Genetical Aspects of Bone Stability in Laying Hens Differing in Phylogeny and Performance Level

Dissertation

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Simon Jansen
born in Cloppenburg

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This thesis was carried out at the Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, 31535 Neustadt, Germany.

1st Referee: Dr. Ahamd Reza Sharifi
Animal Breeding and Genetics Group
University of Göttingen

2nd Referee: Prof. Dr. Armin Otto Schmitt
Breeding Informatics Group
University of Göttingen

3rd Referee: apl. Prof. Dr. Steffen Weigend
Institute of Farm Animal Genetics
Friedrich-Loeffler-Institut

4th Referee: Prof. Dr. Georg Thaller
Institute for Animal Breeding and Husbandry
Christian-Albrechts-University Kiel

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Summary

Skeletal disorders in laying hens is one of the most serious problems facing the egg production industry and is gaining increasing attention due to changes in husbandry systems and overall increased public concern about hen welfare. There are a high number of hens in commercial flocks suffering from bone weakness and fractures due to osteoporosis, which has serious animal welfare implications. Genetics have been found to substantially affect skeletal integrity, although little is yet known about the exact mechanisms. Therefore, the aim of this thesis was to further characterise the influence of genetics on the differentiation of bone stability and to evaluate its potential for improving bone health in laying hens. The experimental part of this work involves three studies, in which an animal model was applied comprising four purebred layer lines differing in their phylogenetic origin (brown vs. white-egg) and egg production level (high vs. moderate performing).

In the first study (Chapter 2), we aimed at analysing the relationship between bone stability and egg production using the four-line animal model. Besides basic characterisation of skeletal traits in these lines, multifactorial models and regression analyses were employed to identify factors determining the bone breaking strength (BBS) and bone mineral density (BMD) of the tibiotarsus and humerus. While the morphometry of the bones had limited effects on their BBS, the BMD was found to be a decisive factor accounting for a high amount of the observed variance in BBS. Strong phylogenetic effects were only observed in relation to bone dimensions, in that the bones of the brown-egg lines were larger and heavier and had a higher BMD than those of the white-egg ones. Although both high performing lines were superior to the moderate performing ones in terms of production traits and inferior in terms of BMD, there was no effect of total eggshell production on BBS or BMD within the lines studied. Contrary to what was suspected, the results did not provide evidence for a negative association between egg production and bone health and we concluded that a high egg number does not necessarily pose a risk for bone weakness. Finally, genetic parameter estimations implied an inherited component of BBS and BMD, supporting the role of genetics in skeletal traits.

The aim of the second study (Chapter 3) was to examine skeletal traits under the metabolically challenging situation of repeated dietary calcium restrictions. Within and among the four chicken lines of our animal model, the hens' adaptation response was characterised with regard to the effects of phylogeny and performance level. Calcium depletions led to a decrease in egg number and eggshell quality in all lines, but recovery occurred after reconversion to adequate supply. Substantial bone demineralisation was observed post mortem. These results may reflect the attempt to maintain reproductive performance at the expense of skeletal integrity. It turned out that the performance level influenced the adaptation response less than phylogeny. In this regard, the white-egg lines showed a more pronounced response whereas the brown-egg ones seemed to be less sensitive towards reduced calcium levels. The latter was explained by a more favourable body constitution of these lines, where higher amounts of calcium could be provided by the skeletal system without severely compromising bone health.

In the third study (Chapter 4), the BBS and BMD measurements obtained in the first experiment were examined from a genomic perspective to see whether genomic regions associated with bone stability could be identified. To this end, the four layer lines were combined to one set. Two alternate approaches were applied for single nucleotide polymorphisms (SNP) selection including single-locus mixed linear model analysis and machine learning-based Random Forests classification. The latter method seem more robust in terms of population stratification bias. Sixteen potential candidate genes located in close proximity to the SNPs were identified by subsequent functional analyses. These genes are supposed to be functionally related to the skeleton in chickens or humans. Moreover, gene set enrichment analysis showed that some of these candidate genes are involved in the same metabolic pathways critical for bone metabolism. The results met our expectations in that they suggest that multiple genes, each of which has a rather small effect size given the calculated SNP effect estimates, determine bone stability. Though the candidates presented in this study are putative and causality has yet to be proven, they are promising in terms of bone biology.

Overall, this work identified genetics as a major determinant of bone stability in laying hens. However, the performance level of the hens does not seem to play a decisive role, as no correlations between hen productivity and bone stability were observed. In contrast, a phylogenetic effect is to be assumed which, according to current knowledge, is most likely based on an advantageous physical constitution of the brown-egg lines. Given moderate inheritance of bone traits, there is the possibility of genetic selection for improved skeletal health. Since multiple genes regulate bone stability, genetic improvement should be achievable through the increased use of genomic information.

Zusammenfassung

Knochenschäden stellen eines der größten Probleme in der Legehennenzucht dar und die Thematik rückt durch veränderte Haltungssysteme sowie ein insgesamt gestiegenes Bewusstsein für den Tierschutz zunehmend in den Fokus der Öffentlichkeit. Der Anteil der kommerziellen Hennen, die an Knochenschwäche und Frakturen aufgrund von Osteoporose leiden, ist hoch, was schwerwiegende Auswirkungen auf das Wohlergehen der Tiere hat. Obwohl die Genetik als einer der entscheidenden Einflussfaktoren für die Knochenstabilität identifiziert wurde, ist bisher nur wenig über die genauen genetischen Mechanismen bekannt. Daher war das Ziel dieser Arbeit, die nähere Charakterisierung des Einflusses der Genetik auf die Differenzierung der Knochenstabilität sowie die Abschätzung ihres Potentials zur Verbesserung der Knochengesundheit bei Legehennen. Der experimentelle Teil der Arbeit umfasst drei Studien, in denen ein Tiermodell verwendet wurde, welches vier Reinzuchtlegelinien beinhaltet. Diese Linien unterscheiden sich hinsichtlich ihrer phylogenetischen Abstammung (Braun- vs. Weißleger) und Legeleistung (hoch- vs. minderleistend).

Das Ziel der ersten Studie (Kapitel 2) war es, den Zusammenhang zwischen Knochenstabilität und Eiproduktion anhand des Vier-Linien-Tiermodells zu analysieren. Neben der grundlegenden Beschreibung von Knochenmerkmalen dieser Linien wurden multifaktorielle Modelle und Regressionsanalysen eingesetzt, um Faktoren zu identifizieren, welche die Knochenbruchfestigkeit (BBS) und die Knochenmineraldichte (BMD) des Tibiotarsus und Humerus beeinflussen. Während die Knochenmaße nur begrenzte Auswirkungen auf die BBS hatten, erwies sich die BMD als entscheidender Faktor, der einen großen Teil der beobachteten Varianz in der BBS erklärte. Deutliche phylogenetische Effekte wurden nur in Bezug auf die Knochenmaße beobachtet, wobei die Knochen der Braunleger größer und schwerer waren und eine höhere BMD aufwiesen als die der Weißleger. Obwohl die beiden Hochleistungslinien den minderleistenden in Bezug auf die Produktionsmerkmale überlegen und in Bezug auf die BMD unterlegen waren, gab es innerhalb der untersuchten Linien keinen Einfluss der Gesamtschalenproduktion auf die BBS oder BMD. Anders als vermutet, lieferten die Ergebnisse keine Hinweise auf einen negativen Zusammenhang zwischen Legeleistung und Knochengesundheit. Wir kamen zu dem Schluss, dass eine hohe Legeleistung nicht zwangsläufig ein Risiko für Knochenschwäche darstellt.

Das Ziel der zweiten Studie (Kapitel 3) war die Untersuchung von Skelettmerkmalen unter den metabolisch herausfordernden Bedingungen einer wiederholten diätetischen Kalziumrestriktion. Innerhalb und zwischen den vier Linien unseres Tiermodells wurde die Adaptationsreaktion der Hennen im Hinblick auf mögliche Effekte von Phylogenie und Leistungsniveau charakterisiert. Die Kalziumrestriktionen führten bei allen Linien zu einer Abnahme der Eizahl und der Schalenqualität, jedoch setzte nach Umstellung auf eine adäquate Versorgung eine Erholung ein. Post mortem wurde eine erhebliche Demineralisierung des Knochens festgestellt. Diese Ergebnisse spiegeln möglicherweise den Versuch wider, die Reproduktionsleistung auf Kosten der Skelettintegrität zu erhalten. Es zeigte sich, dass das Leistungsniveau die Anpassungsreaktion weniger stark beeinflusste als die Phylogenie. Hierbei zeigten die Weißleger eine stärkere Reaktion, während die Braunleger

weniger empfindlich auf den reduzierten Kalziumgehalt zu reagieren schienen. Letzteres wurde mit einer vorteilhafteren Körperkonstitution dieser Linien erklärt, bei der höhere Mengen an Kalzium vom Skelettsystem bereitgestellt werden konnten, ohne die Knochengesundheit stark zu beeinträchtigen.

In der dritten Studie (Kapitel 4) wurden die im ersten Experiment erhobenen Messungen der BBS und BMD aus genomischer Perspektive untersucht, um zu prüfen, ob genomische Regionen identifiziert werden können, die mit Knochenstabilität assoziiert sind. Hierzu wurden die Daten der vier Linien zusammengefasst. Zwei unterschiedliche Verfahren wurden zur Identifikation von Single Nucleotide Polymorphisms (SNP) angewendet, darunter eine Single-Locus Mixed Linear Model Analyse sowie die auf maschinellem Lernen basierende Methode der Random Forests Klassifikation. Letztere Methode scheint robuster in Bezug auf Populationsstratifikation zu sein. Sechzehn potenzielle Kandidatengene, die sich in unmittelbarer Nähe der SNPs befinden, wurden durch anschließende Funktionsanalysen identifiziert. Diese Gene sind mutmaßlich funktionell mit dem Skelett bei Hühnern oder Menschen assoziiert. Darüber hinaus hat das Gene Set Enrichment gezeigt, dass einige dieser Kandidatengene an Stoffwechselwegen beteiligt sind, die für den Knochenmetabolismus von Bedeutung sind. Unsere Ergebnisse deuten erwartungsgemäß darauf hin, dass die Knochenstabilität durch eine Vielzahl von Genen reguliert wird, von denen jedes einen eher geringen Effekt auf das Merkmal hat. Die hier vorgestellten Kandidatengene sind aus knochenbiologischer Sicht sehr vielversprechend, kausale Beziehungen müssen jedoch noch nachgewiesen werden.

Zusammenfassend lässt sich sagen, dass in dieser Arbeit die Genetik als maßgeblicher Bestimmungsfaktor für die Knochenstabilität bei Legehennen identifiziert wurde. Das Leistungsniveau der Hennen scheint allerdings keine entscheidende Rolle zu spielen, da keinerlei Korrelationen zwischen der Produktivität der Hennen und der Knochenstabilität beobachtet wurden. Im Gegensatz dazu ist von einem phylogenetischen Effekt auszugehen, der nach heutigem Kenntnisstand am ehesten auf einer vorteilhaften körperlichen Konstitution der Braunleger beruht. Angesichts der moderaten Erblichkeit der Knochenmerkmale besteht die Möglichkeit einer genetischen Verbesserung der Skelettgesundheit. Da zahlreiche Gene an der Regulation der Knochenstabilität beteiligt sind, erscheint eine verstärkte Nutzung genomischer Informationen als sinnvoll.

1 General Introduction

1.1 A Brief History of Layer Breeding

The domestic chicken (*Gallus gallus domesticus*) descends from the red junglefowl (*Gallus gallus*) and, although to a much lesser extent, from the grey junglefowl (*Gallus sonneratii*) [1]. Its domestication took place at several sites in the Asian region between 2,500 and 6,000 years BCE [2, 3], from which the chicken migrated to Europe along two main trading routes [1]. Archaeological findings suggest that chickens were originally kept for leisure purposes such as cockfighting, whereas the first adoption as food source is attributed to the Romans [4, 5]. An intensive diversification of chicken breeds took place during migration, leading to a broad variety of populations. In China and Europe, traditional breeds were initially formed by selecting for preferred morphological traits, the subgroups of which in turn served as the foundation for standardised breeds [1]. In this way, the founder stocks of today's commercial chicken lines were established and both maintained as purebred populations and used for crossbreeding [6]. While many of the standardised breeds may still be considered dual purpose breeds today, intensive selection for either egg or meat production soon led to the emergence of two specialised chicken types [1, 7, 8]. However, intensive selection also led to a decline in genetic diversity, especially in laying hens. Malomane et al. [6] recently showed that brown-egg layer and broiler lines cluster together with regard to their phylogenetic relationship, while white-egg layer lines form a separate cluster. This represents both domestication and selection effects, as brown and white-egg lines evolved independently during breed history [6, 9] and all white-egg lines have descended from the White Leghorn breed. In contrast, today's brown-egg lines originate from a number of breeds including Rhode Island Red, New Hampshire, Light Sussex and Barred Plymouth Rock [10, 11]. For this reason, there have been completely separate breeding populations for white and brown-shelled eggs since the beginning of commercial layer breeding [12].

Starting in the U.S., chicken breeding developed very rapidly from the 1940s onwards and after a few years, there were a large number of professional poultry breeders, some of whom cooperated with each other, e.g. by swapping stocks to create crossbreeds or attending random sample testing [12–14]. The following decades were marked by the introduction of quantitative genetic principles and concepts such as inbreeding, hybrid vigour and reciprocal recurrent selection [11, 13]. With the increasing professionalisation of layer breeding, the number of primary breeders declined significantly and today there are only two major global players and a few others [14, 15].

Meat and egg-type chicken breeding basically follow the same hybridisation scheme: Commercial breeders maintain some number of pure lines, intensively selected for various performance and behavioural traits and crossed in specific combinations to produce the parent

generation, from which individuals are in turn crossed to deliver the end product. Today, four-way crossing is commonly used for the production of laying hybrids [12, 14, 16]. Field results and information from genetic markers are combined in order to improve both the pure and cross lines [17]. Current selection indexes include a multitude of production and functional traits weighted according to hereditary and economic aspects [16]. Intensive selection for laying performance led to an earlier start of laying as well as to an increased clutch length due to persistent ovulation, which has resulted in an enormous increase in lifetime egg production per hen in the last decades [12, 18, 19]. Another trait that has been massively optimised over the last 20 years is the feed efficiency, which is now around 2 kg of feed per 1 kg of egg mass. This has been achieved through selection for higher egg mass and smaller body size [12, 16]. Continuous improvement in the number of saleable eggs per hen will remain the most important objective. However, this can only be achieved by increasing laying persistency, i.e. extending clutch length [16, 17], as the biological limit of one egg per day is reached at peak production [20]. Recent studies point to the future goal of an extended production period of 100 weeks with 500 saleable eggs [17, 20]. In this regard, Fernyhough et al. [15] caution that an extending cycle length has to go alongside with an improved robustness to ensure that hens can cope with the challenges of a longer laying period. Finally, behaviour and welfare-related traits including those related to suitability for alternative housing systems are gaining more importance in the selection index and complement traditional performance traits [15, 17]. This also applies to parameters of skeletal integrity [16], which are addressed in detail in the following chapters.

1.2 Fundamentals on Avian Bone Biology

Bone Development and Remodelling

The skeleton fulfils a number of important functions. In addition to its protective function for internal organs and its function as an attachment site for muscles, it also serves as a mineral reservoir and contributes to the regulation of calcium homeostasis, both of which are considered particularly important for laying hens [21]. Two major processes are involved in bone growth and development [22]. These are endochondral ossification, which describes the formation of bone on a mineralised cartilage scaffold, and intramembranous ossification, in which bone is formed without the mediation of a cartilage phase [23–26]. The process of ossification is the deposition of a type I collagen matrix by osteoblasts [25], which is subsequently mineralised by the formation of hydroxyapatite crystals in and between the collagen fibres [27].

Once formed, bone undergoes continuous remodelling. This is the process of bone resorption by osteoclast cells followed by the subsequent formation and mineralisation of new bone tissue by osteoblast cells [21, 28, 29]. During ossification, calcium minerals circulating in the blood are fixed [30], while during resorption, calcium is released from the bone into the bloodstream [29]. A balance between osteoclastic and osteoblastic activities is considered crucial for maintaining bone health, including the ability of bone to repair micro-damage and adapt to mechanical loads [31, 32]. An imbalance, on the other hand, can lead to a net loss of bone material [28, 33].

Bone Types in Laying Hens

There are three distinct bone types in laying hens. These include the cortical and cancellous bone, which are together referred to as structural bone, and the medullary bone [23].

Cortical bone tissue is found in the bones of the appendicular skeleton and forms a compact tightly packed outer layer that is adjacent to the periosteum on the inside (Figure 1.1) [21, 22, 34]. The cancellous bone, on the other hand, is predominantly located in the axial skeleton as well as at the epiphyses of the long bones. It has a reticular three-dimensional structure, which is why it is synonymously called trabecular bone [21, 35, 36]. The cortical layer contributes most to the structural integrity of the bone, followed by the cancellous tissue, which was shown to be less calcified and is primarily a site of metabolic activity [21, 22, 28]. Recently, Rodriguez-Navarro et al. [37] observed that it is not the degree of mineralisation, but rather the thickness of the cortical bone that has the greatest contribution to the mechanical bone properties.

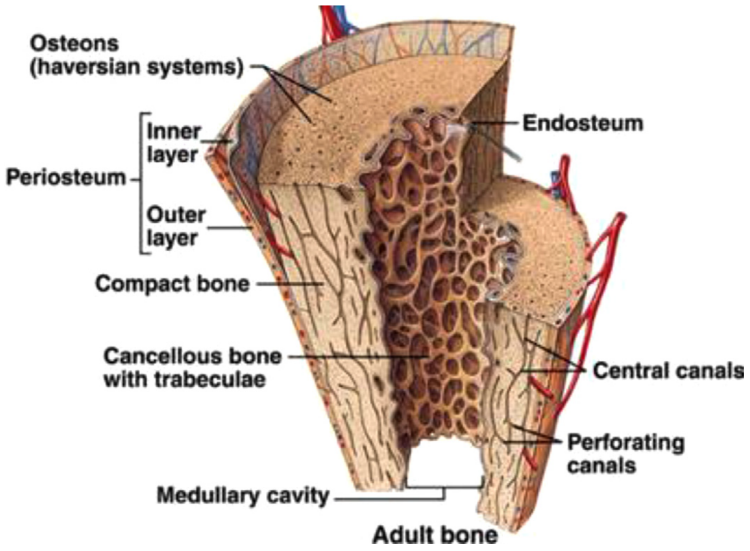

Figure 1.1: Schematic illustration of a long bone cross-section in laying hens showing various types of bone tissue. Reprinted from Toscano [22], with permission from Elsevier.

The medullary bone is the third bone type in the chicken, which, unlike the structural bone found in mammals and male birds alike, is only present in adult female birds and has been detected in dinosaur fossils as well [23, 38, 39]. Medullary bone is a highly labile woven bone [36] located in the medullary cavity and lining the endosteal surface of the structural bone [23, 40, 41]. Its spicules grow from the endosteal surface towards the centre of the bone and can fill the medullary cavities completely [23, 42]. Medullary bone is found mainly in the diaphysis of long bones, including the tibiotarsus, femur and, to varying degrees, humerus, as well as in the keel bone [23, 29, 42–44]. Intrinsic strength of the medullary bone is lower than that of the cortical and cancellous bone [45, 46], due to the irregular arrangement of its collagen fibres and the fact that much of it is present in isolated spicules [23, 29]. Nevertheless, medullary bone contributes to overall bone strength and can increase fracture resistance [37, 43]. Medullary bone formation starts with the initiation of ovarian follicle maturation, i.e. about two weeks before hens first come into lay, and is stimulated by the synergistic effect of oestrogens and androgens [39, 42, 47]. It acts as a labile calcium reservoir for eggshell calcification and represents about 12% of total bone calcium [48]. Medullary

bone is characterised by intense calcification and a high remodelling rate [42, 49, 50]. This is reflected in a constant alternation between osteoclast-mediated resorption during periods of eggshell formation and osteoblast-mediated remineralisation after eggshell formation is completed [23, 36, 41]. Dacke et al. [42] concluded that only the degree of calcification of the medullary bone varies with the egg-laying cycle, but not its volume. After completion of the laying sequence, the follicle-mediated oestrogen level decreases, causing the medullary bone to gradually disappear and the remodelling of the cortical bone to be initiated [28, 51].

Bone Resorption in Favour of Egg Production

Commercial laying hens have been selected for high laying performance, resulting in a virtually daily egg production for a 52-week period [19, 39]. Within the 24-hour laying cycle, eggshell formation takes place in three successive stages in the uterus, which is also referred to as the eggshell gland, and lasts about 19 hours [20, 48]. With a standard lighting programme of 14 h light : 10 h dark, half the time of eggshell formation falls in the dark period [20]. Calcium carbonate ($CaCO_3$) is the main component of eggshells and for shell formation its ionic precursors, ionised calcium (Ca^{2+}) and bicarbonate (HCO_3^-), are supplied in large quantities through the blood [39]. There is a wide variation in egg size and up to three grams of calcium are needed each day for eggshell formation [52], which is 10% of the hen's total body calcium [48]. The amount of calcium deposited in the eggshells throughout a 52-week laying sequence can be 20 to 30 times the body mass of the hens [12, 53]. This places very high demands on their calcium homeostasis and activates efficient mechanisms for Ca^{2+} transfer [47, 54]. During shell calcification, there is a large calcium flow towards the eggshell gland, resulting in a temporarily decreasing plasma Ca^{2+} level, which in turn stimulates the secretion of a number of calcium-regulating hormones, including parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D3 (1,25-$(OH)_2D_3$), also referred to as calcitriol [47, 48]. These lead to increased intestinal mineral absorption and reduced renal excretion [33].

Part of the calcium needed for eggshell formation derives from the feed and passes into the blood via intestinal absorption [33]. Hens show a specific appetite for calcium in the hours before shell formation [48, 55]. However, despite increased feed intake and exceled absorption rates, dietary calcium supply only covers 60-75% of the demand due to limited bioavailability (see chapter 1.5) [23, 33, 56]. Furthermore, external calcium supply is limited because the larger part of eggshell formation takes place during the night, when there is no feed intake and at most a small amount of feed remains in the gastrointestinal tract [20, 33, 57].

Besides enhancement of external supply, resorption of medullary bone is another mechanism intended to increase plasma Ca^{2+} levels for eggshell formation [47]. It is mobilised to provide the remainder of the minerals needed for shell calcification. This proportion is about 20-40%, but varies with the dietary calcium content [48, 56]. Degradation of medullary bone is mediated by 1,25-$(OH)_2D_3$, which stimulates bone resorption by osteoclasts in the presence of PTH [33, 47]. Medullary bone remodelling is directly coupled to the calcification process, so that osteoblasts and osteoclasts periodically change their morphology and function depending on the position of the egg in the eggshell gland [29, 39]. This includes certain restoration of the medullary bone in the short period between two eggs, i.e. between oviposition and the beginning of the subsequent eggshell formation, during which feeding takes place [20, 33]. It should be noted, however, that the ability of hens to store calcium in the skeleton

decreases with age [58]. In principle, calcium supply from this labile source is an effective and natural mechanism, but medullary bone resorption is not without consequences. Degradation of medullary bone is associated with cancellous and cortical bone loss, as osteoclasts are not specific to medullary bone [23, 36]. Furthermore, medullary bone formation coincides with the cessation of structural bone development, which is challenging in terms of maintaining bone health [23, 28, 59, 60]. As a result, the amount of cortical and cancellous bone gradually decreases once medullary bone formation has started, which can lead to considerable skeletal weakening in laying hens, given their long and continuous laying activity [23, 61, 62].

