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Mitochondrial haplotypes, gene expression and nuclear diversity in two strains of laying hens

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List of Abbreviations

ATP	Adenosine triphosphate
LB	Lohmann brown classic
LSL	Lohmann LSL-classic
MIOX	*myo*-inositol oxygenase
mRNA	Messenger RNA
numt	Nuclear mitochondrial DNA sequences
Ca	Calcium
$InsP_6$	Phytic-acid
OXPHOS	Oxidative phosphorylation
P	Phosphorus
qPCR	Quantitative polymerase chain reaction

List of included Publications

Heumann-Kiesler C., Sommerfeld S., Iffland H., Bennewitz J., Rodehutscord M., Hasselmann M (2021) Insights into the Mitochondrial and Nuclear Genome Diversity of Two High Yielding Strains of Laying Hens. *Animals*, **11**(3), 825, https://doi.org/10.3390/ani11030825

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1. General Introduction

Since the domestication of chickens around 5400–8000 BC in Asia (International Chicken Genome Sequencing Consortium, 2004; Nishibori et al., 2005) they have played various and crucial roles in economy, socio culture and the sustaining livelihoods across the world for thousands of years (Kryger et al., 2010). Their domesticated form (*Gallus gallus domesticus*) originated from the red jungle fowl (*Gallus gallus*) (Darwin, 1868) in hybridization with several other species (Eriksson et al., 2008) and serves as one of the main model organisms for avian species (International Chicken Genome Sequencing Consortium, 2004). As the most popular and widely distributed domestic fowl worldwide (Lan et al., 2015), the domestic chicken provides humans with high-quality protein in form of meat and eggs (FAO, 2007). With the growth of the global human population, an accompanying increased need of food is expected. This includes an increase in egg and meat production from poultry, which already is the fastest growing sector in the past decades (FAO, 2009; Mottet & Tempio, 2017). Meat quality and safety have thus been investigated in scientific research for decades, including increasing importance of animal welfare and muscle morphology (Grashorn, 2010). The ongoing demand for a higher and faster production of meat and eggs has resulted in several hundred distinct breeds worldwide, including broilers and layers. These varieties outperform their wild ancestors in meat and egg production, respectively (Qanbari et al., 2019). However, it is known that the breeding for economically beneficial traits such as the growth of broilers is leading to welfare problems (Bessei, 2006), as well as different housing systems can lead to detrimental behaviour such as feather-pecking and other welfare problems in laying hens (Underwood et al., 2021).

This work is embedded in the DFG-funded research unit P-Fowl (“Inositol phosphates and *myo*-inositol in the domestic fowl: Exploring the interface of genetics, physiology, micro-biome, and nutrition”) with the overarching goal to understand the processes within the animals in the context of Phosphorus (P) and Calcium (Ca) utilization.

Phosphorus is an essential element for all living organisms (Elser, 2012) and must be supplied continuously to maintain health. It is mostly available as rock phosphate (Cordell et al., 2009) and accumulated in only a few countries. In addition, rock phosphate is a finite resource, and the deposits could be exhausted within the next 50-100 years (Cordell et al., 2009; Smil, 2000; Steen, 1998). 90% of the global

supply of rock phosphate is used in agriculture as fertilizer (Gross, 2010) or feed supplement (Rothwell et al., 2020). The increasing demand to maintain food availability for a growing population, together with a simultaneously decrease in its availability, makes P utilization one of the greatest challenges of sustainable food production (Gross, 2010; Neset & Cordell, 2012).

In the feed of poultry, P is naturally provided as phytic-acid ($InsP_6$), which can be degraded in limited amounts by the animals (Eeckhout & De Paepe, 1994; Rodehutscord et al., 2016). Due to the high need of this mineral, P derived from rock phosphate or P degrading enzymes (phytases) are added to the feeds. Previous studies have shown that endogenous $InsP_6$ degradation is reduced in broilers when mineral P and Ca are supplemented (Shastak et al., 2014; Sommerfeld et al., 2018; Zeller et al., 2015). In laying hens, Ca is an additional essential mineral (Ahmadi & Rodehutscord, 2012) which is essential for egg shell formation and an interaction of P and Ca content regarding egg-shell quality and quantity of eggs has been shown (Härtel, 1990). Besides the differences in egg quantity and quality of different strains of laying hens (Singh et al., 2009), significant differences in $InsP_6$ degradation between two strains have been shown, too (Abudabos, 2012).
P-Fowl has the objective to understand the processes within the animals from different perspectives including genetics, microbiota, physiology and nutrition instead of breeding (Beck et al., 2016) and the improvement of supplements in the feed (Collins & Sumpter, 2007). As part of P-Fowl this work focusses on mitochondrial genetics and links these findings with performance and gene expression data. In this study, two commercially established strains were used: Lohmann brown classic (LB) and Lohmann LSL-classic (LSL) white leghorn hybrids supplied by Lohmann Tierzucht (Cuxhaven, Germany). Using these strains has several advantages: Both lines are genetically distinct, while they are bred for the same purpose of egg laying and performance data (e.g. eggs per hen, egg mass and weight including body weight and feed consumption) is available from the company, as well as pedigree information.

Mitochondria are commonly known as the powerhouse of the cell, because they provide 90% of the cellular energy in eukaryotes (Marchi et al., 2012). The energy is stored as adenosine triphosphate (ATP) during the process of oxidative phosphorylation (OXPHOS). This process is highly depended on P availability (Elser,

2012) and is also influenced by its cellular concentration (Bose et al., 2003). Their indispensability for eukaryotes and relationship to the availability of P and Ca (Gellerich et al., 2010) as well as their influence on the individuals fitness (Burton et al., 2013) and phenotype (Bevers et al., 2019; Kinoshita et al., 2018) makes mitochondria an important part of the complex framework of the overall project.

Due to their relative high copy number (Shadel & Clayton, 1997), simple molecular structure (Lan et al., 2015), mostly maternal inheritance (Ladoukakis & Zouros, 2017) and presence in nearly all eukaryotic cells (Gray et al., 1999) the mitochondrial genome traditionally serves as a popular molecular marker in population genomics (Harrison, 1989). Due to their endosymbiont origin (Gray et al., 1999) the nuclear and mitochondrial genome need to interact to maintain mitochondrial functionality (Poyton & McEwen, 1996) and thus, the genes of nuclear encoded components and the nuclear genome are co-adapted into mito-nuclear genotypes (Sloan et al., 2017). Although the number of sequenced mitochondrial genomes increased during recent years (Smith, 2016), including domesticated animals such as goats (Colli et al., 2015), horses (Achilli et al., 2012) and pigs (G. S. Wu et al., 2007) the number of studies illuminating the mitochondrial genome of chickens are rather seldom and most studies focus on partial or gene encoding sequences (Lan et al., 2015). Previous studies have shown, that mitochondrial haplotypes and genome diversity has an influence on high altitude adaption in Tibetan chicken (Sun et al., 2013), on the animals resistance to disease, and agricultural important traits such as body weight and egg quality (Li et al., 1998).

In all functionally active organisms, the information encoded in the DNA is transcribed to messenger RNA (mRNA) and then translated into protein sequences to form the different components of the cell. Under changing conditions and during different phases during their life and developmental stages, the expression of mRNA changes. These expression changes may differ between different organisms and can be related to mutations in the DNA or unrelated to them, which makes gene expression analysis an important and commonly used tool to understand organismic function.

Previous studies have shown, that mitochondrial gene expression reacts to energy deficiency (Jäger et al., 2007), nutrient availability (Berdanier, 2006), differs between tissues (Blake et al., 2020) and is declining with age (Anderson & Prolla, 2009; Manczak et al., 2005). In addition, the stimuli from the environment have to be

passed on to the independent mitochondrial genome (Anderson & Prolla, 2009) and the expression of both, nuclear and mitochondrial gene products needs to be coordinated (Scarpulla, 2011; Woodson & Chory, 2008).

2. Overview of the included studies

The overarching objective of this thesis is to obtain a deeper understanding of the links between mitochondrial genetics, gene expression and physiology in the contexts of P utilization and the productive life span in laying hens.

The work is based on two animal experiments, which are described in (Sommerfeld et al., 2020a and Sommerfeld et al., 2020b.The first experiment included 100 animals of five different life stages, ranging from 10 to 60 weeks of age. In the second experiment 80 individuals were distributed at 27 weeks of age into four groups fed diets of different P and Ca content for four weeks. In both experiments an equal number of LB and LSL hens were used.

The first step was to analyse the mitochondrial genome of all 180 individuals to identify mitochondrial haplotypes (Manuscript 1, Heumann-Kiesler et al., 2021). The work included DNA extraction, amplification and NGS sequencing using Illumina technology (Illumina Inc., San Diego, CA, USA), the pre-processing of the raw reads and the reconstruction of the mitochondrial haplotypes as well as analyses of population structure. In addition, the identified haplotypes were combined with performance data such as body weight and P utilization and analysed statistically. After the identification of an unexpectedly low number of haplotypes, an analysis of the nuclear genetic background using genotype data obtained with an Illumina 60K chicken Infinium iSelect chip (Illumina Inc., San Diego, CA, USA) was included to investigate the overall genetic diversity of the included lines and relationships within and between mitochondrial haplotypes.

In Manuscripts 2 and 3 mitochondrial-linked gene expression was investigated focussing on different life stages and diets separately. The same approach was used for both manuscripts: High throughput quantitative polymerase chain reactions (qPCRs) on mitochondrial encoded and mitochondria linked genes were performed on five different tissues from each hen. For this approach both experiments were analysed separately using the Pfaffl method (Pfaffl, 2001) because this method includes individual primer amplification efficiencies, which enhances accuracy in the analysis, especially when many different genes are included. The calculated relative gene expression values were then evaluated statistically using a linear mixed model. The linear mixed model was chosen, because it is the best way to include multiple factors such as the paternal background and the individuals effect itself in the

analysis, especially in a complex network of interacting factors. In addition, hierarchical cluster analyses were performed, to determine and visualize patterns and differences over the whole range of included genes, tissues and strains.

Manuscript 2 focusses on the differences between the different life stages and is the first large-scale study in laying hens focussing on mitochondrial linked gene expression in the context of the productive life span. The main goal was to obtain insights into changes during growth and especially between the phases of growth, egg laying and declining egg laying, which require changes in the whole metabolism of the hens.

In Manuscript 3 the goal was to detect the effects of P and Ca concentrations in the diet on gene expression. The set of genes used in Manuscript 2 was expanded and focused more on differences between both lines but also included the response to the different dietary conditions on the molecular level to detect potential ways the hens are adapting to the changes in nutrient availability.

In combination the manuscripts provide a comprehensive overview about mitochondrial diversity in laying hens in combination with the functional aspects of development during the productive life span and the reaction to dietary Ca and P concentration. The results are complemented with performance and nuclear genetic analyses for integration into a wider context.

3. Included Manuscripts

3.1 Insights into the Mitochondrial and Nuclear Genome Diversity of Two High Yielding Strains of Laying Hens

animals

MDPI

Article

Insights into the Mitochondrial and Nuclear Genome Diversity of Two High Yielding Strains of Laying Hens

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Simple Summary: Mitochondria are commonly known as "the powerhouse of the cell", influencing the fitness, lifespan and metabolism of eukaryotic organisms. In our study we examined mitochondrial and nuclear genomic diversity in two high yielding strains of laying hens. We tested if the mitochondrial genome affects functional traits such as body weight and phosphorus utilization. We discovered a surprisingly low mitochondrial genetic diversity and an unequal distribution of the haplotypes among both strains, leading to limitations of robust links to phenotypic traits. In contrast, we found similar levels of nuclear genome diversity in both strains. Our study explores the potential influence of the mitochondrial genome on phenotypic traits and thus contributes to a better understanding of the function of this organelle in laying hens. Further, we focus on its usefulness as a genetic marker, which is often underestimated in breeding approaches, given the different inheritance mechanism compared to the nuclear genome.

Abstract: Mitochondria are essential components of eukaryotes as they are involved in several organismic key processes such as energy production, apoptosis and cell growth. Despite their importance for the metabolism and physiology of all eukaryotic organisms, the impact of mitochondrial haplotype variation has only been studied for very few species. In this study we sequenced the mitochondrial genome of 180 individuals from two different strains of laying hens. The resulting haplotypes were combined with performance data such as body weight, feed intake and phosphorus utilization to assess their influence on the hens in five different life stages. After detecting a surprisingly low level of genetic diversity, we investigated the nuclear genetic background to estimate whether the low mitochondrial diversity is representative for the whole genetic background of the strains. Our results highlight the need for more in-depth investigation of the genetic compositions and mito-nuclear interaction in individuals to elucidate the basis of phenotypic performance differences. In addition, we raise the question of how the lack of mitochondrial variation developed, since the mitochondrial genome represents genetic information usually not considered in breeding approaches.

Keywords: haplotype; phosphorus utilization; body weight; genetic diversity; relatedness; population structure; genotyping

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1. Introduction

The domestic chicken is the most popular and widely distributed domestic fowl worldwide [1] and hence it is a stable source of protein, including meat and eggs [2]. In contrast to the advances made by high-throughput nuclear genotype analyses in chicken [3,4], the insights into mitochondrial (mt) genome diversity are rather sparse. For many animals it

is known that mt genome diversity can have a remarkable impact on a variety of traits, such as meat quality in pigs [5] and the metabolic capacity of dairy cows [6]. In chickens, previous studies have shown that mutations in the mitochondrial genome can have strong physiological effects. This includes the adaptation to high altitudes of the Tibetan chicken [7,8] and economically important traits such as egg quality and body weight [9]. Even though the whole mitochondrial genome has been used in many studies of domesticated animals such as goats [10], horses [11] and pigs [12], most studies of the chicken relied on partial mitochondrial sequences or the protein coding sequences [1] and thus, studies covering the whole mt genome are underrepresented. Given the importance of the genetic background on organismic physiological performance, beside the nuclear genome, the in-depth study of mitochondrial genomes and their variation are of utmost interest.

Mitochondria are commonly known as the powerhouse of the cell since they contribute to the energy metabolism by generating ATP [13]. During the process of oxidative phosphorylation (OXPHOS) they are a major producer of reactive oxygen species (ROS) and thus contribute to oxidative stress [14]. Therefore, the mitochondrial energy metabolism is directly linked to the availability of phosphorus (P).

Additional to their role as the cellular energy supplier, mitochondria contribute to several other key cellular processes such as the biosynthesis of important macromolecules [13], calcium (Ca) homeostasis [15] and ageing [16] that have been mainly studied within genetic model organisms such as fruit flies or mice [17,18].

Mitochondria carry their own genome, which in chickens is approximately 16 kb long and contains 13 protein coding genes, 22 tRNA genes, two rRNA genes and one regulatory control region [19]. Due to their maternal inheritance, simple molecular structure [1] and relative high copy number [20] they traditionally serve as popular genetic markers in molecular biology [21].

As all living organisms, chickens need P for their growth, health and energy metabolism [22]. For chickens, P in plant-based feedstuff is mainly present as phytic-acid ($InsP_6$) [23,24], which consists of six phosphate groups and one *myo*-inositol ring. While passing through the digestive tract, stepwise $InsP_6$ dephosphorylation results in the release of phosphate and *myo*-inositol [25]. However, the ability of poultry to degrade $InsP_6$ is limited. Feed supplements produced from rock phosphates maintain the P supply. The availability of rock phosphate is finite [26], which makes reducing its use one of the major challenges in food production.

To improve P utilization efficiency, we need to understand the genetic and nongenetic background for the processes influencing the formation of inositol derivates during $InsP_6$ degradation and their relevance for P utilization. It is furthermore important to understand how these processes change over time, since the P demand changes during a chicken's lifetime, depending on factors such as growth or egg laying [27].

We aim to understand the mitochondrial genetic variation in laying hens and hypothesize that different haplotypes lead to physiologically distinct phenotypes. To study this impact, we obtained whole mitochondrial genomes from a large experimental setup of laying hens that had the abovementioned aspects, P and Ca utilization and phytate degradation in focus (Sommerfeld 2020a, 2020b [27,28]). We analyse the body weight and feed intake and hypothesize that the performance of individuals with different mitochondrial haplotypes differs within and between production periods according to the animals' needs. Thus, we can gain insights into the connection between the genetic variation of mitochondrial genomes and their physiological effects and contribute to a better understanding of these effects in laying hens. We enriched our data by nuclear genetic information of a subset of individuals that enable us to gain important insights into the nuclear genetic structure of the population and potential effects of nuclear and mitochondrial interactions [29–31].

2. Materials and Methods

2.1. Animal Experiments

The animal experiments were performed at the Agricultural Experiment Station of the University of Hohenheim, Germany. They were approved by the Regierungspräsidium Tübingen, Germany (Project no. HOH50/17TE) in accordance with the German Animal Welfare Legislation.

We used 180 laying hens: 90 brown (Lohmann Brown Classic) and 90 white (Lohmann LSL-Classic) leghorn hybrids obtained from Lohmann Tierzucht GmbH (Cuxhaven, Germany). The hens originated from two experiments addressing the changes of utilization of P and Ca in different periods of the hens' life (production periods, experiment 1) and under adequate or reduced P and Ca supply during the peak of egg production (experiment 2). The first experiment provided 100 individuals and is described in detail in Sommerfeld et al. 2020a [27]; the second one provided 80 animals and is described in Sommerfeld et al. 2020b [28]. We briefly summarize them in the following.

In experiment 1 (Figure 1), the animals were raised as one group in floor pens on deep litter bedding, with diets according to the specific requirements in each period based on soybean and corn meal, but no difference to the recommendations as given in detail within Table 1 in Sommerfeld et al. 2020a [27]. After 8, 14, 22, 28, and 58 weeks 10 hens per father and strain were chosen and moved into the metabolism units (1 m × 1 m × 1 m, arranged in a randomized block design, where 2 units formed one block, each metabolism unit contained one individual) where after five days of rest excreta and feed intake was measured for four days for each individual. Feed was available for ad libitum consumption and no changes in the diet were made. The animals were weighed and after two days of rest the hens were killed by decapitation following anaesthesia using a gas mixture [32] and samples of blood and liver tissue were taken, thus the hens were 10, 16, 24, 30, and 60 weeks old. In the following the five sampling points will be named as period 1 to period 5.

For experiment 2 (Figure 1), the animals were raised together under the same conditions as in experiment 1. After 27 weeks hens from 20 fathers (10 per strain) were separated into four groups in which each group contained one hen per father and the hens were placed into the metabolism units (1 m × 1 m × 1 m, arranged in a randomized block design, where 8 units formed one block and each metabolism unit contained one individual). For three weeks the individuals received feed for ad libitum consumption with specific diets for each group based on soybean and corn meal, containing recommended or lower concentrations of Ca and P (Table 1). The excreta of each hen were collected and the feed intake was monitored for four days in week 30. The hens were killed at 31 weeks of age (peak of egg production). As in experiment 1 blood and liver tissue were sampled.

Table 1. P and Ca concentrations in the four diets of experiment 2. Values from Table 1 in Sommerfeld et al. 2020 [28]. Recommended concentrations are labelled with +, reduced concentrations with −.

Ingredient g/kg	P−Ca−	P−Ca+	P+Ca−	P+Ca+
Total P, g/kg DM [1]	4.7	4.7	5.3	5.3
Ca, g/kg DM [1]	33.9	39.6	33.9	39.6

[1] Dry mass.

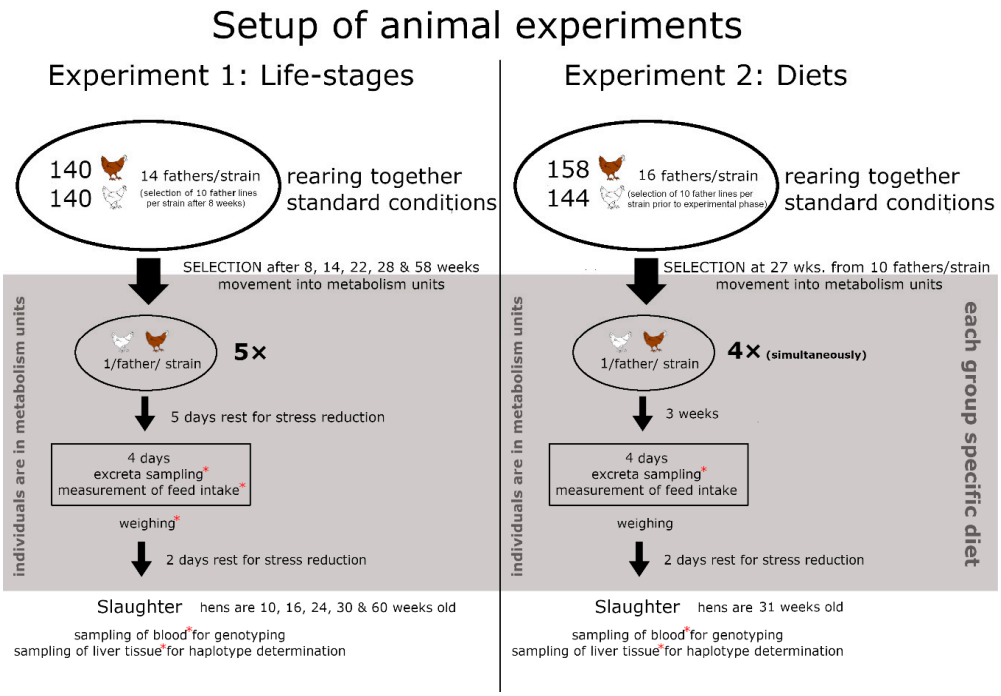

Figure 1. Schematic description of the setup of both animal experiments. Red asterisks mark material and measurements that were used in this study.

2.2. DNA Samples and Extraction

We extracted DNA twice per animal: from liver tissue for the haplotype analyses and from blood for genotyping. For the haplotype reconstruction all 180 individuals were used, while for the nuclear genotyping 52 samples from individuals of the first experiment (26 brown and 26 white, covering period 1 to period 4) and 55 from the second experiment (27 brown and 28 white, covering all four diets) were included. The animals for the genotyping were selected randomly; care was taken to include an even amount of brown and white animals.

Liver samples were taken after slaughtering as described above and directly transferred to dry ice. We extracted high molecular DNA from frozen tissue using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the purification of total DNA from Animal Tissues protocol.

For genotyping, the DNA from 53 brown and 54 white hens was extracted from blood which was directly taken at the sampling sessions as described above, stored in EDTA and further processed using the Maxwell 16 Blood DNA Purification Kit on a Maxwell 16 MDx instrument (Promega, Madison, WI, USA).

2.3. Analysis of Mitochondrial Haplotypes

2.3.1. Long Range PCR and Next Generation Amplicon Sequencing

The mt genome was individually amplified by two overlapping fragments of 8935 bp and 9522 bp length using a Long Range (LR) PCR Kit (Qiagen, Hilden, Germany). Primers were designed and tested if they amplify other fragments of the same size using Primer BLAST [33]. Primer sequences for fragment 1 are: F: ACGCCACAGCTAAAACCCCCAGC R: TGGATGGTGGAGAGGCGGTTGT and fragment 2: F: TCCTGCTTGCCCTCCCCTCCCT R: CGCACCCGCACTGTGAAGGGCAA. The reactions were set up according to the protocol of the manufacturer with primer annealing temperatures of 63 °C for fragment

1 and 60 °C for fragment 2. The elongation phase was set to 9 min for both fragments. Successful amplifications were verified via gel electrophoresis on a 1% agarose gel (VWR International GmbH, Darmstadt, Germany) stained with gel-red (Biotium Inc., Fremont, CA, USA). The PCR products were precipitated with ethanol; quality and quantity of the products were measured using a NanoDrop 2000/2000c Spectrophotometer and Qubit Fluorometer (Thermo Fisher Scientific Inc., Waltham MA, USA). Resulting purified fragments were equimolarly pooled for sequencing. The samples were sequenced by CeGaT (CeGaT GmbH, Tübingen, Germany), using the Nextera XT Kit (Illumina Inc., San Diego, CA, USA) for library preparation, on an Illumina NovaSeq 6000 MiSeq expecting 150 bp paired-end reads.

