1	83185	83185	62.195	0.0001	9935
Type x Line	1	2197.4	2197.4	1.643	0.1369	9924
Section x Line	1	6061.5	6061.5	45.321	0.0033	9940
Type x Section x Line	1	1981.8	1981.8	14.817	0.171	9936
Residual	533	7.13E+09	1337.5			
Total	540	1.65E+09				

B. Ileum digesta

PERMANOVA table of results

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Line	1	4002.1	4002.1	3.1189	0.0046	9937
Residual	179	2.2968E5	1283.2			
Total	180	2.33E+05				

C. Ileum mucosa

PERMANOVA table of results						
Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Unique perms**
Line	1	2803.1	2803.1	1.4978	0.1657	9945
Residual	171	3.2003E5	1871.5			
Total	172	3.2283E5				

D. Caecum digesta

PERMANOVA table of results						
Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Unique perms**
Line	1	4087.4	4087.4	4.8409	0.0001	9903
Residual	92	77680	844.35			
Total	93	81768				

E. Caecum mucosa

PERMANOVA table of results						
Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Unique perms**
Line	1	4714.3	4714.3	5.0187	0.0001	9895
Residual	91	85480	939.34			
Total	92	90194				

Table 2. A-E. Permanova test for the predicted functions based on 16S rRNA gene identified bacterial species obtained from the gut microbiome samples of the mucosa and digesta (type) taken either from the ileum or caeca (section) from the high and low feather pecking laying hen lines (line).

A. Overall test

PERMANOVA table of results						
Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Unique perms**
Type	1	70.081	70.081	3.9665	0.0147	9940
Section	1	464	464	26.262	0.0001	9948
Line	1	5130.5	5130.5	290.38	0.0001	9930
Type x Section	1	13.447	13.447	0.76107	0.4906	9948
Type x Line	1	150.65	150.65	8.5266	0.0001	9944
Section x Line	1	26.922	26.922	1.5238	0.1796	9950
Type x Section x Line	1	11.113	11.113	0.629	0.5837	9931
Residual	533	9417.2	17.668			
Total	540	15268				

B. Ileum digesta

PERMANOVA table of results						
Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Unique perms**
Line	1	27.371	27.371	1.9859	0.1193	9949
Residual	179	2467.1	13.783			
Total	180	2494.4				

C. Ileum mucosa

PERMANOVA table of results						
Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Unique perms**
Line	1	41.369	41.369	1.6259	0.1463	9946
Residual	171	4351	25.444			
Total	172	4392.3				

D. Caecum digesta

PERMANOVA table of results						
Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Unique perms**
Line	1	77.404	77.404	6.7559	0.0015	9952
Residual	92	1054.1	11.457			
Total	93	1131.5				

E. Caecum mucosa

PERMANOVA table of results						
Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Unique perms**
Line	1	73.802	73.802	4.3467	0.0009	9945
Residual	91	1545.1	16.979			
Total	92	1618.9				

GENERAL DISCUSSION

The aim of this thesis was to gain further knowledge about the genetic architecture of feather pecking and its relation to fearfulness, agonistic behavior traits and the gut microbial composition with an additional focus on extreme feather pecking. Therefore, in **chapter one**, a novel model to detect the extreme feather pecking hens was developed and the new trait *posterior probability of a hen to belong to the extreme feather pecking subgroup* (**pEFP**) was defined and analyzed. One third of the hens was detected to be extreme feather peckers and it could be shown that pEFP is positively correlated with fear traits. A genomewide association study (**GWAS**) revealed the quantitative nature of pEFP and fear traits. In the first chapter, an F_2 cross of two laying hen lines divergently selected for feather pecking was analyzed. In the following chapters, the pure high (**HFP**) and low (**LFP**) feather pecking selection lines were considered. In **chapter two,** a region harboring quantitative trait loci (**QTL**) for feather pecking as well as pEFP was found on chromosome one. The putative candidate genes in this chromosomal region are coding for different subunits in the $GABA_A$ receptor and thus belonging to the GABAergic system. In **chapter three** it could be shown that a higher amount of feather pecking comes along with a higher amount of the agonistic behaviors aggressive pecking and threatening. A GWAS revealed the quantitative nature of the agonistic traits and confirmed the QTL region on chromosome one for feather pecking. In **chapter four** it was discovered, that even though the two feather pecking lines differ in some fractions of their gut microbial composition, the gut microbiota does not affect feather pecking or agonistic behavior.