1.3 Skeletal Disorders in Laying Hens

Characteristics and Pathogenesis

Skeletal disorders can occur as a result of impaired bone growth and/or bone metabolism [62]. While the former is more relevant for meat-type chickens, the latter is of particular relevance for laying hens, where bone fragility due to metabolic disorders is a common problem [63]. Osteomalacia and osteoporosis are two distinct severe metabolic skeletal diseases, both of which cause a loss of bone minerals [49, 64].

Osteomalacia, the adult pendant to rickets, is caused by inadequate or disrupted mineralisation of the organic fraction in mature bone, resulting in sparsely mineralised bone matrix [28, 65]. It occurs mainly in response to a disturbance of the vitamin D metabolism [65] or due to a nutritional deficiency of vitamin D, calcium or phosphorus [63].

Osteoporosis, on the other hand, is a more complex disease principally weakening the whole skeleton [28, 66]. Osteoporosis is defined as a systemic progressive loss of fully mineralised structural bone [28, 63], manifested in decreased bone volume and cortical thickness, less well-connected trabeculae and increased skeletal porosity [12, 28, 61]. All this leads to increased bone fragility and high fracture susceptibility [12, 23, 28]. Hence measurements of bone breaking strength [36, 67–70] and bone mineral density [36, 70–72] are commonly performed for bone health assessment. The severity of osteoporosis varies, with milder forms sometimes referred to as osteopenia [63, 65], although this is rather vague as osteopenic bone can occur for reasons other than metabolic [66]. In its most extreme form, structural bone loss can lead to cage layer fatigue, a condition described by Couch [73] after the introduction of battery cage housing, which can lead to bone fractures, paralysis as well as emaciation and sudden death [28, 73, 74]. However, this problem could be reduced through nutritional interventions and generally osteoporosis is not that severe as to result in cage layer fatigue [64, 75]. Nevertheless, osteoporosis is the most widespread skeletal disorder causing bone loss and virtually all modern layer lines are prone to it, even when mineral supply is adequate [63, 64].

The pathogenesis of osteoporosis is multifactorial and includes a strong genetic component as well as external environmental factors [28], both of which are discussed in the following chapters. The main mechanism by which osteoporosis develops is an imbalance in bone remodelling that leads to a net resorption of bone material [28, 31]. This negative balance results from continuous bone degradation, leading to bone loss culminating in osteoporosis [23, 63]. Structural bone loss was shown to begin soon after the onset of laying

and increase subsequently, with the main period of erosion lying between weeks 30 and 50 [28, 61]. Given this obvious link to egg-laying activity, the prevailing view is that the hens' physiological adaptations to high productivity made them susceptible to osteoporosis [23, 76].

Keel bone damage represents another form of skeletal disorder, which is indirectly related to osteoporosis. The term includes both fractures and deviations, i.e. unnatural bends, of the keel bone, which is very susceptible to these disorders due to its prominent anatomical position [77, 78]. The occurrence of these damages is primarily determined by environmental factors [77, 79] (see chapter 1.5), but the gradual but sustained loss of structural bone tissue is assumed as a predisposing factor [66, 70]. This means that the keel bone is weakened by osteoporosis, but the actual fracture or deformity is secondary to trauma such as a collision [66, 80, 81].

Prevalence and Implications

The prevalence of skeletal disorders due to bone weakness in laying hens is extremely high. However, this is by no means a new development, as the first reports of it date far back into the last century [73, 82]. In 1989, one third of battery hens sampled before slaughter were found to have broken bones [83]. Considering a variety of bones of the appendicular and axial skeleton, Budgell and Silversides [84] reported an overall fracture prevalence of 11.1% and 11.7%, respectively, in high performing ISA-Brown and Babcock B300 end-of-lay hens. In a comparison of different genetic strains carried out by Clark et al. [85], an average of 11.2% of the hens in all six lines had at least one fracture, with a maximum value of almost 30%. Larger ranges were reported by the Farm Animal Welfare Council [86] and Sandilands [87], who found fracture prevalence to be 23-44% and 26-53%, respectively. In recent years, increased attention has been paid to the occurrence of keel bone damages. In this regard, Wilkins et al. [88] determined a fracture prevalence of 36-86% covering different housing systems. Considering surveys from several European countries, Canada and the United Kingdom, Toscano et al. [66] recently put the range of hens affected by keel bone fractures at 20-96%. With proportions between 3 and 88%, a similar situation arises with regard to keel bone damage in organic production systems [89].

Skeletal disorders, and in particular fractures, have serious animal welfare implications due to pain, behavioural changes, and mortality [90]. Although the ability of birds to perceive pain is not yet conclusively understood, the fact that they have the necessary anatomical structures, e.g. nociceptors in the bone tissue, is an indication that the painfulness of bone fractures in chickens is similar to that of mammals [86, 90–92]. This includes both acute and chronic pain [90, 92, 93]. Evidence arises from studies in Lohmann Brown (LB) laying hens in which the administration of analgesics after keel bone fracture led to increased mobility [93, 94]. Behavioural changes may occur in response to the pain, including protecting the fracture site from movement by crouching and immobility [77, 90, 95]. Hens with keel bone fractures were observed to spend more time sleeping on the floor and resting in nest boxes [77]. Casey-Trott and Widowski [96] studied the spontaneous behaviour of Lohmann Selected Leghorn (LSL) hens in furnished cages and found that keel bone fracture severity influenced the birds' sitting, standing and resting behaviour, likely representing coping strategies to compensate for pain.

Armstrong et al. [97] concluded that fractures could lead to a depressive-like state in laying hens. Although rare, the most serious welfare impact is the death of the chicken resulting from severe internal injuries due to fractures or from culling for welfare reasons [88, 98–100]. Finally, skeletal disorders are also associated with economic losses mainly resulting from decline in hen productivity due to physiological stress [12, 36, 101]. In this context, egg production, egg weight and egg quality were compromised in hens suffering from keel bone fractures [77, 102]. In a study by Cransberg et al. [61] the laying performance of a group of severely osteoporotic LB hens was reduced by 18% at week 30. A correlation was observed between egg production, the severity of keel bone fractures and age, i.e. fractures caused a higher drop in performance with increasing age of the hens [101]. However, egg quality was not affected by fracture severity.

1.4 Genetics — An Integral Part of Bone Stability

In addition to the external environmental factors of nutrition and husbandry, there is a strong genetic component in skeletal integrity and osteoporosis [12, 46, 64, 103]. The level of genetic determination of bone stability is considered high, as large individual variations have been observed, with some hens maintaining both high productivity and good bone quality at the end of the laying period [12, 23, 28, 104, 105].

It is assumed that bone quality traits are highly polygenic and thus are not regulated by a few major genes, but rather determined by several functional genes acting conjointly [106, 107]. With all the genetic progress obtained in the egg production traits, modern laying hybrids have experienced substantial genetic and phenotypic changes [7]. Due to genetic correlations and linkage of genes, co-selection of undesirable side effects may have occurred in the course of decades of targeted genetic selection for high laying performance. These side effects are likely caused by pleiotropy and epistasis, influencing traits, which were not selected for by the breeding program [7, 108, 109]. In terms of calcium homeostasis, this may have led to an imbalanced allocation of limited mineral resources, with calcium being prioritised in favour of eggshell formation and to the detriment of bone health [8, 110–112]. Compromised skeletal integrity may therefore have been caused by genes that were inadvertently co-selected by genetic hitchhiking during directed selection for efficient egg production, resulting in trade-offs in resource allocation [109, 113].

Inspired by the work of Johnsson [62], this chapter considers (i) findings from comparisons of different breeds and lines, (ii) insights from selection experiments and estimates of genetic parameters, and (iii) genetic regions associated with bone stability.

Findings from Breed and Line Comparisons

Research comparing different chicken breeds or lines kept under similar conditions to show genetic differences probably constitutes the majority of the literature on bone stability in laying hens [62]. There are a number of studies indicating strong breed and line effects on skeletal traits. These findings suggest a genetic potential for bone health in that some lines have better bones than others [85, 103, 114–119].

A large part of the work involved studies comparing brown and white-egg laying hens to draw conclusions about the effects of phylogenetic differences on skeletal traits. According to Riczu et al. [115], phylogenetic differences appear obvious, as they are also observed in parameters of egg production and quality, i.e. in a complex that is presumably closely related to susceptibility to bone weakness. Habig and Distl [120] examined the bone characteristics of the tibiotarsus and humerus in LB (brown-egg line) and LSL (white-eggs line) hens over four consecutive trials. For both bone types, significantly higher breaking strengths were observed in the LB hens. In contrast, LB hens showed a significantly higher prevalence of severe keel bone deformities compared with the LSL hens. Vits et al. [121] previously observed the same phylogenetic effects on breaking strengths of the tibiotarsus and humerus in these lines. Riczu et al. [115] investigated bone stability traits in end-of-lay hens of two phylogenetically divergent lines. They found that although there were only marginal differences in bone mineral density, the bone breaking strength of the brown-egg Shaver 579 line was significantly higher in the humerus (+18%) and femur (+22%) compared to the white-egg Shaver 2000 line. Rayan et al. [122] examined bone quality traits in two layer parent stocks, white-egg Hy-Line W-36 and brown-egg Hy-Line Brown, at the end of the production cycle and observed a 33% higher breaking strength of the tibiotarsus in the brown-egg line. Recently, Sharma et al. [123] performed X-ray microtomography in laying hybrids from the same two lines examined by Rayan et al. [122] and observed higher femoral cortical bone density in W-36 hens. Ali et al. [124] compared two white-egg lines (Hy-Line W36, DeKalb White) and two brown-egg lines (Hy-Line Brown, Bovans Brown) in relation to keel bone damage. Both white-egg lines were found to have a higher probability of keel fractures than hens of the brown strains. The authors explained this in part with different degrees of genetic predisposition and thus confirmed the results of other studies on a phylogenetic effect [81, 116, 125, 126]. All these findings are supportive of a strong phylogenetic divergence between brown and white-egg layer lines.

Another group of studies looked at comparing lines of different performance level or selection intensity. Budgell and Silversides [84] determined the prevalence of bone fractures in commercial Babcock B300 and ISA Brown hens in comparison to a Brown Leghorn line that has been maintained without selection since 1965. High prevalences were observed in both laying hybrids, while the unselected line had a significantly lower prevalence. The authors argued that these differences may be due to the intensive selection that took place in the commercial lines [84]. Rennie et al. [127] compared the bone volume of the proximal tarsometatarsus and free thoracic vertebra of the modern hybrid line Hisex Brown and the Brown Leghorn J-line, which has been maintained at the Roslin Institute for decades without selection. They detected a lower percentage of trabecular bone in the Hisex hens, the majority of which were classified as osteoporotic, in contrast to the J-line hens. Habig et al. [128] performed bone quality measurements in four purebred lines differing in phylogenetic origin and performance level. Within both phylogenetic groups, they observed lower bone strength and mineral density values for the tibiotarsus in the lines selected for high laying performance. It was concluded that there might be an impact of egg-laying performance on bone stability within each phylogenetic group [128]. Hocking et al. [113] studied the genetic variation for skeletal traits between 12 commercial and 13 traditional layer lines. There was moderate genetic variation among and within the lines for bone stability traits. Bones from the traditional lines showed an increased

breaking strength, mineral density and cortical bone dimensions, while the commercial lines showed a lower overall bone stability. These differences were attributed to the maintenance of eggshell quality at the expense of bone health in the lines selected for laying performance [113]. In a subsequent study, these observations were supported by showing that the lines did not differ in bone characteristics until the onset of laying, but diverged thereafter [129]. Given these findings, it is reasonable to assume that the selection of commercial laying hens for increased egg production may have negatively influenced bone stability [23, 36, 113, 126, 130]. However, this assumption is seen critically, as recent findings point to a less straightforward relationship between egg number and osteoporosis [66, 105, 131, 132].

Insights from Selection Experiments and Estimates of Genetic Parameters

As early as 1937, the study by Warren [82] gave indications that the tendency to develop keel bone damage might be hereditary. Previously, divergent selection was carried out in White Leghorn chickens from 1926 to 1935, selecting for the presence or absence of keel bone deformities. Both strains developed clearly differently depending on what they had been selected for. One of the most highly regarded selection experiments on bone stability in layers is certainly the one carried out by Bishop et al. [133], in which the inheritance of bone characteristics was studied over five generations in purebred White Leghorn hens. While the first generation served as the base generation, the hens of the following ones were selected divergently on high and low bone stability traits combined in a bone index. This index included the radiographic density of the keel bone as well as the bone breaking strengths of the humerus and tibiotarsus. Body weight was also considered in the index to prevent its increase. As a result, the lines differed significantly and diverged in the desired direction from the third generation onwards. Finally, the high and low bone index lines differed in keel bone density by 19% and in bone breaking strength by 13% (humerus) and 19% (tibiotarsus), respectively, which was reflected in a significantly lower fracture prevalence in the high bone index line. The authors concluded that bone traits responded quickly, so improving bone health through conventional selection techniques could be promising [133]. After the initial description by Bishop et al. [133], the low and high bone index lines were subjected to a number of follow-up studies including those characterising the bone material composition of these lines in more detail [37, 46, 134]. Furthermore, a higher keel bone mineral density and reduced prevalence of keel bone damage was observed in following generations of the high bone index line [130, 135]. It was also found that there were no differences between the lines in egg production and quality, suggesting that selection for increased bone stability is possible without compromising hen performance [46, 64].

In general, skeletal traits in laying hens are considered weakly to moderately heritable [62, 64]. Preisinger [16] reported a heritability of bone stability, without further specification, of $h^2 = 0.1$ to $h^2 = 0.15$. Dunn et al. [132] recently studied bone quality traits in White Leghorn and Rhode Island Red hens. The heritability estimates for the bone breaking strength of the tibiotarsus and humerus were $h^2 = 0.24$ and $h^2 = 0.30$ in the White Leghorn and $h^2 = 0.51$ and $h^2 = 0.47$ in the Rhode Island Red chickens. The corresponding genetic correlations between these traits were $r_g = 0.76$ and $r_g = 0.81$, respectively. In the study from Bishop et al.

[133], humerus and tibiotarsus breaking strength were moderately heritable at $h^2 = 0.30$ and $h^2 = 0.45$, respectively, and keel bone density at $h^2 = 0.39$. The heritability of the bone index mentioned was $h^2 = 0.40$. The medullary and cancellous bone volume, on the other hand, proved to be rather poorly heritable, with estimates of $h^2 = 0.19$ and $h^2 = 0.0$, respectively. The genetic correlation estimates indicate a rather close relationship between the radiographic density of the keel bone and the breaking strength of the tibiotarsus ($r_g = 0.66$) and humerus ($r_g = 0.49$), as well as the breaking strengths of humerus and tibiotarsus ($r_g = 0.77$) [133]. With a value of $r_g = 0.75$, Hocking et al. [113] reported a similarly close genetic relationship between these long bones. Andersson et al. [136] estimated the heritability of keel bone deviations assessed via palpation and genetic correlations with production parameters in two White Leghorn breeding lines that differed significantly in terms of keel bone damages, showing prevalences of 15% and 75%, respectively. The heritability estimates were $h^2 = 0.30$ (line A) and $h^2 = 0.15$ (line B). They observed that early egg production would be affected by selection on increased keel bone health in these lines, as the corresponding genetic correlations were r_g = -0.24 (line A) and r_g = -0.54 (line B).

Genomic Regions Associated with Skeletal Traits

The last part of this chapter addresses the question of which genes are related to skeletal integrity in laying hens. To date, a number of quantitative trait loci (QTL), i.e. genomic regions with a significant effect on a quantitative trait [7], have been mapped to bone phenotypes and some plausible candidates have been identified [62]. While in the beginning, mapping studies were performed at low-density marker level using microsatellite markers [137–140], technical advances in genotyping and the introduction of modern association analyses enhanced the knowledge about genetic architecture of bone stability in laying hens [106, 107, 141]. Since a comprehensive presentation of candidate genes for osteoporosis will be given in the fourth chapter of this thesis, only a few recent studies are cited here. Guo et al. [106] conducted a genome-wide association study (GWAS) on bone mineral density measures of the femur and identified a novel locus that might be related to bone stability. They confirmed some genes known to be associated with osteoporosis in humans. Raymond et al. [107] performed a GWAS on bone breaking strength measures in LSL hens. They identified five distinct and novel QTLs and the genes located within these QTLs, some of which are known to have functions in bone metabolism. One of the first significant QTL for bone stability in poultry was discovered on chromosome 1 by Dunn et al. [104] using microsatellite markers. The experiment was performed using the aforementioned population of White Leghorn chickens that have been divergently selected for bone stability [133]. De Koning et al. [142] recently performed fine-mapping of this QTL and additional functional studies, which lead to the identification of a gene that may affect the crosslinking of bone collagen matrix. Despite the conciseness of this overview, it is obvious that a number of promising candidate regions have already been discovered. It should be noted, however, that all of these genomic regions are still putative, as none of them has yet been proven to be functionally causal [62].

1.5 Environmental Factors Influencing Bone Stability

Bone stability is also influenced externally by nutrition and housing system, which affect the hens' predisposition to skeletal problems [75, 127, 143]. Calcium is considered the most important mineral in terms of skeletal integrity [143]. Already during the rearing phase, adequate supply is crucial for skeletal development and serves to minimise structural bone loss once medullary bone formation has commenced [52, 144]. Due to their longer retention time in the gastrointestinal tract [33, 127, 145, 146], coarse calcium particles in layer diets are beneficial for bone stability, especially at night when there is no feed intake, but eggshell formation is taking place [147, 148]. Increasing the dietary calcium content leads to improved bone strength [149–152]. However, the calcium bioavailability is limited by the intestinal transit time and absorption rate, which is 40-70% [57, 153, 154]. Furthermore, a too high calcium level can interfere with the digestion of other nutrients [155, 156]. Phosphorus is another mineral essential for bone strength [49, 52, 144] as, together with calcium, it makes up the largest part of the bone mineral structure [157, 158]. The literature is rather contradictory regarding the benefit of additional phosphorus administration on bone stability, however, the bioavailability of dietary phosphorus can be increased by the addition of microbial phytases [143, 144, 157]. Vitamin D is by far the most important vitamin for bone stability [143] and is routinely added as part of premixes to layer diets [150]. However, beneficial effects of vitamin D supplementation beyond the basic requirements remain controversial. There are rather few findings that indicate a positive effect [60, 159, 160] and most studies negate such an effect [117, 150, 161–163]. Adequate vitamin D supply during skeletal development at the pullet stage is considered important, as dietary interventions during the laying period have rather limited effects on bone stability [60, 144, 160].

The effect of different housing systems on bone health has been intensively studied. There is a consensus that chickens from non-cage systems, i.e. aviary as well as floor and free range systems, have a higher bone stability, which is mainly attributed to the beneficial effects of physical exercise that these systems provide [123, 126, 128, 135, 164–169]. Non-caged hens show stimulated bone metabolic activity, a higher turnover rate and better adaptation to mechanical loads, whereas prolonged exercise restriction in caged hens results in bone resorption and adverse structural remodelling [37, 170, 171]. In summary, physical activity has a positive effect on bones' mechanical properties and can thus influence the occurrence and severity of skeletal disorders in laying hens [172, 173]. This also applies to pullets and it is assumed that the positive effect of physical activity carried out during rearing lasts into the laying period, provided that the opportunity for exercise is maintained [169, 174–176]. However, the positive effect of non-cage systems is somewhat diminished by the fact that several authors found a high prevalence of keel bone damages in these systems, mainly involving keel bone fractures [88, 131, 177–181]. This issue, known as the "welfare paradox", is explained by the fact that as the space and internal complexity of a housing system increases, the hens have more opportunity to experience harmful accidents [28, 135, 182, 183]. In this context, the aviary design, including furnishing items such as perches has received particular attention in research, as they influence the hens' risk of falls and collisions when moving throughout the system [80, 183–185].

1.6 Thesis Aim and Objectives

Skeletal integrity in laying hens is an issue of particular relevance. The high incidence of birds suffering from osteoporosis has serious implications for animal welfare and requires urgent intervention. To do this, however, the causative and determining factors of bone stability must be identified. In addition to environmental factors including nutrition and husbandry, there is evidence of a significant contribution of genetics to bone stability.

The overarching aim of this dissertation is to further characterise the influence of genetics on the differentiation of bone stability and to evaluate its potential for improving bone health in laying hens. Different studies were conducted applying an animal model comprising four purebred chicken layer lines (Figure 1.2). This four-line animal model includes two brown (BLA, L68) and two white-egg lines (WLA, R11). Within these phylogenetic groups, both lines differ with regard to their egg-laying performance. The two high performing lines (WLA, BLA) originate from a commercial layer breeding program, whereas their moderate performing counterparts (R11, L68) have been kept as unselected resource populations for more than 25 generations. This two-dimensional divergence not only covers a broad spectrum of laying hen genetics, but is also intended to allow discrimination between effects of performance level and phylogenetic origin. A more detailed description of the layer lines is given in the following chapters.

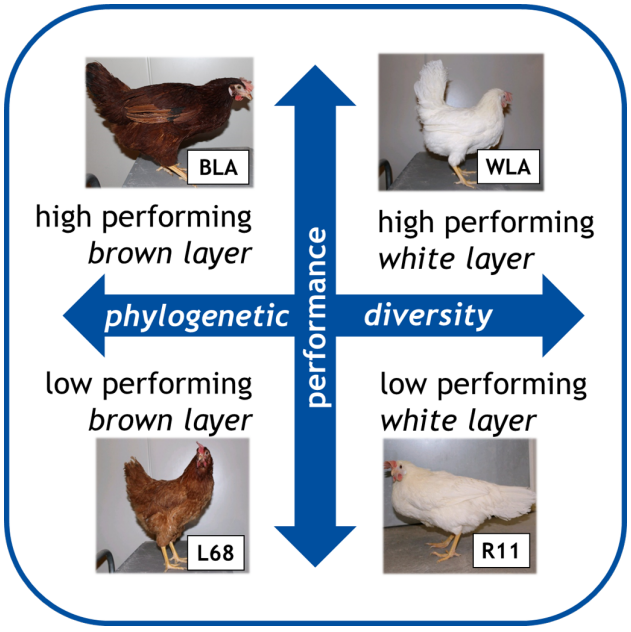

Figure 1.2: Schematic illustration of the four-line animal model comprising four purebred chicken layer lines differing in their phylogenetic origin and egg-laying performance. Modified from Lieboldt et al. [186].

The first objective is to investigate to what extent the factors of phylogeny and egg production level determine bone stability. This is studied in hens under two different metabolic conditions: the normal state and the state challenged by calcium reduction. First, Chapter 2 analyses the relationship between egg production and bone strength in metabolically normal hens with respect to the main factors of phylogeny and performance level. Besides characterising skeletal traits of the four layer lines, the question of whether bone weakness can

be attributed to selection for high laying performance is addressed. In the following Chapter 3, the role of the two main factors on bone properties under the metabolically challenging situation of a nutritive calcium depletion is examined. Repeated administration of a low-calcium diet are employed to investigate whether there are differences in the resilience of the four layer lines in terms of skeletal integrity.