2.3.2. Sequence Analysis

The sequencing reads were demultiplexed and adapter-trimmed by CeGaT using Illumina bclfastq (v2.20; Illumina Inc., San Diego, CA, USA) and Skewer (Version 0.2.2 [34]), followed by a quality-check in FastQC [35] of files containing comparatively high and low numbers of reads. After the removal of primer sequences with Cutadapt (version 2.5 [36]) the reads were quality-trimmed in Trimmomatic (version 0.32 [37]) using the following parameters: SLIDINGWINDOW:4:15 MINLEN:50 CROP:148. The used settings cut the read if the average quality of 4 bases is below 15, discard reads which are shorter than 50 bases and cut the reads that are longer than 148 bases.

We mapped the reads against a published white leghorn mitochondrial genome (AP003317 [38]) using the algorithm implemented in Geneious Prime (version 2020.0.2 https://www.geneious.com, accessed on 5 November 2019). Duplicates were removed before mapping using the Dedupe Duplicate Read Remover 38.37 by Brian Bushnell as implemented in Geneious Prime using default parameters, as well as the variant call with default settings except for minimum coverage, which was set to five. The resulting output was then used to create consensus sequences using GATK FastaAlternateReferenceMaker [39].

The resulting consensus sequences included a poly-C site with 8 to 13 Cytosins (position 3940 and following). The site did not only vary in its length without a pattern between individuals but also within the reads of most individuals. The issue could not be resolved as in both sequencing approaches (Illumina and Sanger-technology) these sites led to read termination. Consequently, we followed the most conserved approach and restricted the length down to 8 Cytosins for all individuals.

For those individuals that showed coverage close to zero in one of the two PCR fragments, the missing parts were sequenced using a PCR based Sanger-sequencing approach. A list of the corresponding individuals, used primers, reagents and PCR protocol can be found in Tables S1–S3, Supplementary Materials. Sanger-sequencing reactions were performed by Microsynth AG (Balgach, Switzerland) and the resulting sequences were edited in Geneious Prime.

2.3.3. Validation of Sequencing Results

To approve the resulting consensus sequences, the primer- and quality-trimmed reads were additionally mapped against a more distant mt genome to the leghorn variant (*Gallus gallus*, NCBI Accession AP003322, [40]) using the same parameters as described above.

To exclude LR-PCR biased artefacts, we resequenced the whole mt genome of one individual with Sanger-technology using mt DNA and a set of 22 oligonucleotides that result in 22 overlapping fragments. Furthermore, the long-range PCR fragments that were used for the library construction were Sanger sequenced using the same set of oligonucleotides (Supplementary Table S1).

2.3.4. Alignment and Haplotype Reconstruction

The consensus sequences were aligned using the implemented algorithm in Geneious Prime with default settings.

Haplotype networks were generated based on the alignment using the TCS algorithm [41] implemented in PopART v. 1.7 [42]. Additionally, a phylogenetic analysis was conducted via Maximum Likelihood, for which we initially identified the nucleotide substitution model that best fit to the data, based on the program Model Test implemented in MEGA X [43]. Based on the lowest Bayesian Information Criterion (BIC) score, the Hasegawa–Kishino–Yano model [44] was chosen for tree construction with 500 bootstrap replicates and default settings in MEGA X.

2.4. Analysis of Nuclear Genotype Data

After the identification of the mt haplotypes, we aimed to gain additional insights into the nuclear genome diversity and by analysing nuclear genotype data obtained with the Illumina 60K chicken Infinium iSelect chip (Illumina Inc., San Diego, CA, USA). For the following analyses we used 54 white and 53 brown individuals from both experiments.

2.4.1. Filtering

The resulting SNPs were filtered for cluster separation $\geq$0.4 in the GenomeStudio software (v. 2011.1, Illumina Inc., San Diego, CA, USA) and then exported for downstream analyses. Prior to the next filtering steps, the SNP positions were transferred to the newest chicken genome version (GRCg6a, Accession: GCF_000002315.6) and the two strains were separated using vcftools [45]. The SNPs were filtered in vcftools for missingness (95%), minor allele count (3) and minor allele frequency (0.03) prior to further analysis. For the population structure analysis with ADMIXTURE [46] Linkage disequilibrium (LD) pruning was performed after filtering as recommended using PLINK version 1.9 [47], where all SNPs with a R^2 value greater than 0.1 with any other SNP within a 50-SNP sliding window (advanced by 10 SNPs each time) is removed as described in the manual. The dataset was filtered without previous separation of the strains for additional ADMIXTURE analyses as described above.

2.4.2. Analysis of Nuclear Genetic Diversity

To gain insights into the nuclear genome diversity and relationships between individuals within the strains, a genomic relationship matrix (G-Matrix) was performed with the *AGHmatrix* [48] package in R (R Core Team 2020, Version 3.0.3) using the VanRaden method [49]. Note that the G-Matrix is usually used to study complex traits, but was used here to obtain some insight of genomic relationships. The two strains were analyzed separately, since we look at them as two distinct lines. After the calculation of the matrices, statistical analyses were performed in JMP Pro (Version 13. SAS Institute Inc., Cary, NC, USA, 1989–2019) to detect the impact of the paternal and possible maternal background and differences between the strains and mt haplotypes. The data was tested for normality using Shapiro–Wilk test. Wilcoxon/Kruskal–Wallis tests were used for the comparison of two groups and Steel–Dwass tests for multiple comparisons.

The differentiation between both strains was estimated using Weir and Cockerham's F-statistics fixation-index (F_{ST}) [50] as implemented in vcftools based on the dataset including both strains filtered together. To analyze the differentiation between the mt haplotypes the F_{ST} between the different haplotypes was estimated the same way based on the file only containing the brown strain.

To estimate population structure ADMIXTURE was used on (1) the merged data set obtained from both strains, and (2) separate data sets for each strain, to gain deeper insights into the strain structure. The number of ancestral populations (K) was set from 2 to 6 for all runs. The best K was estimated using cross validation as suggested in the manual.

2.5. Phenotypic Traits

We included measurements of phenotypic traits from the same individuals, following the overarching hypothesis that mt haplotype variation may impact phenotypic performances in laying hens.

2.5.1. Measurements of Body Weight, Feed Intake and Phosphorus Utilization

Body weight, feed intake and P utilization were measured for all hens in the first experiment ($n = 100$). Feed intake was calculated over the course of 4 days, by measuring the amount of feed in the beginning and the end of the excreta collection phase that is described in Sommerfeld et al. 2020a [27]. Body weight was measured on the last day of the excreta collection phase. P utilization was calculated as the proportion of intake, which was not recovered in excreta (based on quantitative data, the amount of remaining P in the excreta) for the same periods as feed intake was measured [27]. P utilization was additionally measured for all hens of experiment 2 and analyzed for both experiments separately.

2.5.2. Statistical Analyses

Statistical analysis of body weight and feed intake were performed for all individuals from experiment 1 while the distribution of mt haplotypes over the four diets in the second experiment was heterogeneous, limiting robust statistical tests for these individuals.

The overall impact of the haplotypes on body weight and feed intake was evaluated by a statistical model, derived from a linear mixed model developed by Sommerfeld et al. 2020a [27].

$$Y = \text{period} + \text{haplotype} + \text{block} + \text{block} * \text{period} + \text{metabolism unit} + \text{father} + \varepsilon \quad (1)$$

where Y is the response variable, ε is the residual error, period and haplotype are fixed effects, with block, metabolism unit and father as random effects. Statistical significance was declared at $p < 0.05$.

All modelling was performed in R (R Core Team 2019, Version 3.6.1) using the *lmerTest* package [51]. The interaction of period and haplotype was removed, since the model was rank-deficient due to the absence of one haplotype in period 5. To detect the overall influence of period and haplotype on the response variable, a three factorial analysis of variance (ANOVA) was used. A pairwise Tukey post hoc test (package *emmeans* [52]) was performed to detect differences between the haplotypes independent from the periods and between the periods independent of the haplotype.

To detect significant differences in body weight, feed intake and P utilization between the haplotypes within the periods (and for P utilization within the diets of experiment 2), pairwise Tukey–Kramer HSD or Steel–Dwass tests were performed using JMP Pro (Version 15. SAS Institute Inc., Cary, NC, USA, 1989–2019) after testing for normality using Shapiro–Wilk test.

3. Results

3.1. Analyses of Mitochondrial Haplotypes

3.1.1. Next Generation Sequencing

The sequencing resulted in an average number of 53,943 reads per DNA sample (min: 36,146, max: 76,180 reads) with a Q30 value of 91.42%. The mapped reads had a mean coverage of approximately 300×, with 0.02% of the nucleotides covered by less than 5 reads.

The validation via mapping the reads against the more distant reference genome resulted in consensus sequences identical to those obtained from the mapping to the white leghorn genome. Further, the Sanger resequencing from individual 23,676 mtDNA and LR-PCR fragments led to identical sequences. Thus, both validation approaches showed that the resulting sequences are not biased by bioinformatics or LR-PCR errors.

3.1.2. Reconstructed Mitochondrial Haplotypes

We reconstructed the mt genomes of 180 individuals and identified 13 segregating sites in the aligned data set. Eight sites are located in nonprotein-coding regions (control region, tRNA-Phe and rRNA) and five sites in protein coding genes (Cytochrome oxidase subunit II (COII), NADH-ubiquinone oxidoreductase subunit 4 and 5 (ND4 and ND5),

and Cytochrome B (CytB)) (Table 2). Except one SNP that results in an amino acid change from Serine to Glycine in the ND4 gene (position 12689), all SNPs were silent mutations. The mutations in the control region are located outside of the promoter region (Lan et al. 2015 [1], L'Abbee et al. 1991 [53] for exact positions of this region).

Four clearly distinct mt haplotypes were discovered: Surprisingly, all individuals of the white strain share the same haplotype whereas the brown strain comprises three haplotypes (Figure 2A, Table 2). There was no variation within each of the single haplotypes, except for one brown individual (B2_A). The B2_A individual appeared to be heterozygote on position 686 in both, the Illumina and Sanger-sequencing approach, and thus appears as a single individual in both trees and haplotype networks (Figure 2).

Table 2. Overview of the Single Nucleotide Polymorphism (SNPs) of the deduced four haplotypes with number of individuals (*n*), gene/region, position and corresponding nucleotide.

Haplotype		**B1**	**B2**	**B2_A**	**B3**	**W**
n		**13**	**26**	**1**	**51**	**90**
Gene	**Position**					
Control Region	199	C	T	T	T	T
	222	A	G	G	A	A
	243	C	C	C	C	T
	256	T	C	C	C	C
	330	C	T	C	C	C
	342	A	A	G	G	A
	686	G	G	A	A	A
	859					C insertion
$tRNA^{Phe}$	1297	T	C	C	T	T
rRNA	1526					A insertion
COII	8788	C	C	C	C	T
ND4	12013	T	T	T	T	C
	12689	A	G	G	G	G
ND5	13232	A	G	G	A	A
CytB	15840	T	T	T	T	C

Abbreviations: rRNA (Ribosomal ribonucleic acid), COII (Cytochrome oxidase subunit II), ND4 and ND5 (NADH-ubiquinone oxidoreductase subunit 4 and 5), CytB (Cytochrome B).

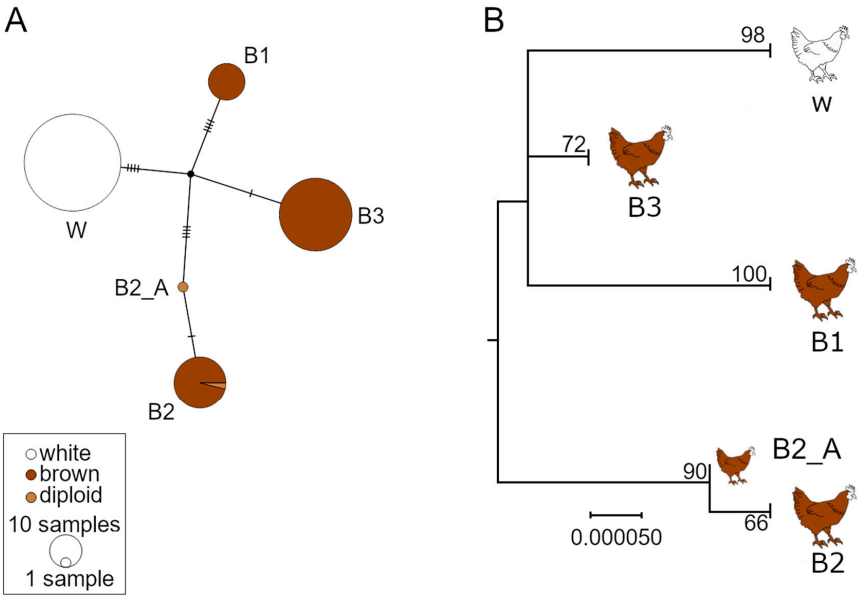

Figure 2. Evolutionary relationship of the mitochondrial genome of laying hens. (**A**) Haplotype network (TCS algorithm) and (**B**) maximum likelihood tree based on the mitochondrial genome (16,784 bp) of 180 laying hens. The tree is based on Hasegawa–Kishino–Yano model with 500 bootstrap replicates and includes all sites.

3.2. Haplotype Distribution among Different Periods and Diets

Since the individuals for the experimental phase were chosen randomly out of a bigger group, without previous genotyping, the haplotypes are not equally distributed throughout the five sampling points (experiment 1) or four diets (experiment 2). Only one individual of haplotype B1 occurred in the first period and none occurred in the last period of experiment 1 (Table 3). In the experiment 2 only three individuals of haplotype B1 were included and the number and distribution of haplotype B2 was uneven as well (Table 4).

Equal sample distribution among groups is an essential part of statistical analysis and thus, the unequal distribution and low number of some mt haplotype influences downstream analyses. Nevertheless, the considerable difference between group B1 and B3 in both experiments might reflect the overall population structure of the brown strain.

Table 3. Number of individuals per haplotype and period obtained from experiment 1.

Haplotype/Period	1	2	3	4	5
B1	1	3	2	4	0
B2	4	3	3	2	4
B3	5	4	5	4	6
W	10	10	10	10	9

Table 4. Number of individuals per haplotype and diet obtained from experiment 2.

Haplotype/Diet	1	2	3	4
B1	1	1	0	1
B2	2	2	5	1
B3	7	7	5	8
W	10	10	10	10

3.3. Nuclear Genotype Data

After filtering for cluster separation, 53,412 SNPs were obtained that were reduced by 1533 SNPs as a consequence of the transfer to the new genome version, resulting in 51,879 SNPs that entered the next filtering steps. During the transfer, all SNP positions changed and 515 SNPs changed the chromosome. Details about remaining SNP numbers per filtering step are given in Supplementary Table S4 for each dataset. Remarkably, a high number of SNPs were identified to be in LD, an observation that is typically found in livestock populations, owing a small effective population size [54]. However, SNPs in LD were only removed for the ADMIXTURE analyses, given by the prerequirements of the algorithm [46], for the calculation of F_{ST} and the G-Matrices these SNPs were included.

3.3.1. Nuclear Diversity between and within the Strains

For the ADMIXTURE analysis of the merged data set (both strains), the algorithm estimated K = 2 as the best number of ancestors. However, K = 3 leads to a separation within the brown strain and rising K to 4 leads to a separation of the white strain, too (Supplementary Figure S1). We calculated a F_{ST} of 0.35 between the two strains, indicating a clear separation of the two strains, too.

If strains were separated, the ADMIXTURE analysis estimated 2 to be the best K for both strains. In both strains, some half siblings belong completely to one cluster and this structure does not break down when the number of K is raised, indicating that these individuals are very closely related to each other. The population structure does not seem to be equal to the mt haplotypes, with some brown individuals belonging equally to the same group, independent of the mt haplotype (Figure 3).

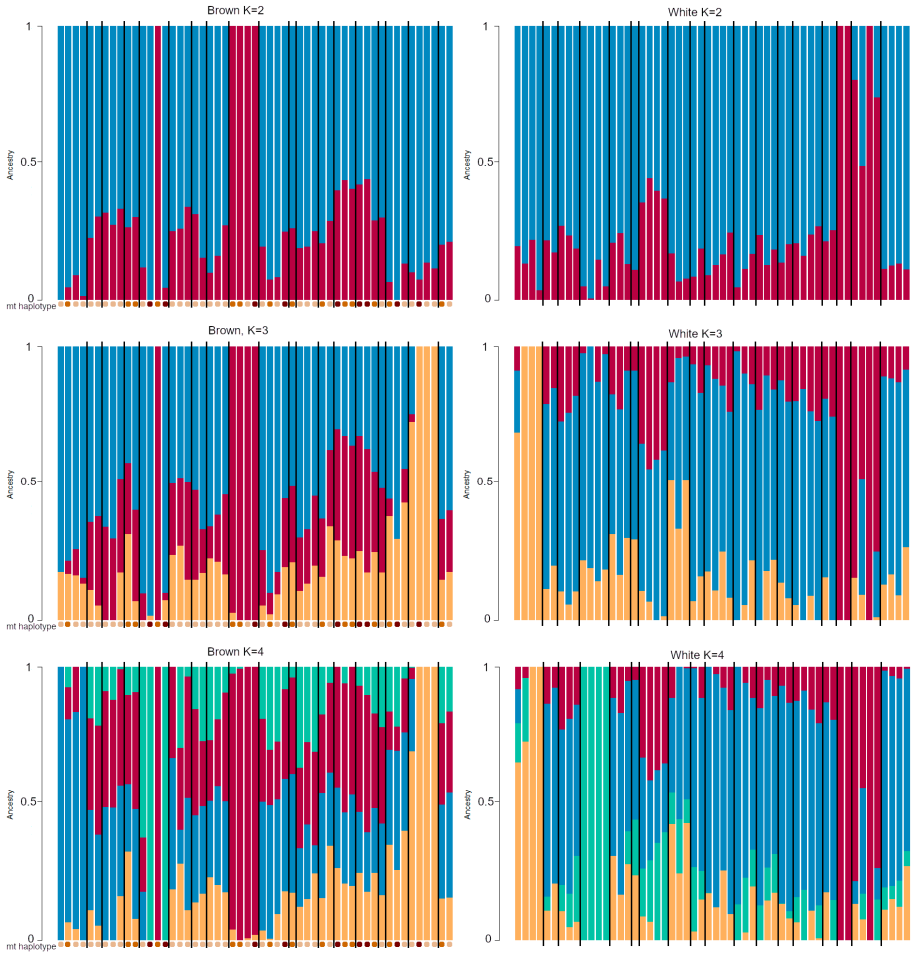

Figure 3. ADMIXTURE plots of the brown and white strain analyzed and filtered separately. Lines separate groups of individuals sharing the same father. Dots under the plots of brown individuals mark the different mt haplotypes.

3.3.2. Nuclear Diversity between Mitochondrial Haplotypes

The calculations of F_{ST}s between the different mt haplotypes of the brown strain were very low (Table 5). The data show that individuals with haplotype B1 are less differentiated from individuals with haplotype B3 on the nuclear level.

Table 5. F_{ST} values calculated between individuals with different mt haplotypes.

Haplotype Groups	F_{ST}
B1 vs. B2	0.00035
B1 vs. B3	0.0044
B2 vs. B3	0.0045

3.3.3. Individual Genomic Relationships

The genomic relationship (g) within the two strains did not differ, ranging from −0.1 to 0.34 in the brown and −0.09 to 0.35 in the white strain (Figure 4).

As expected, individuals sharing the same father (half siblings) have a significantly higher genomic relationship than individuals with different fathers (Table 6, Figure 4).

Table 6. Means of g and *p*-values of compared groups with different haplotype and relationship statuses.

Group 1	Group 2	Mean Group 1	Mean Group 2	*p*
Not related both strains	Half sibling both strains	−0.02	0.16	<0.0001
Not related white	Half sibling white	−0.02	0.16	<0.0001
Not related brown	Half sibling brown	−0.02	0.15	<0.0001
The following only tested in the brown strain				
Same haplotype	Diff. haplotype	−0.0086	−0.0016	0.008
Unrelated same hapl.	Unrelated diff. hapl.	−0.017	−0.022	0.0069
Half sibling same hapl.	Half sibling diff. hapl.	0.137	0.138	ns

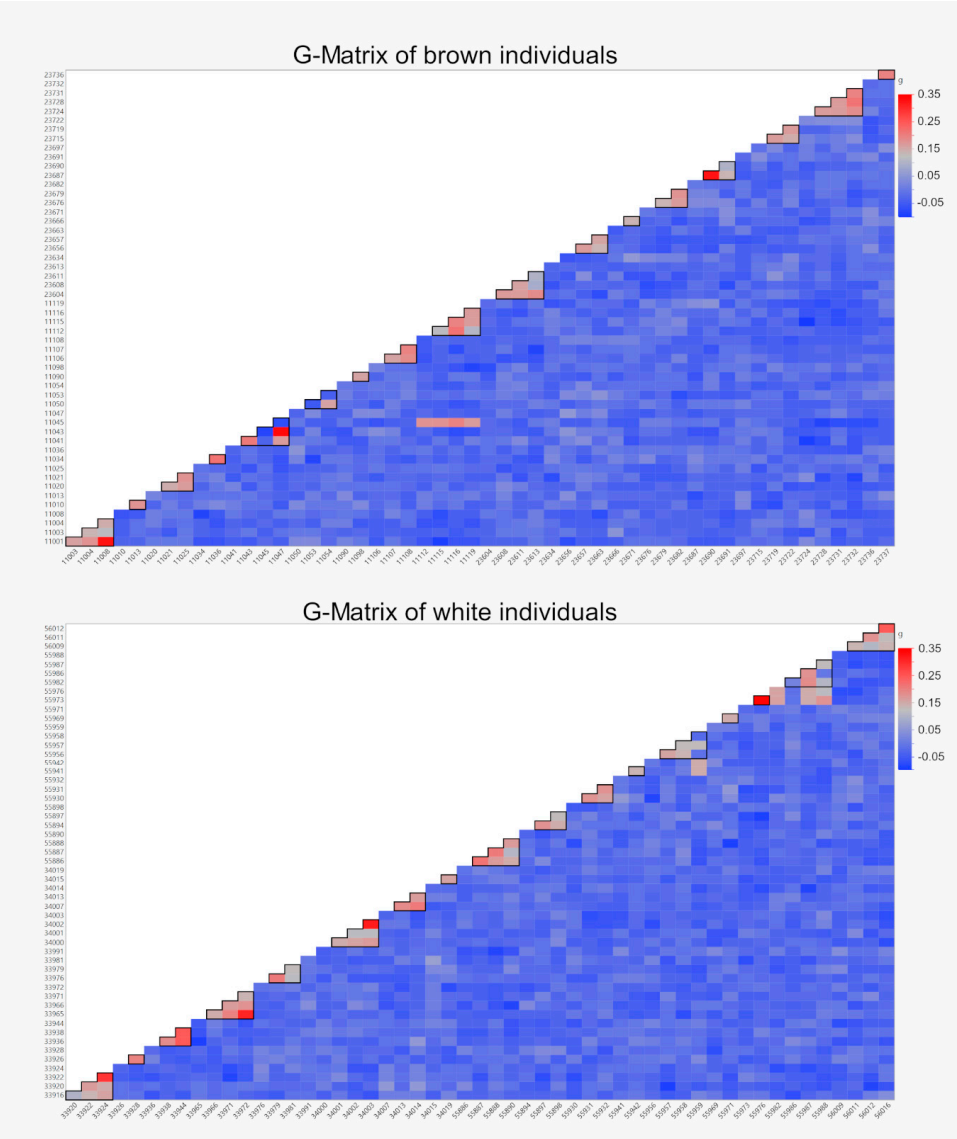

Figure 4. Heat maps of VanRaden G-Matrices for the brown and white individuals. Hens sharing the same father are marked (black lines), individuals are shown in the same order as in the ADMIXTURE plots. The elements of the diagonals are not shown.

Within the brown strain, individuals sharing the same mt haplotype are closer related than individuals with different mt haplotypes (Table 6), impacted by whether the individuals are half siblings or unrelated. It also became apparent that half siblings were always closer related than individuals with different fathers, independent of if they shared the same mt haplotype or not ($p < 0.0001$ in both cases).

3.4. Phenotypic Traits

3.4.1. Phosphorus Utilization

In experiment 1 the P utilization decreased from the first towards the third period, and increased afterwards for all haplotypes (Figure 5). The differences between the highest P utilization (period 1 and 5) to the lowest P utilization (period 3) were significant (Table A2).

The linear mixed model showed that P utilization is not influenced by the mt haplotype but by the period (Table A1). The influence of period was already shown by Sommerfeld et al. 2020a [27]. The pairwise comparison of the haplotypes independent of the period showed no significant difference (Table A2).