In summary the results of this thesis imply that feather pecking is a quantitative trait with many genes with more or less small effects. Some of these genes may have larger effects on this behavior trait as we found a QTL on chromosome one for the extreme form of feather pecking, i.e. pEFP. A reduction of feather pecking through selection would presumably also result in a reduction of aggressive pecking and threatening, as the moderate correlation in chapter three reveals. The underlying mechanisms of feather pecking have been clarified to the extent that the gut microbial composition seems not to influence this behavior. Thus, a selection strategy including gut microbiota would provide no added value.

Based on these findings, the question arises as to how a breeding program can be designed to reduce feather pecking in commercial layer flocks to increase the welfare of hens and decrease economic losses. In the following, direct and indirect approaches to select against feather pecking in poultry are discussed. The discussion ends with the consideration of ethical aspects.

1 Direct Approaches to Select Against Feather Pecking

1.1 Extreme Feather Pecking - pEFP

In chapter two, regarding pEFP, three SNPs on chromosome one reached genomewide significance which revealed the calculation of the genomewide significance level later in chapter three on the same SNP data set. This indicates a greater effect on pEFP of the candidate genes located in this specific region on chromosome one. However, that these genes influence feather pecking to a larger extent has to be confirmed in further studies. In chapter one of this thesis, no genomewide significant region could be found associated with pEFP in the F_2 cross.

Despite the QTL on chromosome one found in chapter one, pEFP seems to be a polygenic trait and thus the variance explained by the QTL is small. The power to detect a QTL in a GWAS depends on the variance explained by the QTL, the frequency of the QTL in the population, the linkage disequilibrium (**LD**) between SNP and QTL and the sample size (Visscher et al., 2017). In the present studies, sample sizes were small for a quantitative trait with 817 and 489 animals. Hence, in subsequent studies it is important to increase the sample size to confirm the QTL found on chromosome one or further putative QTL. This can also be realized by applying a meta-analysis.

Another critical point is SNP density used in a GWAS which is associated with the LD structure of the population. In multi-breed studies, as which the joint analysis of the two feather pecking lines can be interpreted, LD decays fast with increasing distance between two SNPs (Goddard and Hayes, 2009; van den Berg et al., 2016). In the F_2 cross, LD patterns were analyzed in Lutz et al. (2017) and also revealed a rather fast decay of the LD in comparison to F_2 crosses of distantly related breeds where long range LDs are to be expected (Schmid and Bennewitz, 2017). Hence, a higher SNP density would result in a gain in power as well as a higher precision for QTL mapping in these datasets.

This was done by Falker-Gieske et al. (2020). The authors worked with the same two datasets, i.e. the F_2 cross and the feather pecking selection lines. They imputed the genotypes to sequence level and analyzed the trait pEFP within the two datasets. In GWAS they found no genomewide significant SNPs in neither population for pEFP. Due to considerably higher SNP density, the QTL on chromosome one can be displayed in more detail and might be divided into two or more trait associated regions. This can lead to lower SNP effects and p-values. In the corresponding GWAS plot in Falker-Gieske et al. (2020), the significant peak of GWAS results, in chapter two in this thesis, is recognizable, but does not reach genomewide significance.