The second objective of this thesis focuses on the potential of genetics to alleviate bone disorders in laying hens. This is based on heritability and genetic correlation estimates for the bone traits given in Chapter 2, and on the results from a genomic study carried out in Chapter 4. For the latter, the four layer lines are combined to identify and functionally annotate candidate genes associated with bones traits in laying hens.

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2 Relationship between Bone Stability and Egg Production in Genetically Divergent Chicken Layer Lines

Simon Jansen [1], Ulrich Baulain [1], Christin Habig [1], Annett Weigend [1], Ingrid Halle [2], Armin M. Scholz [3], Henner Simianer [4,5], A. Reza Sharifi [4,5] and Steffen Weigend [1,5]

[1] Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut, 31535 Neustadt, Germany.

[2] Institute of Animal Nutrition, Friedrich-Loeffler-Institut, 38116 Braunschweig, Germany.

[3] Livestock Center of the Faculty of Veterinary Medicine, Ludwig-Maximilians-University Munich, 85764 Oberschleißheim, Germany.

[4] Animal Breeding and Genetics Group, Department of Animal Sciences, University of Göttingen, 37075 Göttingen, Germany.

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

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Notice: After publication, it was noticed that a typographic error had been made. However, the data presented are correct and the changes do not alter their interpretation. The published corrigendum is included in the appendix of this thesis (Chapter 6).

Simple Summary

Brittle or fractured bones due to continuous demineralisation cause major welfare and economic problems in laying hens. Bone weakness in laying hens is frequently attributed to long-term selection for increased egg production, but this is controversially discussed in the scientific literature. We aimed at characterizing factors influencing the bone breaking strength of laying hens, focusing mainly on the effect of eggshell production. By examining four different chicken layer lines separately, a genetically diverse spectrum of laying hen origins was included in our study. It was shown that bone strength is primarily influenced by bone mineral density. A strong association between bone strength and eggshell production was not observed within each of the lines studied. This applied to all layer lines. Our results suggest that a high egg number does not generally impair bone stability within layer lines. Findings from this study contribute to the discussion on the improvement of bone stability in poultry breeding programs and thus lead to increased animal welfare in egg production.

Abstract

Impaired animal welfare due to skeletal disorders is likely one of the greatest issues currently facing the egg production industry. Reduced bone stability in laying hens is frequently attributed to long-term selection for increased egg production. The present study sought to analyse the relationship between bone stability traits and egg production. The study comprised four purebred layer lines, differing in their phylogenetic origin and performance level, providing extended insight into the phenotypic variability in bone characteristics in laying hens. Data collection included basic production parameters, bone morphometry, bone mineral density (BMD) and bone breaking strength (BBS) of the tibiotarsus and humerus. Using a multifactorial model and regression analyses, BMD proved to be of outstanding importance for bone stability. Only for the tibiotarsus were morphometric parameters and the bone weight associated with BBS. Within the chicken lines, no effect of total eggshell production on BBS or BMD could be detected, suggesting that a high egg yield itself is not necessarily a risk for poor bone health. Considering the complexity of osteoporosis, the estimated genetic parameters confirmed the importance of genetics in addressing the challenge of improving bone strength in layers.

Keywords: animal welfare; bone mineral density; bone breaking strength; fractures; laying hens; laying performance; osteoporosis; phylogeny

1 Introduction

Although the consideration of functional traits in selection programs to improve animal health has become increasingly important in recent years, the number of saleable eggs and extended persistency of laying are still the main goals in the breeding of laying hens [1]. Since up to three grams of calcium per egg are required for eggshell formation [2], laying more than 300 eggs in 12 months in highly selected commercial hens is an immense challenge for the calcium homeostasis, and as part of it, the skeletal system of the bird. During eggshell calcification, laying hens cover, partially, the temporarily high demand of calcium with increased mobilisation from the bones [3]. In avian species, medullary bone serves as a labile calcium source and its formation increases with the onset of sexual maturity [4, 5]. However, this is accompanied by a decrease of cancellous bone volume under the influence of oestrogen [6–8]. Continuous demineralisation leads to osteoporosis, a pathological condition of progressive loss of structural bone tissue, resulting in brittle and fragile bones being susceptible to fractures [3, 9].

High incidences of birds suffering from osteoporotic or fractured bones have been reported [10–12]. Riber et al. [13] concluded that hens suffering from bone fractures show marked atypical behavioural differences compared to those with healthy bones, suggesting that osteoporosis has serious animal welfare implications. Nasr et al. [14] proved that hens with keel bone fractures do experience pain. Bone weakness can also be a cause of mortality, as shown in a study by McCoy et al. [15], in which it accounted for up to 35% of the deaths. Fracture-associated decline in performance adds an economic dimension to the implications of skeletal disorders [8, 16, 17].

In addition to the important factors of nutrition and husbandry, genetics are considered a decisive factor for bone health [3, 18, 19]. Skeletal problems in layers are frequently attributed to selection for increased egg production, suggesting a negative association between laying performance and bone stability [8, 20–24]. As bone quality traits are supposed to be highly polygenic [18], genetic correlations might lead to an accompaniment of selection for high laying performance by undesirable "co-selectional" side effects [25–27]. In the case of calcium homeostasis, this may have resulted in a prioritization of calcium resources in favour of reproduction and to the disadvantage of bone health [28, 29].

Targeted genetic selection certainly makes the main contribution to changes in performance potential and may be associated with undesirable associated effects on bone stability. However, differences may also be due to the phylogenetic origin of these lines, whose distinct breeding history may have influenced the genetic characteristics before selection for high performance began. Since white and brown-egg laying chicken lines evolved separately after domestication from red jungle fowl several thousands of years ago and underwent genomic changes [30, 31], phylogenetic origin has potential implications for bone characteristic differences [32]. Therefore, in addition to the comparison of genetic lines, it is necessary to assess the association within the genetic lines.

This study is part of a multidisciplinary collaboration initiated at the Friedrich-Loeffler-Institut to investigate the effects of selection on performance efficiency in terms of adaptability to changing environmental conditions in laying hens. The animal model used comprised four chicken lines, two white and two brown-egg layers, which are phylogenetically distant and evolved independently during breed history [31]. Within each phylogenetic group, the two lines differed in performance level, since one of them originated from a contemporary

commercial egg layer breeding line ("high performing"), whereas its counterpart was based on a conservation flock without any selection for many generations ("moderate performing") [33]. Within the framework of these research activities, the phenotypic data on bone stability and egg production were used for genetic analyses in the present study, which were collected from laying hens with complete pedigree in two consecutive generations of four chicken-layer lines. The animals were supplied with different amounts of vitamin D3 (cholecalciferol). The dietary vitamin D3 content was varied in tests, since a relationship between bone stability and vitamin D3 was assumed [4, 9]. We aimed at analysing the relationship between bone stability traits and egg production within the genetically divergent layer lines used in this model. Based on the frequently stated negative association between bone stability and egg production, we hypothesized that the two high performing layer lines show deficits in bone stability compared to their moderately performing counterparts, and that within lines the level of eggshell production significantly contributes to the variation of bone breaking strength (BBS) in humerus and tibiotarsus.

2 Materials and Methods

2.1 Ethical Note

The present experiment was performed in accordance with the German Animal Welfare Law and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES) (33.19-42502-04-15/1988).

2.2 Animals and Housing

The study included four purebred chicken layer lines (*Gallus gallus domesticus*), which differed in respect to their phylogenetic origins and performance levels. Lines WLA and BLA originated from a commercial breeding program of the Lohmann Tierzucht GmbH (Cuxhaven, Germany) selected for high laying performance. These lines have been maintained in a sire rotation program since 2012 and achieve a laying rate of about 320 eggs per year. In contrast, lines L68 and R11 have been maintained as non-selected resource populations at the Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (Neustadt, Germany) for more than 25 generations, R11 even for more than 50 generations. Their laying performance is about 200 eggs per year [33]. In addition to performance differences, the animal model considered a phylogenetic component, since white-egg layer lines WLA and R11 (both originating from White Leghorn) are phylogenetically closely related, but distinct from brown-egg layer lines BLA and L68. BLA originates from Rhode Island Red, while L68 descends from New Hampshire, a breed that has been developed from Rhode Island Red chickens [31].

The experiment was conducted in two consecutive generations with 576 hens in total (72 hens per layer line and generation). All chicks of a respective replicate were hatched on the same day and were reared in floor pens of 24 m^2 until the 16th week of age. Information on the light program and the mean climatic conditions is given in the Supplementary Material (Table S2.1). Usual feeding stuff for chicks (until 6 weeks of age) and pullets (from 7 to 16 weeks of age), which had sufficient content of phosphorus, calcium and vitamin D3, was

offered *ad libitum*. The nutrient compositions of these diets are listed in the Supplementary Material (Table S2.2).

After birds were transferred to the layer facility at the 16th week of age, they were kept in individual cages. The cage dimensions were 50 cm $\times$ 48 cm, which equals 2400 cm^2 of total floor space, and it was equipped with a plastic perch of 3 cm diameter. At the beginning of the 17th week of age, two customary wheat-soya-based diets for layers were fed *ad libitum*. The diets' compositions and their nutrient contents are detailed in the Supplementary Material (Table S2.3). The two diets differed in content of vitamin D3: 300 IU, according to the recommendations of the German Society of Nutrition Physiology [34], or 3000 IU, displaying the maximum content according to the regulation (EC) number 1831/2003 of the European Parliament and of the Council [35]. It turned out, however, that no significant differences were found in terms of this difference in the vitamin D3 content for the traits studied (Table S2.4). It is possible that the difference between 300 and 3000 IU of vitamin D3 was not sufficient to elicit a response reflected in the observed characteristics, as both contents were within the range of what is considered to be adequate for chickens [36]. The results could indicate that laying hens may tolerate a wide range of dietary vitamin D3 supply. However, the present study cannot provide deeper insights into this. With regard to the genetic analysis presented here, data from both vitamin D3 groups were combined.

2.3 Experimental Procedure

The experimental setup, including data collection, is shown in Figure 2.1 and was identical in both generations. The experimental trial lasted 52 weeks from the 18th to the 69th week of age. The individual egg number was recorded daily during weeks 18 to 68; i.e., over 51 laying weeks. Egg weight data (g) were collected every two weeks over four consecutive days each, resulting in a mean egg weight value per individual (based on an average of 78.6 eggs per individual). Eggshell weight (g) was determined six times, at week 28, 36, 44, 52, 60 and 68, on four consecutive days each. For this, the eggs were emptied and shells were dried for 30 s in a microwave (800 watt). A digital table scale with a weighing accuracy of 0.01 g (Type 3709, Sartorius, Göttingen, Germany) was used for egg and eggshell weight determination. Eggshell proportion was calculated as the ratio between eggshell and egg weight. For the eggshell characteristics, mean values were calculated the same way as for the egg weight (based on an average of 18.6 eggs per individual). Total eggshell production was calculated by multiplying the mean eggshell weight and the total egg number. Feed consumption (g) was determined weekly on individual basis by back weighing the remaining feed using a table scale with a weighing accuracy of 20 g (Dexal 3, Epel Industrial, Sant Boi de Llobregat, Spain). Based on this, daily feed consumption (g) was calculated. Feed-to-egg conversion rate was calculated by dividing total feed consumption by the product of mean egg weight and total number of eggs. Feed-to-eggshell conversion rate was calculated analogously. Body weight (g) was measured at hatch and during the experimental period (at week 21, 25, 35 and 69) using a digital table scale (CPA 16001S, Sartorius, Göttingen, Germany) with a weighing accuracy of 0.1 g.

The hens were euthanized by carbon dioxide inhalation after 69 weeks of age. The left tibiotarsus and humerus were extracted and the adherent tissue removed. Bone weight (g), length (mm) and thickness (mm) were recorded, and bone mineral density (BMD) (g/cm^2) was examined by dual energy X-ray absorptiometry (DXA) (GE Lunar *i*DXA scanner, GE

Healthcare, Solingen, Germany). The bones were scanned and analysed by using the small animal mode within the enCore® software version 17 (GE Healthcare, Solingen, Germany). A standardised rice pack positioned between dual energy X-ray source and bones served as the soft tissue standard. All bones were stored, transported and scanned under vacuum conditions in special plastic bags individually for each hen. Manually defined regions of interest helped to analyse the bones separately after the scan. Individual results were stored using the PDF and the DICOM file formats. BBS values (N) of the tibiotarsus and humerus were assessed at the mid-diaphyseal region via three-point bending test (Instron Materials Testing System, Instron Corporation, Canton, MA, USA). Thereby a 5 kN load cell was used and the span length was 40 mm (humerus) or 80 mm (tibiotarsus).

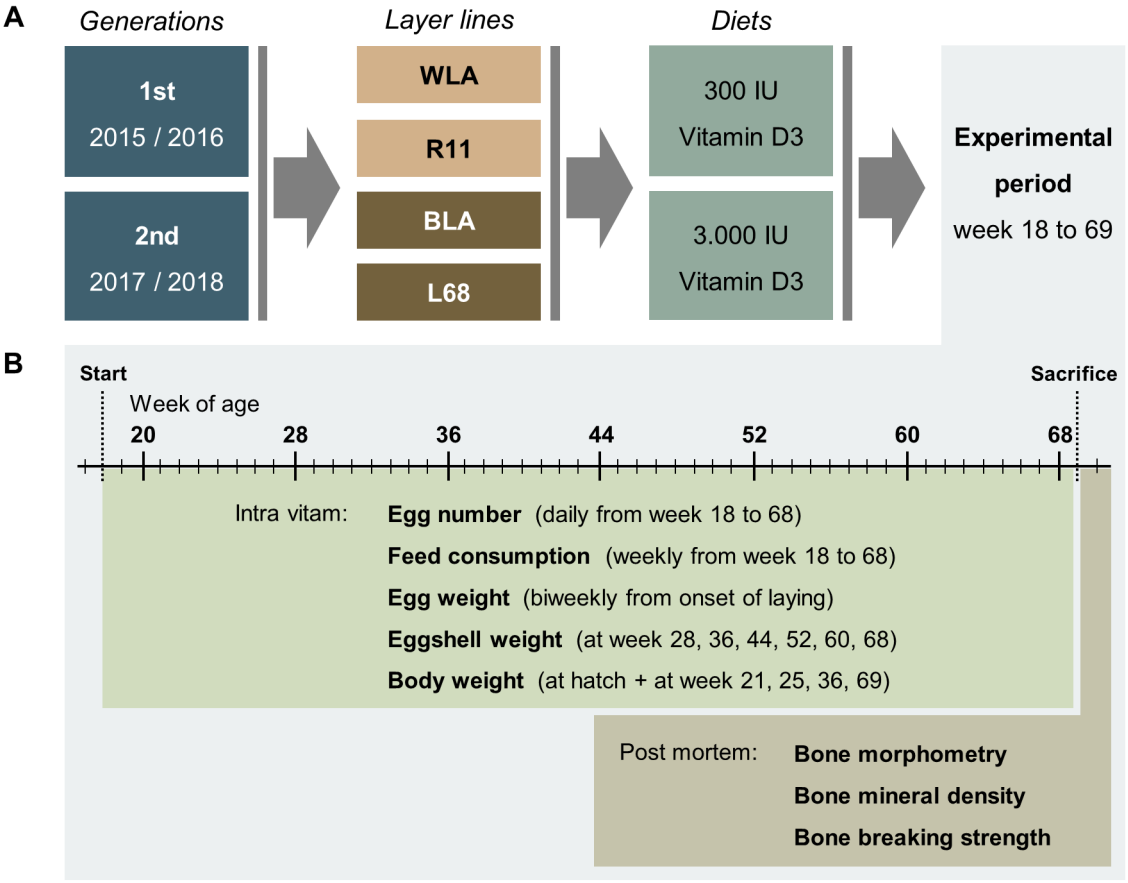

Figure 2.1: Schematic illustration of the experimental setup (**A**) and related data collection (**B**). In two consecutive generations, four chicken layer lines were allocated to a diet containing either 300 or 3000 IU of vitamin D3. During the experimental period, data on egg number, egg quality, feed consumption and body weight were collected as indicated. *Post mortem*, bone morphometry, bone mineral density and bone breaking strength were assessed.

2.4 Statistical Analysis

As bones are supposed to differ noticeably depending on the reproductive status in female birds, exclusion of non-reproducing hens was required [18]. Individuals whose total egg numbers were outside the line specific threefold interquartile range (IQR) ($< X_{0.25} - 3 \times IQR$; $> X_{0.75} + 3 \times IQR$) and who did not lay an egg during the last three consecutive experimental weeks were considered as outliers. After filtering, a total number of 524 animals remained for analyses (WLA: $n = 129$; R11: $n = 134$; BLA: $n = 133$; L68: $n = 128$). The sample size for the statistical analysis varied for the different variables between 125 and 134 observations per layer line (130 observations on average) and was based either on individual records, or, as in the case of egg quality traits, on average values calculated over different points in time as described above. A detailed list of sample sizes separated by genotype for all variables is given in the Supplementary Material (Table S2.5).

The impacts of layer line, generation and their interaction on production parameters, body weight and bone traits were analysed using the GLIMMIX procedure of SAS 9.4 (SAS Institute Inc. Cary, NC, USA, 2017) according to the following model:

$$\gamma_{ijkl} = \mu + LL_i + G_j + LL \times G_{ij} + S_k + \varepsilon_{ijkl} \quad (1)$$

where γ_{ijkl} is the observation for a production parameter, body weight or bone trait; μ is the general mean; LL_i is the fixed effect of layer line ($i = 1$ to 4); G_j is the fixed effect of generation ($j = 1, 2$); $LL \times G_{ij}$ is the fixed effect of interaction between layer line and generation; S_k is the random effect of sire ($k = 1$ to 145); and ε_{ijkl} is the random error variance. Tukey's HSD (honestly significant difference) test was performed for multiple comparisons of means. Statistical significance was set at $p < 0.05$.

To determine the association between the bone characteristics, Pearson's correlation coefficients (r_p) were estimated using the CORR (correlation) procedure from SAS (SAS Institute Inc. Cary, NC, USA, 2017). Results description followed recommendations by Asuero et al. [37].

An analysis of covariance was applied to study the variation of BBS considering bone morphometry, BMD and total eggshell production as covariate terms and the fixed effects of layer line, generation and significant interactions between main factors and the covariates [38]. In a backward elimination approach, the Wald F-statistics were used to determine the significance of fixed effects [39], resulting in the following model:

$$\begin{aligned}\gamma_{ijklmnopq} = \mu + LL_i + G_j + LL \times G_{ij} + BMD_k + LL \times BMD_{ik} + G \times BMD_{jk} \\ + LL \times G \times BMD_{ijk} + W_l + T_m + L_n + TEP_o + S_p + \varepsilon_{ijklmnopq}\end{aligned} \quad (2)$$

where $\gamma_{ijklmnopq}$ is the observation of BBS; μ is the general mean; LL_i is the fixed effect of layer line ($i = 1$ to 4); G_j is the fixed effect of generation ($j = 1, 2$); $LL \times G_{ij}$ is the fixed effect of interaction between layer line and generation; BMD_k is the effect of BMD; $LL \times BMD_{ik}$ is the effect of interaction between layer line and BMD; $G \times BMD_{jk}$ is the effect of the interaction between generation and BMD; $LL \times G \times BMD_{ijk}$ is the effect of the interaction between layer line, generation and BMD; W_l is the effect of bone weight; T_m is the effect of bone thickness; L_n is the effect of bone length; TEP_o is the effect of total egg number; S_p is the random effect of sire ($p = 1$ to 145); and $\varepsilon_{ijklmnopq}$ is the random

error variance.

The bone data were converted to standardised z-scores to have a standard deviation of 1.0 and a mean of 0.0. Univariate regression analyses were performed using the MIXED procedure from SAS (SAS Institute Inc. Cary, NC, USA, 2017) according to the following model:

$$\gamma_i = \beta_0 + \beta_1 x_i + \varepsilon_i \tag{3}$$

where γ_i is the BBS or the BMD; β_0 is the intercept; β_1 is the slope; x_i is a morphometric parameter (in case of BBS analysis) or total eggshell production (in case of BMD analysis); and ε_i is the random error variance.

Genetic parameters were estimated using ASReml 4.1 [40] according to the following animal model:

$$\gamma_{ij} = \mu + A_i + G_j + \varepsilon_{ij} \tag{4}$$

where γ_{ij} is the BBS or the BMD; μ is the general mean; A_i is the random direct genetic effect of the hen; G_j is the fixed effect of the generation ($j = 1, 2$); and ε_{ij} is the error term. Within lines, univariate analyses were conducted to estimate the heritability of BMD and BBS. Bivariate analyses were used for estimation of genetic correlations between these traits.

3 Results

3.1 Basic Production Parameters

Table 2.1 summarises the least squares means and the significance of layer line, generation and their interaction for various basic production parameters. For all traits, a highly significant effect of the layer line was observed. With exception of total egg number and feed-to-egg conversion rate, the generation was also identified as a significant explanatory variable. However, only in respect to egg- and eggshell weight, the effect of layer line by generation interaction proved to be significant. Here, in line WLA heavier eggs were observed in the second generation, whereas in R11, BLA and L68, slightly higher eggshell weights were seen in the first generation. With the first eggs being laid at 20.56 (WLA) and 20.69 (BLA) weeks of age, in both high performing lines the onset of laying was significantly earlier than in their moderate performing counterparts, as L68 and R11 reached laying maturity only at 23.12 and 24.66 weeks of age, respectively. Within the 357 days lasting laying period, lines WLA and BLA achieved laying performances of 316.34 and 317.32 eggs respectively, and differed significantly from lines R11 (average of 226.25 eggs) and L68 (average of 215.94 eggs). In terms of egg weight, eggshell weight and proportion of the eggshell, the high performing genotypes showed significantly higher values than their corresponding moderate performing lines. This pattern continued for the total eggshell production. However, there was a clear ranking of genotypes, with WLA producing the largest amount of eggshell, followed by BLA, R11 and L68. The mean difference in total eggshell production within the phylogenetic groups was 821.94 g between the white-egg lines, and 812.63 g between the brown-egg lines. Despite significantly higher daily feed consumption of both high performing lines, the feed-to-egg and feed-to-eggshell conversion rates were about one third lower in both BLA and WLA hens than in their counterparts. Results on body weight development are shown in the Supplementary Material

(Table S2.6). The fact that WLA and BLA hens were heavier at hatch reversed during rearing. The brown-egg lines were both significantly heavier during the following measurements, and at final weighing in, week 69, line L68 had the highest average body weight.

3.2 Bone Characteristics

The least squares means and the significance level for layer line, generation and their interaction for the examined traits of tibiotarsus and humerus are shown in Table 2.2. The layer line had a highly significant effect on all bone traits studied. With exception of weight and thickness in tibiotarsus, the same applies to the generation effect. The layer line by generation interaction was significant for the weight of tibiotarsus. However, post-hoc comparison did not detect any significant deviation between the two generations within lines. Hens from the brown-egg lines displayed a higher humerus BBS than the white-egg strains. For the tibiotarsus, line L68 was characterised by a high BBS, whereas the other lines differ only slightly amongst each other. Mean BMD of both bone types was significantly higher in the brown-egg lines BLA and L68, while hens of line WLA showed the lowest values. It turns out that BLA and L68 do have significantly heavier, thicker and longer bones than their white-egg-laying counterparts. Line L68 especially stands out in relation to the tibiotarsus, being the line with the highest values for all traits.