The pairwise tests of the haplotypes within each period showed no significant differences as well.

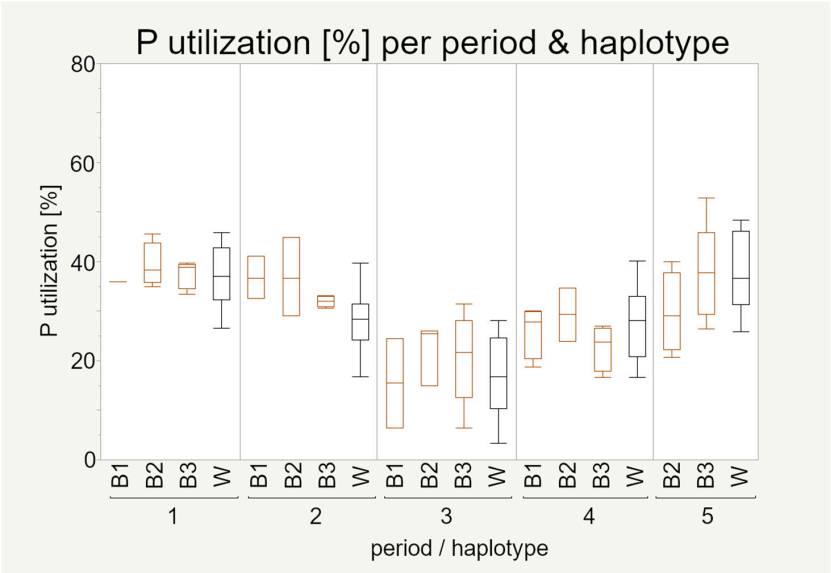

Figure 5. Phosphorus utilization (%) of 100 laying hens by haplotype and period (experiment 1). Boxes represent 50% of the data points (median with interquartile ranges) whiskers show minimum and maximum. Sample numbers are given in Table 3. Statistical significance was declared when $p < 0.05$. P utilization data were first studied by Sommerfeld et al. 2020a [27] in the context of strain differences.

In the context of different P and Ca concentrations in the diet (experiment 2), it was not possible to observe significant differences between the mt haplotypes, too (Figure 6). However, it became apparent that under high P concentrations individuals of mt haplotype B3 seem to have a higher P utilization than individuals of mt haplotype B2. The most notable observation is the scattering of the brown strain, which is higher than in the white strain under most conditions, even if the number of individuals in the white strain is higher. There were no significant differences between the different diets.

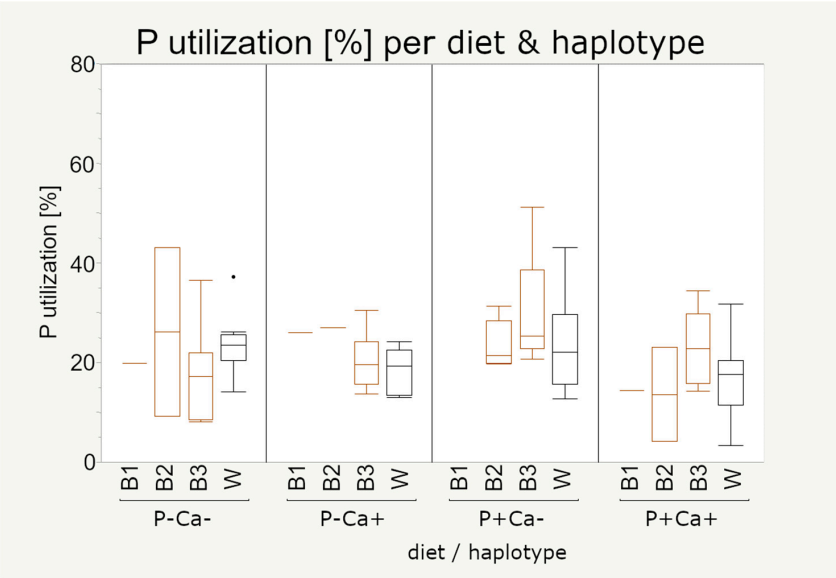

Figure 6. Phosphorus utilization (%) of 80 laying hens by haplotype and diet (experiment 2). Boxes represent 50% of the data points (median with interquartile ranges) whiskers show minimum and maximum. Sample numbers are given in Table 4. Statistical significance was declared when $p < 0.05$. P utilization data were first studied by Sommerfeld et al. 2020b [28] in the context of strain differences.

3.4.2. Body Weight

By implementing the multifactorial linear mixed model, there is evidence that the body weight is significantly influenced by both period and mt haplotype (Table A1). However, it must be noted that both strains were analyzed together and that this effect might originate from the high number of white individuals in the dataset rather that the mt haplotypes within the brown strain. Independent of the period, mt haplotype B2 and B3 have a significantly higher body weight than haplotype W, while there was no difference observed between W and B1 (Table A3).

The body weight increased from period to period for all mt haplotypes and the white strain accumulated less body mass than at least one brown haplotype in all periods (Figure 7). Notably, there was a tendency of lighter individuals carrying B3 haplotype at younger age (period 1, slightly in period 2 and 3) while at the later stages (period 4 and 5), this haplotype showed more variation, including lighter and heavier individuals compared to B2 individuals (Table A4).

Regarding differences between mt haplotypes within the periods, there were no differences except between mt haplotype B2 and W in period 2 and period 3, where individuals of haplotype B2 had a higher body weight (Figure 7). This is a difference between a specific group of brown individuals defined by their mt haplotype with individuals from the white strain, while the body weight of brown individuals with different mt haplotypes is not different to the body weight of the white strain.

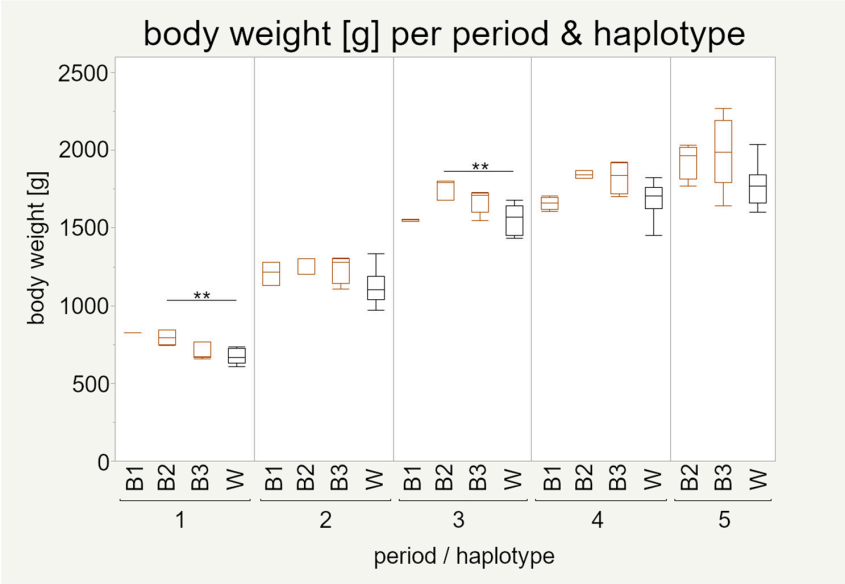

Figure 7. Pairwise Tukey–Kramer HSD or Steel–Dwass tests were used to test for significance within each period. Sample numbers are given in Table 3. Boxes represent 50% of the data points (median with interquartile ranges) whiskers show minimum and maximum. Asterisks indicate significance: ** $p < 0.01$. Body weight was first studied by Sommerfeld et al. 2020a [27] in the context of strain differences.

3.4.3. Feed Intake

The model showed that the overall feed intake was significantly influenced by period but not by the different haplotypes (Table A1). In the first two periods, in which the metabolism of the hens was focused on growth [27], the individuals' feed intake was lower, while in the following three periods (onset and continuation of egg laying), the feed intake was higher (Figure 8). The feed intake differed significantly between all periods, except between period 4 and period 5 (Table A2). In the first and last period the feed intake differed significantly between haplotypes. In the first period the pattern was the same as for body weight (Figure 8) while in the last period, the feed intake of individuals with mt haplotype B2 was significantly less than of the white strain. Additionally, the variance in feed intake in period four was comparably high, which was not reflected in the body weight. Again, these differences reflect the differences between the two strains; nevertheless, they also depict that these strain differences are not present for all haplotypes.

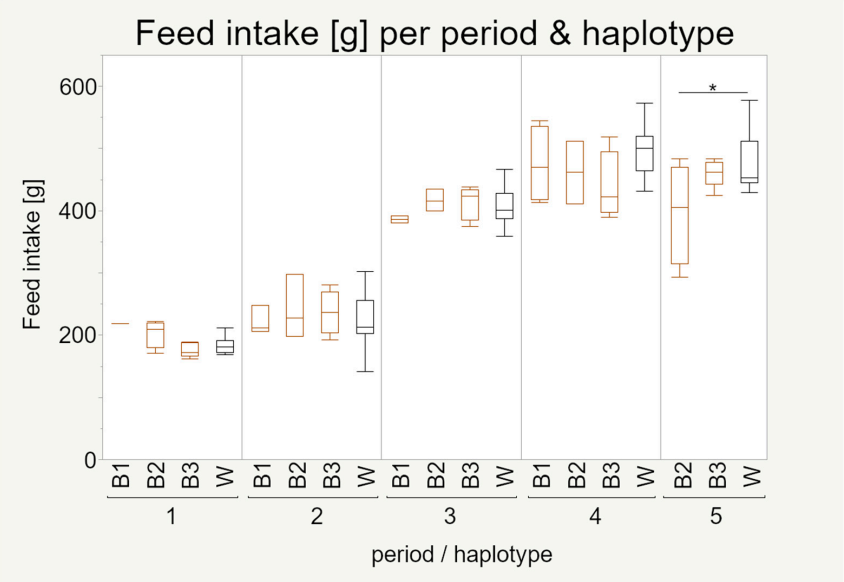

Figure 8. Feed intake (g) of 100 laying hens by haplotype and period. Boxes represent 50% of the data points (median with interquartile ranges) whiskers show minimum and maximum. Pairwise Tukey–Kramer HSD (for normal distributed data) or Steel–Dwass (for nonnormal distributed data) tests were used to test for significance within each period. Sample numbers are given in Table 3. Significance level * = $p < 0.05$. Feed intake was first studied by Sommerfeld et al. 2020a [27] in the context of strain differences.

4. Discussion

4.1. Mitochondrial Haplotypes

The goal of this study was to identify (individual) mt haplotypes and link them to physiological phenotypes.

4.1.1. Low Number of Mt Haplotypes

Our analysis showed that both strains have a surprisingly low number of mt haplotypes: three haplotypes were found in the brown and one in the white strain.

Other studies showed higher variability in mt haplotypes, but have focused on the highly variable mitochondrial control region (CR). A comparison is possible, since our CR haplotypes are similar to the whole mt haplotypes. Guan et al. 2007 [55] identified two mt haplotypes of CR in 20 white leghorn individuals and Liu et al. 2006 [56] identified nine divergent clades of CR in Eurasian populations. There exist several other studies that have used genetic material from Africa or Asia [1,55,57,58]; however, most likely these individuals were not selected as strongly as the ones in the present study, which would explain the difference in genetic variation. Even though most studies focused on partial mt genomes, there are several complete genomes available such as given by the study of Miao et al. 2013 [59] that includes 60 individuals of different breeds from Asia. Our identified haplotypes cluster well into the published genomes, and the white haplotype is similar to a haplotype identified in Miao et al. 2013 [59]. A maximum likelihood tree with 22 published mt genomes, selected for their similarity to our genomes (via BLAST search) and the references used for the bioinformatics analysis and validation is provided in Appendix B Figure A1.

Due to the maternal inheritance of the mt genomes, our data shows that all white hens can be traced back to one female and the brown hens to three females of origin. This low diversity brought us to question whether the nuclear genetic background is poor due to

the breeding history, or if the lack of mt variance is not representative for the whole genetic background of the two strains.

4.1.2. Signs of Heteroplasmy

We observed not only a general low number of mt haplotypes, but also a lack of individual mutations in both strains. Only one individual carrying mt haplotype B2 showed a heterozygous site. Heterozygous appearing sites in the normally haploid mt genome can be a sign of nuclear mitochondrial pseudogenes (numts) [60] or heteroplasmy. Due to our approach amplifying fragments of a size around 9 kb and the low number of known numts in the mt genome of chicken [61], it is rather unlikely that the observed heterozygous site is caused by the sequencing of a numt, supporting heteroplasmy that can be commonly found in mt genomes [62] and has also been observed in chicken [63].

4.1.3. Characterisation of the Identified Haplotypes

The identified haplotypes were characterized by synonymous mutations and mutations in the noncoding mt CR.

Only haplotype B1 contains an amino acid changing mutation in the ND4 gene, which also changes the polarity of the amino acid (serine to glycine). ND4 is part of complex 1 of the respiratory chain and this subunit is not directly part of the electron transport, but anchors complex 1 in the mitochondrial membrane [64]. It is known that mutations in ND4 affect human health [65,66]. In chickens, Li et al. 1998 [9] linked a silent mutation in ND4 with resistance to Marek's disease, a viral infection affecting the birds' eyes. The used individuals in our study do not carry this mutation, but the notable impact of a silent mutation in ND4 underlines the potential impact of mutations in this region.

Unfortunately, B1 is the smallest group in our setup, which reduces statistical power. It remains to be elucidated whether the small number of individuals is the result of lower individual performance or rather by coincidence. However, the amino acid change is interesting and needs to be investigated further to gain insights into potential functional effects.

Not only amino acid changing mutations can affect an organism; the influence of synonymous and noncoding mutations has been underestimated for a long time [67]. Now it is known that these mutations can have an impact on, for example, the substrate specificity of the multidrug resistance 1 gene [68] and diseases including cancer [69]. The two main ways silent mutations affect functionality are either through linkage disequilibrium or allele specific differences in mRNA folding and splicing (as reviewed from Kimchi-Sarfaty et al. 2007 and Bali and Bebok 2015) [68,70].

4.2. Does the Low Number of Mitochondrial Haplotypes Affect Phenotypic Traits?

None of our included traits is significantly influenced by the mt haplotype—the positive result for body weight is most likely derived from the prominent white strain that has a proven lower body weight [27]. However, our data of the diverse brown strain are more scattered, making an mt haplotype-specific effect more likely. Unfortunately, the unequal sample distribution between the haplotypes in our setup does not allow a robust test for these differences.

The impact of mt haplotypes on body weight is well known in other organisms such as in human [71] or rainbow-trout [72]. This indicates that body weight is a suitable example to demonstrate the importance of the mt genome and its influence on the body (and, thus, for the following experiments) a genotyping previous to the selection of the animals will be included to correct for this and give the analyses more power.

Feed intake and body weight seem to be connected in a way; more feed intake leads to a higher body weight. However, there are some significant differences, since haplotype B2 had a higher feed intake than the white strain in period 5 while not differing in body weight in this time period, but differing in earlier periods with the same feed intake (Figures 7 and 8).

The utilization of P was of the highest interest in our study and, thus, data from both experiments are presented here. In both experiments we were not able to observe significant differences regarding the mt haplotypes but a rather plastic response was found among all groups, especially under the changes of P and Ca concentrations provided in the diets of the second experiment.

However, the link between P utilization and the mt haplotypes is rather complex, which requires additional data from different disciplines such as e.g., from gene expression analyses or metabolites that result from OXPHOS regulatory pathway. The candidates for these analyses are, for example, mt ATPase or ND4, which shows an amino acid change in one of our identified haplotypes, nuclear genes such as AMP-activated protein kinase (AMPK) or Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), which play a role in mitochondrial biogenesis and energy metabolism [73,74]. Myo-inositol as an end product of complete phytate degradation is also discussed to have an impact on mitochondrial biogenesis and function in cell culture cells [75]. As another example, acylcarnitines are indicators of the mitochondrial function with enhanced concentrations in plasma as a consequence of the dysregulation of fatty acid oxidation [76].

Additionally, little is known on how the laying hens and their metabolisms react to the changing dietary conditions related to P bioavailability. Recent studies in broilers have shown an remarkable effect on the plasma metabolome after phytase and myo-inositol supplementation [77].

As a conclusion we were not able to show significant differences between the mt haplotypes but were able to illustrate interesting tendencies that need to be followed up in more stringent experimental design. Furthermore, this would address the current underrepresentation of studies in this field.

4.3. Low Mitochondrial Diversity and the Nuclear Genome of the Strains

The observation of little to no mitochondrial variation aroused our interest in the nuclear genome by the usage of the SNP-chip data, enabling insights into the genetic background of the individuals. From the breeding scheme and the way individuals were selected for the experiments (see Figure 1) we know that both strains originate from the same number of fathers and thus, should have a somehow equal genetic background from the paternal side.

From these findings we expected the white strain to be genetically less diverse compared to the brown strain and a general low genetic diversity.

4.3.1. Nuclear Genetic Distance between and Genomic Relatedness within the Strains

Regarding the differentiation between the two lines, the ADMIXTURE analysis showed a clear separation, which was also confirmed by the F_{ST} value (0.35) between them. Gholami et al. 2014 [78] calculated a slightly lower F_{STs} between the three lines of white leghorns derived from Lohmann and a brown layer breed (Rhode Island Red) (F_{ST} = 0.24), but their work includes more lines and SNPs than our work.

To our surprise, the level of genetic relatedness was similar within both strains, giving no indication that the lower mitochondrial diversity of the white strain also exists on the nuclear level. In addition, it became clear in the ADMIXTURE analysis that both strains most likely originated from two lines (K = 2, Figure 3). This leads to an increased number of possible interacting gene products between the mitochondrial and the nuclear genome. The compatibility of nuclear and mitochondrial gene products is known to limit individual fitness [79], which indicates that the higher number of possible combinations can lead to better or worse performing animals. This might explain the often-observed higher variance in measurements (e.g., P utilization) within brown haplotypes, compared to the more similar values within the white haplotype even considering the higher number of white individuals. In addition, the comparable nuclear genomic diversity of the two strains increases the plausibility of effects of the differences in mitochondrial genetic diversity.

4.3.2. Mitochondrial Haplotypes and the Nuclear Genome

The ADMIXTURE analysis showed that some individuals from the brown strain are highly similar independent of their mt haplotype, but often according to their paternal origin (Figure 3). The same individuals appeared as most related on the genomic level in the G-Matrix (Figure 4), which is also a sign that the removal of a high number of SNPs during LD pruning prior to the ADMIXTURE analysis did not disturb the results. The analysis of the population differentiation (F_{ST}) confirmed these findings with values close to zero between the three brown haplotypes (Table 5).

Contrasting to these findings, the genomic relationship between individuals with the same mt haplotype is significantly closer than individuals with different mt haplotype (Table 6). However, the effect vanishes when looking only at half siblings. These results show that the maternal genetic background represented by the mt haplotype seems to have a limited role on the genomic relationship compared to the impact of the known father. However, given our experimental design, sharing a mt haplotype does not necessarily imply the same nuclear genomic background provided by the mother. Thus, it becomes obvious that individuals with the same mt haplotype are not as closely related as individuals with the same father, providing potential for modifications in mito-nuclear interactions [80].

5. Conclusions

Linking mt haplotypes and phenotypic traits is of high interest but was not fully possible in this study. Nevertheless, the rather surprisingly low mitochondrial diversity is still interesting, given the contrasting and high-yielding performance of these two strains. In addition, this study examined a part of the genome that is normally not used in breeding approaches, and even if the mt genome does not seem to be representative for the whole genetic background, the low diversity is worrying and provides important information about the breeding history of the strains. However, further analyses including the determination of the nuclear genetic background from the maternal side might lead to a better understanding of the mito-nuclear interaction, an interesting and so far, less explored topic in the breeding of laying hens.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2615/11/3/825/s1: Table S1: Primer used for resequencing and validation with position, length and according long-range fragment; Table S2: PCR conditions used for all PCR reactions.; Table S3: PCR conditions used for all PCR reactions; Figure S1: ADMIXTURE plots of the brown and white strain analyzed and filtered together; Table S4: SNPs left after filtering for all three datasets, filters were applied one after another from top to bottom.

Author Contributions: Conceptualization, C.H.-K., V.S., J.B., M.R. and M.H.; methodology, all authors; validation, C.H.-K., V.S., H.I. and M.H.; formal analysis, C.H.-K.; investigation, C.H.-K., V.S.; resources, M.R. and M.H.; data curation, C.H.-K. and V.S.; writing—original draft preparation, C.H.-K.; writing—review and editing, all authors; visualization, C.H.-K.; supervision, M.H.; project administration, M.H., M.R. and J.B.; funding acquisition, M.R., J.B. and M.H. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal experiments were performed at the Agricultural Experiment Station of the University of Hohenheim, Germany. They were approved by the Regierungspräsidium Tübingen, Germany (Project no. HOH50/17TE) in accordance with the German Animal Welfare Legislation.

Data Availability Statement: All sequencing data obtained in this study were deposited on GenBank under the accession numbers MT800324–MT800504.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Influence of period and haplotype on body weight, feed intake and phosphorus utilization. *p*-values are from the evaluation of the statistical model using a three factorial anova.

Response Variable	Body Weight	Feed Intake	P Utilization
Period	$<2.2 \times 10^{-16}$	$<2 \times 10^{-16}$	5.965×10^{-11}
Haplotype	4.925×10^{-5}	ns [1]	ns

[1] not significant.

Table A2. *p*-values of pairwise comparisons of body weight, feed intake and phosphorus utilization between periods independent of the haplotype. Tukey post hoc was used for testing.

Difference	Body Weight	Feed intake	P Utilization
1 vs. 2	<0.0001	0.0094	ns
1 vs. 3	<0.0001	<0.0001	<0.0001
1 vs. 4	<0.0001	<0.0001	0.0002
1 vs. 5	<0.0001	<0.0001	ns
2 vs. 3	<0.0001	<0.0001	<0.0001
2 vs. 4	<0.0001	<0.0001	ns
2 vs. 5	<0.0001	<0.0001	ns
3 vs. 4	0.0118	<0.0001	ns
3 vs. 5	<0.0001	0.0016	<0.0001
4 vs. 5	0.0123	ns	0.0012

Table A3. *p*-values of pairwise comparisons of body weight, feed intake and phosphorus utilization between haplotypes independent of the period. Tukey post hoc was used for testing.

Haplotype	Body Weight	Feed Intake	P Utilization
B1 vs. B2	0.0581	ns	ns
B1 vs. B3	ns	ns	ns
B1 vs. W	ns	ns	ns
B2 vs. B3	ns	ns	ns
W vs. B2	0.0007	ns	ns
W vs. B3	0.0009	ns	ns

Table A4. *p*-values of the comparison of body weight of the different haplotypes per period. Pairwise Tukey–Kramer HSD or Steel–Dwass tests were used for testing depending on the distribution of the data.

Haplotype/Period	1	2	3	4	5
B1 vs. B2	ns	ns	ns	ns	- [1]
B1 vs. B3	ns	ns	ns	ns	-
B1 vs. W	ns	ns	ns	ns	-
B2 vs. B3	ns	ns	ns	ns	ns
W vs. B2	0.0045	ns	0.0067	ns	ns
W vs. B3	ns	ns	ns	ns	ns

[1] no statistical test performed, no animals of group B1 present.

Appendix B

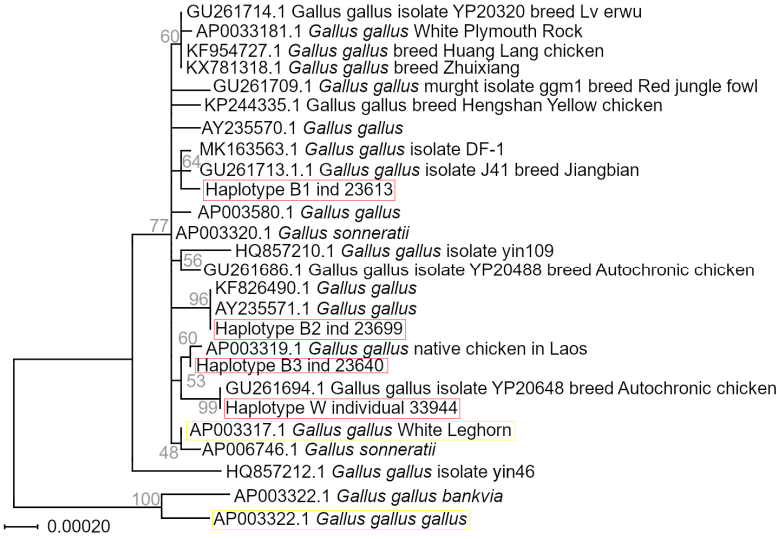

Figure A1. Maximum likelihood tree based on 26 mitochondrial genomes. The tree is based on Hasegawa–Kishino–Yano model including gamma distribution with 200 bootstrap replicates and includes all sites. The alignment was generated with clustalo [81,82], the tree was generated in MEGA X [43]. The tree includes all four detected haplotypes (red), the references used for mapping and validation (yellow) and the five genomes with the highest sequence identity with each haplotype using BLASTn search [83]. Sequences which were under the top five for several haplotypes were only included once and the next highest hits were used to fill up to the final number of five sequences per haplotype.