1.2 Meta-Analysis of pEFP for the F_2 cross and the Feather Pecking Selection Lines

In addition to the GWAS of pEFP in the F_2 cross in chapter one and both feather pecking selection lines in chapter two, a meta-analysis was carried out to combine the GWAS results of both studies to achieve more statistical power due to a higher number of animals. This was done using the METAL software by Willer et al. (2010) with the sample size based approach. The benefit is for example, that, in contrast to a directly pooled dataset, it can be accounted for study-specific covariates as it was the case in these studies. GWAS results of 1,306 hens were combined with 25,983 common SNPs in both populations. The Manhattan plot of the meta-analysis for pEFP is shown in Figure 1. One nominal ($p_{nominal} \leq 5*10^{-5}$) significant SNP on chromosome 20 (Gga_rs15177217) was found which was not identified as significant in any of the two single studies (Table 1). Since there are no neighboring SNPs flanking the significant one, it is indicated that Gga_rs15177217 is to be classified as untrustworthy whereby the reason for a lack of flanking SNPs can also be a low SNP density.

In Table 1, the significant SNPs of the GWAS of pEFP of chapter one and two with their $-\log_{10}$ p-values and SNP effect directions of the meta-analysis are shown. Three of them only segregated in the feather pecking selection lines and thus no combined p-values were estimated for them in the meta-analysis (symbolized by the forward slash).

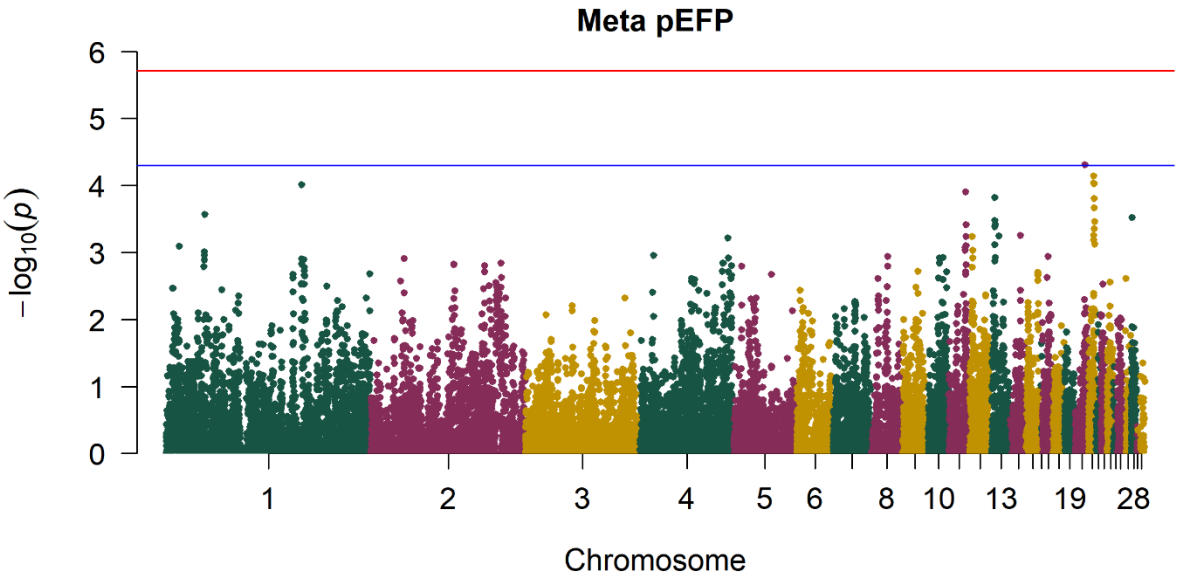

Figure 1 Manhattan plot of the $-\log_{10}$ p-values for association of SNPs (n_{SNP} = 25,983) for the posterior probability of a hen to belong to the extreme feather pecking subgroup (pEFP) of the meta-analysis of the F_2 cross and the feather pecking selection lines ($n_{animals}$ = 1,306). The bottom line indicates the nominal level of significance $p_{nominal} \leq 5*10^{-5}$ and the top line indicates the genomewide level of significance $p_{genomewide} \leq 0.05$.

Table 1 Significant SNPs from GWAS of chapter one and chapter two for the posterior probability of a hen to belong to the extreme feather pecking subgroup (pEFP) with their -$\log_{10}$ p-values of the meta-analysis and the direction the SNP effect has on pEFP. The left character in the direction column symbolizes the direction of the SNP effect in the F_2 cross and the right character in the feather pecking selection lines. The forward slash (/) symbolizes SNPs which did not segregate in both populations.