Figure 2.2 shows the Pearson's correlation coefficients (r_p) between the bone traits examined in the tibiotarsus and humerus of the genetic lines WLA (Figure 2.2A), R11 (Figure 2.2B), BLA (Figure 2.2C) and L68 (Figure 2.2D). With values varying from $r_p = 0.43$ (Figure 2.2C) to $r_p = 0.70$ (Figure 2.2D), BBS and BMD were moderately correlated for the tibiotarsus. Slightly weaker correlations ranging from $r_p = 0.33$ (Figure 2.2A) to $r_p = 0.66$ (Figure 2.2B) were observed between these traits for the humerus. Except for moderate correlations between BBS and weight in the tibiotarsus of WLA ($r_p = 0.68$, Figure 2.2A) and L68 ($r_p = 0.62$, Figure 2.2D), rather low and non-significant correlations were observed regarding BBS and morphometric traits. The same applies to the relationship between BMD and morphometry in the tibiotarsus. In both bone types, BMD and weight were moderately to strongly associated. With values from $r_p = 0.64$ (Figure 2.2C) up to $r_p = 0.86$ (Figure 2.2B), the lengths of the two different bone types were strongly associated. While the correlation of the thicknesses of the two bone types varied only between $r_p = 0.38$ (Figure 2.2B,C) and $r_p = 0.43$ (Figure 2.2D), it differed considerably for the weight. However, a general pattern of correlating characteristics that applies to all lines and/or to phylogenetic groups could not be identified.

Table 2.1: Least squares means ± standard errors for production parameters for the effect of layer line (LL), generation (Gen) and their interaction, and the significance levels of the effects.

Effect	Laying Maturity (Weeks)	Total Number of Eggs [1]	Egg Weight (g) [2]	Eggshell Weight (g) [2]	Eggshell Proportion (%) [2]	Total Eggshell Production (g) [3]	Daily Feed Consumption (g)	Feed-to-Egg Conversion Rate	Feed-to-Eggshell Conversion Rate
Layer line (LL)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
WLA	20.56 ± 0.15^{c}	316.34 ± 2.57^{a}	57.63 ± 0.41^{b}	6.38 ± 0.04^{a}	11.10 ± 0.06^{a}	2019.89 ± 16.23^{a}	93.82 ± 0.97^{b}	1.84 ± 0.02^{c}	16.64 ± 0.29^{d}
R11	24.66 ± 0.15^{a}	226.25 ± 2.53^{b}	51.54 ± 0.41^{c}	5.29 ± 0.04^{c}	10.28 ± 0.06^{b}	1197.95 ± 16.00^{c}	79.58 ± 0.96^{c}	2.47 ± 0.02^{b}	24.14 ± 0.29^{b}
BLA	20.69 ± 0.15^{c}	317.32 ± 2.55^{a}	60.09 ± 0.41^{a}	6.14 ± 0.04^{b}	10.25 ± 0.06^{b}	1950.23 ± 16.08^{b}	102.46 ± 0.98^{a}	1.92 ± 0.02^{c}	18.80 ± 0.29^{c}
L68	23.12 ± 0.15^{b}	215.94 ± 2.57^{c}	53.05 ± 0.41^{c}	5.29 ± 0.04^{c}	9.95 ± 0.06^{c}	1137.60 ± 16.27^{d}	91.32 ± 0.98^{b}	2.88 ± 0.02^{a}	29.04 ± 0.29^{a}
Generation (Gen)	< 0.0001	0.8384	0.0032	< 0.0001	< 0.0001	< 0.0001	0.0017	0.3673	< 0.0001
Gen1	21.81 ± 0.11	269.23 ± 1.82	54.97 ± 0.29	5.94 ± 0.03	10.80 ± 0.05	1619.31 ± 11.53	90.25 ± 0.69	2.27 ± 0.02	21.14 ± 0.21
Gen2	22.70 ± 0.11	268.70 ± 1.79	56.19 ± 0.29	5.62 ± 0.03	9.99 ± 0.05	1533.53 ± 11.30	93.33 ± 0.68	2.29 ± 0.02	23.17 ± 0.20
LL × Gen	0.4859	0.2202	0.0470	0.0028	0.4750	0.1823	0.6130	0.9523	0.2921
WLA × Gen1	20.01 ± 0.22	319.87 ± 3.64	56.16 ± 0.59^{b}	6.41 ± 0.06^{a}	11.42 ± 0.09	2048.99 ± 22.99	91.57 ± 1.38	1.82 ± 0.03	16.00 ± 0.41
WLA × Gen2	21.10 ± 0.22	312.80 ± 3.63	59.11 ± 0.58^{a}	6.37 ± 0.06^{a}	10.78 ± 0.09	1990.80 ± 22.91	96.06 ± 1.37	1.86 ± 0.03	17.27 ± 0.41
R11 × Gen1	24.42 ± 0.21	224.59 ± 3.58	51.44 ± 0.58^{c}	5.51 ± 0.06^{c}	10.71 ± 0.09	1237.57 ± 22.66	78.85 ± 1.36	2.47 ± 0.03	23.09 ± 0.41
R11 × Gen2	24.90 ± 0.22	227.92 ± 3.64	51.64 ± 0.58^{c}	5.08 ± 0.06^{d}	9.84 ± 0.09	1158.33 ± 22.58	80.30 ± 1.37	2.47 ± 0.03	25.20 ± 0.41
BLA × Gen1	20.20 ± 0.22	319.53 ± 3.65	59.28 ± 0.59^{a}	6.33 ± 0.06^{a}	10.70 ± 0.09	2023.14 ± 23.10	101.39 ± 1.41	1.91 ± 0.04	17.85 ± 0.42
BLA × Gen2	21.19 ± 0.21	315.12 ± 3.56	60.91 ± 0.58^{a}	5.95 ± 0.06^{b}	9.80 ± 0.09	1877.31 ± 22.38	103.53 ± 1.35	1.93 ± 0.03	19.74 ± 0.40
L68 × Gen1	22.62 ± 0.22	212.90 ± 3.70	53.00 ± 0.59^{c}	5.49 ± 0.06^{c}	10.37 ± 0.09	1167.53 ± 23.45	89.21 ± 1.41	2.86 ± 0.04	27.62 ± 0.42
L68 × Gen2	23.62 ± 0.22	218.99 ± 3.58	53.10 ± 0.58^{c}	5.06 ± 0.06^{d}	9.54 ± 0.09	1107.67 ± 22.56	93.43 ± 1.36	2.89 ± 0.03	30.46 ± 0.40

[a,b,c,d] Means with different letters within an effect differ significantly (Tukey's HSD-test, $p < 0.05$). [1] Laid between weeks 18 and 68 (357 days). [2] Mean value of the eggs laid from 18 to 68 weeks of age. [3] Product of total number of eggs and mean eggshell weight.

Table 2.2: Least squares means ± standard errors for characteristics of tibiotarsus and humerus for the effect of layer line (LL), generation (Gen) and their interaction and the significance levels for the effects.

	Tibiotarsus					Humerus				
Effect	**Bone Breaking Strength (N)**	**Bone Mineral Density (g/cm^2)**	**Weight (g)**	**Length (mm)**	**Thickness (mm)**	**Bone Breaking Strength (N)**	**Bone Mineral Density (g/cm^2)**	**Weight (g)**	**Length (mm)**	**Thickness (mm)**
Layer line (LL)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
WLA	137.34 ± 3.62 ^{b,c}	0.211 ± 0.005 ^{d}	8.12 ± 0.11 ^{c}	116.78 ± 0.44 ^{c}	5.27 ± 0.04 ^{c}	90.81 ± 3.43 ^{c}	0.136 ± 0.003 ^{d}	3.76 ± 0.10 ^{c}	75.81 ± 0.25 ^{c}	5.33 ± 0.03 ^{c}
R11	149.40 ± 3.54 ^{b}	0.231 ± 0.005 ^{c}	8.23 ± 0.11 ^{c}	116.63 ± 0.44 ^{c}	5.17 ± 0.04 ^{c}	109.94 ± 3.40 ^{b}	0.156 ± 0.003 ^{c}	4.07 ± 0.10 ^{c}	75.38 ± 0.25 ^{c}	5.19 ± 0.03 ^{d}
BLA	124.23 ± 3.58 ^{c}	0.265 ± 0.005 ^{b}	10.90 ± 0.11 ^{b}	119.41 ± 0.44 ^{b}	5.94 ± 0.04 ^{b}	138.64 ± 3.40 ^{a}	0.197 ± 0.003 ^{a}	6.53 ± 0.10 ^{a}	79.69 ± 0.25 ^{a}	5.59 ± 0.03 ^{b}
L68	211.57 ± 3.61 ^{a}	0.327 ± 0.005 ^{a}	12.03 ± 0.11 ^{a}	121.37 ± 0.44 ^{a}	6.19 ± 0.04 ^{a}	146.02 ± 3.45 ^{a}	0.180 ± 0.003 ^{b}	4.93 ± 0.10 ^{b}	76.85 ± 0.25 ^{b}	5.71 ± 0.03 ^{a}
Generation (Gen)	< 0.0001	0.0369	0.7077	0.0002	0.1231	< 0.0001	< 0.0001	< 0.0001	0.0021	0.0327
Gen 1	139.23 ± 2.55	0.264 ± 0.004	9.80 ± 0.08	117.74 ± 0.31	5.61 ± 0.03	111.27 ± 2.43	0.174 ± 0.002	5.60 ± 0.07	76.54 ± 0.18	5.42 ± 0.02
Gen 2	172.04 ± 2.53	0.253 ± 0.004	9.84 ± 0.07	119.36 ± 0.31	5.67 ± 0.03	131.43 ± 2.40	0.161 ± 0.002	4.05 ± 0.07	77.32 ± 0.18	5.49 ± 0.02
LL × Gen	0.0925	0.1725	0.0155	0.0892	0.4924	0.5249	0.8472	0.5376	0.2413	0.0665
WLA × Gen1	113.51 ± 5.10	0.210 ± 0.007	7.84 ± 0.15 ^{c}	115.03 ± 0.62	5.20 ± 0.05	77.13 ± 4.85	0.142 ± 0.004	4.51 ± 0.15	75.00 ± 0.36	5.27 ± 0.04
WLA × Gen2	161.16 ± 5.15	0.214 ± 0.007	8.40 ± 0.15 ^{c}	118.54 ± 0.62	5.35 ± 0.05	104.48 ± 4.85	0.131 ± 0.004	3.02 ± 0.15	76.61 ± 0.36	5.40 ± 0.04
R11 × Gen1	132.92 ± 5.02	0.233 ± 0.007	8.25 ± 0.15 ^{c}	116.22 ± 0.61	5.15 ± 0.05	103.33 ± 4.78	0.162 ± 0.004	4.89 ± 0.15	74.99 ± 0.35	5.21 ± 0.04
R11 × Gen2	165.87 ± 5.01	0.229 ± 0.007	8.21 ± 0.15 ^{c}	117.05 ± 0.62	5.20 ± 0.05	116.55 ± 4.83	0.151 ± 0.004	3.24 ± 0.14	75.77 ± 0.35	5.18 ± 0.04
BLA × Gen1	112.38 ± 5.12	0.275 ± 0.007	11.10 ± 0.15 ^{b}	119.07 ± 0.63	5.93 ± 0.05	127.87 ± 4.89	0.205 ± 0.004	7.20 ± 0.15	79.44 ± 0.36	5.59 ± 0.04
BLA × Gen2	136.07 ± 5.01	0.255 ± 0.007	10.71 ± 0.15 ^{b}	119.75 ± 0.61	5.94 ± 0.05	149.40 ± 4.72	0.190 ± 0.004	5.86 ± 0.14	79.94 ± 0.35	5.59 ± 0.04
L68 × Gen1	198.11 ± 5.20	0.338 ± 0.007	12.02 ± 0.15 ^{a}	120.62 ± 0.63	6.18 ± 0.05	136.75 ± 4.94	0.188 ± 0.004	5.79 ± 0.15	76.74 ± 0.36	5.62 ± 0.04
L68 × Gen2	225.03 ± 5.03	0.315 ± 0.007	12.04 ± 0.15 ^{a}	122.12 ± 0.62	6.20 ± 0.05	155.29 ± 4.83	0.171 ± 0.004	4.07 ± 0.14	76.97 ± 0.35	5.79 ± 0.04

^{a,b,c,d} Means with different letters within an effect differ significantly (Tukey's HSD-test, $p < 0.05$).

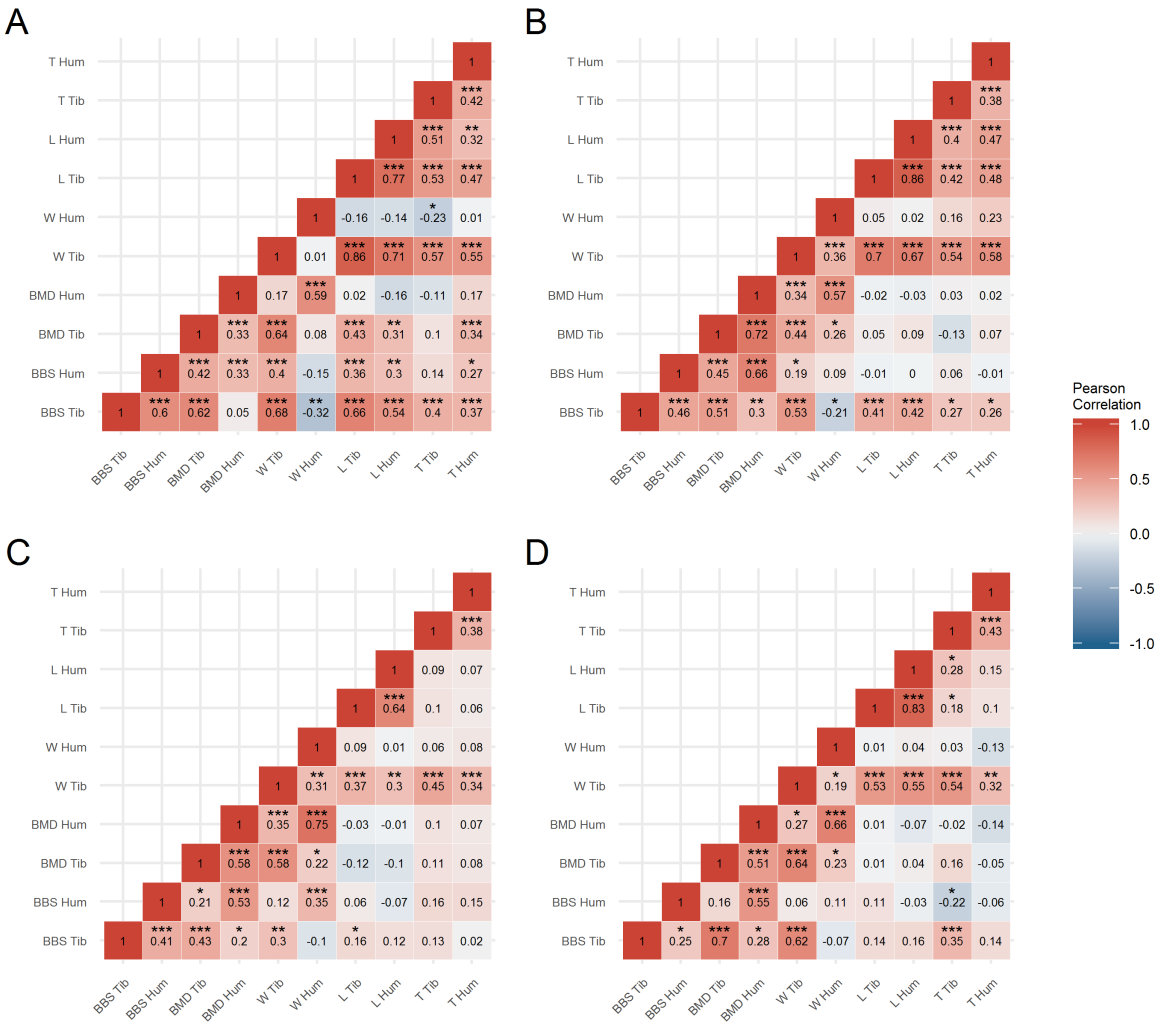

Figure 2.2: Heatmap of Pearson's correlation coefficients between bone traits (BBS, bone breaking strength; BMD, bone mineral density; W, bone weight; L, bone length; T, bone thickness) of the tibiotarsus (Tib) and humerus (Hum) in laying hens of the genetic lines WLA (**A**), R11 (**B**), BLA (**C**) and L68 (**D**). Red indicates a positive correlation; white represents no correlation and blue represents a negative correlation. Significant correlation coefficients are marked with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3.3 Factors Affecting Bone Strength

Table 2.3 shows the effects of the main factors and covariates, and significant interactions on the BBS of tibiotarsus and humerus. As an extension of the basic statistical model (1), which is shown in Table 2.2, in model (2) different covariates are considered additionally to assessing the effects of bone morphometry and total eggshell production on BBS. The bone types studied were influenced by the layer line and the generation. However, the interaction of layer line and generation was only significant for the humerus. The analysis revealed that a high amount of the observed variance in the hens' BBS is attributable to its BMD, as indicated by comparatively high F values being 243.50 (tibiotarsus) and 281.92 (humerus), respectively. The bone types differed with regard to the influence of morphometry on fracture strength, as an effect of bone thickness and length was only observed for the

tibiotarsus, while bone weight did not play a role at all. Within lines, an effect of total eggshell production on the BBS was also not detectable, leading to the assumption that this variable does not contribute to the variance in BBS within the lines studied.

Table 2.3: The effects of layer line (LL), generation (Gen), bone mineral density (BMD), bone weight, bone thickness, bone length, total eggshell production and significant interactions on bone breaking strengths of the tibiotarsus and humerus in laying hens.

	Tibiotarsus		**Humerus**	
Effect	**F Value**	***p*-Value**	**F Value**	***p*-Value**
Layer line (LL)	9.10	< 0.0001	8.13	< 0.0001
Generation (Gen)	13.22	0.0003	8.92	0.0030
LL $\times$ Gen	1.75	0.1568	5.58	0.0009
Bone mineral density (BMD)	243.50	< 0.0001	281.92	< 0.0001
BMD $\times$ LL	24.71	< 0.0001	10.53	< 0.0001
BMD $\times$ Gen	33.96	< 0.0001	26.59	< 0.0001
BMD $\times$ LL $\times$ Gen	2.08	0.1025	7.23	< 0.0001
Weight	0.00	0.9927	3.42	0.0654
Thickness	23.33	< 0.0001	0.62	0.4319
Length	10.90	0.0011	0.09	0.7660
Total eggshell production[1]	0.13	0.7196	0.07	0.7879

[1] Total eggshell production = number of eggs $\times$ eggshell weight.

Figure 2.3 shows the regression coefficients (β) between standardised bone traits; i.e., between BBS and BMD or BBS and morphometric traits. Highly significant regression coefficients varying from $\beta = 0.53$ (WLA) to $\beta = 0.76$ (L68) among the layer lines illustrate that the BMD is strongly associated with the variability of the tibiotarsal BBS (Figure 2.3A). On average across lines, a change in BMD by one standard deviation results in a 0.64 standard deviation increase in BBS. For the humerus, regression coefficients between BBS and BMD were proved to be significant and BMD was detected as primary explanatory variable of the BBS, although coefficients widely ranged from $\beta = 0.43$ (WLA) to $\beta = 0.84$ (R11) among the lines (Figure 2.3B). Line R11 stands out in this regard, while the average coefficient among the layer lines was $\beta = 0.61$. If bone weight is considered as an explanatory variable, the analysis attributed a relatively large and highly significant effect on BBS, at least for the tibiotarsus, where the average coefficient was $\beta = 0.53$. In contrast, the coefficients vary greatly in the case of the humerus, resulting in an average value of $\beta = 0.22$. A significant effect of bone weight on humeral BBS was only seen in the brown-egg lines BLA and L68. With average values of $\beta = 0.31$ and $\beta = 0.25$ respectively, the length and thickness of the tibiotarsus were only weakly correlated with BBS. However, these correlations were significant for the majority of lines. For the length ($\beta = 0.04$) and thickness ($\beta = 0.09$) of the humerus, a rather weak influence was observed, it being only occasionally significant.

Figure 2.4 shows the regression coefficients (β) of the total eggshell production in relation to the BMD of the tibiotarsus (Figure 2.4A) and humerus (Figure 2.4B). Overall, rather low negative regression coefficients were obtained for the two bone types, averaging $\beta = -3.5 \times 10^{-5}$ (tibiotarsus) and $\beta = -1.7 \times 10^{-5}$ (humerus) respectively. In both bone types, regression coefficients were only significant in the low performing white-egg line R11. However, other significant relationships between BMD and eggshell production, could not be

observed. Figure 2.4C (tibiotarsus) and Figure 2.4D (humerus) show the trend of BMD with increasing total eggshell production within the chicken lines studied. Considering the range of variation in BMD (see Table 2.2), an increase of total eggshell production appears to have only limited effects on BMD, especially for hens of layer lines WLA, BLA and L68.