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3.2 The dynamics of mitochondrial-linked gene expression among tissues and life stages in two contrasting strains of laying hens

PLOS ONE

RESEARCH ARTICLE

The dynamics of mitochondrial-linked gene expression among tissues and life stages in two contrasting strains of laying hens

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Abstract

The cellular energy metabolism is one of the most conserved processes, as it is present in all living organisms. Mitochondria are providing the eukaryotic cell with energy and thus their genome and gene expression has been of broad interest for a long time. Mitochondrial gene expression changes under different conditions and is regulated by genes encoded in the nucleus of the cell. In this context, little is known about non-model organisms and we provide the first large-scaled gene expression analysis of mitochondrial-linked genes in laying hens. We analysed 28 mitochondrial and nuclear genes in 100 individuals in the context of five life-stages and strain differences among five tissues. Our study showed that mitochondrial gene expression increases during the productive life span, and reacts tissue and strain specific. In addition, the strains react different to potential increased oxidative stress, resulting from the increase in mitochondrial gene expression. The results suggest that the cellular energy metabolism as part of a complex regulatory system is strongly affected by the productive life span in laying hens and thus partly comparable to model organisms. This study provides a starting point for further analyses in this field on non-model organisms, especially in laying-hens.

Introduction

Understanding the impact of the genetic background and developmental processes on gene expression are of broad general interest to understand organismic function. Through the process of oxidative phosphorylation (OXPHOS), mitochondria are generating 90% of the cellular energy [1]. In addition, these organelles are involved in the maintenance and regulation of intracellular energy metabolism and signalling [2], apoptosis [3, 4] and have major importance in cell cycle regulation [5] and ageing processes [6]. Mitochondria received particular attention in relation to ageing as a modulator of both, the production of damaging reactive oxygen species (ROS) that can cause cellular damage [1] and the synthesis of energy in form of adenosine triphosphate (ATP) via electron transfer mediated by nicotinamide adenine dinucleotide + hydrogen (NADH) [7]. The nuclear encoded Superoxide dismutase (*SOD2)* is converting

superoxide anions ($O_2^{\bullet -}$), (which are the most produced ROS during OXPHOS [8]) to hydrogen peroxide [6, 9]. An increase in the production of ROS in the mitochondrion is linked to the process of ageing in many species [10] and the increase of *SOD2*-expression protects the mitochondrion from damage, which would otherwise lead to the death of the cell (Santos et al., 2018 [10], Yin et al., 2018 [11] and cited references within).

Evidence of diminished performance of mitochondria during the course of life span have been frequently found [12, 13]. To maintain their functionality and their ability to react to external influences, the mitochondrial gene expression is part of a network of transcription factors and other gene products from the nuclear genome [14, 15], supporting the view of an intimate interaction of mitochondria with nuclear genomes.

Within this complex framework of interaction, mitochondria play a crucial role as powerhouse of the cell that have been studied in a variety of model organisms such as in fruit flies (*Drosophila*) and mice [16, 17] but in a much smaller proportion of livestock such as cattle or pigs [18, 19].

In our experimental setup, we use two different strains of laying hens, with the benefit of having a highly bred species for the specific purpose of egg laying, but represented in two genetically distinct lineages at the same time [20]. Since the focus and thus the need for energy and metabolites changes during the hens' life span, we sampled at five different points, covering growth and egg laying as well the shift between these two phases, and the changes during the laying phase towards its end. Related to this, it is an utmost importance to gain a better understanding of the utilization of phosphorus (P) and calcium (Ca) and the metabolism of *myo*-inositol (MI) during life span within the animals [21, 22]. Since the mitochondrial process of OXPHOS is directly linked to the availability of P [23], and the energy metabolism is closely linked to the animals' fitness, mitochondrial gene expression is of highest interest in this framework. Furthermore, we included nuclear encoded subunits of OXPHOS complexes such as UQCRC1, which is a component of the ubiquinol-cytochrome c oxidoreductase and thus part of the mitochondrial electron transport chain [24], and other nuclear genes such as e.g. AMP-activated protein kinase (AMPK), which is linked to the regulation of mitochondrial biogenesis and the energy metabolism [25]. AMPK acts as an activator of PGC1α in case of energy deficit, to increase mitochondrial gene expression [26]. In addition, its activation inhibits MTOR [27], which in addition regulates the expression of PGC1α and other transcription factors linked to mitochondria [28] and a decrease in its expression has been linked to an extension of life span in *Drosophila* [29] and yeast [30] as a response to nutrients. The reactions of PGC1α, subunits of AMPK and MTOR have also been described in broilers in the context of feed efficiency and muscle growth [31]. To cover not only the mitochondrial gene expression itself, but also the complex regulatory network, these genes were included in the study as well.

We follow three main hypotheses:

1. Due to the different (mitochondrial) genetic background [20] and different measured performance [21] of the two strains, differences in gene expression are expected as well.
2. Due to the observation in other species [12, 13] and the metabolic changes during the observed time period a change or decrease in mitochondrial gene expression with the ongoing productive lifespan is expected.
3. These changes include a visible change when the hen's focus shifts from growth to egg laying (period 2 to 3) and after the peak of egg laying towards the end (periods 4 and 5).

In this study, we explore the gene expression differences and changes during life span of two strains of laying hens and provide insight into the genetic machinery linked to the

complex energy metabolism of a domesticated animal. Our study benefits from the known genetic background and low mitochondrial diversity of the two strains [20], which provides a robust data base for the analysis of gene expression differences between groups.

Material and methods

Animals and experimental setup

The animal experiments were performed at the Agricultural Experiment Station of the University of Hohenheim, Germany. They were approved by the Regierungspräsidium Tübingen, Germany (Project no. HOH50/17TE) in accordance with the German Animal Welfare Legislation.

We used 100 laying hens: 50 brown (Lohmann brown classic) and 50 white (Lohmann LSL classic) white leghorn hybrids contributed by Lohmann Tierzucht (Cuxhaven, Germany). The hens originated from an experiment addressing the utilization of P and Ca in different periods of the hens' life [21]. The experimental setup is described in detail in Sommerfeld et al., 2020 [21] and will only be outlined briefly in the following.

The hens were reared together under standard conditions, with diets according to the requirements of each period, based on soy and corn meal with no difference to the recommendations as described in Sommerfeld et al., 2020 [21]. Ten father lines per strain were selected based on the average bodyweight of the female offspring prior to the start of the first experimental phase. After 8, 14, 22, 28, and 58 weeks ten hens per strain were selected and placed into metabolism units, to monitor feed intake and collect excreta on an individual basis. The hens were weighed at the beginning and end of this period, to calculate changes in body weight. After the end of this period (10, 16, 24, 30, and 60 weeks) the animals were slaughtered at the Agricultural Experiment Station of the University of Hohenheim [21].

Samples and RNA extraction

We used fives tissues from each individual: breast muscle, duodenum, ileum, liver and ovary. Samples were directly taken after slaughtering when the hens were 10, 16, 24, 30 and 60 weeks old (denoted in the following as period 1 to 5, respectively) as described by Sommerfeld et al., 2020 [21] and were immediately placed on dry ice. The samples were stored at -80˚C until the extraction of RNA.

RNA was extracted from 25mg tissue using TRIzol Reagent (Thermo Fisher scientific Inc., Massachusetts, USA) according to the manufacturers' instructions. The samples were homogenized using steel beads at 5.5m/s for 40 seconds on a FastPrep24 (MP Biomedicals, Thermo Fisher scientific Inc., Massachusetts, USA) and a centrifugation step was included afterwards, as recommended for samples with high fat content. Samples were dissolved in nuclease-free water and RNA concentration and quality was measured using a NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher scientific Inc., Massachusetts, USA). In addition to the 260/280 and 260/230 ratios provided by NanoDrop for all samples the integrity of the extracted RNA was checked via gel-electrophoresis [32] and on a Qubit 4 (Thermo Fisher scientific Inc., Massachusetts, USA) using the Qubit RNA IQ Assay Kit (Thermo Fisher scientific Inc., Massachusetts, USA) of a random but representative subset (including all tissues, strains an periods as well as different concentrations and 260/230 ratios). The samples were stored at -80˚C until further processing.

Real time PCR

We selected 33 genes including all mitochondrial encoded genes (13), nuclear encoded genes (20), which includes genes important for life span and mitochondrial biogenesis. During the course of PCR evaluation and quality checks, six genes (two mitochondrial and four nuclear

encoded ones) needed to be excluded due to suboptimal performance or due to low number of successful reactions (the final subset of genes can be found in Table A in S1 File). A list of the final genes and their abbreviations used in this work can be found in Table 1. Thus, for all subsequent analyses we remain with a set of 27 gene assays. Additionally, we included three potential nuclear encoded reference genes: Actin beta (*ACTB*), Peptidyl-prolyl cis-trans isomerase A (*PPIA*) and Glycerinaldehyd-3-phosphat-Dehydrogenase (*GAPDH*) that have been used in previous studies for this purpose [33, 34].

Assay design and evaluation. All primers were designed using Primer3 [35] based on reference sequences from NCBI except the primer-pair for *GAPDH* [34] (Table A in S1 File). Prior to real time PCR all primers were tested using standard PCR with the same conditions as used for the final analysis (Table B in S1 File). DreamTaq Green (Thermo Fisher Scientific) was used according to the manufacturers manual, and the resulting fragments were visualized by standard agarose gel-electrophoresis and sequenced bidirectional using Sanger technique (performed by Microsynth AG (Balgach, Switzerland)) to test for specificity.

All real time PCR analyses were performed on a Biomark HD system (Fluidigm Corporation, San Francisco, USA), following the protocols of the supplier for gene expression analysis. The evaluation of the performance of the assays was done on FlexSix GE integrated fluidic circuits (IFCs) in duplicates per assay. A 12-step 10-fold dilution series (starting with a concentration of 16.6ng/μl) of the previously sequenced specific PCR product was used to determine limits of detection, linear dynamic detection range, variation at detection limit, PCR efficiency and melting curves of the products (via standard curve as described in Bustin et al., 2009 [36]). The PCR cycling conditions were the same as for the final experiments and can be found in supplementary Table C in S2 File. The efficiency was above 90% for 27 primers and above 80% for four of our primer pairs (Table A in S1 File). Melting and standard curves can be found in the S2 File.

Final qPCR runs. The analysis was performed on six 96.96 IFCs using the Delta Gene Assays protocol with the manufacturers standard protocol for fast PCR and melting curve as described in supplementary Table C in S1 File. Remaining DNA was digested using DNAse I (Thermo Fisher scientific Inc., Massachusetts, USA) using 2μg RNA extract in each reaction. For reverse transcription 1μl (= 166.67ng) of this RNA was used in each reaction using the Fluidigm Reverse Transcription Master Mix containing a mixture of poly-T and random

Table 1. Genes used in this study with abbreviations and genome in which they are encoded.

Abbreviation	Genome	Gene
ACTB	Nuclear	Actin beta
ATP6, ATP8, ATP5F1	Mitochondrial Nuclear	ATP-synthase F_0 subunits
COX1, COX2, COX3, COXC6, COX5A	Mitochondrial Nuclear	Cytochrome oxidase subunits
CytB	Mitochondrial	Cytochrome b
GAPDH	Nuclear	Glycerinaldehyd-3-phosphat-Dehydrogenase
IGF-1α	Nuclear	Insulin-like growth factor 1α
MTOR	Nuclear	mechanistic target of rapamycin
ND1, ND4, ND4L, ND5, ND6, NDUFB6	Mitochondrial Nuclear	NADH:ubiquinone oxidoreductase subunits
PGC1α	Nuclear	Peroxisome proliferator-activated receptor gamma coactivator 1-α
PPIA	Nuclear	Peptidyl-prolyl cis-trans isomerase A
AMPK (PRKAA1, PRKAA2, PRKAB2, PRKAG2)	Nuclear	AMP-activated protein kinase and its α1, α2, β2 and γ2 subunits
SDHA. SDHB	Nuclear	Succinate dehydrogenase complex subunits
SOD2	Nuclear	Superoxide dismutase
UQCRC1, UQCRC2	Nuclear	Cytochrome b-c1 complex subunits 1 and 2

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oligonucleotides. Pre-amplification with the Fluidigm Preamp Master Mix was performed with 1.25μl (~41.6ng) cDNA for 10 cycles using pooled primers that were the same as used for the final qPCR runs. A multiplex control was performed including five samples of different tissues, strains, period and RNA qualities with and without the pre-amplification step. After Exonuclease I digest of the primers the samples were diluted five-fold. In each well of the IFC 2.25μl of the diluted Exo I digested sample were added, resulting in 3.015nl in each reaction chamber. Negative controls were included throughout all preparation steps and on the final qPCR runs as well to test for contamination of the primers and reagents. Additionally, an internal control was used on each chip, to detect potential intra-run variance. All qPCRs were performed in duplicates and the samples were placed randomly on the chips, only grouped by individual to avoid any bias of sample arrangement.

Data preparation

Quality controls. For data evaluation and quality controls the Fluidigm Real-Time PCR analysis software (version 4.5.2) was used. Only Cq-values from reactions with logarithmic increase of fluorescence and specific melting points were used for the following analyses. After the automatic quality check of the software, the results were evaluated by eye and revised manually if necessary. The quality threshold was set to 0.65 and the peak ratio threshold to 0.8.

The results of the internal control were used to detect possible variation due to technical issues.

Reference gene evaluation. To evaluate if the three candidate genes for normalization (*ACTB*, *PPIA* and *GAPDH*) were constant under our experimental conditions, Normfinder [37] was used. As input we used the quality checked data of one brown and one white individual per period and included all five tissues to cover all factors of the experiment. Normalization was tested for tissue type and period.

Calculating relative gene expression. Means of duplicates were calculated of all samples with two successful runs. For samples that only had one successful duplicate this run was used. Gene expression relative to the reference genes was calculated using the Pfaffl-method [38] as optimized for multiple reference genes [39, 40]:

$$rel.\,gene\,expression = \frac{RQ_{GOI}}{geomean[RQ_{refs}]}$$

Where RQ = $E^{\Delta ct}$ and E = (primer efficiency [%]/100) + 1.

Δct was calculated as the difference between the average cycle threshold (ct) of the internal control to the ct of the corresponding sample.

Statistical analyses

Hierarchical clustering. Two-way hierarchical clustering analyses were performed in JMP Pro (Version 15, SAS 199 Institute Inc., Cary, NC, 1989–2019) using Ward's minimum variance method [41] based on relative gene expression values of all 29 genes. The data were not standardized and only samples without missing data were included. To estimate the best number of clusters the cubic clustering criterion [42] was used as implemented in the program. The process was done for both strains together, and for each strain separate.

Analysis of individual genes. A linear mixed effects model was implemented and used for all genes:

$$Y \sim strain + period + tissue + tissue * strain + tissue * period + strain * period + tissue * strain * period + individual + father + \varepsilon$$

Where Y is relative gene expression, ε is the residual error, strain, period and tissue are fixed effects, with individual and father as random effects. All modelling was performed in R (R Core Team 2019, Version 3.6.1) using the *lmerTest* package [43]. A three factorial analysis of variance (anova) was performed to evaluate the influence of fixed effects and pairwise tukey *posthoc* tests (package *emmeans* [44]) were performed to detect differences between strain, period and tissues in various combinations based on the estimated marginal means (emmeans) derived from the model. The fulfilling of normal distribution and the homogeneity of variance were evaluated using QQ and residual plots. Outliers were removed for each dataset using the interquartile range prior to the statistical analysis except for *SDHA*, *MTOR*, *PRKAG2* and *GAPDH* where a removal would lead to a strong bias of the analyses.

Means and standard derivations were calculated over the emmeans of all genes to compare the gene expressions between tissues and periods in general. To avoid performing statistical tests on the results of other statistical tests and generating hardly interpretable results, the means were used only for visualization.

Results

Sample quality

RNA concentrations ranged from 340ng/μl to 10556.5ng/μl. The results of the NanoDrop and Qubit measurements can be found in the (S3 File). The 260/280 ratios for all samples were close to the optimum of 2, while the ratios were lower for older individuals from period 5 in comparison with the other periods. The same observation was made for the 260/230 ratio where the values decreased with increasing age of the animals and were more diverse in general. Since all samples were treated equally, the differences might result from the age of the individuals themselves, since age related changes in tissues are described for connective tissues [45] and in context of lipofuscin [46]. During the downstream processes no dependencies between lower ratios and qPCR success were observed. The IQ values were above 8 for all except one tested sample independent of the concentration, 260/230 or 260/280 ratios. The high IQ value indicate a proportion from more than 80% of large or/and structured RNA (mRNA, tRNA, rRNA). Samples with high concentrations were diluted previously to the DNAse treatment, which also dilutes the concentration of potential contaminants such as phenol or carbohydrates. Due to the dilutions, no pattern of low performance in combination with low 260/230 ratios or RNA degradation was observed; thus all samples were used for the statistical analysis.

Final dataset

We identified gene expression of *PPIA* and *ACTB* to be most consistent for using them as reference genes for the normalization of our dataset. The expression of *GAPDH* showed high variation between tissues, and was thus included in our study as nuclear candidate gene instead as reference gene.

After the removal of low-quality Ct values, we received a dataset of 12,628 relative gene expression values including 493 from 500 samples and 28 candidate genes. For 252 samples all genes were run successful, for all other samples values for at least one gene were missing. Missing values originated from the previous filtering or failed runs, whereas we observed no dependencies between sample quality, RNA concentration or sample group (tissue, strain, period) and missing values. Due to the stringent filtering criteria applied to the dataset prior to the analysis, and the filtering for outliers of each gene separately during statistical analysis, together with the high number of samples, we decided to analyse the complete dataset. On

average 447.5 samples per gene (min. 296 for *ND5* and max. 486 for *CytB*) entered the final analysis. A detailed table including sample numbers per gene can be found in S2 Table.

The calculated ΔCt values ranged from -7.339 (min for *COX1*) to 11.89 (max for *GAPDH*). The calculated ΔCt values can be found in S5 File.

Hierarchical clustering

The hierarchical cluster analysis of the merged dataset was performed on 252 samples, containing 54 breast muscle, 55 duodenum, 60 ileum, 44 liver, and 39 ovary samples, 134 samples from brown, and 118 from white individuals. All five periods were included. The CCC estimated 26 as the best number of clusters. All breast muscle samples formed one coherent cluster, containing no other tissue type (Fig 1). A second coherent cluster was built by 39 liver samples. The other tissue types formed smaller clusters but were more admixed compared to breast muscle and liver. The same observation was made for strain and period: no bigger cluster contained only samples of one strain or period. The clusters containing breast muscle tissue showed higher gene expression values compared to the other tissues, especially in *PRKAA2*, *PRKAB2* and *GAPDH*. The hierarchical cluster analyses on the separated datasets showed the same pattern as described above for both strains but the number of clusters decreased to 14 in brown and 12 in white (S1 Fig). In the white strain, the ovary samples formed a third larger cluster.

Influence of strain, period and tissue

The linear mixed model showed that all genes were significantly influenced by the tissue, while the influence of the period was affecting 17 and strain only four genes. The detailed results of all analysed genes can be found in S1 Table. The most frequent interaction was between tissue and period (20 genes), followed by strain*tissue*period (7), and the interaction of strain*tissue (4) while strain*period was only influencing the expression of one gene (*NDUFB6*).

Gene expression differences between tissues

Independent of the period, the gene expression was highest in breast muscle tissue, followed by liver tissue and was lowest in the ileum (Fig 2). Since gene expression differed between periods for many genes in our dataset, the scattering represented by the standard derivation is high but general trends can be observed.

The gene expression was highest in breast muscle tissue for all genes, except for *IGF-1α* and *PRKAG2* where liver tissue had the highest expression. However, the difference between breast muscle and all other tissues was significant for all tested genes ($p<0.0001$).

Gene expression differences between periods

The period had less influence on gene expression than the tissue, and not all tissues behaved the same way during the periods (Fig 3). In breast muscle tissue the mean gene expression of all genes decreased from period 1 to period 2 followed by an increase and peak in period 4. In liver, the expression was lower in period 4 and 5 compared to the first three periods, in ovary and duodenum the gene expression increased in period 5. The ileal gene expression declined in period 2 and reached a peak in period 3. As described before, the gene expression was highest in breast muscle and liver tissue throughout all periods.

From the 17 genes that were influenced by period, the expression of *ATP5F1*, *GAPDH*, *IGF-1α*, *MTOR*, *PRKAA1*, *PRKAB2*, *UQCRC1*, and *PGC1α* decreased, while the expression of *ATP6*, *COX1*, *COX3*, *ND1*, *ND4*, *ND4L*, *CytB*, *NDUFB6* and *SOD2* and increased with period

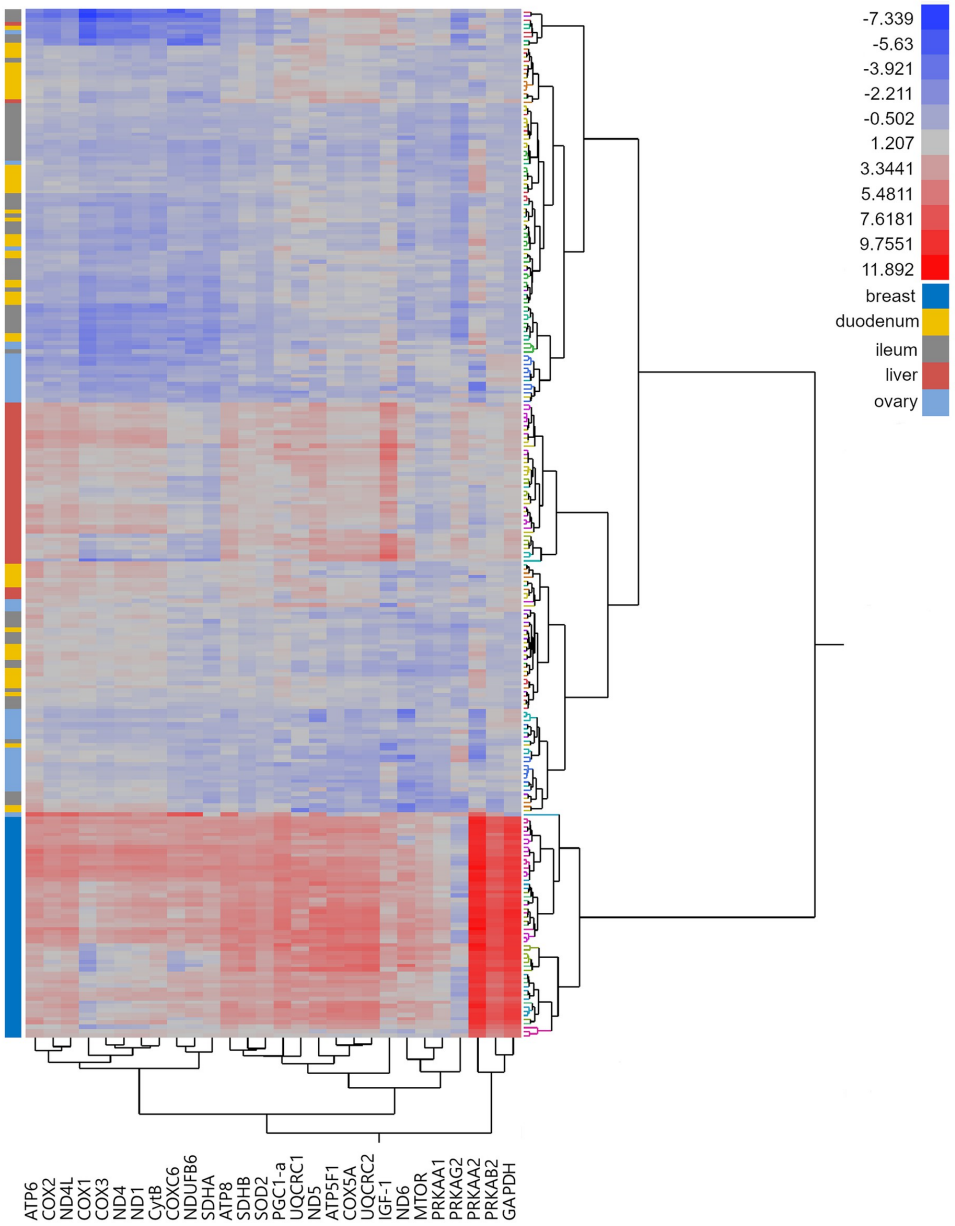

Fig 1. Heat map of two-way hierarchical cluster analysis of the gene expression of 252 samples. 28 Mitochondrial and nuclear genes (Table 1) were used for five tissues obtained from 94 laying hens. Ward's minimum variance method [41] was used, the number clusters was estimated using the cubic clustering criterion [42]. Colours of branches of the right indicate clusters, coloured bars on the left tissue types.