Population with significant SNP	Chr	SNP	Position	-$\log_{10}$ (p)	Direction of SNP effect
F_2 cross	11	Gga_rs15622328	15,910,351	3.07	+-
	11	Gga_rs14027234	15,934,863	3.03	-+
	11	GGaluGA079200	16,878,999	2.82	+-
	13	GGaluGA093070	8,072,026	3.25	+-
Feather Pecking Selection Lines	1	Gga_rs13938103	131,055,669	4.01	++
	1	Gga_rs14887858	132,015,352	/	/
	1	GGaluGA044500	132,686,520	2.28	+-
	1	Gga_rs14888608	132,789,468	/	/
	1	GGaluGA044531	132,792,863	/	/
	1	Gga_rs13940234	132,960,547	2.90	++
	1	Gga_rs13624646	133,345,452	1.29	+-
	17	Gga_rs15792349	8,366,984	1.22	-+
	17	Gga_rs14098115	8,458,039	2.08	++
	17	Gga_rs14097650	8,891,679	2.05	-+
	26	Gga_rs16203090	3,684,301	1.99	+-
	28	Gga_rs15249217	1,623,905	1.66	+-

Most of the other SNP effects, except three, point to different directions in the two studies. Two of those three SNPs are in the significant QTL on chromosome one in chapter two. This region was confirmed by considering the nominal level of significance -$\log_{10}$(p) > 5 in Falker-Gieske et al. (2020) who also performed a meta-analysis with the sequence level genotypes. In addition, all SNPs in that region (131,766,790 bp -134,135,880 bp) segregated in both populations and the effects pointed in the same directions (Additional File 2 of Falker-Gieske et al. (2020)). Nevertheless, the candidate genes related to GABAergic signaling found in the QTL on chromosome one in chapter two of this study were not confirmed. Falker-Gieske et al. (2020) found four genes (NIPA1, KIAA1211L, AFF3 and TSGA10) in the region by applying a variant effect prediction and identified them as probable candidate genes influencing pEFP. However, neither that QTL nor any of the other nominal significant SNPs in their study reached

genomewide significance. Additionally, the region on chromosome 20 as in this study could not be confirmed by Falker-Gieske et al. (2020).

Hence, even though the number of animals and SNP density were increased, no SNPs reached the level of genomewide significance. This might be because sequence data allows to zoom more deeply into the genome and thus QTL effects can be distributed over several SNPs. This might lead to lower SNP effects and p-values. Additionally, sample size is probably still not large enough to detect QTL reliably. Another point is that not all SNPs segregate in both populations.

In total, the results shed some light onto the underlying mechanisms of pEFP and its genetic architecture. The trait pEFP is a polygenic one like feather pecking and thus the use of marker-assisted selection is not possible to reduce its occurrence.

1.3 Traditional Pedigree-Based Selection

A promising direct approach to reduce feather pecking on basis of present results is to apply a selection program, tailored for the polygenic nature of the trait. In the past, one of the biggest issues, why feather pecking was not considered in poultry selection schemes, was that the birds were solely held in single cages. But for phenotyping feather pecking they need to be held in groups. Additionally, phenotyping feather pecking is expensive as long as it has to be processed manually either by direct observation or video recording and subsequent analyses. What has changed today is that hens are housed in groups during performance testing. Thus, one issue is solved and if one is willing to bear the additional financial burden of the manual observation, the trait can be phenotyped during the performance tests and be integrated into the breeding programs. Hence, it can be applied nearly directly into existing breeding structures.

1.4 Genomic Selection

In order to reduce cost of manual phenotyping, the application of genomic selection would be a suitable method because one advantage of genomic selection is, that a breeding value of an animal can be estimated solely based on its genotype (Goddard and Hayes, 2009). It is assumed for quantitative traits that each SNP or gene which is in LD with the SNP, influences the considered trait. Thus, for each SNP an effect on the trait is estimated regardless of whether it was significant in a putative previous association study (Meuwissen et al., 2016). For the estimation of SNP effects, a large reference population is needed with individuals being pheno- as well as genotyped. Estimated SNP effects from the reference population can then be combined with the genotypes of the selection candidates and are forming the genomic estimated

breeding values, without the need for phenotyping the selection candidates (Meuwissen et al., 2016).