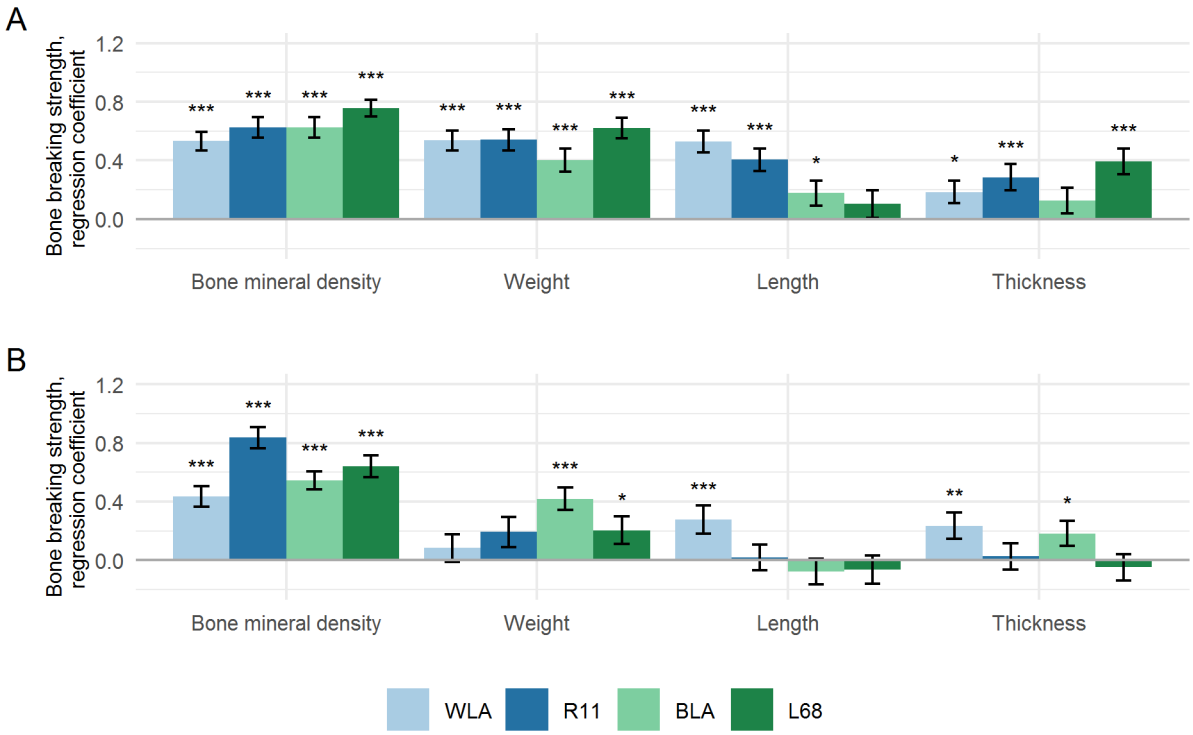

Figure 2.3: Standardised regression coefficients (β) $\pm$ standard errors of regression of bone mineral density, bone weight, bone length and bone thickness pertaining univariately to the bone breaking strengths of the tibiotarsus (**A**) and humerus (**B**) in four different chicken layer lines (WLA, R11, BLA, L68). Significant regression coefficients are marked with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

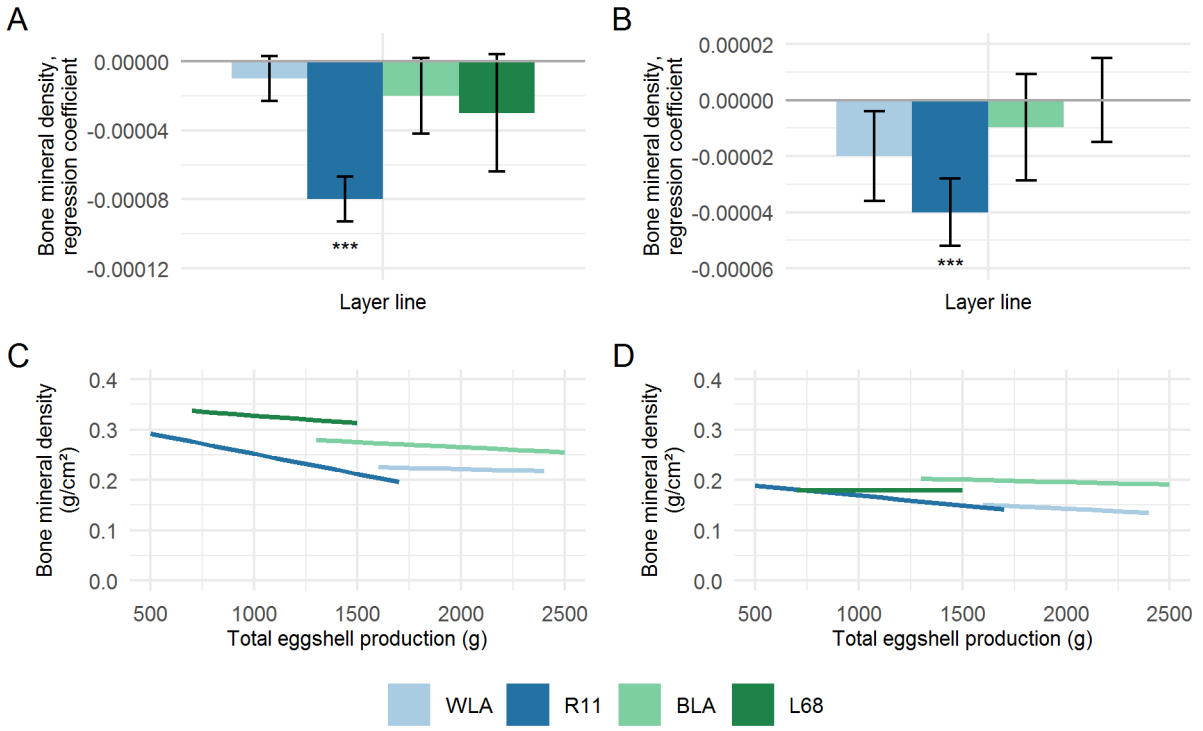

Figure 2.4: Regression coefficients (β) $\pm$ standard errors of regression of total eggshell production pertaining to the bone mineral densities of the tibiotarsus (**A**) and humerus (**B**), and the effect of total eggshell production on the bone mineral densities of the tibiotarsus (**C**) and humerus (**D**) in four different chicken layer lines (WLA, R11, BLA, L68). Significant regression coefficients are marked with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3.4 Genetic Parameters

Results of heritability (h^2) estimates for BBS and BMD and the genetic correlations (r_g) between these traits are shown in Table 2.4. Due to the lack of convergence of the model, no h^2 estimations were possible for the BMD of the tibiotarsus of line R11 and for the humerus of the WLA line. Accordingly, the genetic correlation coefficients could not be estimated in these cases. The h^2 values estimated for BBS vary rather strongly among lines. In case of the tibiotarsus, for example, they vary from $h^2 = 0.17$ (BLA) to $h^2 = 0.58$ (WLA). A similar situation was found for the humerus, for which the values range from $h^2 = 0.26$ (WLA) to $h^2 = 0.50$ (BLA). The h^2 estimation for the BMD values of the tibiotarsus and humerus resulted in similarly fluctuating values, among which the line WLA stands out at $h^2 = 0.75$ for the tibiotarsus and $h^2 = 0.73$ for the humerus. Estimated r_g coefficients suggest a moderate to close genetic relationship between BBS and BMD, except for the tibiotarsus of the BLA line, where it was estimated to be only $r_g = 0.16$.

Table 2.4: Heritability (h^2; ± standard error) and genetic correlation (r_g; ± standard error) estimated for bone breaking strength (BBS) and bone mineral density (BMD) of tibiotarsus and humerus in four chicken layer lines.

	Tibiotarsus			Humerus		
Layer Line	**h^2 BBS**	**h^2 BMD**	**r_g**	**h^2 BBS**	**h^2 BMD**	**r_g**
WLA	0.58 ± 0.23	0.75 ± 0.23	0.93 ± 0.23	0.26 ± 0.22	N.A.	N.A.
R11	0.29 ± 0.22	N.A.	N.A.	0.40 ± 0.20	0.73 ± 0.21	0.81 ± 0.18
BLA	0.17 ± 0.19	0.55 ± 0.20	0.16 ± 0.37	0.50 ± 0.26	0.25 ± 0.17	0.54 ± 0.30
L68	0.46 ± 0.23	0.51 ± 0.23	0.74 ± 0.17	0.44 ± 0.21	0.46 ± 0.25	0.79 ± 0.18

N.A., not analysable.

4 Discussion

The aim of the current study was to analyse the relationship among bone stability traits and egg production in four phylogenetically divergent layer lines differing in their performance levels. The phylogenetic divergence provides insights into the impacts of different breeding histories, which may have affected bone stability before commercial poultry breeding began; the difference in performance level may provide a hint as to the effect of selection for high egg yield within groups of brown and white-egg layer lines.

4.1 Phenotypic Characterization

We observed significant differences among the layer lines regarding all examined production parameters, which are consistent with previous reports [21, 33]. Both high performing lines were superior to their counterparts, which was expected, as commercial lines have long been selected for age at laying maturity, peak production and laying persistency [41, 42]. The results on body weight and feed efficiency clearly reflect the efforts made toward improving feed conversion [1, 42]. Our results indicated significantly higher amounts of calcium required for eggshell formation in the high performing genotypes that can compensate for this by stimulated bone resorption and/or better intestinal calcium absorption. The latter should probably be reflected in an increased expression of epithelial calcium transporting proteins [43]. However, further assumptions on this require a detailed investigation of calcium homeostasis, which could be addressed in further studies.

In accordance with Riczu et al. [44] and Habig et al. [21] our results on bone measurements revealed a strong phylogenetic divergence between brown and white-egg layer lines. Consistent performance-related differences were only found for the BMD, as within the phylogenetic groups the moderate performers possessed a significantly higher BMD. With regard to the BBS, we observed rather inconsistent results. Nevertheless, our results confirmed the tendency for the two high performing genotypes to have lower bone stability [21]. Contrary to the findings of Bishop et al. [19], we did not observe a strong correlation between the tibiotarsal and the humeral BBS, which was evident across all lines. Rather, the correlation varied depending on the layer line. Taken together, the results on the production parameters and bone characteristics reflect remarkably phenotypic differences among the layer lines.

4.2 Determinants of Bone Stability

The analyses clearly turned out that the BMD significantly contributes to the variation in BBS, which is consistent with an earlier study on White Leghorn hens [45]. According to our observations, this can probably be extended to laying hens in general. Our findings are in line with those from others who have associated BMD with biomechanical strength, which is why it plays an important role in osteoporosis [4, 46]. A histological differentiation of cortical and medullary bone tissue, e.g., by means of quantitative computed tomography [47], would be helpful for a more detailed insight into the components affecting bone stability.

Since the bone stability and the whole bone properties are inseparably linked by the bone's architecture and geometry [48, 49], morphometric bone traits were considered as covariates of the BBS. Only in the tibiotarsus, could a rather small effect of morphometry on fracture strength be accounted. However, this is marginal, given the tremendous influence of the BMD. Contrary to our assumption, bone weight did not contribute to the variance in BBS at all. This could be because BMD is already considered in the statistical model and is strongly correlated with bone weight and/or because BMD indirectly integrates bone dimensions as it relates to the scanned bone area [50].

Interestingly, the total eggshell production had no significant effect on BBS or BMD of tibiotarsus and humerus. This was not expected, because although this assumption is controversially discussed, the level of egg production is frequently claimed to be detrimental [51]. Considering our findings and those from other studies that have reported an absence of relationship between bone stability and egg production [47, 51–54], evidence for a strong association within chicken lines seems rather questionable. However, if we only compare lines differing in performance level, our results would suggest that osteoporosis is mainly caused by a high laying rate, supporting earlier conclusions that differences of bone quality characteristics between genotypes should not be oversimplified [54].

We observed individuals that produced high amounts of eggshells and at the same time had high BBS values, which may indicate that high laying rate and good bone quality are not mutually exclusive. However, some studies pointed to the laying persistency causing continuous degradation of structural bone tissue, rather than the precise number of eggs [4, 7, 54]. This likely applies to our moderately performing lines, since their reduced egg number necessarily involved periods of laying inactivity, during which they were able to regenerate. An adverse effect of a premature onset of laying, at which the ossification is possibly not yet sufficiently complete, was also suggested [3, 53]. Possibly, these two factors will ultimately have a combined effect.

4.3 Genetic Perspectives

The results regarding genetic parameters indicate a rather close genetic relationship between BBS and BMD in all layer lines, completing our findings from the phenotypic analyses. With an average h^2 estimate of 0.39, we can confirm the moderate inheritance of BBS [19]. However, the h^2 estimates were quite variable and considerable differences among lines were observed. This might reflect diverse genetic composition or distinct breeding history of the lines studied [30, 31]. Given the large individual variation in bone characteristics and the implied inherited component of susceptibility to osteoporosis, the problem of skeletal damage is assumed to be alleviated by genetic selection [18, 19, 22, 54]. Our results support this assumption based on the h^2 estimates of the BBS presented. The current study emphasizes the great importance of animal breeding, offering promising possibilities to counteract the loss of bone strength. At that, the eggshell quality must continue to be considered in the selection index to improve bone stability without compromising eggshell quality.

5 Conclusions

In this study, we analysed the variation of bone breaking strength (BBS) within phylogenetically divergent chicken layer lines, differing in their levels of egg production. The current results support earlier findings that bone mineral density (BMD) is of particular importance for the BBS. Results do not provide evidence of a strong association between the total eggshell production and bone stability traits within the genetic lines studied. Finally, the estimation of genetic parameters revealed an inherited component of BBS and BMD. A rather weak correlation between laying performance and bone stability was observed, opening up the possibility to select for improved bone stability without adverse effects on laying performance. Due to the line specificity in the various phenotypic characteristics, generalised statements about a possible superiority of a certain phylogenetic group or performance level are not justified.

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

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Supplementary Materials

Table S2.1: Light program and mean climatic conditions.

Week of age	Time		Day length (h)	Light intensity (Lux)	Temperature (°C)	Relative humidity (%)
	Start	End				
Day 1	-	-	24.0	20.0	27.0	66.0
1	4:00 am	7:00 pm	15.0	20.0	27.6	64.8
2	4:30 am	7:00 pm	14.5	20.0	26.4	67.9
3	5:30 am	7:00 pm	13.5	20.0	24.5	66.8
4	6:00 am	7:00 pm	13.0	20.0	22.1	68.5
5	7:00 am	7:00 pm	12.0	20.0	19.4	69.0
6	7:00 am	6:00 pm	11.0	10.0	18.2	75.3
7	7:00 am	5:00 pm	10.0	10.0	18.8	77.8
8	7:30 am	4:30 pm	9.0	10.0	18.4	73.1
9	7:30 am	3:30 pm	8.0	10.0	18.2	76.1
10	7:30 am	3:30 pm	8.0	10.0	18.0	75.6
11	7:30 am	3:30 pm	8.0	10.0	18.2	76.4
12	7:30 am	3:30 pm	8.0	10.0	18.2	77.4
13	7:30 am	3:30 pm	8.0	10.0	18.8	78.7
14	7:30 am	3:30 pm	8.0	10.0	18.7	78.6
15	7:30 am	3:30 pm	8.0	10.0	18.1	78.8
16	7:30 am	3:30 pm	8.0	10.0	18.3	53.1
17	7:00 am	3:30 pm	8.5	10.0	18.1	54.0
18	6:30 am	3:30 pm	9.0	10.0	17.5	63.7
19	6:30 am	4:00 pm	9.5	10.0	17.8	54.8
20	5:30 am	4:00 pm	10.5	10.0	18.3	61.2
21	4:30 am	4:00 pm	11.5	10.0	18.0	62.6
22	4:00 am	4:30 pm	12.5	10.0	17.8	62.3
23	3:30 am	5:00 pm	13.5	10.0	17.8	58.9
24 - 69	3:30 am	5:30 pm	14.0	10.0	-	-

Table S2.2: Calculated nutrient compositions of the diets fed to the chicks during the first and second generations, respectively, and of the diet fed to the pullets during both generations.

Diet	Chicks		Pullets
Generation	1	2	1 + 2
Crude protein (%)	21.00	21.00	15.50
Crude fat (%)	4.00	4.00	4.00
Crude fibre (%)	3.20	3.50	4.50
Crude ash (%)	6.00	6.00	5.00
ME/kg DM (MJ)	11.80	11.80	11.40
Calcium (%)	1.00	0.95	0.85
Phosphorus (%)	0.70	0.65	0.55
Natrium (%)	0.15	0.15	0.15
Lysine (%)	1.20	1.20	0.65
Methionine (%)	0.48	0.48	0.34
Vitamin D3 (IU/kg)	3000.00	3000.00	1950.00

Table S2.3: Ingredients and analysed nutrient compositions of the experimental layer diets of the first and second generations.

Diet	**Layers** 300 IU Vit D3		**Layers** 3000 IU Vit D3	
Generation	1	2	1	2
Ingredients (%)				
Wheat	39.80	39.74	39.80	39.74
Corn	20.00	20.00	20.00	20.00
Soybean, toasted	10.63	10.63	10.63	10.63
Soybean meal, toasted	8.00	8.00	8.00	8.00
High protein soybean meal, toasted	5.00	5.00	5.00	5.00
Lucerne pellets	2.44	2.44	2.44	2.44
Soybean oil	2.00	2.00	2.00	2.00
Calcium phosphate	2.16	2.46	2.16	2.46
Calcium carbonate	7.53	8.15	7.53	8.15
Sodium chloride	0.29	0.42	0.29	0.42
DL-Methionine	0.15	0.16	0.15	0.16
Silica (Sipernat®)	1.00	-	1.00	-
Premix 74237	1.00	1.00	-	-
Premix 74118	-	-	1.00	1.00
Nutrient composition				
Crude protein (%)	15.90	16.80	15.80	17.10
Crude fat (%)	5.60	6.20	6.20	6.20
Starch (%)	40.70	38.40	38.90	38.00
Sucrose (%)	3.60	3.00	4.00	3.40
ME/kg DM (MJ)	11.60	11.50	11.60	11.60
Vitamin D3 (IU/kg)	430.00	2000.00	2240.0	3400.00
Calcium (%)			3.38	
Phosphorus (%)			0.65	
Natrium (%)			0.26	

Table S2.4: The effects of layer line, diet and their interaction on the observed bone traits and basic production parameters calculated within generations.

	Layer Line		**Diet**		**Layer Line × Diet**	
	F Value	*p*-Value	F Value	*p*-Value	F Value	*p*-Value
Generation 1						
Laying maturity	169.42	< 0.0001	1.20	0.2743	0.39	0.7618
Total number of eggs	346.92	< 0.0001	3.39	0.0669	0.55	0.6509
Egg weight	69.00	< 0.0001	0.68	0.4112	0.31	0.8152
Eggshell weight	95.23	< 0.0001	0.50	0.4816	0.59	0.6203
Eggshell proportion	36.18	< 0.0001	3.22	0.0740	0.30	0.8250
Total eggshell production	516.80	< 0.0001	3.22	0.0739	0.33	0.8041
Daily feed consumption	76.43	< 0.0001	0.24	0.6248	0.58	0.6308
Feed-to-egg conversion rate	234.46	< 0.0001	0.73	0.3933	0.58	0.6271
Feed-to-eggshell conversion rate	245.59	< 0.0001	2.78	0.0967	0.39	0.7589
Bone breaking strength Tibiotarsus	120.59	< 0.0001	0.27	0.6054	1.60	0.1909
Bone mineral density Tibiotarsus	87.07	< 0.0001	0.25	0.6155	2.86	0.0376
Weight Tibiotarsus	305.57	< 0.0001	0.05	0.8319	0.33	0.8005
Length Tibiotarsus	38.36	< 0.0001	0.47	0.4949	0.56	0.6442
Thickness Tibiotarsus	197.36	< 0.0001	0.00	0.9924	0.28	0.8416
Bone breaking strength Humerus	55.72	< 0.0001	1.66	0.1986	0.79	0.4994
Bone mineral density Humerus	43.75	< 0.0001	2.50	0.1148	0.29	0.8315
Weight Humerus	63.97	< 0.0001	0.03	0.8599	0.28	0.8417
Length Humerus	62.34	< 0.0001	0.02	0.8808	2.02	0.1112
Thickness Humerus	40.52	< 0.0001	1.03	0.3112	0.70	0.5538
Body weight 69th week of age	151.27	< 0.0001	0.03	0.8624	1.52	0.2097
Generation 2						
Laying maturity	159.93	< 0.0001	1.50	0.2211	0.10	0.9611
Total number of eggs	461.62	< 0.0001	0.16	0.6909	0.51	0.6781
Egg weight	119.07	< 0.0001	0.74	0.3890	0.43	0.7298
Eggshell weight	217.06	< 0.0001	1.05	0.3061	1.88	0.1341
Eggshell proportion	55.17	< 0.0001	0.03	0.8526	0.53	0.6613
Total eggshell production	826.22	< 0.0001	1.32	0.2524	2.07	0.1049
Daily feed consumption	109.61	< 0.0001	1.52	0.2181	0.99	0.3997
Feed-to-egg conversion rate	278.68	< 0.0001	0.28	0.5960	3.35	0.0197
Feed-to-eggshell conversion rate	259.92	< 0.0001	0.56	0.4538	2.37	0.0708
Bone breaking strength Tibiotarsus	70.35	< 0.0001	0.03	0.8692	1.37	0.2536
Bone mineral density Tibiotarsus	94.81	< 0.0001	0.13	0.7201	1.69	0.1706
Weight Tibiotarsus	291.45	< 0.0001	0.19	0.6649	0.52	0.6672
Length Tibiotarsus	21.25	< 0.0001	0.02	0.8904	0.08	0.9717
Thickness Tibiotarsus	179.68	< 0.0001	0.04	0.8395	0.08	0.9711
Bone breaking strength Humerus	32.15	< 0.0001	0.08	0.7807	1.30	0.2744
Bone mineral density Humerus	56.76	< 0.0001	1.44	0.2315	0.19	0.9033
Weight Humerus	99.83	< 0.0001	0.03	0.8528	0.21	0.8864
Length Humerus	49.52	< 0.0001	0.01	0.9390	0.37	0.7765
Thickness Humerus	60.71	< 0.0001	0.53	0.4689	0.61	0.6062
Body weight 69th week of age	137.20	< 0.0001	1.82	0.1786	2.13	0.0963

Table S2.5: Sample sizes for the analysis.

Variable	Total	Layer Line			
		WLA	R11	BLA	L68
Laying maturity	524	129	134	133	128
Total number of eggs	524	129	134	133	128
Egg weight	524	129	134	133	128
Eggshell weight	524	129	134	133	128
Eggshell proportion	524	129	134	133	128
Total eggshell production	524	129	134	133	128
Daily feed consumption	513	128	131	129	125
Feed-to-egg conversion rate	513	128	131	129	125
Feed-to-eggshell conversion rate	513	128	131	129	125
Bone breaking strength Tibiotarsus	518	126	134	131	127
Bone mineral density Tibiotarsus	524	129	134	133	128
Weight Tibiotarsus	524	129	134	133	128
Length Tibiotarsus	524	129	134	133	128
Thickness Tibiotarsus	524	129	134	133	128
Bone breaking strength Humerus	516	128	131	132	125
Bone mineral density Humerus	519	129	134	128	128
Weight Humerus	521	127	134	132	128
Length Humerus	523	129	134	132	128
Thickness Humerus	524	129	134	133	128
Body weight at hatch	523	129	133	133	128
Body weight at week 21	524	129	134	133	128
Body weight at week 25	524	129	134	133	128
Body weight at week 35	524	129	134	133	128
Body weight at week 69	524	129	134	133	128

Table S2.6: Least squares means ± standard errors and level of significance for body weight measured at hatching, and different weeks of age under the effect of layer line (LL), generation (Gen), and their interaction.

Effect	Body weight(g)				
	Hatch	Week 21	Week 25	Week 35	Week 69
Layer line (LL)					
WLA	38.35 ± 0.37 [a]	1420.02 ± 15.79 [b]	1468.38 ± 16.19 [b]	1497.54 ± 20.46 [b]	1504.23 ± 22.26 [c]
R11	33.17 ± 0.36 [c]	1040.84 ± 15.60 [c]	1236.40 ± 15.99 [c]	1309.28 ± 20.21 [c]	1362.79 ± 21.99 [d]
BLA	39.35 ± 0.37 [a]	1584.15 ± 15.71 [a]	1663.55 ± 16.11 [a]	1821.81 ± 20.34 [a]	1838.10 ± 22.13 [b]
L68	34.84 ± 0.37 [b]	1568.91 ± 15.81 [a]	1714.92 ± 16.21 [a]	1837.91 ± 20.48 [a]	1923.44 ± 22.29 [a]
Generation (Gen)					
Gen 1	35.86 ± 0.26	1379.01 ± 11.17	1485.67 ± 11.45	1567.49 ± 14.47	1616.21 ± 15.76
Gen 2	37.00 ± 0.26	1427.95 ± 11.07	1555.96 ± 11.36	1665.79 ± 14.34	1698.07 ± 15.59
LL × Gen					
WLA × Gen1	37.77 ± 0.52	1376.72 ± 22.36	1415.84 ± 22.91	1460.33 ± 28.96	1443.48 ± 31.51
WLA × Gen2	38.93 ± 0.52	1463.31 ± 22.32	1520.93 ± 22.88	1534.75 ± 28.91	1564.98 ± 31.45
R11 × Gen1	32.64 ± 0.51	1027.33 ± 21.99	1222.77 ± 22.53	1284.20 ± 28.49	1338.12 ± 31.02
R11 × Gen2	33.69 ± 0.52	1054.36 ± 22.14	1250.04 ± 22.71	1334.37 ± 28.66	1387.45 ± 31.17
BLA × Gen1	38.84 ± 0.52	1549.66 ± 22.41	1627.91 ± 22.97	1767.90 ± 29.03	1804.33 ± 31.60
BLA × Gen2	39.87 ± 0.51	1618.63 ± 22.02	1699.19 ± 22.59	1875.73 ± 28.50	1871.86 ± 30.98
L68 × Gen1	34.18 ± 0.52	1562.32 ± 22.61	1676.16 ± 23.16	1757.53 ± 29.31	1878.89 ± 31.92
L68 × Gen2	35.51 ± 0.51	1575.50 ± 22.11	1753.67 ± 22.68	1918.30 ± 28.62	1968.00 ± 31.12

	ANOVA significance level (p value)		
	Layer line	Generation	LL × Gen
Hatch	< 0.0001	0.0019	0.9908
Week 21	< 0.0001	0.0020	0.3097
Week 25	< 0.0001	< 0.0001	0.3907
Week 35	< 0.0001	< 0.0001	0.2486
Week 69	0.0003	< 0.0001	0.6892

Means within a column with different letters differ significantly (Tukey's HSD-Test, $p < 0.05$).