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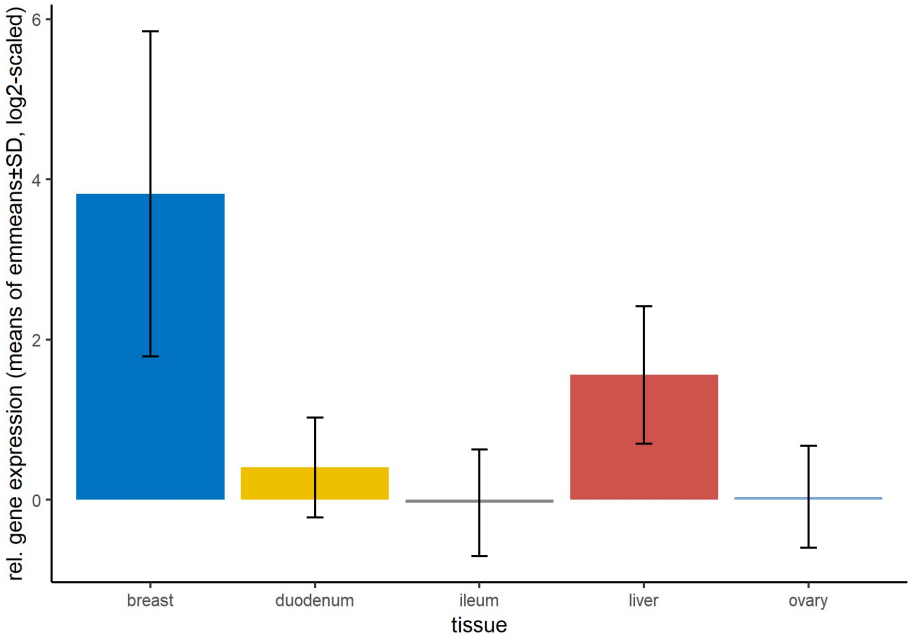

Fig 2. Relative gene expression of all analysed genes per tissue. Shown are means and standard derivation of emmeans over the course of strain and period, calculated using the linear mixed model. Number of samples per group can be found in S2 Table. No statistical test was applied on the shown means of emmeans.

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(Fig 4). In all decreasing genes (except *PRKAA1* and *PRKAB2*), the difference between period 1 and 5 was significant (p values in Table A in S4 File). In *ATP5F1* the expression in period 2 was significantly higher as in period 5 ($p = 0.0181$) as well as in *PRKAA1* ($p = 0.0085$). In *PGC1α* the expression in period 1 was significantly lower than in period 4 ($p<0.0001$). Most significant differences between periods were observed for *IGF-1α*: the expression in period 5 was significantly lower than in all other periods and the expression in period 4 was significantly lower than in the first two periods.

All genes with increasing gene expression showed decreasing gene expression in period 2 and a significantly lower expression compared to period 5. In *COX3* and *ND1* the difference in expression between period 2 and 3 was significant ($p = 0.0329$ for *COX3* and $p = 0.0208$ for *ND1*), too. A table with detailed gene expression and p-values can be found in Table B in S4 File.

Gene expression differences between strains

From our variables of interest, strain had the lowest effect on gene expression. Over all periods and tissues only *SOD2*, *GAPDH*, *ND6* and *PGC1α* were differently expressed between the two strains, with significant higher expression in the brown strain (Fig 5). When testing on the period level, the number of differently expressed genes between the strains increased from two or three genes in the first three periods towards five and nine genes in the last two periods. Most differences between the strains were found in breast muscle tissue, not exclusively for genes that were influenced by strain in general.

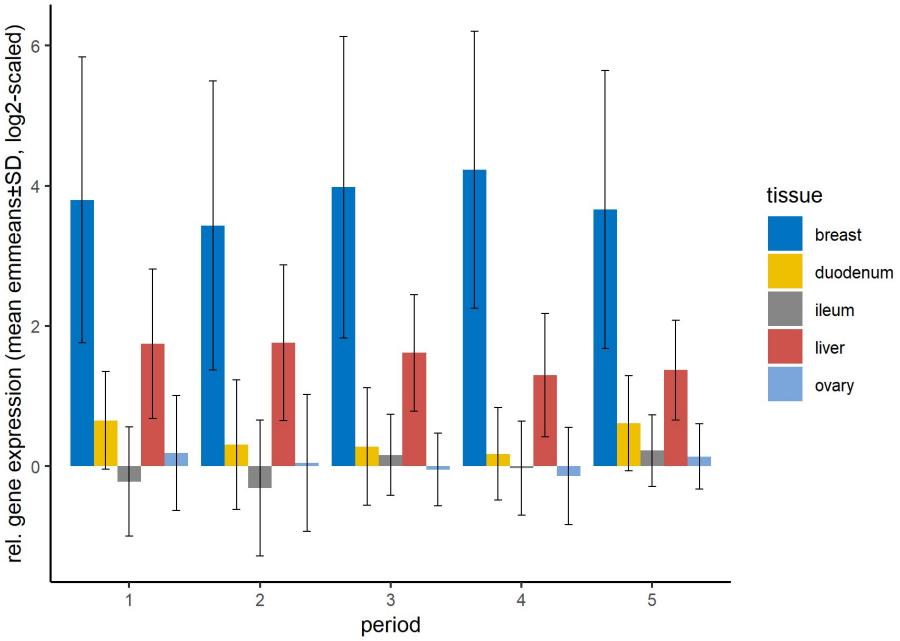

Fig 3. Relative gene expression of all analysed genes per tissue and period. Shown are means of emmeans over the level of strain, estimated by the statistical model with standard derivations. Number of samples per group can be found in S3 Table. No statistical test was applied on the shown means of emmeans.

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More in detail, most of the strain differences appeared in PGC1α expression (Table 2): The gene expression was significantly higher in the brown strain in all periods, except for period 2 and was also higher in liver and breast muscle tissue. The expression of *SOD2* was significantly higher in the brown strain in period 4, period 5 and in liver tissue (Table 2). The only gene expression differences with lower gene expression in the brown strain compared to white appeared in *IGF-1α* in period 5 (Table 2).

Discussion

In our experimental setup we were able to analyse a vast number of mitochondrial-linked genes in the context of changes during life span and differences between representative tissue samples in two strains.

Contrasting gene expression of mitochondrial and nuclear genes during life span

Contrary to our expectation, we observed no decrease in mitochondrial gene expression in the course of our experiment. Instead, the expression of mitochondrial genes that were influenced by period, increased. Interestingly, all the genes followed the same pattern: a decrease in period 2 followed by a constant increase. Manczak et al., 2005 [13] described a similar pattern for genes of the complexes I, III, IV and ATP6 in mice brains: an increase in 12 and 18 months old individuals, followed by a decrease in 24 month old mice (compared to the expression with

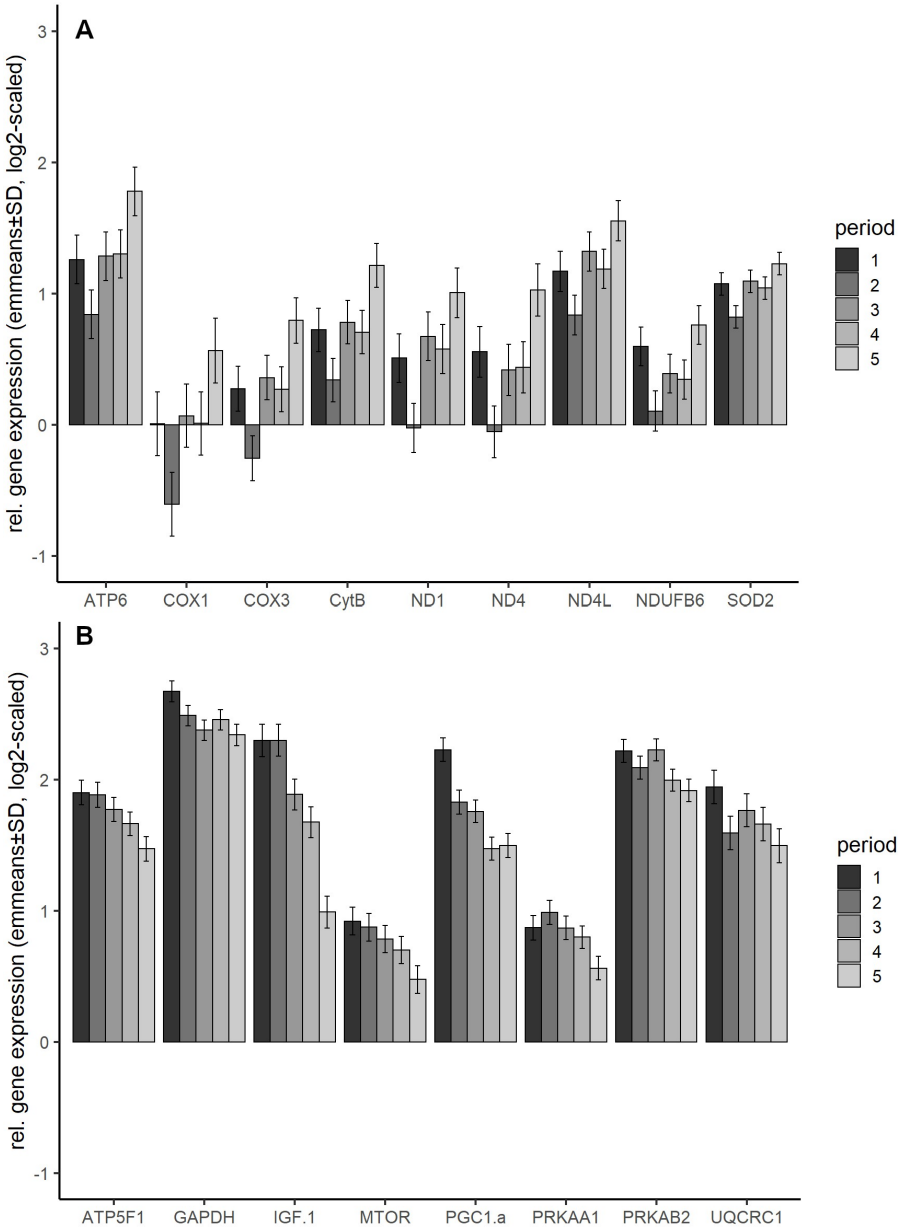

Fig 4. Relative gene expression of genes influenced by period. (A) increasing gene expression (B) decreasing gene expression. Shown are emmeans and standard error, averaged over strain and tissue, calculated using the statistical model. Number of samples per group can be found in S4 Table.

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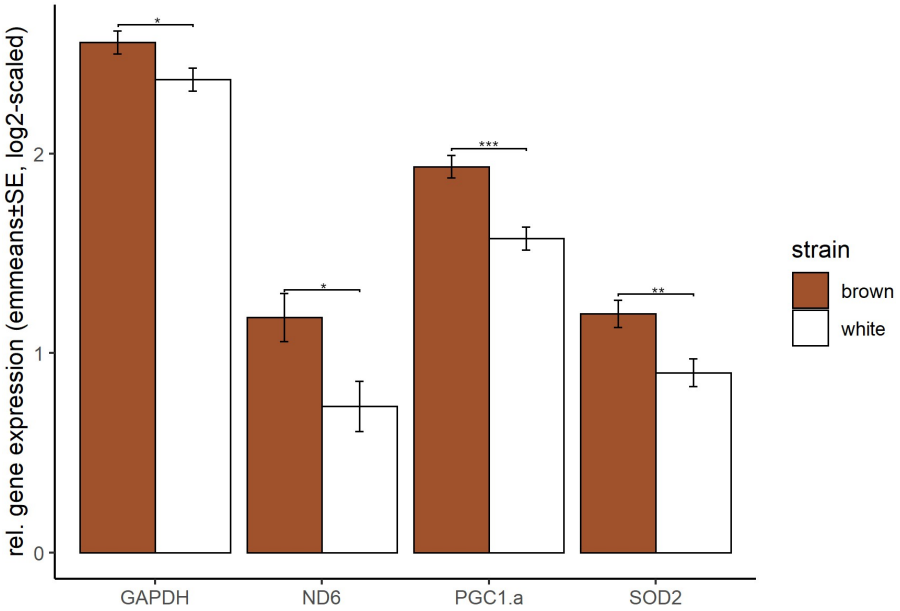

Fig 5. Gene expression differences between the strains. Shown are emmeans and standard errors averaged over the levels of tissue and period for genes with different relative gene expression between both strains. Statistical significance was declared when $p < 0.05$.

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two month). Even if the time points chosen in the studies differs, the different life expectations of mice and hens suggest, that the observed increase in gene expression might be followed by a decrease later in life, since our study covers the life span that is of agricultural interest, and not the complete potential life span of the birds.

The nuclear encoded *SOD2* gene was also decreasing in the second period and increased in the following periods. *SOD2* protects the cell from oxidative damage deriving from the produced ROS during oxidative phosphorylation, which might explain the increase in gene expression in later periods. This theory is supported by the simultaneously increase of the expression of subunits of complex I and III, which are the main producers of ROS in mitochondria [6]. The observed higher increase of *SOD2* expression in the brown strain, especially in the later periods indicates a different reaction to oxidative stress and might suggest, that the brown strain is better coping with this situation.

The observed increase in mitochondrial gene expression during the productive life span, towards the peak of egg laying (period 4) and towards the end of the laying phase might suggest, that the energy-requirement and related need for ROS detoxification increases with ongoing egg-laying.

Beside the genes that are involved in the process of OXPHOS and ROX detoxification, six genes showed a decrease with ongoing life-stages, which all belong to a complex network: PGC1α, IGF-1α, two subunits of *AMPK* and *MTOR*

PGC1α is a known key regulator of both, the expression of genes involved in the respiration chain and mitochondrial biogenesis [15] and the expression of detoxifying ROS such as SOD2 [47, 48]. The higher expression of both *PGC1α* and *SOD2* in the brown strain in the later

Table 2. Gene expression differences in four genes that are influenced by strain. Shown are emmeans with standard error and p-values, calculated by the statistical model.

	Brown (emmean± SE)	White (emmean± SE)	p
	GAPDH		
Period 5	2.52±0.111	2.16±0.117	0.0273
Breast muscle	9.1389±0.113	8.5348±0.119	0.0003
	ND6		
Period 1	1.191±0.247	0.458±0.273	0.0489
Period 5	1.127±0.227	0.426±0.242	0.0379
Ileum	0.27145±0.189	-0.39683±0.215	0.0214
	PGC1α		
Period 1	2.46±0.126	2±0.129	0.0124
Period 3	1.95±0.124	1.56±0.121	0.0275
Period 4	1.68±0.126	1.26±0.122	0.0197
Period 5	1.69±0.119	1.3±0.135	0.0322
Breast muscle	5.16±0.123	4.287±0.121	<0.0001
Liver	1.808±0.126	1.251±0.141	0.0036
	SOD2		
Period 4	1.308±0.121	0.774±0.120	0.0024
Period 5	1.429±0.119	1.023±0.125	0.0208
Liver	1.81813±0.11	1.23718±0.112	0.0003
	IGF-1α		
Period 5	0.707±0.166	1.271±0.178	0.0229

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periods support our hypothesis that the different strains are reacting differently in the course of their development. Consequently, with an increase of ROS production during life span the expression of enzymes coping with the oxidative stress is needed. On the other hand, *PGC1α* is down regulated in our experimental setup, while the expression of many genes that are affected by the PGC1 family including *SOD2* are up regulated or not affected by the age of the birds.

IGF-1α is another important player within this network, which can be inhibited by AMPK [49] or increase the expression of AMPK [50] under different conditions and work as a nutrient sensitive regulator [6]. However, the regulatory mechanisms between IGF-1, AMPK and PGC1α are poorly understood yet [50]. IGF-1α is an important growth-factor, which is a plausible explanation for the decreasing expression after the first two periods (with significant differences between the first and the last two periods), when the physiology of the hen switches from growth to egg laying.

Breast and liver tissue represent high levels of gene expression

As expected, we observed a strong influence of tissue on the expression of all genes in our study. However, we were surprised to find the highest gene expression in breast muscle tissue, especially since we are investigating laying hens, which are not bred to primarily gain weight. Interestingly, we observed no increase of gene expression in ovary tissue during the shift from growth to egg laying (period 2 to 3), but an increase towards the end of the laying period (Fig 3).

Gene expression differences between the strains

We hypothesized to observe differences between the two strains, and also included the father as a random factor, because the individual genetic background might influence gene

expression. Interestingly, the strain had the least influence on gene expression as shown by the statistical model. However, we observed that even if both strains are following the same pattern of tissue and period differences, there are differences in some genes. The differences in *SOD2* have already been discussed in the section about contrasting gene expression. In all four genes with significant strain differences independent of period and tissue (*GAPDH*, *ND6*, *PGC1α*, and *SOD2*) the brown strain shows higher expression. The fact, that *GAPDH* is one of these genes supports our initial decision to exclude it as reference gene, additionally to the observed differences between tissues, which have already been shown in human [51]. Interestingly, *PGC1α* showed the most strain differences, while the genes which are regulated by this factor do not differ. However, the fact that two important genes regulating mitochondrial biogenesis (*PGC1α*) and the reduction of oxidative stress (*SOD2*) are significantly higher expressed in the brown strain suggests, that both strains differ in the way they react to changes during the productive life span, especially in the later periods and in highly active tissue such as liver.

An important aspect we expected but did not observe in the broad panel of our data set were differences in the expression of *IGF-1α*. Despite the significant differences in body weight while showing no difference in feed intake observed by Sommerfeld et al., 2020 [21] in the same animals, we only observed strain differences in period 5, where the lighter white strain shows significantly higher expression (Table 2). As a growth factor, *IGF-1α* has been linked to body weight in chicken [52] but seems not to be one of the key players in our experimental setup.

Gene expression of subunits from the same complex differs

Interestingly, not all subunits of a complex followed the same expression pattern over the different periods. For most complexes, the affected subunits (*COX1* and *COX3*, *ND1*, *ND4*, *ND4L* and *NDUFB6*) followed the same pattern, however, the rest of the subunits of the same complex were not affected by time. The only exception was *CytB* and *UQCRC1*, where the expression of mitochondrial subunit was increasing, while the expression of the nuclear subunit decreased. For the complexes of the respiration chain, studies showed, that the majority of the genes belonging to the same complex seem to be co-expressed and thus, follow the same pattern among different conditions [53]. However, the expression regulation of mitochondrial genes depends on several factors, and expression differences between subunits of complex I have been observed in mice brains [13]. The authors suggested, that the up regulated subunits might be more sensitive to oxidative damage, and thus the organism tries to compensate the resulting loss of mitochondrial function with increased gene expression. In addition, it is known that not all subunits of protein-complexes are regulated in the exact same way [53] and thus, the expression of single subunits can work as a regulatory mechanism of the whole complex [54, 55].

Conclusion

We performed the first large scale study investigating mitochondrial gene expression in the course of productive life span of laying hens. Our data provided insights into the complexity of this regulatory network by including both, mitochondrial and mitochondrial-linked nuclear genes. In addition, we were able to show, that mitochondrial gene expression is increasing during the productive life span of laying hens, including the ROS detoxifying gene *SOD2*. These findings suggest, that the energy requirements might change during the phase of egg-laying and the organism reacts with an increase in mitochondrial gene expression. The reaction to this increased oxidative stress differs in case of the expression of *SOD2*. The complexity and number of included genes provide a first, initial insight into mitochondrial linked gene

expression, whereas for exploring the full expression pattern and underlying regulatory netorks more in detail, transcriptomic analyses are the next logical step as recently shown in Omotoso et al., 2021 [56].

Supporting information

S1 File. Information about primer pairs and cycling conditions.
(DOCX)

S2 File. Standard and melting curves of all primer pairs included in the study.
(XLSX)

S3 File. NanoDrop measuremnts of all extracted samples. Samples that were used in the hierarchical cluster analysis and for that all genes run successful are marked.
(XLSX)

S4 File. Genes that were influenced by period, with significant period differences.
(DOCX)

S5 File. Relative gene expression values (log2) calculated using the Pfaffl method and used as input for statistical analyses.
(XLSX)

S1 Table. Significant influence of strain, period, tissue and all possible interactions on gene expression per gene. p-values from the three-factorial anova obtained from the linear mixed model. Statistical significance was declared when $p < 0.05$.
(DOCX)

S2 Table. Number of samples per gene and tissue after the removal of outliers used to calculate emmeans from the statistical model.
(DOCX)

S3 Table. Number of samples per gene, tissue and period after the removal of outliers used to calculate emmeans from the statistical model.
(DOCX)

S4 Table. Number of samples per gene and period after the removal of outliers used to calculate emmeans from the statistical model. Included are only genes influenced by period shown in Fig 4.
(DOCX)

S1 Fig. Heat maps of two-way hierarchical cluster analyses of both strains analysed separately. Ward's minimum variance method [41] was used, the number clusters was estimated using the cubic clustering criterion [42].
(TIF)

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3.3 The impact of dietary calcium and phosphorus on mitochondrial-linked gene expression in five tissues of laying hens

PLOS ONE

RESEARCH ARTICLE

The impact of dietary calcium and phosphorus on mitochondrial-linked gene expression in five tissues of laying hens

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Abstract

Mitochondria and the energy metabolism are linked to both, the availability of Ca and P to provide the eukaryotic cell with energy. Both minerals are commonly used supplements in the feed of laying hens but little is known about the relationship between the feed content, energy metabolism and genetic background. In this study, we provide a large-scaled gene expression analysis of 31 mitochondrial and nuclear encoded genes in 80 laying hens in the context of dietary P and Ca concentrations. The setup included five tissues and gene expression was analysed under four different diets of recommended and reduced Ca and P concentrations. Our study shows, that mitochondrial gene expression is reacting to a reduction in P and that an imbalance of the nutrients has a higher impact than a combined reduction. The results suggest, that both strains (Lohmann Brown and Lohmann Selected Leghorn) react in a similar way to the changes and that a reduction of both nutrients might be possible without crucial influence on the animals' health or gene expression.

Introduction

Phosphorus (P) is an essential mineral for all living organisms which must be continuously supplied and is needed in poultry for growth, health and the energy metabolism [1]. The ability of poultry to degrade the natural present phytic-acid ($InsP_6$) is limited [2, 3] and thus feed supplements derived from rock phosphate are added to maintain the P supply. The availability of rock phosphate is limited [4] and thus a reduction of its usage is of utmost interest.

Another important mineral essential for laying hens is calcium (Ca) [5] which is needed e.g. to form eggshells. Recent studies in broiler chickens have shown, that endogenous $InsP_6$ degradation is reduced when mineral P and Ca are supplemented [6–8] and there is a strong interaction of P and Ca content regarding egg-shell quality and the number of produced eggs [9]. Many studies suggested, that the recommended dietary P content in the feed of laying hens might be too high, and can be reduced, without significant negative effects on performance and health of the animals [5, 10–12]. Hence, a better understanding of the effects of dietary P and Ca is necessary to implement these suggestions and adjust their dietary provision accordingly.