Beside several benefits, there are certain difficulties in applying genomic selection in the poultry industry.

1.4.1 Benefits and Difficulties in the Poultry Industry

Based on the equation for genetic gain as a function of selection intensity, accuracy of selection, additive genetic standard deviation and the generation interval by Rendel and Robertson (1950), Schefers and Weigel (2012) summarized the advantages of genomic selection as a) a higher accuracy of predicted breeding values, b) a reduced generation interval and c) an increased selection intensity. For example in dairy cattle genomic selection is successfully established, because the strong reduction of the generation interval, amongst others, led to a clear increase in genetic gain (Schefers and Weigel, 2012; García-Ruiz et al., 2016).

In laying hens, the implementation of genomic selection faces several specific characteristics as listed by Wolc et al. (2016). For example, the generation interval of about 1 year is already short and the selection intensity is already high, with a large number of selection candidates generated in each generation but only a few percent of selected males. Moreover, commercial laying hens are crossbred animals of four purebred lines which means that the genetic gain in each purebred line only contributes one quarter to the crossbred animals. In addition, building a reference population is more complex than in dairy cattle because commercial crossbred animals are neither recorded on their production traits nor is there any pedigree information about them. To build up a reference population of purebred animals in a sufficient size is hard to achieve as well because there are only relatively few of them per line. Additionally, the combination of several lines is not suitable because they are genetically too different to achieve high accuracies of genetic breeding values (Sitzenstock, 2012). Nevertheless, some studies (Sitzenstock et al., 2013; Wolc et al., 2015) actually proved that genomic selection in laying hens, as in dairy cattle, led to an increase in genetic gain compared to traditional pedigree-based breeding programs.

1.4.2 Genomic Selection Under Manual Phenotyping - Reference Population made of Purebred Lines

One approach to use the advantages of genomic selection might be to phenotype the reference population for feather pecking only in a certain cycle, for example every two or three years. Thus select the purebred lines, regarding this trait, only based on their genotypes. Hence, cost for manual phenotyping of feather pecking would at least be halved. However, it has already

been shown in dairy cattle (Habier et al., 2010) as well as in laying hens (Wolc et al., 2011) that the reference population needs a retraining on a regular basis because otherwise the accuracies of the genomic breeding values decrease rapidly over generations. Specifically in laying hens, Wolc et al. (2011) concluded that, the reference population should be retrained every generation to avoid the immense decrease in accuracy during decreasing pedigree relationship.

1.4.3 Genomic Selection Under Automated Phenotyping - Reference Population made of Crossbred Layers

It is likely to be that automation of phenotyping feather pecking will become practically feasible in future. Ellen et al. (2019) reviewed several technical methods, which have been shown in various studies, to have different strengths and weaknesses. One big challenge is to implement these techniques in standard housing systems, because equipment like metals or also liquids can negatively interfere the function of the systems (Ruiz-Garcia and Lunadei, 2011). Further difficulties are that hens in contrast to other livestock species are using their space in three dimensions, are difficult to distinguish because of their great similarity in appearance and are held in large flocks (Ellen et al., 2019). Nevertheless, some promising approaches indicate that these challenges can be overcome. Ellen et al. (2019) discussed that a combination of an ultra-wideband system and accelerometers seem to be the best technical approach yet to phenotype feather pecking automatically. As already discussed in chapter two, this is based on the knowledge that activity is correlated with feather pecking and a study already showed the possibility to phenotype feather pecking automatically (Rodenburg et al., 2017). In detail, the movement patterns of hens were recorded using an ultra-wideband system where the hens had an active tag on their back which communicated with sensor beacons. This technology allows to track the activity of individually identifiable animals. The recording was done on HFP, LFP and unselected control hens. It was shown, that HFP hens moved as twice the distance as other individuals and even within the HFP line, individual differences were recognizable and could be associated with the amount of feather pecking (Rodenburg et al., 2017). Additionally, with accelerometers it is possible to record directional movement and speed of the birds (Ellen et al., 2019). Rodenburg et al. (2017) discussed that an application of ultra-wideband techniques could be well possible in commercial selection lines during performance testing because of the rather small groups. In combination with genomic selection this should be a feasible option to breed against feather pecking.