3 Bone Health or Performance? Adaptation Response of Genetically Divergent Chicken Layer Lines to a Nutritive Calcium Depletion

Simon Jansen [1,*], Mara Bues [2,*], Ulrich Baulain [1], Christin Habig [1], Ingrid Halle [3], Stefanie Petow [4], A. Reza Sharifi [5,6], Annett Weigend [1], Mirja R. Wilkens [2] and Steffen Weigend [1,6]

* *These authors contributed equally to this work.*

[1] Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut, 31535 Neustadt, Germany.

[2] Institute of Physiology and Cell Biology, University of Veterinary Medicine Hannover, Foundation, 30173 Hannover, Germany.

[3] Institute of Animal Nutrition, Friedrich-Loeffler-Institut, 38116 Braunschweig, Germany.

[4] Institute of Animal Welfare and Animal Husbandry, Friedrich-Loeffler-Institut, 29223 Celle, Germany.

[5] Animal Breeding and Genetics Group, Department of Animal Sciences, University of Göttingen, 37075 Göttingen, Germany.

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

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Simple Summary

Selection for high egg production in laying hens has led to an increased calcium requirement and consequently to an intensified calcium mobilization from the bones. However, excessive demineralization can lead to osteoporosis, which is manifested by a high incidence of bone-damaged hens. In this study, we characterized the adaptation response of laying hens to a repeated dietary calcium restriction (1.09% instead of 4.26% calcium) by means of egg production, eggshell quality, body weight and bone stability. The animal model included four layer lines differing in performance level (high vs. moderately performing lines) and phylogenetic origin (white-egg vs. brown-egg layers). We assumed that the high performing lines would respond by maintaining egg production level at the expense of eggshell quality and bone health. Egg production and eggshell quality declined considerably and bone demineralization occurred in all lines. Contrary to our hypothesis, there was evidence that phylogeny rather than performance level influenced the hens' response. The brown-egg lines appeared to be more tolerant to the calcium depletion, while the white-egg lines were more sensitive. Our findings demonstrate the influence of genetics on the adaptive capacity of chickens and underline the importance of preserving genetic variability to cope with potential future environmental challenges.

Abstract

In modern laying hybrids, calcium (Ca) homeostasis is immensely challenged by daily eggshell calcification. However, excessive mobilization of Ca from bones may lead to osteoporosis, which then manifests in a high incidence of poor bone quality. The aim of this study was to characterize the hens' adaptation response to an alternating dietary Ca restriction. The animal model consisted of four purebred layer lines, differing in laying performance (high vs. moderately performing lines) and phylogenetic origin (white- vs. brown-egg lines). According to the resource allocation theory, hens selected for high egg production were assumed to show a different response pattern to cope with this nutritive challenge compared to moderately performing lines. Data collected included egg number, egg quality traits, body weight and bone characteristics. The Ca depletion led to a temporary drop in egg production and shell quality and a loss of bone stability due to Ca mobilization. The white-egg lines response was more pronounced, whereas the brown-egg lines were less sensitive towards reduced Ca supply. Our study shows that the hens' responsiveness to coping with a nutritive Ca depletion is not ultimately linked to genetic selection for increased egg production but rather to phylogenetic origin.

Keywords: bone strength; egg production; eggshell quality; calcium depletion; laying hens; phylogeny; recovery

1 Introduction

While various functional traits have now been introduced into the selection index of laying hens, the main focus of the breeding companies remains the number of saleable eggs [1]. In female birds, there is a competitive situation, as calcium (Ca) is needed for eggshell formation and maintaining bone stability. Since up to three grams of Ca are required for each eggshell calcification process [2], modern laying hybrids' demand for Ca is particularly high and their Ca homeostasis is stressed continually [3]. The required Ca is provided by increased intestinal absorption and stimulated bone resorption in an approximate relation of 1:0.6 [4, 5]. With the onset of sexual maturity, the formation of medullary bone, which serves as a labile Ca source that can be replenished quickly [6], develops under the influence of estradiol-17β. At the same time, there is a decrease of cancellous bone volume [7, 8]. This enables laying hens to meet the temporarily high demand of Ca during the periods of eggshell formation by elevated mobilisation from the bones [9]. However, if this process exceeds physiological dimensions, continuous demineralisation leads to progressive loss of not only medullary but also structural bone tissue resulting in osteoporotic bones susceptible to fractures [9, 10]. High incidences of bone damage have been demonstrated [11, 12] indicating major animal welfare problems and economic losses [10, 13, 14].

It has been reported that selection for high production efficiency in livestock species might be associated with undesirable side effects such as deficiencies in physiological and functional traits [15], due to an imbalance in resource allocation [16]. Accordingly, chicken layer lines selected for high egg production might have a reduced capacity to compensate for unfavourable physiological conditions, e.g. limited mineral resources, compared to moderately performing genotypes [17–19]. Differences in adaptation responses can also be attributed to phylogenetic origin, as brown- and white-egg layer lines evolved separately over a long period of time and underwent genomic changes, which may have had effects on genetic characteristics even before directional (artificial) selection for high performance began [20, 21].

In the current study, we investigated the effect of a repeated dietary Ca restriction on performance traits and bone stability in four genetically divergent chicken layer lines. To address the potential implications of both the performance level and the phylogenetic origin, the animal model consisted of two brown-egg and two white-egg chicken layer lines. Within each phylogenetic group, the two lines differed in terms of egg-laying performance [22–24]. By characterizing the lines' adaptation responses to dietary Ca restriction, we aimed to test line-specific responses and assess whether genetic lines reacted differently, depending on phylogenetic origin or performance level, or both. In addition, repeated Ca reductions allowed us to study if the lines' responses were temporary and recovery occurred after supplementing feed with Ca again, or if changes were rather permanent.

We assumed that the long-term selected breeding lines for high egg production would respond by retaining their laying performance at the expense of eggshell quality and bone stability. In contrast, the moderately performing lines were supposed to respond with a decrease in performance in favour of bone health.

2 Materials and Methods

2.1 Ethical Note

The experiment was performed in accordance with German Animal Welfare Law and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES) (33.19-42502-04-15/1988).

2.2 Animals and Housing

The experiment included four purebred chicken layer lines (*Gallus gallus domesticus*) differing in terms of egg production performance and phylogenetic origin, two white-egg layers and two brown-egg layers. The high performing lines WLA and BLA originate from a commercial breeding program (Lohmann Tierzucht GmbH, Cuxhaven, Germany) and achieve an annual egg production of about 316 eggs [22]. The moderately performing lines R11 and L68 are maintained as resource populations without any selection at the Institute of Farm Animal Genetics of the Friedrich-Loeffler-Institut (Neustadt, Germany) and achieve a laying performance of 226 (R11) and 216 (L68) eggs per year. Besides performance divergence, the animal model included a phylogenetic dimension, as the two white-egg lines (WLA and R11) are both of White Leghorn type and are closely related, but distinct from the brown-egg ones (BLA and L68), which originated from Rhode Island Red and New Hampshire breeds, respectively. The latter breed was derived from the Rhode Island Red, explaining the close phylogenetic relationship of both [21].

All chicks were hatched on the same day. The chicks were tagged with wing bands at hatch for identification and sorted by sex. They were reared in a floor system under standard conditions. Information on the light program is given in the Supplementary Table S3.1. From the beginning of the 24th week of age, the birds were exposed to a light-dark cycle of 14 h L : 10 h D. Customary complete feeds for chicks (until 6 weeks of age; 11.8 MJ AME_N/kg dry matter (DM), 210.0 g/kg crude protein, 40.0 g/kg crude fat, 35.0 g/kg crude fiber, 60.0 g/kg crude ash, 9.5 g/kg Ca, 6.5 g/kg phosphorous) and pullets (from 7 to 17 weeks of age; 11.4 MJ AME_N/kg DM, 155.0 g/kg crude protein, 40.0 g/kg crude fat, 45.0 g/kg crude fiber, 50.0 g/kg crude ash, 8.5 g/kg Ca, 5.5 g/kg phosphorous) were offered *ad libitum*. At 16 weeks of age, 132 pullets (33 birds per layer line) were transferred to six 8 m^2 floor pens each littered with wood-shavings and equipped with nipple drinkers, two feeding troughs and four nest boxes. Each pen was occupied in equal proportions with 22 randomly chosen hens of WLA/L68 or BLA/R11 combination. The lines were combined to meet the limited number of nest boxes in the pens, as brown-egg lines use the nests earlier during the day than white-egg lines [25]. Furthermore, combining white-egg and brown-egg lines enabled a separate recording of the egg data for each chicken line even when kept together in the same floor pen. In this way, four Ca restricted pens and two control pens were formed resulting in two temporarily Ca deficient (DEF) and one control group (CON) per chicken layer line.

2.3 Experimental Procedure

The experiment lasted from the beginning of the 31st to the end of the 51st week of age. Two customary wheat-soya-based diets for layers were fed *ad libitum*, which only varied in

terms of Ca content. The ingredients and chemical composition of the layer diets are listed in Supplementary Table S3.2. With regard to nutritional recommendations for high performing laying hens [26], the diets' Ca content can be classified as deficient (Ca-, 1.09%) and adequate (Ca+, 4.26%). The Ca+ diet was fed to all hens from 18 to 30 weeks of age. While the Ca+ diet was fed to the control groups continuously during the whole experiment, the Ca deficient groups were provided with both the Ca+ and the Ca- diet, alternatingly. In the latter case, a 21-day period of Ca depletion (Ca-) was followed by a 44-day recovery phase (Ca+) twice, followed by a third period of Ca restriction (Ca-). This resulted in a preliminary period (Pre, week 18–30), three periods of Ca depletion (D1, week 31–33; D2, week 40–42; D3, week 49–51) with two intermediate recovery periods (R1, week 34–39; R2, week 43–48). Figure 3.1 gives an overview on the experimental periods and the related procedures.

Data collection included traits on production performance and bone stability. Pen-level egg production, including eggshell breakages and defects, was recorded daily. The total laying rate and rate of broken eggs were calculated on a daily basis by dividing the number of eggs by the number of hens. Feed consumption (g) was recorded weekly on pen-level as the difference between the feed weighed in the feeding trough and the refusals. However, due to technical obstacles, feed consumption is only available from period D2 onwards. Immediately prior to changing the diet, the body weight (g) was measured using a digital table scale (CPA 16001S, Sartorius, Göttingen, Germany). Egg quality measurements included all eggs laid during Ca depletion periods (D1, D2 and D3) as well as those eggs laid within the last three consecutive days of the preliminary period (Pre), and the two recovery periods (R1 and R2). Eggshell breaking strength (N) was determined using a testing machine that showed the maximum load that was required to break the eggshell. Egg weight (g) and eggshell weight (g) were recorded using a digital table scale (Type 3709, Sartorius, Göttingen, Germany). For the latter, the eggs were emptied with a spoon and the shells were dried for 30 s in a microwave (800 watt). Eggshell thickness (mm) was measured near the equator using a caliper with an accuracy of 0.01 mm after removing the shell membranes.

At the end of the 51st week of age, the hens were euthanized by carbon dioxide inhalation. Keel bones, tibiotarsi and humeri of the birds were extracted and the adherent tissue removed. The bones were vacuum-packed and stored frozen (-20 °C) until further examination. After measuring the bone weight (g), length (mm) and thickness (mm) of the left tibiotarsus and the left humerus, their radio density was determined given as millimetres of aluminum equivalent (mm Al eq) [27]. The diaphyseal cortical bone proportion ("cortical area") of the left tibiotarsus cut surface was assessed planimetrically [28]. The right long bones were used for assessing bone breaking strength (N) via a three-point bending test as described by Jansen et al. [22]. At slaughter, the keel bones were visually examined for the presence of fractures, indicated by fracture lines or callus formation.

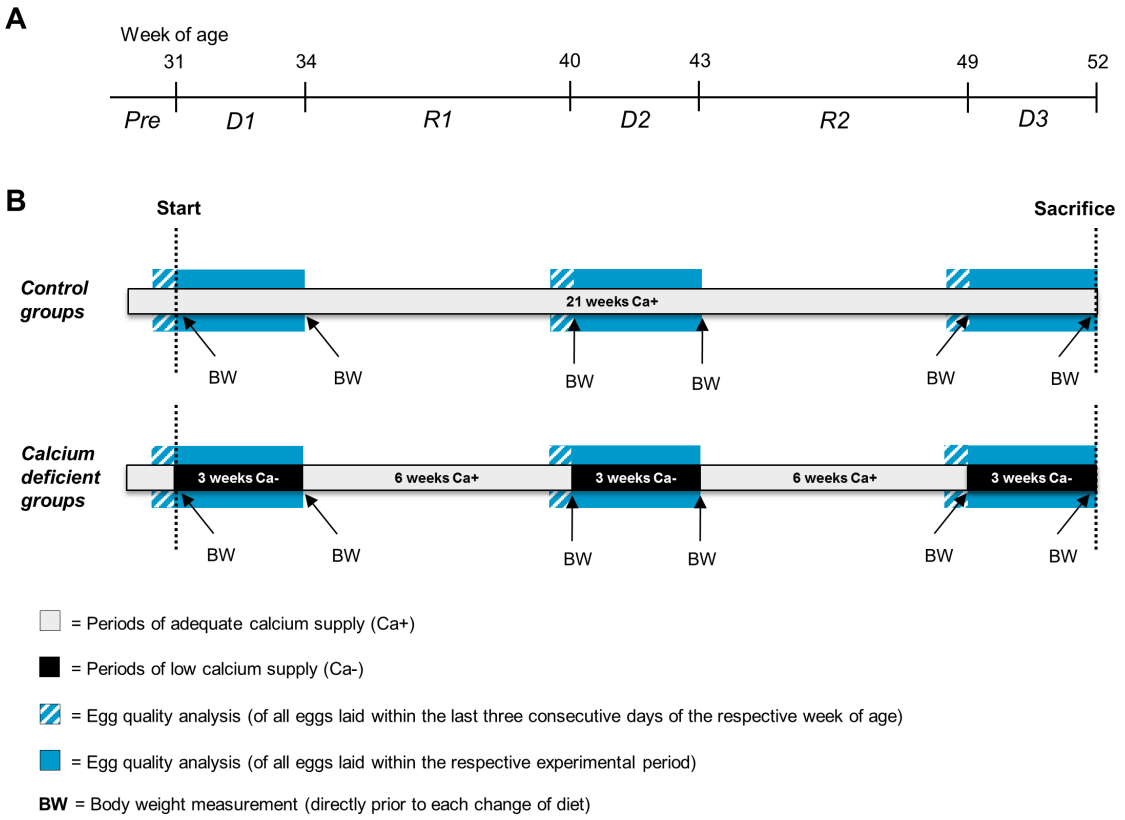

Figure 3.1: Schematic illustration of the experimental periods and related procedures. (**A**) Age of the animals in weeks and experimental periods (Pre: preliminary period; D1: first calcium depletion period; R1: first recovery period; D2: second calcium depletion period; R2: second recovery period; D3: third calcium depletion period). (**B**) Dietary calcium supply and experimental procedures. Control groups were fed with adequate calcium diet (Ca+) continuously. Calcium deficient groups were fed alternatingly with low calcium diet (Ca-) (black sections) and adequate Ca+ feed. Egg quality analysis was performed on all eggs laid within the last three consecutive days of periods Pre, R1 and R2 (blue striped sections), as well as during the entire phases of Ca- (blue sections). Body weighing (BW) was performed directly prior to each change of diet.

2.4 Statistical Analysis

Data analysis was carried out using SAS 9.4 (SAS Institute Inc. Cary, NC, USA, 2017). For each layer line, the sample size was $n = 22$ (DEF groups) and $n = 11$ (CON groups).

Both the total laying rate and the rate of broken eggs were analyzed within the layer lines, applying a univariate regression approach using the MIXED procedure, modelling the linear relationship between the laying rate or rate of broken eggs and the day of depletion according to the following model:

$$\gamma_i = \beta_0 + \beta_1 x_i + \varepsilon_i \quad (1)$$

where γ_i is the trait under consideration; β_0 is the intercept; β_1 is the slope; x_i is the independent variable "day of depletion"; and ε_i is the random error variance.

Concerning egg quality traits, we first examined whether there were significant differences between the dietary groups of the layer lines in the preliminary (Pre) or recovery phases

(R1, R2). For the depletion periods (D1, D2, D3), an analysis of covariance was applied in order to fit regression curves considering the time during the depletion as a covariate term up to 4 polynomial degrees and the fixed effect of dietary treatment as well as significant interactions between the dietary treatment and the covariate (day of depletion) up to degree 4 of polynomials [29]. For the analysis of the eggshell weight data, the egg weight was considered as a covariate. In a backward selection approach, the Wald F-statistics were used to determine the significance of fixed effects. Egg quality data were analyzed with the MIXED procedure of SAS. Least squares means (LSM) were estimated by applying the LSMEANS statement. Significant differences between LSM were tested using Tukey's HSD (honestly significant difference) test. Statistical significance was set at $p < 0.05$. Standard errors of LSM were calculated as described by Littell et al. [29].

The impact of time of measurement, dietary treatment and their interaction on the body weight was analyzed within layer line using the GLIMMIX procedure according to the following model:

$$\gamma_{ijkl} = \mu + T_i + D_j + T_i \times D_j + S_k + \varepsilon_{ijkl} \quad (2)$$

where γ_{ijkl} is the trait under consideration; μ is the general mean; T_i is the fixed effect of time of measurement ($i = 1$ to 6); D_j is the fixed effect of the diet ($j = 1, 2$); $T_i \times D_i$ is the fixed effect of interaction between time of measurement and diet; S_k is the random effect of sire ($k = 1$ to 66); and ε_{ijkl} is the random error variance. Tukey's HSD-Test was performed for multiple comparisons of means. Statistical significance was set at $p < 0.05$.

Bone analysis took place at the end of the study. At this point, the impact of layer line, dietary treatment and their interaction on the bone characteristics were analyzed using the GLIMMIX procedure according to the following model:

$$\gamma_{ijkl} = \mu + LL_i + D_j + LL_i \times D_j + S_k + \varepsilon_{ijkl} \quad (3)$$

where γ_{ijkl} is the trait under consideration; μ is the general mean; LL_i is the fixed effect of layer line ($i = 1$ to 4); D_j is the fixed effect of the diet ($j = 1, 2$); $LL_i \times D_j$ is the interaction between layer line and diet; S_k is the random effect of sire ($k = 1$ to 66); and ε_{ijkl} is the random error variance. Tukey's HSD-Test was performed for multiple comparisons of means. Statistical significance was set at $p < 0.05$.

The results of the keel bone examination have been dichotomized, differentiating between "at least one fracture was present" (score 1) and "no fractures were present" (score 0). The effect of the dietary treatment on the fracture occurrence was analyzed by means of Fisher's exact test separately for each layer line using the FREQ procedure.

3 Results

3.1 Laying Performance

Figure 3.2 illustrates the total laying rate of the dietary groups of the four layer lines. In addition, the linear relationship between the total laying rate and the day of the respective depletion period is indicated. At 19 (BLA) and 20 (WLA) weeks, laying maturity was reached earlier in the high performing lines than in the moderately performing ones, whose first eggs

were laid at week 21 (L68) and 23 (R11). The DEF groups of WLA, R11 and BLA showed a marked decline in egg production among all depletion periods. Highly significant, negative regression coefficients (β_1) between time of progressive Ca depletion and the laying rate were found in these lines, resulting in average values across the three depletion phases of $\beta_1 = -1.94$ (WLA), $\beta_1 = -1.75$ (R11) and $\beta_1 = -1.93$ (BLA). In the case of the line WLA, for example, this means that per day of Ca depletion, the laying rate decreased by 1.94%. After reconversion to adequate Ca supply, the initial performance level was regained consistently in these lines, as the intercept (β_0) of the regression curves varied only a little between the depletion periods (Figure 3.2). This indicates a recovery in laying performance. Since both dietary groups of the moderately performing brown-egg line L68 declined more or less equally in the course of the experimental period, there was not such a strong response to Ca depletion in this line as there was for the other lines. The corresponding regression coefficients were not significant.

Figure 3.2 also shows the rate of broken eggs, i.e., eggshell breakages and defects, and the linear relationship between this variable and the day of the depletion period. Increased incidences of eggshell breakages and defects were observed in the DEF groups of all lines. In this instance, the high performing white-egg line WLA showed a considerably higher incidence, as reflected by average regression coefficients of $\beta_1 = 2.48$ (WLA), $\beta_1 = 0.91$ (R11), $\beta_1 = 0.80$ (BLA) and $\beta_1 = 0.56$ (L68). In all lines, however, the rate of broken eggs declined to the initial level of below 1.5% within two weeks of the recovery phases.