Both used strains (Lohmann Brown and Lohmann Selected Leghorn) are commercial important and selected for egg production [13–15]. Despite their similarities in egg production previous studies have shown differences between them concerning body weight, gene expression and phytate degradation in the context of the productive life span and changes in dietary Ca and P concentrations [10, 16, 17]. These results suggest differences in the strains reaction to dietary changes, which will be analysed in this work.

In this study we focus on mitochondrial gene expression since mitochondria are linked to P and Ca availability as well as to the animals' fitness and energy metabolism. Mitochondria are the main energy producers of the cell through the process of oxidative phosphorylation (OXPHOS) [18] and this process depends on the availability [1] and is influenced by the concentration [19] of P. In addition, they play a major role in regulating Ca^{2+} homeostasis [20], which is an important factor in cell signalling since Ca^{2+} controls many cellular functions [21] including gene expression [22] and the regulation of OXPHOS [23].

In our experimental setup, we included all mitochondrial encoded OXPHOS subunits, as well as nuclear encoded ones such as *NDUFB6*, which is a subunit essential for the electron transport in complex I of the respiration chain [24] and *SOD2*, which detoxifies reactive oxygen species (ROS) produced during OXPHOS in mitochondria [25, 26]. In addition, we included nuclear genes which are part of the regulatory network, linking mitochondrial gene expression and biogenesis to external stimuli, such as *PGC1α* [27], nutrient sensitive factors such as *MTOR* as well as subunits of *AMPK*, which is part of the adaptive response to energy deficit [27]. Another important key player is *IGF-1α*, which has been described as participating in P transport [28], and a reduced IGF1 expression increase the effect of Ca deficiency on bone accretion in mice [29]. *IGF-1α* has also been linked to body weight in chickens [30], which makes it a promising gene in our experimental setup.

Preliminary studies mostly focus on physiological traits important for agricultural purposes, but also try to understand the mechanisms behind the effects of dietary P and Ca. In a study focusing on phytate degradation, transcellular mineral transporters, and mineral utilization of the same hens [10] no P-mediated effects were identified and a major question rising from this studies is, how the animals react to compensate the reduced amount of P and Ca. In this study, we focus on the expression of mitochondrial-linked and nutrient sensitive genes and test the following hypotheses:

1. Mitochondrial gene expression reacts to the reduction of P and Ca
2. The strains react differently to the changes in the diet composition
3. Genes regulating mitochondrial gene expression and biogenesis react to the dietary changes

Our study benefits from the already published analysis of phenotypic traits [10] as well from the known genetic background of the two strains [31]. Together with the possibility of a controlled environment during the experimental procedure it was possible to detect and analyse gene expression changes in the context of dietary adjustment.

Material and methods

Animals and experimental setup

The animal experiments were performed at the Agricultural Experiment Station of the University of Hohenheim, Germany. They were approved by the Regierungspräsidium Tübingen, Germany (Project no. HOH50/17TE) in accordance with the German Animal Welfare Legislation.

Table 1. P and Ca content (g/kg, dry mass) of the four diets. A table containing detailed information about the nutrients in the diets can be found in Sommerfeld et al., 2020.

Ingredient, g/kg	P+Ca+ (diet 1)	P-Ca- (diet2)	P+Ca- (diet3)	P-Ca+ (diet 4)
Calculated concentrations				
Total P	5.3	4.7	5.3	4.7
Ca	39.6	33.9	33.9	39.6
Analysed concentrations				
Total P	5.3	4.7	5.3	4.7
Ca	39.5	34.4	35.1	40.3

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We used 80 laying hens: 40 Lohmann brown classic (LB) and 40 Lohmann LSL-classic (LSL) white leghorn hybrids contributed by Lohmann Tierzucht (Cuxhaven, Germany). The hens originated from an experiment addressing the utilization of phosphorus (P) and calcium (Ca) under different dietary conditions [10]. The experimental setup is described in detail in Sommerfeld, Omotoso, et al., 2020 [10] and will only be outlined briefly in the following.

The hens were reared together under standard conditions, with diets according to the requirements of each period, based on soy and corn meal. Ten father lines per strain were selected prior to the start of the experimental phase. After 27 weeks four hens per rooster were chosen and placed individually in metabolism units (1m × 1m × 1m) where the hens received specific diets for the following three weeks. Four different feed compositions were used (Table 1) and fed *ad libitum*, each group contained one hen per father line.

Samples and RNA extraction

Samples of five tissues (breast muscle, ileum, duodenum, liver and ovary) were taken on four consecutive days with random distribution of the four diets in week 31. The animals were individually stunned with a gas mixture of 35% CO_2, 35% N_2, and 30% O_2 and killed by decapitation at the Agricultural Experiment Station of the University of Hohenheim [10]. The samples were directly taken after slaughtering and were immediately placed on dry ice and stored at -80˚C until RNA extraction.

RNA was extracted using TRIzol Reagent (Thermo Fisher scientific Inc., Massachusetts, USA) according to the manufacturers' instructions with modifications described in Dreyling and Hasselmann 2022 [32]. Samples were dissolved in nuclease-free water, RNA concentration and quality in form of 260/280 and 260/230 ratios were measured using a NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher scientific Inc., Massachusetts, USA). In addition to the provided NanoDrop values for all samples, a representative subset including samples of different quality and quantity was measured on a Qubit 4 (Thermo Fisher scientific Inc., Massachusetts, USA) using the Qubit RNA IQ Assay Kit (Thermo Fisher scientific Inc., Massachusetts, USA). The samples were stored at -80˚C until further processing.

Real time PCR

The primer design and assay evaluation are already published in Dreyling and Hasselmann (2022) [32], and were not repeated specific for this experiment. The experimental procedure is identical and only described in brief in the following. In this study, 28 candidate genes were used, whereas now five additional genes have been integrated into the final set of primers: *ATPF0*, *ND2*, *ND3*, *PRKAB1*, and *PRKAG3*. A list with the gene names and their abbreviations used in this study can be found in Table 2.The used primer set including product size, primer efficiency and accession numbers of the reference sequences can be found in S1 Table. In

Table 2. Genes used in this study with abbreviations and genome in which they are encoded.

Abbreviation	Genome	Gene
ACTB	Nuclear	Actin beta
ATP6, *ATP8*, *ATP5F1*, *ATPF0*	MitochondrialNuclear	ATP-synthase F_0 subunits
COX1, *COX2*, *COX3*, *COXC6*, *COX5A*	MitochondrialNuclear	Cytochrome oxidase subunits
CytB	Mitochondrial	Cytochrome b
GAPDH	Nuclear	Glycerinaldehyd-3-phosphat-Dehydrogenase
IGF-1α	Nuclear	Insulin-like growth factor 1α
MTOR	Nuclear	Mechanistic target of rapamycin
ND1-ND4, *ND4L*, *ND5*, *ND6*, *NDUFB6*	MitochondrialNuclear	NADH:ubiquinone oxidoreductase subunits
PGC1α	Nuclear	Peroxisome proliferator-activated receptor gamma coactivator 1-α
PPIA	Nuclear	Peptidyl-prolyl cis-trans isomerase A
AMPK (PRKAA1, *PRKAA2*, *PRKAB1*, *PRKAB2*, *PRKAG2*, *PRKAG3)*	Nuclear	AMP-activated protein kinase and its α1, α2, β1, β2, γ1, and γ2 subunits
SDHA, *SDHB*	Nuclear	Succinate dehydrogenase complex subunits
SOD2	Nuclear	Superoxide dismutase
UQCRC1, *UQCRC2*	Nuclear	Cytochrome b-c1 complex subunits 1 and 2

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addition, melting and standard curves are provided which were produced the same way as described in Dreyling and Hasselmann (2022) [32].

All analyses were performed on a Biomark HD system (Fluidigm Corporation, San Francisco, USA), following the protocols for gene expression analysis using five 96.96 IFCs using the Delta Gene Assays protocol with the manufacturers standard protocol for fast PCR and melting curve as described in S2 Table. The protocol includes a DNase digestion prior to the reverse transcription and a pre-amplification with multiplexed primers followed by an Exonuclease I treatment. Pre-amplification bias was already described and discussed in Dreyling and Hasselmann (2022) [32]. All qPCRs were performed in duplicates and the samples were placed randomly on the chips, only grouped by individual to avoid any bias of sample arrangement. An internal control and a negative control (throughout all preparation steps) were included to detect variance between the runs and potential contamination. In each well of the IFC 2.25μl of the diluted Exo I digested sample were added, resulting in 3.015nl in each reaction chamber. Information about the primer-pairs and thermal cycling conditions can be found in Table A-C in S1 File.

Data preparation

Quality control. For data evaluation and quality control the Fluidigm Real-Time PCR analysis software was used. Only Cq-values from reactions with logarithmic increase of fluorescence and specific melting points were used for the following analyses. After the automatic quality check of the software, the results were evaluated by eye and revised manually if necessary. The quality threshold was set to 0.65 and the peak ratio threshold to 0.8.

The results of the internal control were checked to detect possible variation due to technical issues.

Reference gene evaluation. We used *PPIA* and *ACTB* as reference genes for normalization. Our previous evaluation already showed that *GAPDH* is strongly influenced by tissue and thus we included it as candidate gene in our study. To verify that *GAPDH* shows high variance between tissues in this experiment as well, we performed a reference gene evaluation using Normfinder [33]. As input one individual of each strain and diet was used, including all five tissues per individual to cover all our variables of interest. Normalization was tested for tissue type and diet.

Calculating relative gene expression. Means of duplicates were calculated of all samples with two successful runs. For samples that only had one successful duplicate this run was used.

Gene expression relative to the reference genes was calculated using the Pfaffl-method [34] as optimized for multiple reference genes [35, 36]:

$$rel.gene\ expression = \frac{RQ_{GOI}}{geomean[RQ_{refs}]}$$

Where $RQ = E^{\Delta ct}$ and $E = \left(\frac{primer\ efficiency\ \%}{100}\right) + 1$.

Δct was calculated as the difference between the average cycle threshold (ct) of the internal control to the ct of the corresponding sample. E refers to the converted primer efficiency and GOI to gene of interest. RQ are relative quantity values calculated using E and Δct. *PPIA* and *ACTB* were used as reference genes (refs). The data was log2 transformed prior to statistical analysis.

Statistical analysis

Linear mixed models were implemented and adjusted to each of the genes.

$$Y \sim strain + diet + tissue + tissue * strain * diet + tissue * strain + tissue * diet + strain + diet + individual + father + \varepsilon$$

Where Y is relative gene expression, ε is the residual error; strain, diet and tissue are fixed effects, with individual and father as random effects. All modelling was performed in R (R Core Team 2019, Version 3.6.1) using the *lmerTest* [37]. A three factorial analysis of variance (anova) was performed to evaluate the influence of fixed effects and pairwise tukey *posthoc* tests (package *emmeans* [38]) were performed to detect differences between strain, diet and tissues in various combinations. The output of the model is estimated marginal means (emmeans), which are used for statistical analyses throughout the whole study. The fulfilling of normal distribution and the homogeneity of variance were evaluated using QQ and residual plots. Outliers were removed for each dataset using the interquartile range, except for *SDHA*, *PRKAA2*, *PRKAB2* and *GAPDH* because for these datasets a removal of outliers would have included too many samples to perform a proper analysis.

Results

After the quality filtering, we received a dataset of 13,487 ct values from 35 Genes and all 400 samples. From 184 samples we obtained high-quality ct values for all genes, whereas for the remaining samples at least one gene was missing. In the final analyses all samples were included since no relationship between sample quality and the successful run of all genes was recognizable. Tables containing concentrations and quality of the RNA extracts can be found in S3 File.

The calculated Δct values ranged from -6.49 (min. for *PRKAA2*) to 13.38 (max for *PRKAG3*).

The statistical model revealed, that no included gene was significantly influenced by the strain, two genes were influenced by diet and all genes by the tissue. The most frequent interaction was between strain:tissue (6), followed by strain:diet and strain:tissue:diet (1) while there was no significant interaction of tissue:diet. The results of the tests derived from the statistical models for all genes and factors can be found in S1 Table. Sample numbers per gene, strain, tissue and diet can be found in S2 and S3 Tables.

Strain differences

The two-way hierarchical clustering analyses revealed a different number of clusters within each strain of laying hens. Two clusters were calculated by the cubic clustering criterion [39]

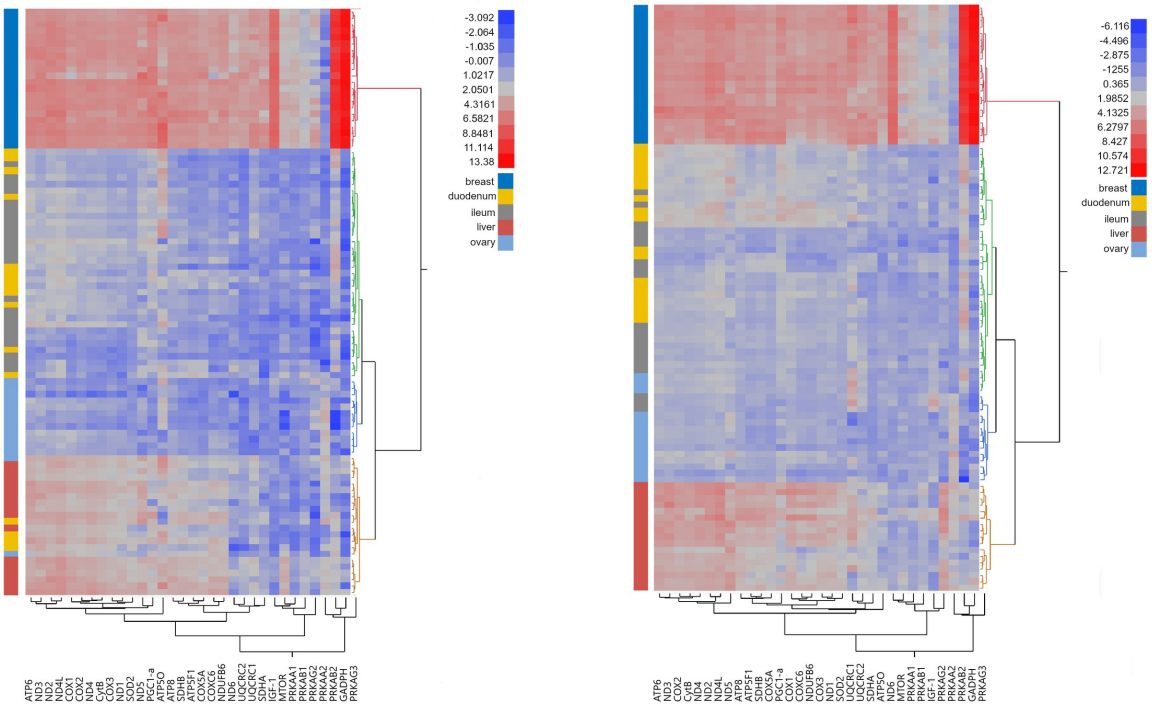

Fig 1. Heat maps of two-way hierarchical cluster analysis for individuals of the LB (left) and LSL (right) strain using Ward's minimum variance method [40], the number clusters was estimated using the cubic clustering criterion [39].

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for both strains (Fig 1). The analysis was performed on 92 samples of each strain, including 19 to 25 samples per diet per strain and 14 to 24 samples per tissue and strain. In general, both strains showed differentiation different tissue types, especially breast and liver, with more pronounced tissue specific expression pattern in the LSL strain. Consistent for both strains, a strikingly high up-regulation of three subunits of AMPK (*PRKAA2*, *PRKAB2* and *PRKAG3*) and *GAPDH* are found within breast tissue. The number of clusters was two in the LB and four in the LSL strain, which reflects the clearer separation of tissue types in the LSL strain.

Using our statistical linear mixed model, we tested for the overall impacts of strain, tissue and diet on gene expression. We observed no difference between the two strains for all genes under all four nutritional conditions, except for *IGF-1α* and *UQCRC1* (Fig 2). Both genes were showing higher gene expression in the LSL strain for all four diets, with a significant difference under low P and high Ca (diet 4) for *IGF-1α* ($p = 0.0076$), and low Ca and low P conditions (diet 2) for *UQCRC1* ($p = 0.0214$).

For *IGF-1α* these strain differences are most pronounced in three tissues: breast (higher in LB hens under low P conditions ($p = 0.0227$ for P-Ca- and $p = 0.0415$ for P-Ca+)), liver (always higher in the LSL strain, $p = 0.003$ for P+Ca+, $p = 0.0001$ for P-Ca- and $p<0.0001$ for P+Ca- and P-Ca+) and ovary tissue with contrasting pattern among the diets under P+Ca- ($p = 0.0116$) and P-Ca+ ($p = 0.0037$) (Fig 3). In ovary tissue, the significant decrease of gene expression in the LB strain under P-Ca+ is consistent with the overall trend (Fig 5) while the increase in the LSL strain does not follow this pattern.

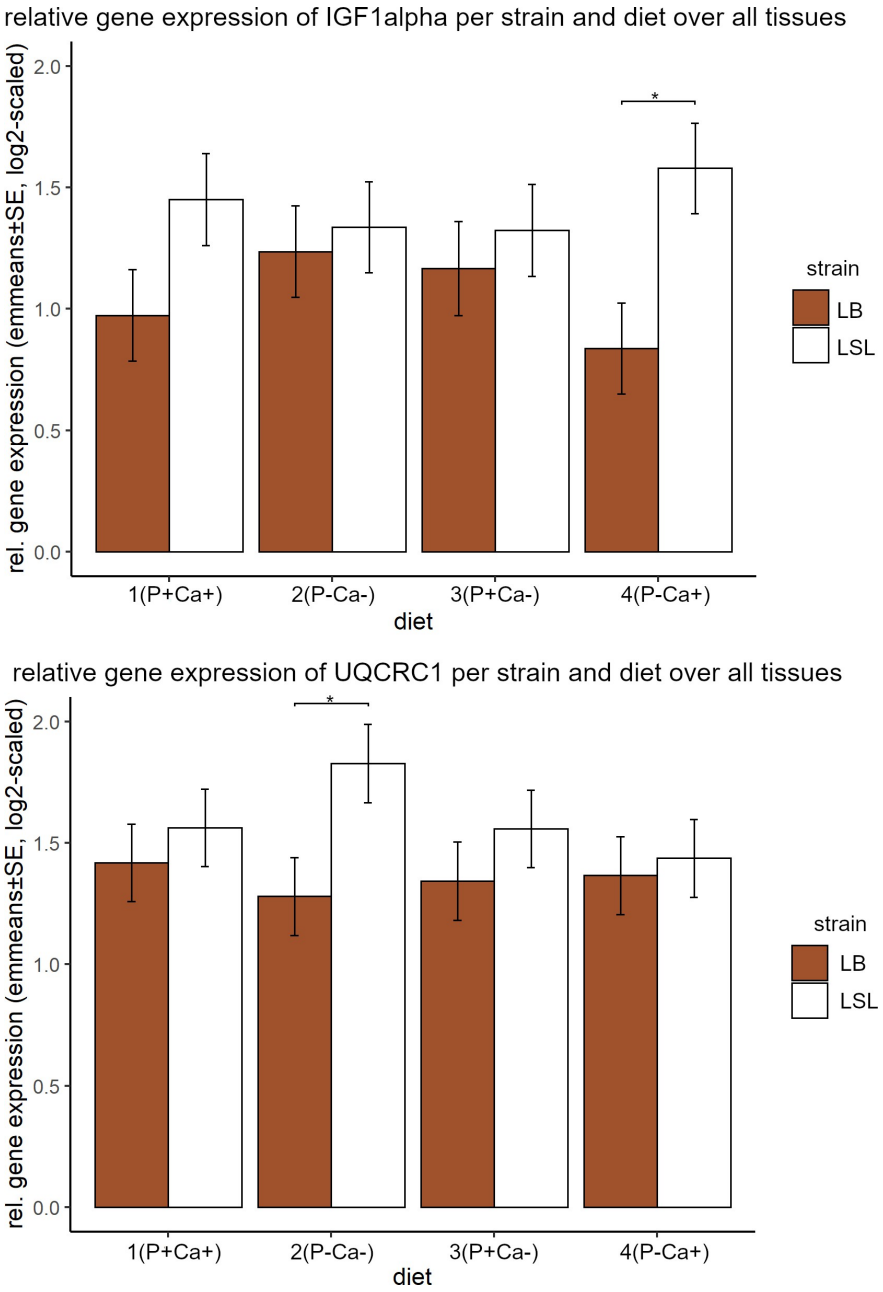

Fig 2. Relative gene expression of *IGF-1α* and *UQCRC1* of both strains for all diets. Shown are emmeans and standard errors estimated by the statistical model over all tissues. Statistical significance was declared when $p<0.05$.

https://doi.org/10.1371/journal.pone.0270550.g002

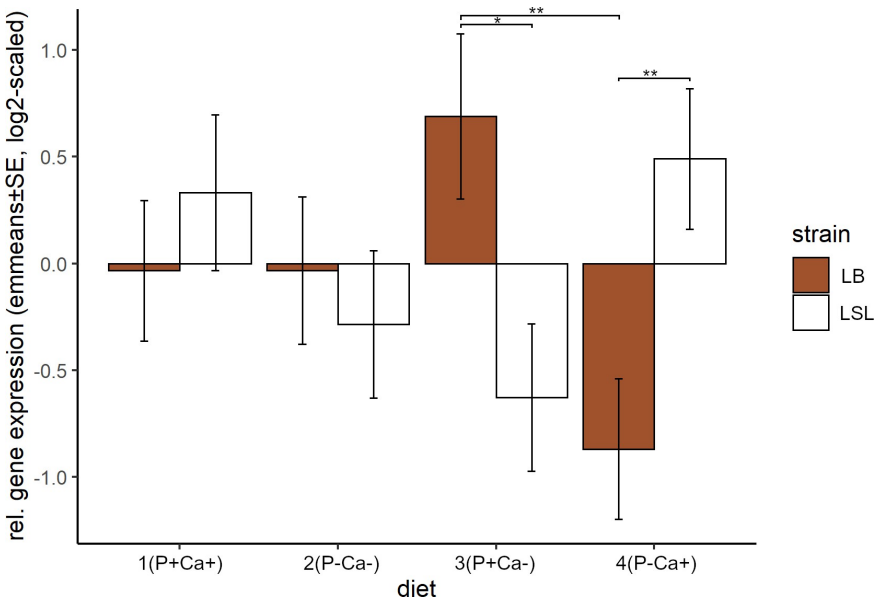

Fig 3. Relative gene expression of *IGF-1α* per strain and diet in ovary tissue. Shown are emmeans and standard errors estimated by the statistical model over all tissues. Statistical significance was declared when $p<0.05$.

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Differences between the diets

The overall gene expression (mean of all genes, tissues and strains per diet) was lowest under low P and high Ca conditions; however, the differences between the diets were not significant. The statistical model revealed, that the expression of only two genes (*SOD2* and *NDUFB6*) was affected by the diet. Nevertheless, with pairwise comparisons of the diets, more genes showed significant gene expression differences: *ND3*, *CytB*, *SOD2*, *COXC6*, and *NDUFB6*. For all these genes, the expression was lowest under low P and high Ca conditions. The expression of *SOD2*, *COXC6* and *NDUFB6* was significantly higher under high P compared to low P under high Ca conditions (Fig 4) for both strains analysed together. In addition, the LB strain showed expression differences in *CytB*, where the expression was higher under high P and Ca levels as well as under low P and Ca levels compared to diet 4 (P- Ca+).