Nevertheless, with an optimistic view in the technology development in the near future it might be possible to apply automatic recording techniques in larger groups of laying hens as well. Hence, to make the best use of the advantages of observation techniques and to consider the

urgency of selection against feather pecking to increase the welfare of laying hens, an innovative approach would be to implement test flocks following the example of test herds in dairy cattle breeding. Test herds serve to record hard-to-measure-traits like feed efficiency or health related traits on some representative animals. Therefore, the participating farms are equipped with additional recording techniques in order to collect data of the respective traits. It might be possible to implement this system in the poultry industry as well. The test flocks could be made up of either purebred animals or production hybrid layers. Due to the relatively low number of animals in the purebred lines and the difficulty of combining different lines due to their genotypic differences as described above, test flocks made up of the hybrid layers would be more recommendable. Hence, the test flocks can be nearly standard production flocks held in barn systems but equipped with modern recording techniques to be able to phenotype feather pecking automatically. Many studies have already shown that genomic selection on purebreds for crossbred performance is possible especially with a crossbred reference population (Dekkers, 2007; Ibánez-Escriche et al., 2009; Esfandyari et al., 2015; Duenk et al., 2019). In laying hens, it might be a bit more challenging because only a quarter of the genes of each line is present in the hybrids due to the four purebred lines. This may result in lower accuracies in estimated breeding values of the purebred lines in comparison to a two- or three-line cross. Ibánez-Escriche et al. (2009) showed in their simulation study, that with a higher number of animals in the reference population and a higher SNP density, higher accuracies in a four-breed cross are possible to achieve as well. Once test flocks of the hybrid layers are set up, they can also be phenotyped on production traits as this would increase the reference population for these traits.

2 Indirect Approaches to Select Against Feather Pecking

Beside these strategies to select directly against feather pecking, based on the results of this thesis and other studies, it as well seems possible to select against it in an indirect way, either with a traditional or genomic approach.

2.1 Fear Traits

In chapter one, the correlation between pEFP and fear was studied because there was evidence, that hens showing high feather pecking as well show a higher level of fear (Rodenburg et al., 2004). It could be revealed, that pEFP is medium genetically correlated with fearfulness measured by the emerge box test in juvenile as well as in adult age. Hence, the emerge box tests in both ages could be used as proxy traits in a breeding program whereas the correlation is higher in adult age. The disadvantage is, that genetic correlations are not high and thus the

response to selection would be lower in contrast to a direct selection approach on feather pecking. Furthermore, the results refer only to pEFP and not feather pecking as a whole. For feather pecking at adult age, Rodenburg et al. (2004) found a strong negative genetic correlation with activity in an open field test at juvenile age, whereas Grams et al. (2015) found a less strong genetic correlation with tonic immobility at juvenile age. Hence, more research is needed here to confirm a fear test as a proxy trait for feather pecking.

2.2 Feather Eating

As introduced in chapter four, it could already be shown that feather pecking hens ate more feathers than non or low feather pecking hens (McKeegan and Savory, 2001; Harlander-Matauschek and Bessei, 2005; Harlander-Matauschek et al., 2006; Harlander-Matauschek and Häusler, 2009). In the studies of Meyer et al. (2012) and Meyer et al. (2013) it was shown that the gut microbial composition and microbial metabolites changed after feather ingestion. Lutz et al. (2016) found a causal effect from feather eating on feather pecking by using structural equation models. It was concluded, that feather eating is one of the drives for a hen to peck feathers (Bessei and Kjaer, 2015; Lutz, 2016). As a result of the study findings so far, there was evidence that the gut microbiota plays a role in the development of feather pecking. Nevertheless, in chapter four it could be revealed that although differences in the gut microbiome of the HFP and LFP lines exist, the gut microbiota does not influence feather pecking or agonistic behavior in that dataset. Weishaar et al. (2020) showed in a novel two-step procedure how microbiota data can be integrated into a selection index combined with the classical genomic breeding values and the benefits of this method. This hologenomic approach, where the host genome and the gut metagenome are considered in estimating breeding values, only makes sense when there is proven evidence that the microbiome influences the trait. Hence, due to the already mentioned results of chapter four, it is not useful to include gut microbiota information in the estimation of breeding values for feather pecking.