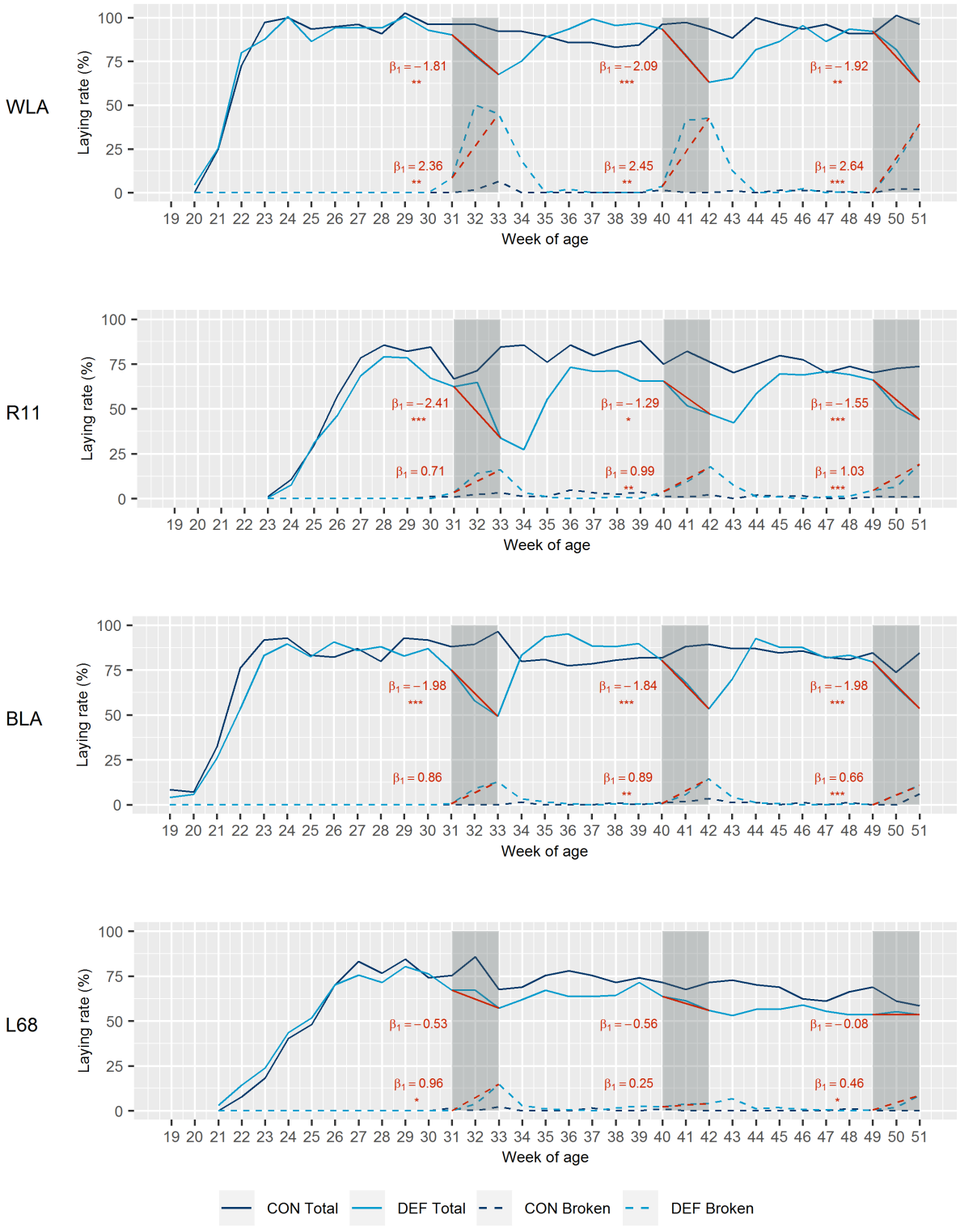

Figure 3.2: Total laying rate (solid lines) and laying rate of broken eggs (dashed lines) in control (CON) and calcium (Ca) deficient (DEF) groups of four layer lines (white-egg layers (WLA, R11), brown-egg layers (BLA, L68)). Grey-shaded sections represent the periods of Ca depletion. Linear regression curves and the corresponding coefficients (β_1) between day of depletion and total laying rate or laying rate of broken eggs are given in red. Significant regression coefficients are marked with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). DEF groups: $n = 22$; CON groups: $n = 11$.

3.2 Egg Quality

The progression of egg quality traits during the periods of Ca depletion is shown in Figure 3.3 (egg weight), Figure 3.4 (eggshell thickness) and Figure 3.5 (eggshell breaking strength). Taking all periods into account, significant differences between DEF and CON groups of line WLA were observed consistently and mostly earlier than in all other layer lines. While the DEF group of line WLA responded significantly for most traits within the first three days, the dietary groups of line R11 only tended to differ in terms of eggshell breaking strength and thickness. The only significant differences found in line R11 were in egg weight in periods D1 and D3. However, these differences were rather minor. In Ca deficient BLA hens, a significant egg weight decline was observed from the ninth day on (except in D3), whereas for the other traits, a significant response occurred even within the first five days. Although Ca depletion also caused a decrease in egg quality in line L68, a more pronounced decline was observed in its high performing counterpart BLA. These observations are supported by the results on the eggshell weight shown in Supplementary Figure S3.1.

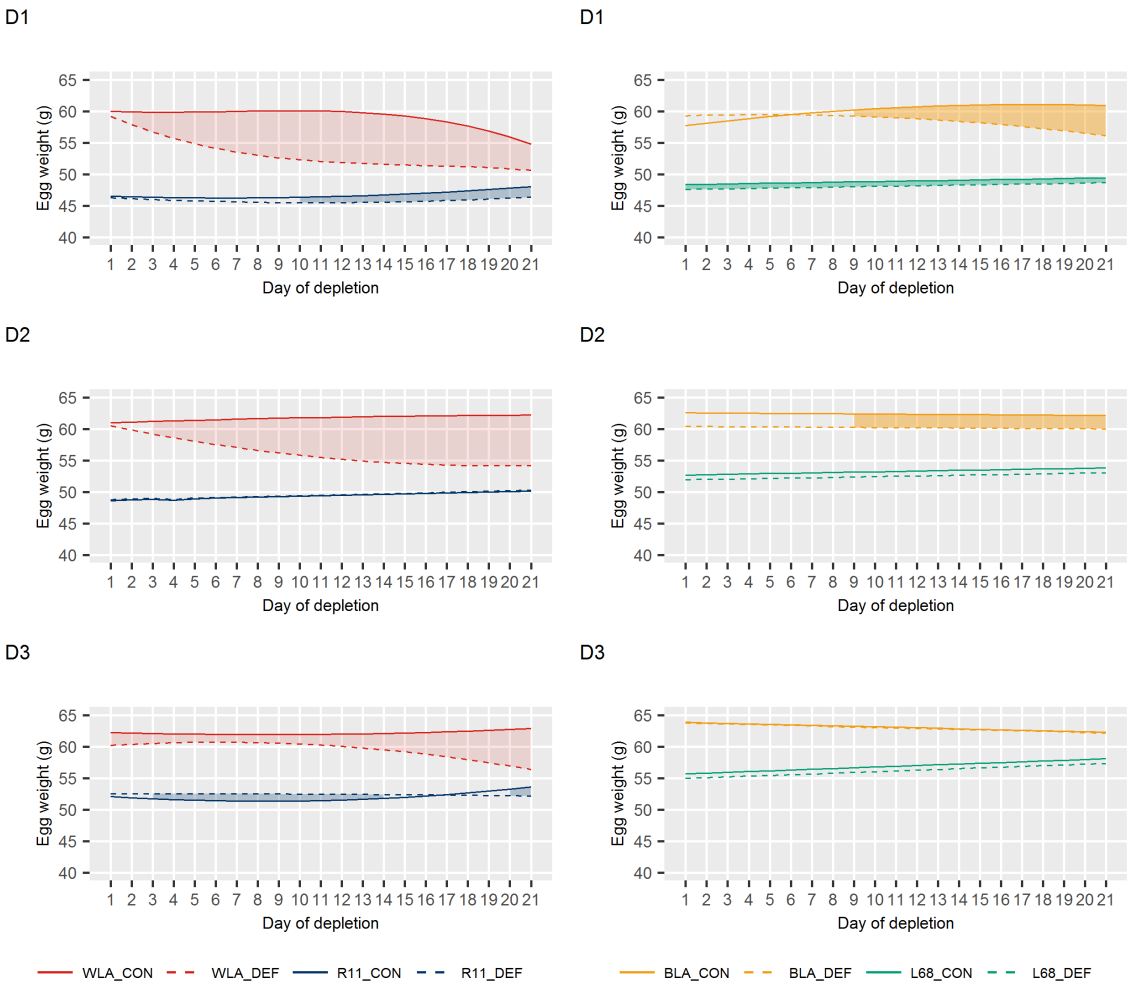

Figure 3.3: Least squares means for egg weight in control (CON) and calcium (Ca) deficient (DEF) groups of four layer lines (WLA, R11, BLA, L68) during periods of Ca depletion (D1, D2, D3). The filled in areas indicate when both dietary groups of the lines differ significantly at $p < 0.05$.

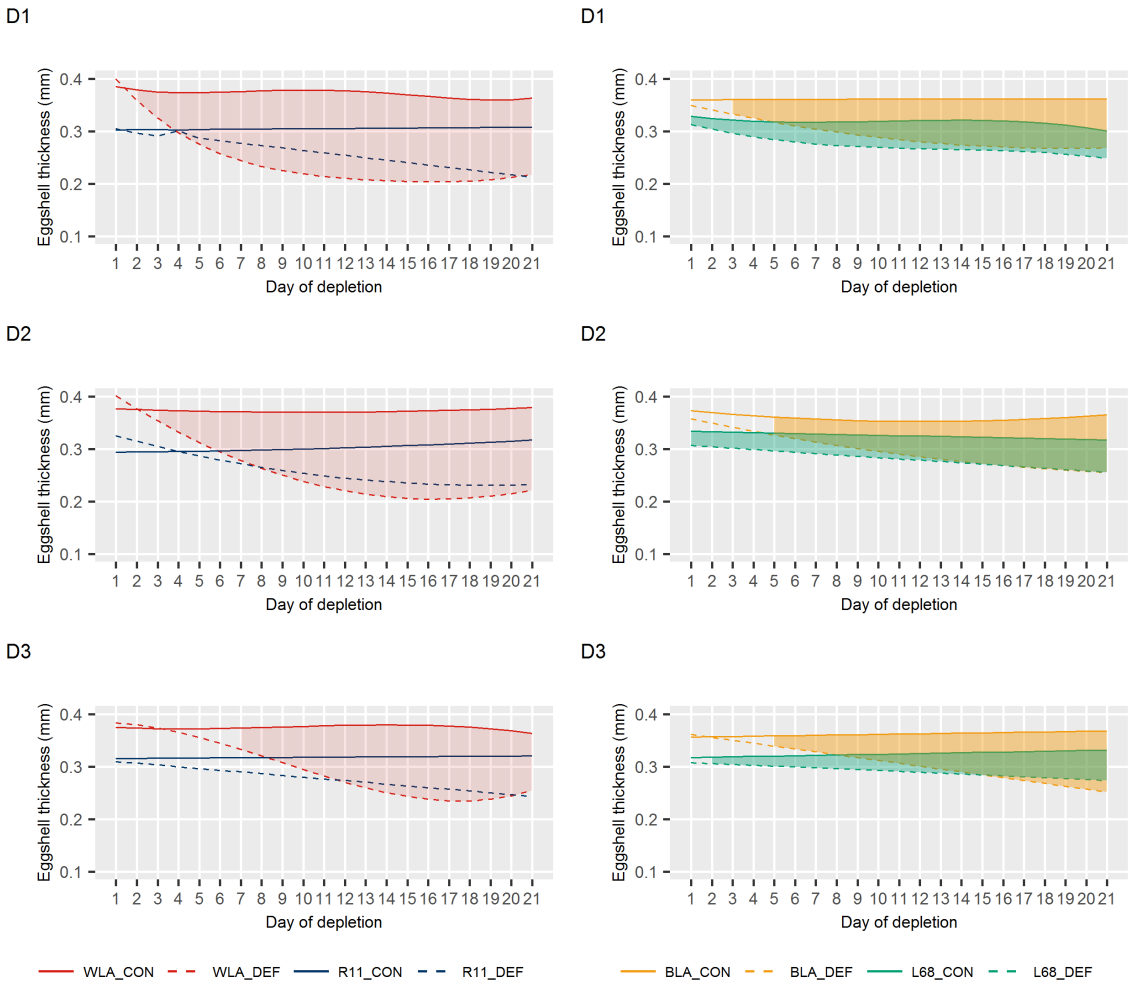

Figure 3.4: Least squares means for eggshell thickness in control (CON) and calcium (Ca) deficient (DEF) groups of four layer lines (WLA, R11, BLA, L68) during periods of Ca depletion (D1, D2, D3). The filled in areas indicate when both dietary groups of the lines differ significantly at $p < 0.05$.

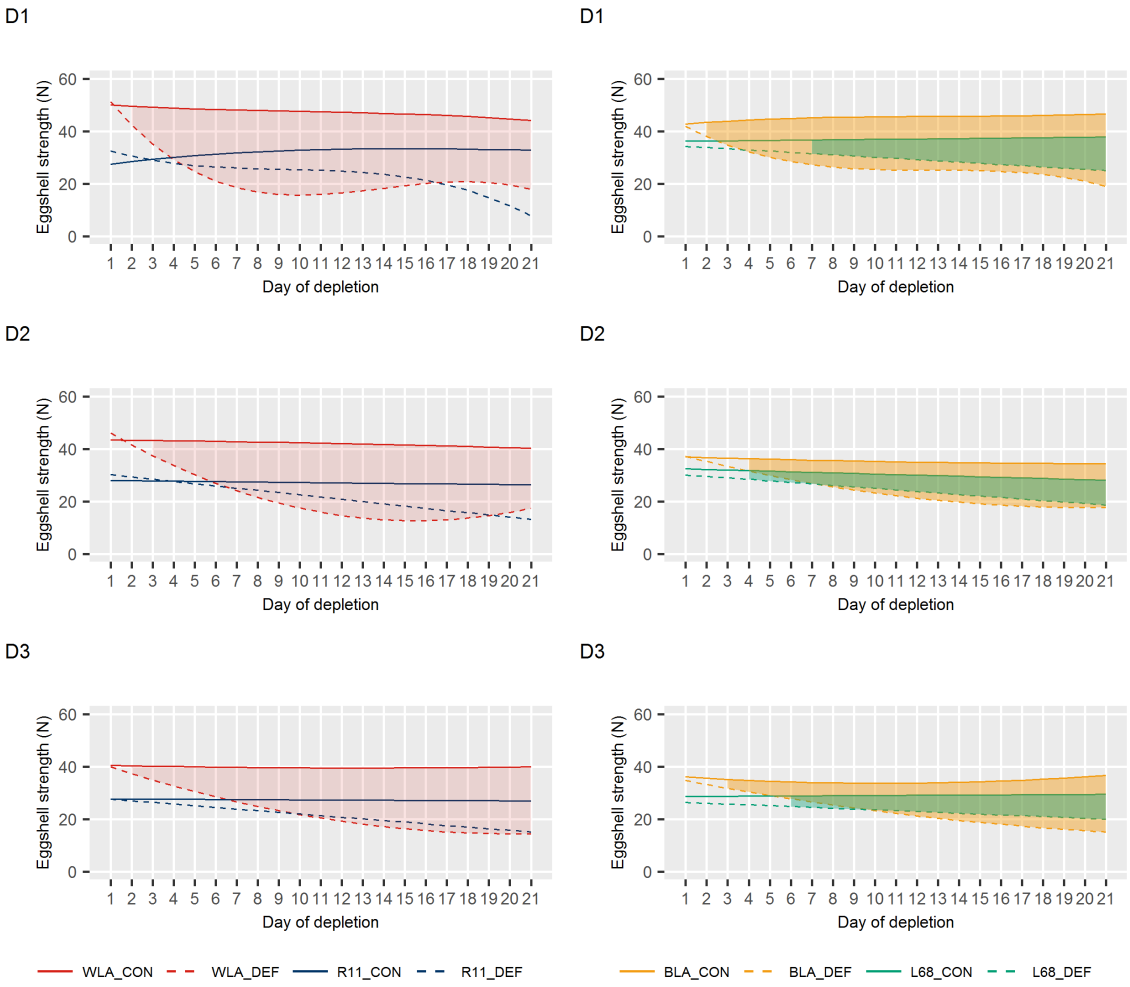

Figure 3.5: Least squares means for eggshell breaking strength in control (CON) and calcium (Ca) deficient (DEF) groups of four layer lines (WLA, R11, BLA, L68) during periods of Ca depletion (D1, D2, D3). The filled in areas indicate when both dietary groups of the lines differ significantly at $p < 0.05$.

No significant differences in egg quality were observed between the two dietary groups within each of the layer lines WLA, R11 and BLA at the end of the periods with a sufficient Ca supply, i.e., at the end of the periods Pre, R1 and R2 (Supplementary Table S3.4). Thus, egg quality during periods D2 and D3 has not been affected by prior depletions, suggesting a recovery from restricted Ca supply. Only in line L68, rather small but significant differences in eggshell weight and eggshell thickness were observed in period R1. Whether this represents an aftereffect of period D1 cannot be ruled out, but it seems rather unlikely, since no differences occurred in both the Pre and R2 periods.

3.3 Body Weight

Figure 3.6 illustrates the body weight prior and after the depletion periods according to model (2). Within layer lines and over all time points, the ANOVA revealed a significant dietary effect on the body weight (WLA: $p < 0.001$; R11: $p < 0.0001$; L68: $p = 0.0381$). In contrast, no dietary effect was found for line BLA ($p = 0.7457$). Significant body weight reduction was only observed in the DEF groups of the white-egg lines, whereas no weight changes were evident in the brown-egg lines. While the dietary groups of line R11 differed only in period D1, the DEF group of line WLA had a significant weight reduction in all depletion periods suggesting a distinct response to the Ca depletion. However, the lines fully recovered from this, as no significant differences between the body weight values at the end of the periods Pre, R1 and R2 were found.

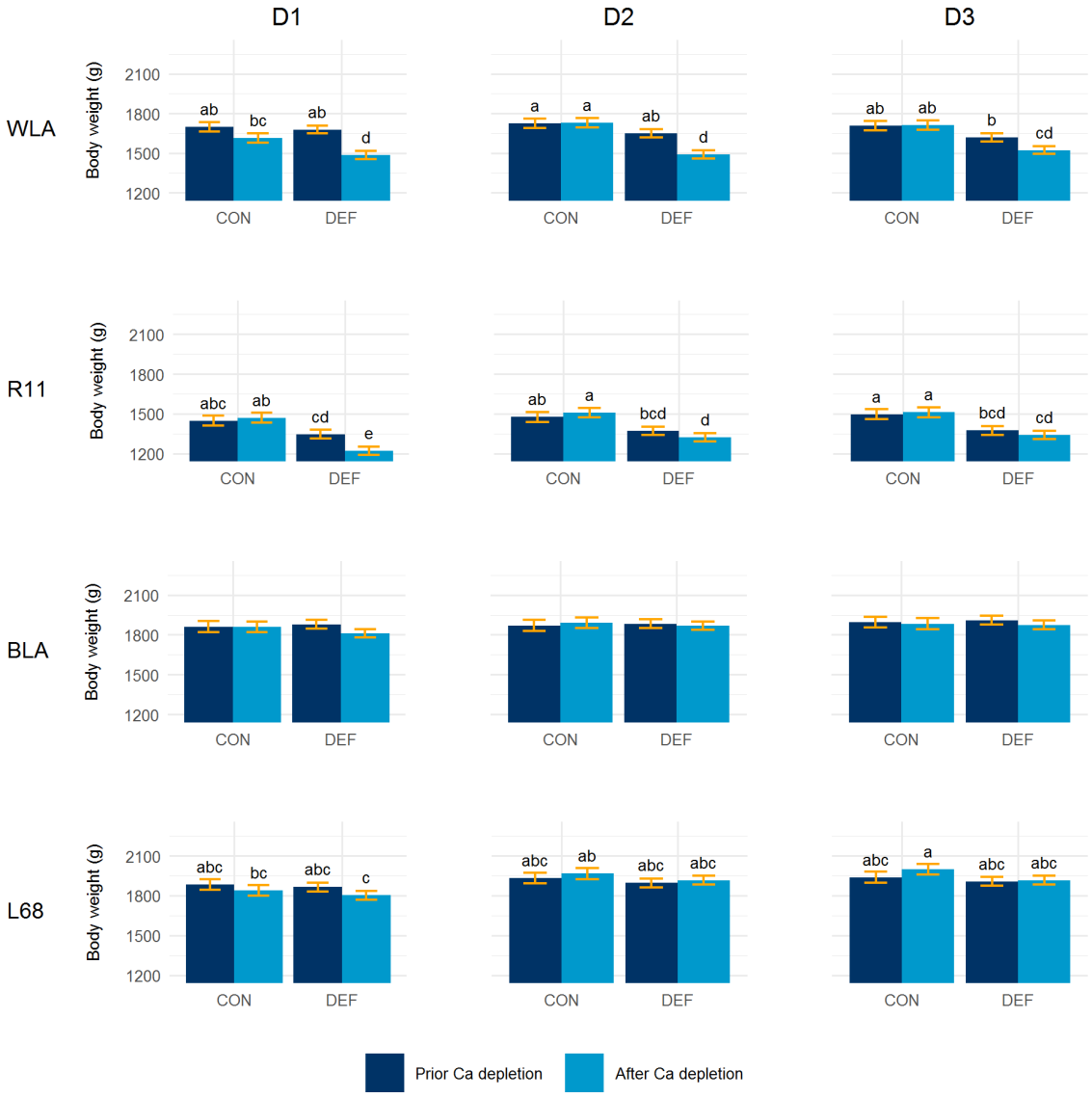

Figure 3.6: Least squares means ± standard errors for body weight prior and after calcium (Ca) depletion during the three restriction periods (D1, D2, D3), in control (CON) and Ca deficient (DEF) groups of four layer lines (WLA, R11, BLA, L68). DEF groups: $n = 22$; CON groups: $n = 11$. ^{a,b,c,d,e} means with different letters within layer lines differ significantly at $p < 0.05$.

3.4 Feed Consumption

The feed consumption for the WLA/L68 or BLA/R11 combination is shown in the Supplementary Table S3.3. Overall, the feed consumption ranged between 100 g and 120 g per animal and day. Apart from certain general fluctuations, the descriptive analysis suggested a reduced feed consumption of the DEF groups during all depletion periods, and this was reversed in the following recovery phases.

3.5 Bone Characteristics

The LSM of examined bone characteristics are shown in Figure 3.7. All bone parameters were significantly influenced by the layer line ($p < 0.0001$). The breaking strength, radio density, weight and cortical area of the tibiotarsus ($p < 0.0001$) as well as the breaking strength ($p < 0.0001$) and radio density ($p = 0.0193$) of the humerus were significantly influenced by the dietary treatment. The layer line by diet interaction was only significant for the breaking strength ($p = 0.0218$) and cortical area ($p = 0.0018$) of the tibiotarsus. With the exception of the humerus in line L68, bone breaking strength was significantly decreased in the DEF groups of all layer lines. The comparison of means further showed that radio density was only affected in the tibiotarsus, where the DEF groups possessed significantly lower bone density. The DEF groups of the white-egg lines WLA and R11 showed significantly declined cortical area of the tibiotarsus, while for both brown layers the difference was rather small and not significant. The Ca deficit led to a slight weight reduction of the tibiotarsus in all lines, which was only significant in line L68.

Results of post mortem examination of the keel bones are shown in the Supplementary Figure S3.2. Only in the DEF group of line R11 was the proportion of hens with at least one fracture significantly higher than in the CON group. The dietary groups of the other lines did not differ significantly regarding the occurrence of keel bone fractures.