In addition to the already described differences between the strains in the expression of *IGF-1α*, the differences between P+Ca- and P-Ca+ in ovary tissue were significant in the LB strain ($p = 0.0062$) and strong but not significant in the LSL strain ($p = 0.0563$) (Fig 3). Additionally, the expression in the LB strain was significantly higher in liver tissue under P-Ca- compared to P-Ca+ ($p = 0.0088$)

Tissue differences

As already discussed in the context of time dependent gene expression [32], the tissue had the strongest influence in our experimental setup, influencing the expression of all included genes. The gene expression was significantly highest for all genes in breast muscle compared to the remaining four tissues ($p<0.001$ for all except *PRKAA1* where $p = 0.048$ when breast compared to liver), except for *IGF-1α* and *PRKAG2*, where there was no difference compared to

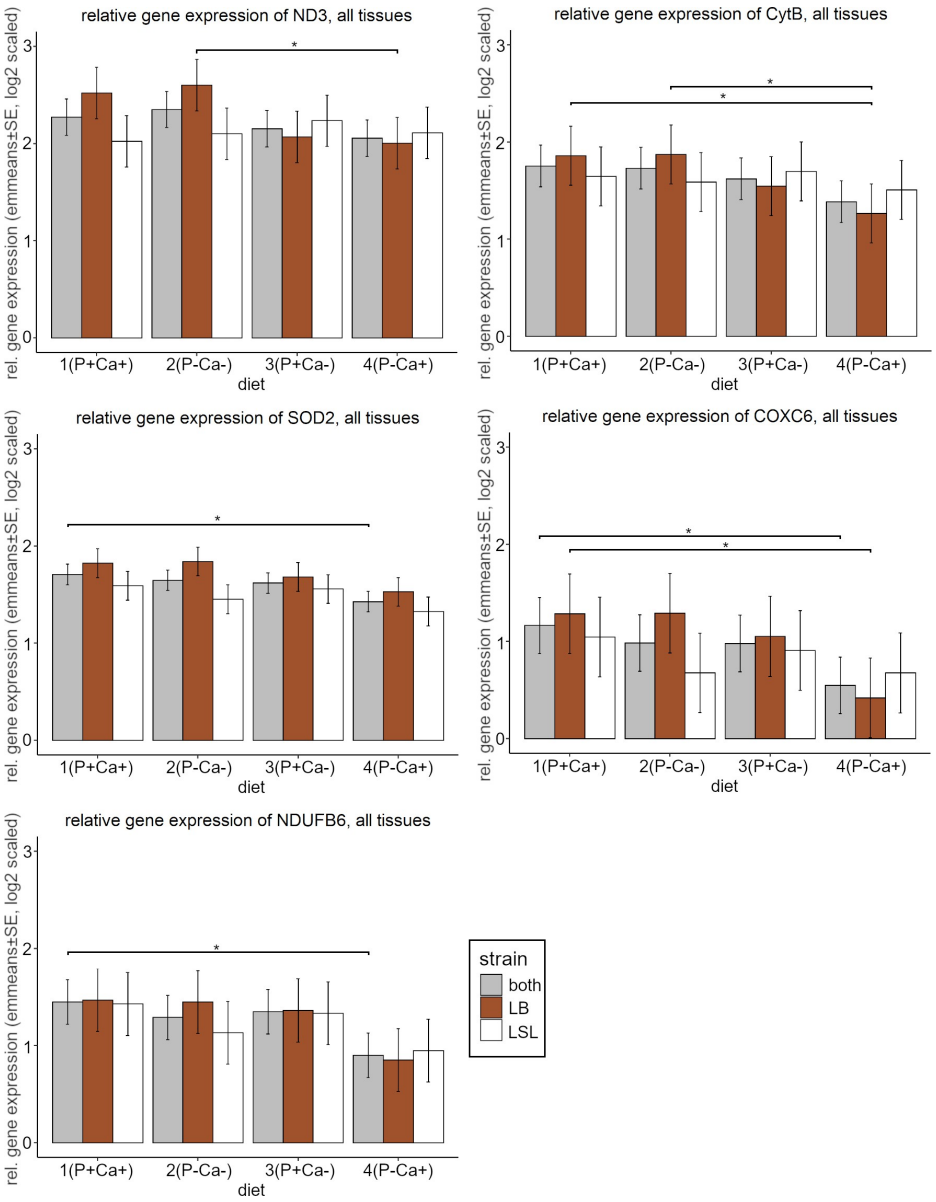

Fig 4. Relative gene expression of *ND3*, *CytB*, *SOD2*, *COXC6* and *NDUFB6* for both, the LB and the LSL strain for all diets. Shown are emmeans and standard errors estimated by the statistical model over all tissues. Statistical significance was declared when $p < 0.05$.

https://doi.org/10.1371/journal.pone.0270550.g004

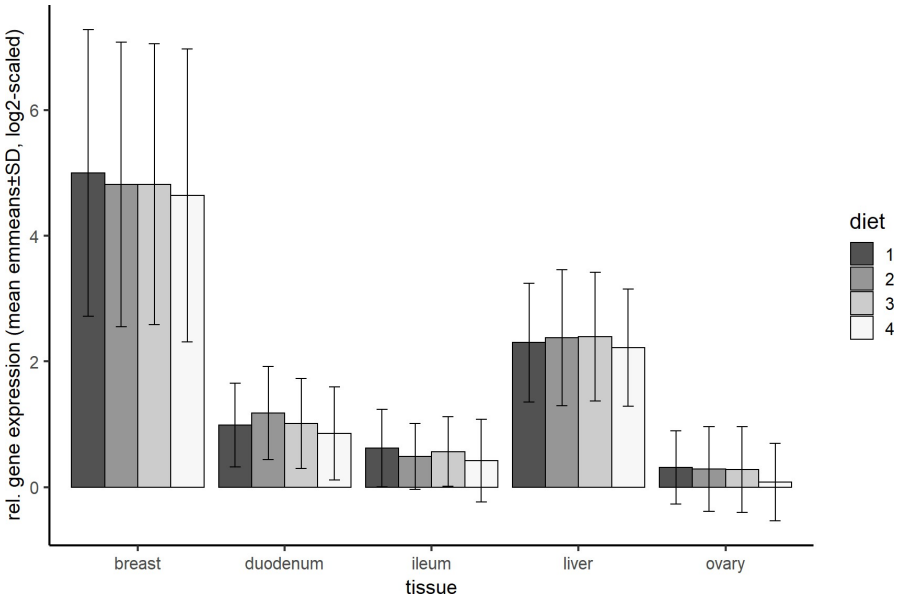

Fig 5. Relative gene expression in five tissues and diets. Shown are means and standard derivations of the emmeans of all genes calculated by the statistical model.

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liver tissue. The expression was lowest in all tissues when fed diet 4 (P-Ca+) (Fig 5). The already described diet-dependent gene expression changes in *SOD2* and *NDUFB6* were only observed in breast muscle tissue, where the expression was significantly lower under P-Ca + than under P+Ca+ levels (for more details see S3 Table). In *GAPDH* highly significant differences between all pairwise tissue comparisons were observed ($p<0.0001$), which supports the decision to abandon it as a reference gene in our study.

Discussion

In our experimental setup we were able to analyse a vast number of mitochondrial and nutritional linked genes in the context of dietary changes in P and Ca contents. We hypothesized, that one compensatory mechanism of changes in the diets is the adaption of mitochondrial gene expression, since it is directly linked to the availability of P and the fitness of the individuals.

Differences between the strains

For none of our candidate genes, the gene expression differed between the strains, and only two genes (*IGF-1α* and *UQCRC1*) showed different gene expression in specific diets (under low P concentration). These observations indicate, that both strains react to the changes in dietary P and Ca content the same way and also in the hierarchical clustering analyses, the pattern of both strains was similar. A genome wide gene expression analysis comparing the same strains as included in this work identified genes related to the GO-cluster of phosphorous metabolism (GO-IDs: GO:006468, GO:0006793, GO:0006796, GO:0016310) to be down regulated in hens of the LSL strain [14] and Sommerfeld et al., 2020 [10] identified two sodium/

phosphate co transporters to be higher expressed in the LSL strain using the same individuals as in this work. These results indicate, that there are differences between the strains related to P metabolism, however the genes included in this work showed no general differences between the two strains. It must also be noted, that Sommerfeld *et al*. 2020 [10] identified no interaction of strain and diet on the included genes, which supports the hypothesis that the reduction of P and Ca content in the used diets was not sufficient to improve the expression levels of our gene targets significantly. Even if we were able to show differences in gene expression between strains and diets in some genes in our study, the majority of the included genes showed no reaction to the dietary changes. A significant change in the mineral concentrations with an detrimental effect on the whole animal might lead to stronger effects on the animals as described in this work or by Sommefeld *et al*. 2020 [10].

Mitochondrial gene expression in the context of dietary changes

Since both nutrients are linked to the mitochondrial energy-metabolism [1, 23] we suggested an adaption of mitochondrial gene expression as a compensatory reaction to the changing amount of the minerals. Our data revealed, that most genes showed no significant difference in gene expression according to the changes in the diet. However, the gene expression was lowest under P-Ca+ conditions, while the reduction of both minerals or only Ca had a smaller effect on gene expression. These results suggest, that the effect of reduced P concentrations is stronger, when there is an imbalance of the proportion, especially under low P.

Four of the five genes showing significant differences were part of the electron respiratory chain, representing complexes I, III, and IV and the ROS detoxifying gene *SOD2*. The gene expression was significantly lower under P-Ca+ compared to P+Ca+ in genes representing subunits from Complex I and IV of the respiration chain and the ROS detoxifying gene *SOD2*. The same observation was made for *CytB* in the LB strain (Fig 4). This observation suggests that the reduced availability of P impacts distinct parts of the respiratory system, resulting in reduced gene expression. Since it is known, that the expression of whole complexes can be regulated by the expression of individual subunits [41, 42], the reduced expression of single subunits might regulate the amount of the whole complex. Additionally, previous studies showed pattern of co-expression of the OXPHOS complexes [43], which is also shown in our analysis under low P and high Ca conditions.

A general reduction of assembled OXPHOS complexes might be the result of the observed expression pattern. The potential reduced production of ROS resulting from diminished OXPHOS activity leads to a reduced need of SOD2. SOD2 expression has been linked to impact immunity against bacterial infections in zebrafish [44] and ROS are known to play a role in the reaction to inflammatory disease [45–47]. Thus, the differences in SOD2 expression in laying hens might indicate differences in resistance to infections as well. In addition, an increase in the production of ROS in the mitochondrion is linked to the process of ageing in many species [48] and the increase of *SOD2*-expression protects the mitochondrion from damage, which would otherwise lead to the death of the cell (as stated in Santos et al., 2018 [48], Yin et al., 2018 [49] and cited references within). Regarding the missing of differences in gene expression between the other treatments suggests that a reduction of P alone is more crucial than a reduction of P and Ca or Ca.

Gene expression differs between different tissue types

As described in the context of life span [32] the gene expression in breast muscle was significantly higher than in all other tissues, followed by liver tissue. Studies in humans and chimpanzees have shown, that the differences in gene expression are higher between tissues than

between species and suggests to include different tissue types in functional genomics studies [50]. In liver, the high rates of gene expression might be explainable by the high amount of functions of the tissue, ranging from the conversion of glucose into glycogen, the filtration of blood to the production of cholesterol [51]. In the duodenum and ileum the mucosa and microbiome are important participants in the process of digestion and uptake of nutrients [52], which might explain the lower rates in the tissue itself. However, the high activity in breast muscle tissue is surprising, especially since the process of growing was already finished during sampling and the purpose of the strains focuses on egg laying instead of meat production. In general, there are not many studies comparing gene expression rates between tissues since most studies focus on differences between different treatments, diseases or developmental states.

IGF-1α in the context of nutritional changes

We observed most differences in gene expression between strains and diets in the growth factor *IGF-1α*. This gene plays a major role in a variety of tissues and functions, e.g. metabolic homeostasis [53, 54], and growth [55, 56] and nutrition has been identified as a key factor of IGF1 regulation in humans [57, 58]. This sensitivity might be the reason of the significant differences in expression between the diets observed in this study, especially since malnutrition is known to reduce circulating IGF1 in mice [59]. Additionally, malnutrition has been linked to decreasing IGF1 expression in liver tissue [60], which is reflected by the significantly lower expression under P-Ca+ compared to P-Ca- in liver tissue of the LB strain in this study. This observation also leads to the conclusion, that an imbalance of the minerals is detrimental compared to the reduction of both minerals, and is thus a form of malnutrition. An interaction of P and Ca content have also been shown in the context od egg-shell quality and quantity of eggs [9]. Simultaneously the gene expression is significantly lower under P-Ca+ conditions in the LB strain, which is another indication of differences in the reaction to the reduction of Ca in both strains. The most prominent difference between the strains was the contrary expression of the strains under P+Ca- and P-Ca+ conditions. Even if it is long known, that IGF-1α plays a role in avian ovaries [61], we could not observe any changes in egg weight between the strains matching the change in expression pattern [10].

Expression changes in nutrient sensitive and mitochondrial regulatory genes

We included nutrient sensitive genes such as *MTOR* and *AMPK* and the nutrient sensitive mitochondrial regulator *PGC1α* in our study and the missing reaction to our dietary changes is striking. All of these genes have been analysed in the context of changes during the productive life span of laying hens [32] using the same technical approach, which makes it rather unlikely, that our setup is failing in detecting expression changes. The same hens have been analysed in the context of performance traits such as body weight, feed intake, average egg weight and P/Ca efficiency, where no diet specific changes could be observed [10]. In accordance to other studies [5, 11, 12] the authors conclude, that a 20% reduction of P is not affecting the animals and thus, the recommended concentrations in the feed of these animals might be too high. The missing effect on genes that are part of the mitochondrial regulatory network supports this hypothesis.

Conclusion

We performed a large-scaled analysis of mitochondria-linked gene expression in laying hens in the context of P and Ca content in the diets. Our study revealed interesting differences of

gene expression of subunits covering most OXPHOS complexes under low P and normal Ca concentrations in the diets. Together with the decrease in the expression of the ROS detoxifying gene *SOD2*, an interesting part of the regulation of mitochondrial gene expression has been revealed. In addition, the effects on the growth factor IGF-1α showed that the reduction of P in the diet has an effect on the mitochondrial regulatory network as well. We also observed that an imbalance of both minerals seems to have greater influence of gene expression than the reduction of both nutrients, especially under low P conditions.

Supporting information

S1 File. Information about primer pairs and cycling conditions.
(DOCX)

S2 File. Standard and melting curves of the primers for ATP50, ND2, ND3 and PRKAG3.
(XLSX)

S3 File. NanoDrop measuremnts of all extracted samples. Samples that were used in the hierarchical cluster analysis and for that all genes run successful are marked.
(XLSX)

S1 Table. Significant influence of strain, diet, tissue and all possible interactions on gene expression per gene. p-values from the three-factorial anova obtained from the linear mixed model. Statistical significance was declared when $p < 0.05$.
(DOCX)

S2 Table. Number of samples per gene and tissue after the removal of outliers used to calculate emmeans from the statistical model.
(DOCX)

S3 Table. Number of samples per gene, tissue and diet after the removal of outliers used to calculate emmeans from the statistical model.
(DOCX)

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Methodology: Clara Dreyling.

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Supervision: Martin Hasselmann.

Validation: Clara Dreyling, Martin Hasselmann.

Visualization: Clara Dreyling.

Writing – original draft: Clara Dreyling.

Writing – review & editing: Clara Dreyling, Martin Hasselmann.

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4. General Discussion

The aim of this work was, to explore the mitochondrial genetic influence on phenotypic traits and to explore mitochondrial-linked gene expression in the context of the productive life span and dietary changes in laying hens. Two experiments were set up of which the genetic analyses were performed together while the gene expression analyses were executed for each experiment separately. As a result, a low number of mitochondrial haplotypes was identified, which influenced further analyses of this work. In addition, a low effect of dietary changes of P and Ca content on mitochondrial-linked gene expression was identified. The analysed of gene expression in the context of the productive life span revealed contrary gene expression of mitochondrial and regulatory genes. These findings are discussed in the following paragraphs and contribute to a better understanding not only of the genetic structure of the used strains, but also reveal the complexity of the regulatory gene expression network of laying hens. The findings of this work can be used to enhance future experiments on laying hens and provide an interesting insight into the molecular processes in the context of P and Ca utilization and resource conservation.

4.1 Methodological considerations of the genetic analyses

Compared to natural populations the use of domesticated animals has several benefits: pedigree information is usually available and experiments can be performed under highly controlled conditions, which are relatively similar to the usual living conditions of the animals, apart from additional requirements for animal experiments. However, the experimental setup of this project led to some unexpected issues:

Based on previous studies (Guan et al., 2007; Lan et al., 2015; Liao et al., 2016; Liu et al., 2006; Miao et al., 2013; Osman et al., 2016) it was expected to identify several haplotypes including individuals with unique haplotypes to combine them with expression and performance data. However, the genetic analysis revealed a surprisingly low number of mitochondrial haplotypes for both strains (Manuscript 1, Heumann-Kiesler et al., 2021), which were unequally distributed among the experimental groups in both experiments. This distribution led to a loss of statistical power, since we could not include it properly in the statistical modelling, which was a major challenge throughout the whole project. The problematic distribution occurs especially when the group size of the different treatments is highly unequal. A minimal invasive genotyping approach e.g. from plucked feathers of the individuals

(Segelbacher, 2002) prior to the subdivision into different groups would bypass this phenomenon while providing genetic information of the whole population independently from their usage in the experimental phases.

4.1.1 The importance of choosing an algorithm fitting to the data

Another important aspect that was considered during the genetic analyses is the estimation of genomic relatedness. Due to the low number of mitochondrial haplotypes, the nuclear genomic relationships became increasingly interesting. Prior to the G-Matrix approach (VanRaden, 2008) presented in Manuscript 1, I additionally tested the *relatedness2* command of the program vcftools (Danecek et al., 2011). This analysis is based on the KING algorithm which estimates the probability that two alleles sampled at random from two individuals are identical by descent (Manichaikul et al., 2010). The algorithm thus includes pairwise comparisons of all individuals, and estimates the genomic relationship without considering additional background information. As a result, all individuals were declared to share at least a second degree relationship, which is equal to being half siblings, while all known half siblings were estimated to be as related as full-siblings or parents to their offspring. In addition, statistical tests revealed, that the mean relatedness within the white strain was significantly higher than in the brown strain ($p<0.0001$, Kruskal-Wallis test performed in JMP Pro (Version 13. SAS Institute Inc., Cary, NC, USA, 1989–2019)). While the observation of closer relationships within the white strain fit well into the observed mitochondrial haplotype diversity, the general high relationships between the individuals did not fit into the pedigree information supplied by Lohmann. All individuals sharing at least a second degree relationship would indicate a high level of inbreeding and nearly no genomic variation left in the strains. The full-sibling relationships of individuals sharing the same father would indicate that these individuals either share the same mother, a generally low number of mothers or closely related mothers. Since the pedigree information stated that the hens used in the parental generations were unrelated and thus the relationships estimated by the KING algorithm were questioned. For verification, a G-Matrix (VanRaden, 2008) was used as described in Heumann-Kiesler et al., 2021, which is a commonly used approach from the field of animal breeding genetics. The results are described in Manuscript 1, and show the same pattern of relationships between individuals as the KING algorithm: Individuals with the same paternal background appeared to be closer related to each other than other individuals, but the overall relatedness was

lower and more similar between the strains. In contrast to the KING algorithm, this approach includes genetic information of the whole population into the calculations, and is thus designed to analyse more complex genomic relationships. Since I was working on a strongly selected domesticated population, the KING estimation might be influenced by the different background compared to natural populations for which it was designed. Furthermore, it is known that animal breeding can reduce genetic diversity (Notter, 1999) and produces additional selective pressure on traits favoured by the breeders. In subsequent analysis the G-Matrix approach was used instead, since it was more suitable for the data, especially considering this background information and our knowledge of the population. Still, the clear difference of the estimates is remarkable and shows the importance of algorithm choice. It needs to fit the research questions, the population type in question and finally the collected data, to avoid biases and over- or under interpretation of the results.

4.1.2 Limitations of the qPCR approach

In the experimental setup, the sampling and storage of tissue samples as well as the RNA extraction and its storage were performed under standardized and controlled conditions. These conditions minimize known problems such as RNA degradation or bias introduction through sampling and storage conditions. In addition, the usage of a high-throughput machine including between-run and negative controls reduces the probability of experimental bias. Furthermore, the experimental structure, with phases in which the animals were separated and monitored individually, provided a high level of standardization from the beginning of the experiment. However, after the evaluation of the gene expression data some points occurred which should be considered in further experiments: i) The number of mitochondria has not been measured or considered. It is known, that the number of mitochondria per cell can vary between tissues (Veltri et al., 1990). Through model correction for tissue and individual effects, biases introduced by both could be limited. Yet it would be valuable to gain quantitative insights into the mitochondrial occurrence. This can be achieved through qPCRs based on mitochondrial DNA (Rooney et al., 2015) and would add more important detail to the analyses. Nevertheless, the detected differences in mRNA expression are of interest, regardless of their origin, might it be varying number of mitochondria or variance in gene expression. ii) Changing RNA expression is only one part of a potential reaction on the molecular level and only explains a fraction of variation in protein abundance (Vogel & Marcotte, 2012). However, the

concentration of mRNA is an adequate measurement for the presence of a protein in the cell (Ramakrishnan et al., 2009). Including a measurement of protein activity could complement the overall picture of functional reactions on the molecular level (Greenbaum et al., 2003; Hatzimanikatis et al., 1999). But after all, changing gene expression is the first step in a complex molecular regulatory system and depicts a profound basis for further research.

4.2 Genetic diversity of the populations

The aim of this part of the thesis was, to identify mitochondrial haplotypes and examine their link to phenotypic traits such as body weight, feed intake and P utilization. The analyses revealed a surprisingly low level of mitochondrial diversity, including three haplotypes within the LB and one within the LSL strain. Due to the long-range PCR approach and several validation steps on the laboratory and bioinformatics level, errors or bias as through nuclear mitochondrial DNA sequences (numts) (Lopez et al., 1994) or laboratory errors could be excluded. As derived from other studies, we expected a higher level of genetic diversity and individual mutations. A higher breeding pressure, compared to African or Asian lines included in the other studies, might explain the absence of this diversity. This pressure would also explain the non-appearance of individual mutations, except for one LB hen, that showed signs of heteroplasmy, which is common in chicken (Alexander et al., 2015). Considering the mostly uni-parental haploid inheritance of mitochondria (Ladoukakis & Zouros, 2017) the numbers of identified haplotypes could indicate, that all included LB hens originated from three and the LSL hens from one single female ancestor. This observation is even more striking, considering that the hatchlings were delivered on different time points four months apart, including different fathers and thus most likely a different group of mothers as well. The detected low mitochondrial diversity raised the question, whether this diversity is representative for the nuclear genetic background as well. The analyses of the genotype data revealed, that both strains are clearly separated and that the nuclear genetic diversity is similar within both lines. This discovery shows, that the general genetic diversity is higher as estimated exclusively by the mitochondrial genome, and that the mitochondrial haplotype cannot be equated with the distinct mothers. In addition, individuals with the same mitochondrial haplotype were not closer related than individuals with different haplotypes which underlines that sharing a mitochondrial haplotype is not equal to sharing a mother.

The genomic differentiation (F_{ST} = 0.35) between both lines is similar to other published data (F_{ST} = 0.346 (Brekke et al., 2020)) and higher as indicated by the mitochondrial genomes. Nevertheless, some half-siblings appeared to be highly similar in the ADMIXTURE analysis, which calculates individual ancestries using a maximum likelihood algorithm (Alexander & Lange, 2011), and also showed high scores in the G-Matrix. This observation together with the low mitochondrial diversity in general underlines that more research is needed to determine the maternal background of the lines and potential effects of the loss of mitochondrial diversity.

4.3 Linking mitochondrial haplotypes with phenotypic traits

Linking mitochondrial haplotypes to phenotypes has become a very active field of research and analyses were performed on different domesticated species in the context of meat quality (Fernández et al., 2008; Mannen et al., 1998) and metabolic capacity (Kinoshita et al., 2018) in cattle. As stated in Heumann-Kiesler et al., 2021 (Manuscript 1) and in chapter 4.1 the low number of mitochondrial haplotypes and a suboptimal distribution among the different treatments and groups did not allow robust testing of the relationship between mitochondrial haplotype and the included phenotypic traits. Since not all haplotypes appeared in all diets and life stages and the number of individuals per group varied drastically, statistical tests would not lead to trustworthy results. However, the high variance of body weight, feed intake and P utilization within the brown strain might be a sign of haplotype-specific effects, especially since body weight is a well-known trait affected by the mitochondrial genome in bison (Derr et al., 2012) and pigs (St John & Tsai, 2018) as well as in the rainbow-trout (Danzmann & Ferguson, 1995) and humans (Ebner et al., 2015).