However, the association between feather eating and feather pecking can still be worthwhile by using feather eating as a proxy trait for feather pecking as it could already be shown that feather eating is heritable and correlated with feather pecking (Bennewitz et al., 2014; Lutz et al., 2016). The benefit is, that eating feathers can be recorded directly in single cages and thus directly on the selection candidates. On the other hand, the correlations are not consistently strong and the genetic gain would not be presumably as high as through a direct selection approach. Nevertheless, the usage of feather eating as a proxy trait seems as well to be able to reduce feather pecking in a notable manner.

2.3 Plumage Condition

Another indirect approach might be to select for a denser plumage. The assumption is, that a certain amount of received feather pecks would not negatively affect a hen because the formation of bald spots would take much longer due to the denser plumage. Thus, the decrease of animal welfare would appear later than normal and might stick to an acceptable level. This would also lead to a decrease in economic losses because the hens do not have to eat more to remain their body temperature and might live longer during laying period due to an assumed decrease of cannibalism. Thus, the survival time of the hens would increase. The advantage is that like the aforementioned proxy traits, plumage density could be recorded on single birds. However, it must be urgently considered that laying hens have a high metabolic activity and thus are producing much heat. With a denser plumage, the hens would presumably eat less and produce fewer eggs as a result. Hence, housing conditions must be adjusted if the plumage density changes.

Another approach might be to select for a good plumage condition or survival time after group housing because these hens seem to be the ones which can successfully avoid a high number of pecks received. However, the exclusive selection on a good plumage condition and survival time is risky because of the indirect genetic effects one animal has on its conspecifics. Peeters et al. (2012) showed in their study that hens with a positive direct breeding value for their own survival time usually have a negative indirect breeding value for the survival time of their conspecifics (Ellen and Bijma, 2019). Brinker et al. (2014) revealed that the genetic variation of plumage condition is substantially due to indirect genetic effects. Hence, selection solely based on the direct genetic effects of a hen, regarding her survival time or plumage condition, would result in more feather pecking instead of less in the following generations. Therefore, it is necessary to take the indirect genetic effects of the conspecifics into account when it comes to an indirect selection against feather pecking with survival time or plumage condition as proxy traits. The benefit of this selection approach is that the hens do not have to be phenotyped for feather pecking but only for survival time which would be cheaper to achieve. Ellen et al. (2014) reviewed the challenges for application of direct-indirect effect models under practical conditions and pointed out the opportunities arising under genomic selection.

3 Practical Recommendations

To implement genomic selection against feather pecking in commercial layer lines, the first step is to find out how the trait must be phenotyped in order to obtain a good repeatability of the trait record and a sufficient variance in the population. Note, that with a (too) short

observation time many individuals show no feather pecking activity and thus variance is small. With increasing observation time, more and more individuals will show the behavior pattern and thus can be distinguished from each other, increasing variance. Hence, for a given budget there is an optimum regarding the length of the observation period (the longer the better) and the number of individuals being observed (the more the better). It should be noted that the number of animals per observer must not be too high, otherwise feather pecking events might not be noticed. In general, the less feather pecking occurs in the group, the more animals can be observed by one person. Since trait expressions are population specific, the first step probably has to be done for each line separately if it is expected that the lines differ in their feather pecking frequency. Thus, some lines may need to be observed shorter or longer, respectively, depending on how much feather pecking is shown.