The LB strain showed higher variance in all analysed traits (body weight, feed intake and P utilization) compared to the LSL hens and showed high variation within and differences between the different haplotypes as well. Especially in the context of P utilization LB haplotypes showed higher variation while having distinctly less individuals per group compared to the LSL hens. In combination with the comparably diverse nuclear genetic background, the higher number of mitochondrial haplotypes enables more combinations of different mito-nuclear genotypes and thus, has a higher probability of beneficial or detrimental combinations. These combinations might influence different traits in different ways, especially since a relationship of the

mitochondrial haplotype and body weight has been shown (Danzmann & Ferguson, 1995; Ebner et al., 2015; St John & Tsai, 2018).

The presented genetic results can also be used as a starting point for further research. Especially in the context of P utilization, where the connection of its availability, usage and molecular processes linked to mitochondria is rather complex. A part of this complex network, consisting of gene expression of mitochondrial and mitochondria-linked genes is analysed in Manuscript 2 and Manuscript 3.

4.4 Differences in gene expression between the two strains

Gene expression was analysed in the context of the productive life span in five different periods (100 individuals, 10 per strain and period) and under four different dietary conditions, including high and low P and Ca contents in all possible combinations (80 individuals, 10 per strain and group). Due to the low number of haplotypes and their distribution in both experiments, we analysed differences between the two strains instead of between the haplotypes. The objective of this part of the work was, to combine the genetic with functional findings and identify differences between both strains. Previous analyses of the individuals revealed strain differences in several phenotypic traits, which make gene expression differences between the strains more likely.

The expression of the majority of the included genes was not influenced by strain in both experiments. The hierarchical cluster analyses showed, that the expression follows the same pattern in both strains, but also revealed small differences which hint at differences between the strains. In general, the strain differences are minor especially since strain differences in other traits (e.g. feed intake, body weight and P utilization) were observed (Gonzalez-Uarquin et al., 2021; Sommerfeld et al., 2020a; Sommerfeld et al., 2020b). Due to the differences in body weight, we expected expression differences in the growth factor *IGF-1α*, which has been linked to body weight in chicken in the past (Bhattacharya et al., 2015). In our experimental setup, the expression of *IGf-1α* does not seem to be one of the key genes especially in context of the productive life span. However, a significantly higher expression in LSL hens was also observed in the second experiment under low P and high Ca conditions, and large differences were observed in ovary tissue, especially between P+Ca- and P-Ca+. These observations fit well into the results of Sommerfeld *et al*. 2020b, where the average egg weight was influenced by both, strain and P content.

IGF-1α is known to be higher expressed in ovary tissue of laying compared to nesting Muscovy ducks (Wu et al., 2016). These results illustrate, that the expression of *IGF-1α* is an important modulator in laying hens, however its influence in the context of weight gain during the productive life span is supposedly minor. In this experiment, no gene was significantly influenced by strain and only *IGF1α* and *UQCRC1* showed significant differences between the strains, but only under low P conditions. Yet, the expression was higher in the LSL hens in all diets for both genes but with no significant difference, while the expression was higher in LB hens in all other genes and diets (with no significant difference).

In the context of the productive life span, four genes were significantly influenced by strain: *GAPDH*, *ND6*, *PGC1α* and *SOD2*. The expression of all genes was higher in the LB strain. The higher expression of *SOD2* as a nuclear encoded detoxifier of reactive oxygen species (ROS) (Bratic & Larsson, 2013; Kokoszka et al., 2001) in LB hens might be a sign of higher levels of oxidative stress. Particularly during the laying period, where the metabolism of the hens switches to reproduction and eggs are produced frequently. Accordingly, more pronounced strain differences were observed. The higher expression of the transcriptional co-activator *PGC1α* in LB hens in nearly all experimental phases underlines the hypothesis that this strain might experience higher metabolic stress and has to adjust gene expression on a higher level compared to the LSL hens. These observations are consistent with other findings from the same experiment, where the LB hens showed a higher metabolic body size and *myo*-inositol oxygenase (MIOX) concentration in the kidney (Gonzalez-Uarquin et al., 2021).

The higher expression of *PGC1α* and *SOD2* in the LB hens in later periods underline the already described differences between the two strains. Not only is gene expression and body weight more diverse within the brown strain, the higher expression of *SOD2* in later periods suggest a higher level of oxidative stress. These findings match the observation made by Gonzalez-Uarquin et al., 2021, who suggest a higher level of metabolic stress in the LB hens.

The significant differences in gene expression of *GAPDH* in this experiment are of major interest, since it is commonly used as a reference in gene expression analyses (Barber et al., 2005) and was initially included as a potential reference gene in this study, too. During the evaluation of the candidate genes strong tissue differences

appeared, which are also described in human (Barber et al., 2005) and the gene was excluded as a reference gene. The observed differences between the strains underline the importance of the selection of reference genes according to the question and experimental setup.

The absence of strain differences in the context of different diets indicates, that at 27-30 weeks the strains display similar gene expression, independent of the varying diet while strain differences become more pronounced during the productive life span. These results suggest to include several time points of interest into future experiments focusing on gene expression in laying hens, instead of focusing on a specific time point.

4.5 Gene expression differences between tissue types

To understand the complex mechanisms of P and Ca utilization, several tissues were included into the gene expression analysis. The expression of all genes was highly influenced by tissue, independent of strain, experiment or group of the individuals making it to the strongest influence in the whole setup. Tissue specific gene expression is well described in many species (Barber et al., 2005; Blake et al., 2020) and was also observed in both experiments, which is one of the reasons *GAPDH* was excluded as a reference gene in both studies. Despite the suggestion that tissue specific gene expression might be more population- than species specific (Whitehead & Crawford, 2005) it has been proposed to include several tissue types into functional genomic studies (Blake et al., 2020). The observed differences might be explainable by the distinct functions of the tissues (Whitehead & Crawford, 2005) and are useful to get a better understanding of mitochondria linked gene expression in the context of P and Ca utilization. The high activity of liver tissue, with multiple functions ranging from turning glucose into glycogen, the filtration of blood to the production of cholesterol (Campbell & Reece, 2008), might explain the high gene expression. Contrastingly, the low gene expression of duodenum and ileum could be explained by the central role of mucosa and their microbiome as shown by Nicholson et al., 2012. The importance and interaction of the microbiota with the host in different gut-tissues have been shown in Japanese quails in the context of P and Ca utilization (Borda-Molina et al., 2020; Ponsuksili et al., 2020). The significantly higher gene expression in breast muscle tissue is interesting, especially since it appears

independent of growth or development and the hens used in this study were bred for egg laying, instead of high muscle mass and body weight for meat production.

4.6 Changing gene expression during the productive life span and under different diets

Assessing changes during the productive life span on the tissue level, it becomes apparent, that the expression does not change in the same way. This can also be seen in the high amount of genes influenced by an interaction of tissue and period (n=20 out of 30). While the mean expression of all genes was constantly high in liver and breast muscle tissue, the expression in ileum and ovary changed more. In the ovary, the mean gene expression declined from period one to four and increased in the last period. Intuitively one would expect increasing gene expression towards the start and decreasing towards the end of the laying period, however the formation of eggs is regulated by a complex network of hormones interacting with several genetic factors (Mishra et al., 2019) of which none were included in this study. Regarding expression changes during the different periods for individual genes, only seventeen genes were influenced by period. These genes fall into two different categories: Genes related to OXPHOS and ROS detoxification with increasing gene expression, and genes that are part of the mitochondrial regulatory network, that decreased with ongoing period.

The analyses of gene expression under different Ca and P concentrations revealed, that most genes except for *SOD2* and *NDUFB6* were not affected by the changes. This observation suggests, that a reduction of 20% is not enough to challenge the individuals' metabolism. However, gene expression was lowest under P-Ca+ conditions (without significance) indicating that the reduction of P has a stronger effect on gene expression than reducing both minerals simultaneously. In addition, most significant expression changes were observed between P+Ca+/P-Ca+ (and P-Ca- /P-Ca+) which also emphasizes the hypothesis that an imbalance of the minerals is more crucial than a reduction of both. Most differences appeared under the reduction of P which underlines it's importance in terms of availability (Elser, 2012) and concentration (Bose et al., 2003) for the production of energy. The absence of differences between the diets indicates, that the suggested amounts of P and Ca in the feed of laying hens should be reconsidered. The same suggestion was made based on data from the same experiment by Sommerfeld et al., 2020b and in other

studies focusing on laying hens, where a reduction of P was possible without significant negative effects on performance and health of the animals (Ahmadi & Rodehutscord, 2012; Jing et al., 2018; Pongmanee et al., 2020).

4.7 Changes in gene expression in genes related to OXPHOS and ROS detoxification

Genes encoding for proteins involved in OXPHOS were the main target of both studies, to add functional information to the haplotypes identified in the genetic analyses. In addition, nuclear encoded subunits were included, as well as *SOD2* as the main mitochondrial ROS detoxifier in mitochondria, preventing them from major oxidative damage (Yin. et al., 2018).

In the context of the productive life span a decrease in gene expression was expected for these genes, instead an increase has been observed for these genes. Manczak et al., 2005 observed increasing gene expression for OXPHOS complexes I, III, IV and *ATP6* in mice brains in a comparable time frame. A potential reason of the missing decline in gene expression might be the timeframe chosen, which does not reflect the total lifespan of the hens but only their productive lifespan. However, as a domesticated animal bred for the purpose of egg laying, the productive lifespan is the most relevant in agricultural science, because the general life span is shortened. In addition, the high frequency of egg laying (the performance data specifies more than one egg every second day) requires not only enough nutrients and calcium, but also an increasing energy demand with increasing productivity, which might be reflected by the increasing gene expression.

During the process of OXPHOS complexes I and III are the main producers of ROS as a by-product (Bratic & Larsson, 2013). Along with the increase in gene expression of the mitochondrial OXPHOS genes with ongoing productive life span, the expression of *SOD2* followed the same pattern. SOD2 is one of the main detoxifiers of ROS in the mitochondrion (Missirlis et al., 2003) and thus protect the cell from dying due to the resulting oxidative damage (Santos et al., 2018; Yin. et al., 2018).

In the context of dietary changes, *SOD2* was affected by the diet and showed significant difference between high and low P content under stable Ca+ conditions. Since ROS play a major role in ageing and cell death these findings contribute to a better understanding of the effects of ongoing life span and oxidative phosphorylation

on laying hens. The same observation was made for subunits of OXPHOS complexes I and IV with complex I being one of the major ROS producers (Bratic & Larsson, 2013) as already mentioned in the context of the productive life span. Other studies have shown, that the mitochondrial complexes show patterns of co-expression (van Waveren & Moraes, 2008), which can also be seen in this experiment under low P and high Ca conditions (P-Ca+). Together with the observation of a lack of co-expression of different subunits of the same protein, the complexity of the regulatory mechanisms becomes apparent and these results suggest to include several subunits and complexes into mitochondrial gene expression to depict OXPHOS related gene expression and their potential effects instead of focusing on individual subunits.

4.8 Expression changes in nutrient sensitive and mitochondrial regulatory genes

As a second target, genes of the mitochondrial regulatory network were added, since the processes within the mitochondrion are regulated and affected by external stimuli. The down regulation of *MTOR*, *PGC1α*, *IGF-1α*, and two subunits of *AMPK* with ongoing productive lifespan highlight the complexity of the mitochondrial regulatory network. *PGC1α* as the key regulator of mitochondrial biogenesis (Scarpulla, 2011) was down regulated, despite proteins regulated by the PGC1 family such as *SOD2,* being up regulated or not affected by period. In general, contrasting expression of regulating genes and their targets illustrates the complexity of this network and clearly shows that more research is needed to understand the mechanisms behind the regulation of OXPHOS during the productive life span. However, some of the findings in this work show, that mitochondrial gene expression is, in part, comparable to model organisms such as mice and fruit fly.

In the context of different diets, no diet dependent differences were observed for these genes, except for *IGF-1α* in LB hens, where the expression is significantly lower under P-Ca- than under P+Ca- in ovary tissue, and higher in liver tissue. These observations show, that there are differences according to the diets, since *IGF-1α* plays a major role in metabolic homeostasis (Broughton & Partridge, 2009; Saltiel & Kahn, 2001) and malnutrition has been linked to decreasing IGF1 expression in liver tissue (Fox et al., 2006). The appearance of expression differences of *IGF-1α* in both experiments make it an interesting target of future studies, especially since it's important role in the regulation of ovarian function has already been shown in

different avian species (Onagbesan et al., 1999). However, the low number of expression differences as a reaction to different diets support the hypothesis, that the reduction of P and Ca was not enough to challenge the hens' metabolism, and that in reverse, it's concentrations in the diets could be reduced to save resources.

The analysis of mitochondrial-linked genes with little effects of the different diets and inverse changes during the productive life span compared to mitochondrial gene expression reflects the complexity of this regulatory network, which is still not completely understood (Goffart & Wiesner, 2003).

4.9 Conclusions

Even if the original plan to include the mitochondrial haplotypes in each analysis did not work out, the three studies together depict mitochondrial diversity and functionality from different perspectives. In Heumann-Kiesler et al., 2021 (Manuscript 1) I was able to identify signs of heteroplasmy, four mitochondrial haplotypes, and showed tendencies of possible relationships between them and phenotypic traits. Besides the insight into a poor genetic diversity, this work is a suitable foundation to further research: An additional collection of information about the maternal background in the future and its estimation for this population would deepen the knowledge about the genetic background and breeding history of both lines. The selection of animals can be adjusted to the detected low mitochondrial diversity to allow robust testing and the identification of potential effects on phenotypic traits. This would also make further analyses possible, which include the here presented expression analyses to deepen the understanding of the already detected differences in gene expression and differences in the mitochondrial genome.

The findings about mitochondria linked gene expression show the complexity of the regulatory network but also underline the importance and potential effect on the whole individual. The low number of strain effects and differences suggests, that other factors than the mitochondrial haplotype or strain are the main regulating factors. Both, OXPHOS and regulatory genes are not affected by the dietary changes, indicating a possible adjustment and reduction of P and Ca in the hens feed. In addition, the results show, that an imbalance the minerals is more crucial than a 20% reduction of both. In addition to the higher variance in the analysed phenotypic traits and mitochondrial genome in LB hens, they showed signs of increased oxidative stress compared to LSL hens. In the context of the productive life

span, a potential higher demand for energy is suggested, since OXPHOS related gene expression is increasing.

In combination this work provides an insight into the mitochondrial genome and provides the first large scaled analysis of mitochondrial linked gene expression in laying hens.

5. Summary

The domesticated chicken (*Gallus gallus domesticus*) is the most popular and widely spread domestic fowl worldwide, providing human with a stable source of protein in form of meat and eggs for centuries. The ongoing growth of human population increases the need for food and made poultry production one of the fasted growing sectors in the past decades. This need for food has resulted in several different strains which outperform their wild ancestors in terms of meat and egg production. During the past decades not only animal welfare gained importance but also ecological aspects such as global warming and the shortage of resources are becoming more important to society. One important resource for mankind which is becoming shortened is phosphorus (P), whose deposits in form of rock phosphate could be exhausted within the next 50-100 years. 90% of P supply is used in agriculture as fertilizer, whose demand will increase as well with growing population.

This thesis focuses on the mitochondrial genetic background and mitochondrial related gene expression in the context of the productive life span and different diets in two contrasting high-yielding strains of laying hens, Lohmann Brown-Classic (LB) and Lohmann LSL-Classic (LSL).

Mitochondria, which are commonly known as the powerhouse of the cell due to their role as the main producer of energy, play roles in other processes from cellular homeostasis to the process of ageing. The process of oxidative phosphorylation depends on the availability of P and thus, they become an important part of the complex framework of P utilization. In addition, mitochondrial haplotypes are known to affect physiological traits such as body weight in laying hens or important traits such as e.g. the metabolic capacity in dairy cows. It is known, that single mutations in the mitochondrial genome lead to a better adaptation to height in the Tibetan chicken or play a role in diseases from Alzheimer to obesity or lead to resistance to disease such as Marek's disease in birds.

This work provides insight into the whole mitochondrial genome of 180 laying hens of two commercial strains and links this information to physiological traits and genetic diversity. In addition, the first large-scaled gene expression analyses in the context of the productive life span and different P and Ca contents in laying hens is implemented.

The analysis of mitochondrial haplotypes revealed a low level of genetic diversity with only three haplotypes within the LB strain while all LSL hens shared the same mitochondrial genome. Following from this observation, the nuclear genome was analysed based on genotyping data to reveal the whole genetic diversity of both strains. On the nuclear genetic level, both strains appeared as clearly distinct and equally diverse, while some individuals appear as strikingly close related. These individuals are mostly half-siblings sharing the same mitochondrial haplotype, underlining the need for more analyses about the genetic structure about the parental generation, especially the maternal background. Although there were no strong associations were found between the mitochondrial haplotypes and the analysed phenotypic traits (feed intake, body weight, P and Ca utilization), the differences between the strains indicate a potential involvement of the mitochondrial genetic background.

The gene expression analyses revealed tissue type and point of the productive life span as the main influencers on gene expression while the influence of the strain is secondary. In addition, the expression of the gene *GAPDH*, which is frequently used as a reference gene for normalization in gene expression studies, was influenced by tissue and strain, leading to the decision to exclude it as a reference, that should be considered for in further studies. Further, no influence of the changes in dietary P and Ca on gene expression could be observed, suggesting that a reduction of 20% of both minerals is possible without the need to adapt gene expression. However, the results show, that a reduction of both minerals has less effect than a reduction of P alone, leading to an imbalance. In the context of the productive live span, mitochondrial and mitochondrial regulatory genes react contrary, illustrating the complexity of mitochondrial gene expression and regulation.

In addition to the higher variance in the analysed phenotypic traits and mitochondrial genome in LB hens, they showed signs of increased oxidative stress compared to LSL hens. In the context of the productive life span, a potential higher demand for energy is suggested, since OXPHOS related gene expression is increasing. As a conclusion this work provides an insight into the mitochondrial genome and provides the first large scaled analysis of mitochondrial linked gene expression in two contrasting laying hen strains.

6. Zusammenfassung

Das Haushuhn (*Gallus gallus domesticus*) als bekanntestes und weltweit verbreitetste Geflügel ist seit Jahrhunderten eine beständige Proteinquelle in Form von Fleisch und Eiern. Das fortlaufende Bevölkerungswachstum und der daraus hervorgehende steigende Bedarf an Nahrung hat den Geflügelsektor zum am stärksten gewachsenen Bereich der Tierhaltung der letzten Jahrzehnte weltweit gemacht. Mit der Zeit sind so verschiedene Zuchtlinien entstanden, die auf maximale Legeleistung oder die Fleischproduktion gezüchtet wurden und ihre wilden Vorfahren in diesen Leistungen weit übertreffen. In den letzten Jahren hat nicht nur das Thema Tierwohl an Bedeutung gewonnen, sondern auch ökologische Themen wie Erderwärmung und Ressourcenknappheit sind in den Fokus der Öffentlichkeit gerückt. Eine für den Menschen wichtige Ressource ist Phosphor, dessen Vorrat in Form von Rohphosphat bereits in den nächsten 50-100 Jahren aufgebraucht sein könnte. 90% des verbrauchten Phosphors wird in der Landwirtschaft als Düngemittel benötigt, dessen Bedarf mit dem anhaltenden Bevölkerungswachstum weiter steigen wird.

Diese Arbeit analysiert das mitochondrielle Genom der Tiere und verbindet diese Informationen mit Genexpressionsanalysen im Zusammenhang mit der produktiven Lebensspanne und verschiedenen Futterzusammensetzungen in zwei unterschiedlichen Hochleistungs-Legehennen-Linien.

Mitochondrien sind als zelluläre Energieproduzenten gemeinhin als die Kraftwerke der Zelle bekannt, spielen zusätzlich in zahlreichen weiteren Prozessen wie beim zellulären Gleichgewicht und dem Alterungsprozess eine Rolle. Die oxidative Phosphorylierung ist abhängig von der Verfügbarkeit von Phosphor und daher wichtiger Bestandteil im Zusammenhang mit der Phosphorverwertung. Zusätzlich ist bekannt, dass mitochondrielle Haplotypen physiologische Merkmale wie Körpergewicht in Legehennen oder wichtige Merkmale wie die metabolische Kapazität in Milchkühen beeinflussen. Schon einzelne Mutationen im mitochondriellen Genom können einen großen Einfluss haben, wie zum Beispiel in Krankheiten wie Alzheimer oder der Resistenz gegen die Marek-Krankheit in Vögeln oder die Anpassung an Höhen im Tibetanischen Huhn.

Diese Arbeit gibt Einblicke in das mitochondrielle Genom von 180 Legehennen aus zwei Zuchtlinien und verbindet sie mit physiologischen Merkmalen und genetischer

Diversität. Zusätzlich beinhaltet sie eine umfassende Genexpressionsanalyse in Legehennen im Kontext der produktiven Lebensspanne sowie der Phosphor und Calcium Nutzung.

Die Analyse der mitochondriellen Haplotypen hat eine geringe Diversität gezeigt, mit drei Haplotypen in LB Hennen während alle LSL Hennen denselben Haplotyp teilen. Aufgrund dieser Beobachtung wurde auch das Kerngenom basierend auf Genotypisierungsdaten betrachtet, um die genetische Diversität innerhalb beider Linien zu erfassen. In diesen Analysen sind beide Linien klar getrennt und ähnlich divers, während einige Tiere als nah verwandt erscheinen. Diese Tiere sind meistens Halbgeschwister die sowohl denselben Vater als auch den gleichen mitochondriellen Haplotyp teilen. Dieses Ergebnis verdeutlicht die Notwendigkeit weiterer Analysen der Elterngeneration, besonders in Bezug auf die Mütter der Tiere. Obwohl es nicht möglich war die mitochondriellen Haplotypen mit den analysierten phänotypischen Merkmalen (Körpergewicht, Futteraufnahme und Phosphor und Calcium Nutzung) in allen Bereichen eindeutig zu assoziieren, zeigen die Unterschiede zwischen den Linien einen möglichen Einfluss des mitochondriellen Genoms.

Die erste groß angelegte Genexpressionsanalyse in Legehennen in diesem Kontext hat gezeigt, dass der Gewebetyp und Zeitpunkt innerhalb der produktiven Lebensspanne den größten Einfluss auf Genexpression haben, während der Einfluss der Linie zweitrangig zu sein scheint. Zusätzlich wurde ein Einfluss von Gewebstyp und Linie auf das Gen GAPDH festgestellt, welches häufig als Referenzgen in Genexpressionsanalysen genutzt wird. Aufgrund dessen wurde es als Referenzgen ausgeschlossen, was auch für zukünftige Studien von Bedeutung ist. Des Weiteren wurde beobachtet, dass sich die verschiedenen Futterzusammensetzungen nicht auf die Genexpression auswirken, was darauf schließen lässt, dass eine 20%ige Reduktion von Phosphor und Calcium möglich ist, ohne dass die Tiere ihre Genexpression anpassen müssen. Die Ergebnisse zeigen aber auch, dass die gleichzeitige Reduktion beider Minerale einen geringeren Effekt hat, als die Reduktion nur eines der Mineralien. Im Zusammenhang mit der produktiven Lebensspanne haben die Analysen gezeigt, dass mitochondrielle und mitochondriell regulierende Gene gegensätzlich reagieren, was die Komplexität mitochondrieller Genexpression und ihrer Regulierung verdeutlicht.

Zusätzlich zu der höheren Varianz der phänotypischen Merkmale und der mitochondriellen Diversität in LB Hennen gab es Anzeichen für erhöhten oxidativen Stress im Vergleich zu LSL Hennen. Die steigende Genexpression von Genen in Zusammenhang mit oxidativer Phosphorylierung im Verlaufe der produktiven Lebensspanne lässt auf einen höheren Energiebedarf schließen.

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Affidavit

Anlage 3

Eidesstattliche Versicherung über die eigenständig erbrachte Leistung

gemäß § 18 Absatz 3 Satz 5 der Promotionsordnung der Universität Hohenheim für die Fakultäten Agrar-, Natur- sowie Wirtschafts- und Sozialwissenschaften

1. Bei der eingereichten Dissertation zum Thema

Mitochondrial haplotypes, gene expression and nuclear diversity in two strains of laying hens

handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

3. Ich habe nicht die Hilfe einer kommerziellen Promotionsvermittlung oder -beratung in Anspruch genommen.

4. Die Bedeutung der eidesstattlichen Versicherung und der strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt.

Die Richtigkeit der vorstehenden Erklärung bestätige ich. Ich versichere an Eides Statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

Frankfurt am Main, den 25.8.2012

Ort, Datum

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