In a second step, the required size of the reference population can be calculated to ensure a sufficiently high accuracy of the estimated breeding values which depends on the heritability of the trait and the effective population size (Goddard, 2009; Goddard and Hayes, 2009). As already discussed above, one of the main issues will be to provide a sufficiently large reference population within the lines. The lines differ genetically, and thus a multi-breed approach has to be tested before it can be recommended. This could be a chance to implement genomic selection against feather pecking in the poultry breeding industry.

4 Ethical Aspects

Finally, the decision for a selection against feather pecking in poultry breeding programs, either traditional or genomic, is not only a question of cost but of ethics. Of course it will need investments to implement hard-to-measure-traits in breeding programs but at some point, there should simply be no excuse when it comes to animal welfare. Fernyhough et al. (2020) discussed in detail the ethics of laying hen genetics. They extended the well-known sustainable triangle which visualizes the three pillars of sustainability, i.e. ecology, economy and social by a fourth pillar called animal welfare and made it a sustainable square using the example of egg production. They emphasized thereby that the consideration of animal welfare is one of the basic requirements in order to act in the sense of sustainability and thus intergenerationally equitable. If animals are used for the purpose of human nutrition, it should at least be assured that they experience a life without unnecessary stress, suffering or harm. As it is known that it is possible to select against feather pecking as a harmful behavior, it should be done. Breeding companies could develop a new layer line which then can be advertised as e.g. robust with higher welfare and take on a pioneering role in their industry (Fernyhough et al., 2020). This

might as well be a chance for a new breeding company to position itself on the market as it is predictable that animal welfare will become more important in future livestock.

In addition to the breeding companies, farmers also have to play their part as it is well known that feather pecking is influenced by several environmental factors (Nicol et al., 2013; Rodenburg et al., 2013) and can thereby be reduced by adjusting housing or feeding conditions of laying hen flocks (van Hierden et al., 2004; Lambton et al., 2013).

The consumers are as well responsible for making their contribution by being willing to pay more money for staple foods and above all animal products which have been produced sustainably. It could already be shown in a study of Bennett et al. (2016) on British consumers, that they are willing to pay more money for eggs from hens which have experienced fewer reductions in animal welfare due to less feather pecking after they were educated of the feather pecking problem in commercial egg production. However, it is well-known that there is a difference between what people answer in survey studies and how they actually behave in reality. Therefore, it cannot be assumed that after an appropriate education of the consumers, the purchase decisions solely turn out for the well-being of animals.

Another important key in the supply chain of food is the retail food industry. On the one hand, the oligopolistic structure of this industry is part of the problem, as there is a price war between them over food products like for example butter, milk, meat or even eggs. This contributes to a low financial appreciation of food, which in turn has a negative effect on food production like insufficient animal welfare. On the other hand, the food retail industry uses their market power in positive ways as for example in Germany, they support alternatives to killing the male brothers of laying hens. When a leading company in food retailing starts doing so, it puts pressure on its competitors and can thus influence the supply on the market.

Finally, politics has to play its part as well in achieving positive change by adopting regulations and laws based on the latest scientific findings and ethical discussions.

At the end, all stakeholders considered here involved in egg production and consumption must share the cost and do their part whereas the poultry industry must be aware of their responsibility as a nearly monopolistic industry, to bring the appropriate product of low feather pecking hybrid layers to the market. Possible other incentives are governmental regulations to oblige the reduction of feather pecking as well as subsidies to initiate the start of breeding against feather pecking.

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Iffland, H., Wellmann, R., Preuß, S., Tetens, J., Bessei, W., Piepho, H.P., Bennewitz, J. A Novel Model to Explain Extreme Feather Pecking Behavior in Laying Hens. *Behavior Genetics* **2020**, *50*, 41–50. https://doi.org/10.1007/s10519-019-09971-w.

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Lutz, V., Kjaer, J.B., Iffland, H., Rodehutscord, M., Bessei, W., Bennewitz, J. Quantitative genetic analysis of causal relationships among feather pecking, feather eating, and general locomotor activity in laying hens using structural equation models. *Poultry Science* **2016**, *95*, 1757-1769. http://dx.doi.org/10.3382/ps/pew146.

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CONFERENCE CONTRIBUTIONS

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