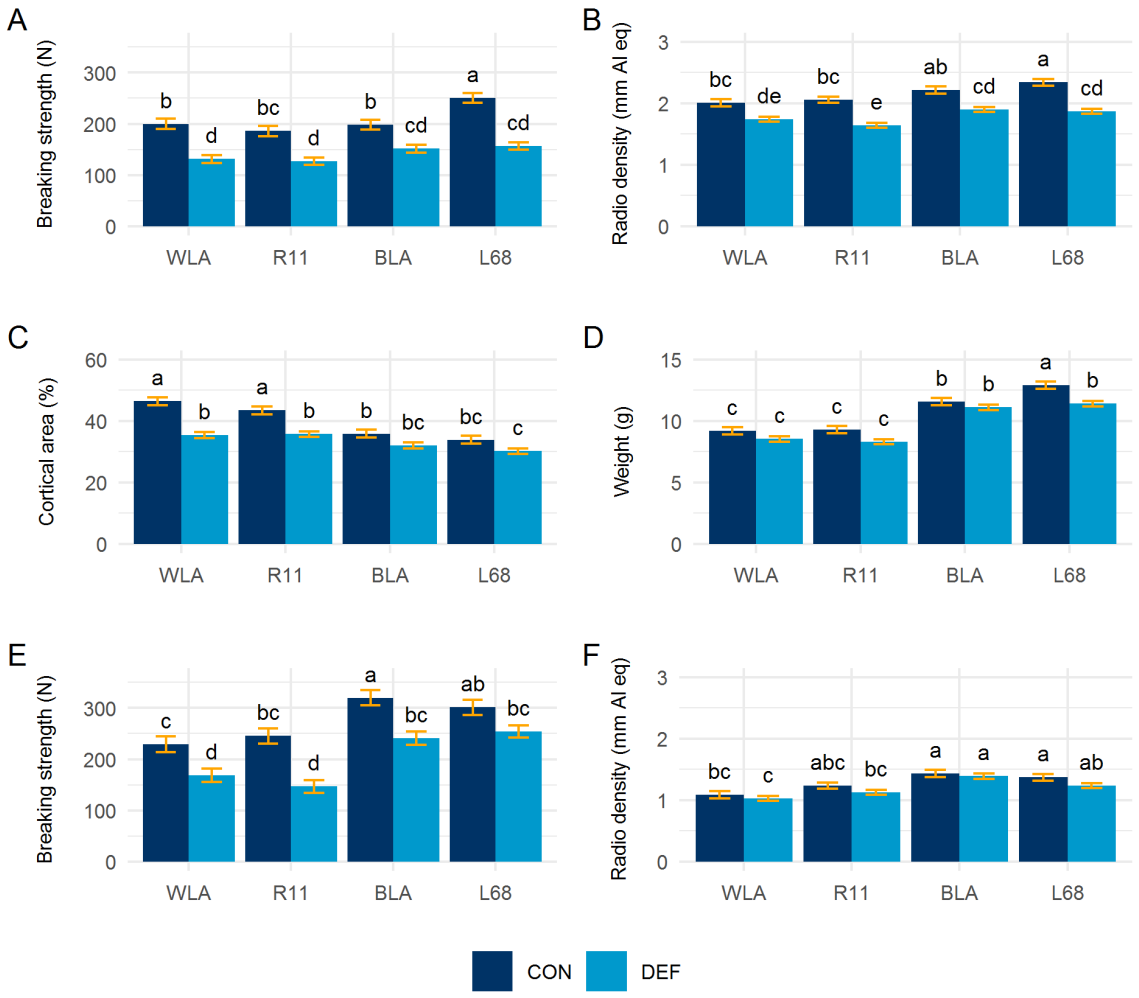

Figure 3.7: Least squares means ± standard errors for (**A**) bone breaking strength, (**B**) radio density, (**C**) cortical area and (**D**) weight of the tibiotarsus; (**E**) bone breaking strength and (**F**) radio density of the humerus in control (CON) and calcium deficient (DEF) groups of four layer lines (WLA, R11, BLA, L68). DEF groups: $n = 22$; CON groups: $n = 11$. $^{a, b, c, d, e}$ means with different letters differ significantly at $p < 0.05$.

4 Discussion

Considerable response to Ca depletion was observed among the layer lines. Our results support the findings of previous studies, in which dietary Ca restriction led to marked and sudden reduction of egg production [30–32]. Consistent with the literature, we also found that hypocalcaemia resulted in progressive reduction of eggshell production [32–34]. In line with Jiang et al. [35], our study showed that decreased eggshell quality leading to increasing incidence of egg breakages and defects was most likely the result of an inadequate Ca supply.

In contrast to Gilbert and Blair [30] and Luck and Scanes [36], who fed a diet of 0.05% Ca for six weeks and 0.03% Ca for three weeks, respectively, no evidence of cessation of laying activity was observed. A possible explanation for this might be that only drastically reduced Ca contents cause a suspension, while minor depletions, like in our case, merely lead to a performance decline [30, 37].

Although all lines showed a certain performance depression, there were differences between

the layer lines. Here, the white-egg lines seem to have been more sensitive to the Ca depletion, as they showed not only a greater drop in egg production, but also a higher proportion of defective eggs reflecting impaired eggshell quality. Conversely, despite the same dietary stress, the brown-egg lines retained a higher proportion of intact eggs, possibly because their skeletal system was a relatively larger Ca reservoir compared to the white-egg lines. Moreover, there was evidence that within the phylogenetic groups, a greater decline in egg production and quality occurred in the high performing lines. For example, eggshell quality traits were more decreased in the high performing WLA and BLA lines than in their moderately performing counterparts.

We observed substantial recovery in all layer lines after returning to the adequate Ca supply. Our results therefore indicate that the physiological stress induced by the administration of 1.09% Ca provoked adaptation response but did not cause permanent impairment of the hens' performance. This is in accordance with previous studies, as Summers et al. [31] found significantly heavier eggs with stabilized eggshells after changing from a previous 28-day supply of 1.50% Ca to 2.96% Ca. Immediate improvement, namely increased eggshell strength and laying performance, was also reported for repeated eight-week periods of Ca depletions [32].

The Ca deficiency led to a decrease in body weight and tended to reduce feed consumption. While a tendency of lower feed intake was observed in all DEF groups, significant body weight reduction only occurred in the white-egg lines. This may reflect phylogenetic differences. There seems to be a consensus that Ca deficiency leads to reduced feed intake [31, 32, 38]. In contrast, reports on body weight are inconsistent. Different studies have shown a decreasing [34], increasing [39] or even missing effect of diet on body weight [40]. Irrespective of the underlying mechanism, the body weight of the WLA and R11 hens recovered each time after reconversion to adequate Ca supply.

We observed a significant decrease in bone breaking strength and, to some extent, radio density, which is in accordance with previous studies [32, 38, 41]. However, our investigations revealed layer-line-specific differences. This especially applied to the white-egg lines, both of which showed a significant degradation of cortical bone tissue. While the medullary Ca reserves were sufficient to buffer temporary Ca fluctuations, structural bone, i.e., cortical and trabecular bone, was demineralized during prolonged Ca reduction [4, 42]. Cortical bone resorption therefore suggests that the medullary Ca reservoir was insufficient in the WLA and R11 lines. The lines BLA and L68, on the other hand, had larger and thicker bones [22], which probably also had a higher absolute medullary content that provided sufficient Ca so that no cortical bone had to be resorbed. Therefore, the brown-egg lines probably had a higher capacity to tolerate Ca depletion thus reflecting a phylogenetic component. That the bone breaking strength was equally impaired in both phylogenetic groups emphasized that medullary bone contributed to overall fracture resistance [8, 43].

Taken together, Ca depletion caused both decreased eggshell production and increased bone demineralization. Given the ongoing debate about mismatched resource allocation, according to which, egg production is prioritized [19, 44], our results may reflect the hens' attempt to maintain reproductive performance at the expense of bone stability. Contrary to our assumptions, this was the case for all layer lines. However, the response to the Ca deficit was differently pronounced, which possibly represented the line-specific adaptation potential of the layer lines examined here. For this, lines WLA and L68 responded most differently, as the Ca restriction had the most striking effects on WLA hens. On the other hand, only

minor effects were observed for line L68, but this may have been caused by a lower Ca demand in general. Despite this line-specificity, there is evidence that the hens responded differently depending on their phylogenetic origin.

While the present study phenotypically focuses mainly on egg production traits and bone characteristics, further studies at the molecular level may help to characterize the adaptation response and explain the differences between the layer lines observed in the current experiment. Here, the description of blood parameters relevant for Ca homeostasis, such as ionized Ca, total blood Ca, vitamin D3 and phosphorus, and the expression level of epithelial Ca transport proteins may be used for a more comprehensive characterization of the adaptation response. In a follow-up study, the use of single cage housing instead of a floor management system would allow the administration of Ca restrictions adapted to the egg production of the individual layer lines. Moreover, cage housing would avoid the hens eating eggs, which, although not observed in the present study, could lead to a bias in the data.

5 Conclusions

In this study, we characterized the adaptation response of genetically divergent chicken layer lines to repeated transient periods of calcium (Ca) depletion. It could be shown that laying hens apparently compensate for a temporary lack of Ca in the characteristics studied, albeit in different ways. Contrary to our hypothesis, our results did not indicate a major influence from selection for high egg numbers on the response to Ca depletion. Although layer-line-specific responses were observed, overall, the phylogenetic origin tended to be one of the determining factors with the brown-egg lines showing a higher tolerance to the Ca deficit. This was probably due to a more favorable body constitution in which the skeletal system was able to provide a higher amount of Ca without severe health restrictions. It seems essential to maintain the hens' adaptability to cope with changing or disadvantageous environmental conditions. This inevitably requires the preservation of genetic variation for adaptive performance.

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

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Supplementary Materials

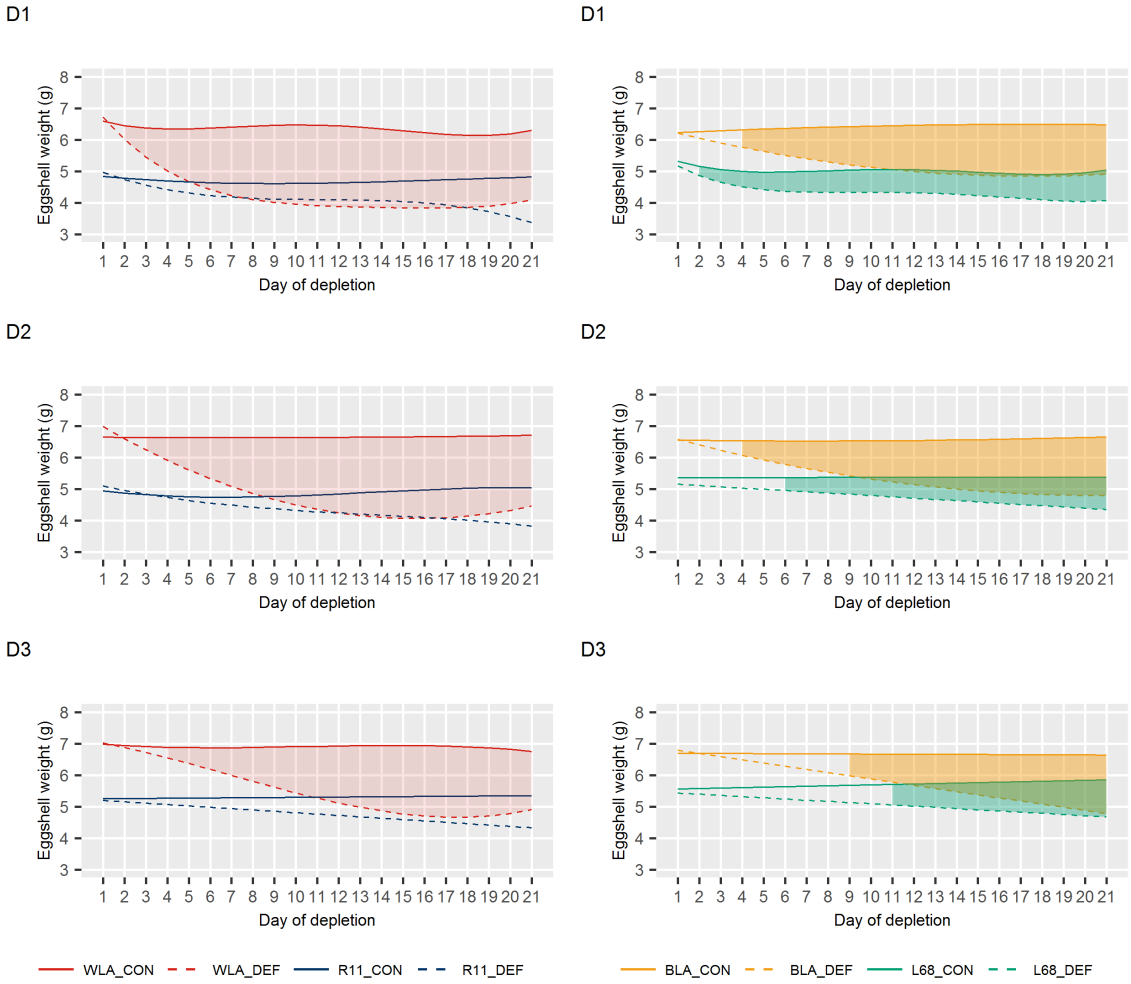

Figure S3.1: Least squares means for eggshell weight in control (CON) and calcium deficient (DEF) groups of four layer lines (WLA, R11, BLA, L68) during periods of calcium depletion (D1, D2, D3). The filled in areas indicate when both dietary groups of the lines differ significantly at $p < 0.05$.

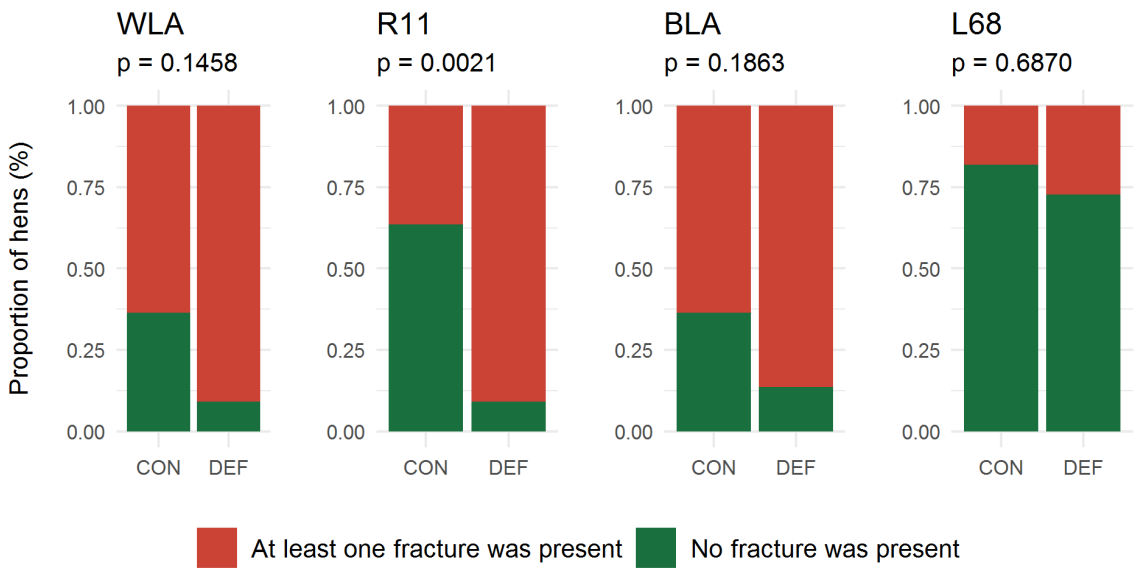

Figure S3.2: Occurrence of keel bone fractures in control (CON) and calcium deficient (DEF) groups of four layer lines (WLA, R11, BLA, L68). Each bar represents the total amount of examined keel bones of one layer line × diet combination (Fisher's exact test, $p < 0.05$).

Table S3.1: Light program.

Week of age	Time		Day length [h]	Light intensity [Lux]
	Start	End		
Day 1	-	-	24.0	20.0
1	4:00 am	7:00 pm	15.0	20.0
2	4:30 am	7:00 pm	14.5	20.0
3	5:30 am	7:00 pm	13.5	20.0
4	6:00 am	7:00 pm	13.0	20.0
5	7:00 am	7:00 pm	12.0	20.0
6	7:00 am	6:00 pm	11.0	10.0
7	7:00 am	5:00 pm	10.0	10.0
8	7:30 am	4:30 pm	9.0	10.0
9	7:30 am	3:30 pm	8.0	10.0
10	7:30 am	3:30 pm	8.0	10.0
11	7:30 am	3:30 pm	8.0	10.0
12	7:30 am	3:30 pm	8.0	10.0
13	7:30 am	3:30 pm	8.0	10.0
14	7:30 am	3:30 pm	8.0	10.0
15	7:30 am	3:30 pm	8.0	10.0
16	7:30 am	3:30 pm	8.0	10.0
17	7:00 am	3:30 pm	8.5	10.0
18	6:30 am	3:30 pm	9.0	10.0
19	6:30 am	4:00 pm	9.5	10.0
20	5:30 am	4:00 pm	10.5	10.0
21	4:30 am	4:00 pm	11.5	10.0
22	4:00 am	4:30 pm	12.5	10.0
23	3:30 am	5:00 pm	13.5	10.0
24 - 52	3:30 am	5:30 pm	14.0	10.0

Table S3.2: Ingredients and nutrient composition of the layer diets.

Diet	Adequate Calcium diet (Ca+)	Low Calcium diet (Ca-)
Ingredients (%)		
Wheat	39.74	36.81
Wheat bran	-	11.00
Corn	20.00	25.00
Soybean toasted	10.63	-
Soybean meal toasted	8.00	10.71
High protein soybean meal toasted	5.00	5.00
Lucerne pellets	2.44	5.58
Soybean oil	2.00	2.00
Calcium phosphate	2.46	1.79
Calcium carbonate	8.15	0.53
Sodium chloride	0.42	0.40
DL-Methionine	0.16	0.15
Lysine HCL	-	0.02
Premix[1]	1.00	1.00
Nutrient composition (on dry matter basis)		
Crude protein (%)[2]	17.10	16.60
Crude fat (%)[2]	6.40	5.00
Starch (%)[2]	38.20	42.70
Sucrose (%)[2]	3.30	3.40
AME_N/kg DM (MJ)[3,4]	11.60	11.90
Calcium (%)[2]	4.26	1.09

[1] Premix – hens: feed additives (per kg premix): Vitamin A, 1,000,000 IU; Vitamin D3, 250,000 IU; Vitamin E, 2,000 mg; Vitamin B1, 250 mg; Vitamin B2, 700 mg; Vitamin B6, 400 mg; Vitamin B12, 2,000 μg; Vitamin K3, 400 mg; Nicotin amide, 4,000 mg; Calcium-D-pantothenate, 1,000 mg; Folic acid, 60 mg; Biotin, 2,500 μg; Choline chloride, 40,000 mg; Fe, 4,000 mg; Cu, 1,000 mg; Mn, 10,000 mg; Zn, 8,000 mg; I, 120 mg; Se, 25 mg; Co, 20.5 mg; Butylated hydroxy toluene (BHT), 12,500 mg; Beta-carotene, 400 mg; Canthaxanthin, 400 mg; [2] Analyzed; [3] Calculated; [4] Apparent metabolizable energy concentrations corrected to zero nitrogen balance (AME_N), calculated according to the energy estimation equation of the World's Poultry Association (Vogt, 1986).

Table S3.3: Mean ($\bar{x}$) daily feed consumption (g) during periods of calcium depletion (D2, D3) and recovery (R1, R2) in control (CON) and calcium deficient (DEF) groups of combinations of two layer lines each.

	WLA / L68 combination		BLA / R11 combination	
Period	**CON**	**DEF**	**CON**	**DEF**
D1	115.50	113.20	121.80	101.20
R1	115.80	114.90	110.30	115.50
D2	104.30	104.40	110.70	101.00
R2	119.30	123.90	118.10	123.40
D3	117.20	112.10	121.20	107.90

Table S3.4: Least squares means ± standard errors for egg quality traits during the last three consecutive days of preliminary (Pre), first (R1) and second recovery period (R2) in control (CON) and calcium deficient (DEF) groups of four layer lines (WLA, R11, BLA, L68).

	WLA		R11		BLA		L68	
Period	**CON**	**DEF**	**CON**	**DEF**	**CON**	**DEF**	**CON**	**DEF**
Egg weight (g)								
Pre	59.16 ± 0.87	58.19 ± 0.62	46.13 ± 0.42	45.91 ± 0.34	58.83 ± 0.86	59.83 ± 0.59	47.72 ± 0.69	47.62 ± 0.45
R1	60.12 ± 0.78	60.65 ± 0.50	49.34 ± 0.41	49.18 ± 0.30	61.57 ± 1.23	60.36 ± 0.83	52.68 ± 0.79	51.47 ± 0.49
R2	59.43 ± 0.80	53.68 ± 3.23	51.79 ± 1.00	52.48 ± 0.72	62.30 ± 2.45	63.00 ± 1.72	51.79 ± 1.00	52.48 ± 0.72
Eggshell weight (g)								
Pre	6.65 ± 0.12	6.68 ± 0.08	4.63 ± 0.08	4.71 ± 0.06	6.20 ± 0.13	6.32 ± 0.09	4.77 ± 0.17	4.73 ± 0.12
R1	6.94 ± 0.16	7.08 ± 0.11	5.01 ± 0.07	5.28 ± 0.06	6.52 ± 0.14	6.63 ± 0.10	5.42 ± 0.10[a]	5.14 ± 0.06[b]
R2	7.11 ± 0.16	7.14 ± 0.11	5.40 ± 0.15	5.60 ± 0.11	6.61 ± 0.49	6.87 ± 0.35	5.40 ± 0.15	5.60 ± 0.11
Eggshell thickness (mm)								
Pre	0.39 ± 0.01	0.39 ± 0.01	0.32 ± 0.01	0.31 ± 0.01	0.36 ± 0.01	0.37 ± 0.01	0.32 ± 0.01	0.31 ± 0.01
R1	0.39 ± 0.01	0.37 ± 0.01	0.32 ± 0.01	0.32 ± 0.01	0.36 ± 0.01	0.36 ± 0.01	0.33 ± 0.01[a]	0.31 ± 0.01[b]
R2	0.38 ± 0.01	0.39 ± 0.01	0.33 ± 0.01	0.33 ± 0.01	0.36 ± 0.01	0.37 ± 0.01	0.33 ± 0.01	0.33 ± 0.01
Eggshell breaking strength (N)								
Pre	49.26 ± 1.89	50.70 ± 1.35	34.36 ± 1.30	34.60 ± 0.98	44.12 ± 1.72	45.95 ± 1.15	38.05 ± 2.67	37.29 ± 1.85
R1	42.96 ± 2.86	43.70 ± 1.91	28.70 ± 1.59	29.89 ± 1.26	38.33 ± 1.47	38.78 ± 0.99	30.28 ± 2.00	29.91 ± 1.26
R2	37.97 ± 2.41	38.14 ± 1.73	25.64 ± 3.18	27.39 ± 2.27	31.60 ± 2.67	34.00 ± 1.86	25.64 ± 3.18	27.39 ± 2.27

[a,b] Means with no common superscript differed significantly within each parameter, experimental period and layer line (Tukey's HSD-test, $p < 0.05$).

4 Identification and Functional Annotation of Genes Related to Bone Stability in Laying Hens Using Genome-wide Association Analyses

Simon Jansen [1], Ulrich Baulain [1], Christin Habig [1], Faisal Ramzan [2], Jens Schauer [1], Armin O. Schmitt [2,3], Armin M. Scholz [4], A. Reza Sharifi [3,5], Annett Weigend [1] and Steffen Weigend [1,3]

[1] Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut, 31535 Neustadt, Germany.

[2] Breeding Informatics Group, Department of Animal Sciences, University of Göttingen, 37075 Göttingen, Germany.

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

[4] Livestock Center of the Faculty of Veterinary Medicine, Ludwig-Maximilians-University Munich, 85764 Oberschleißheim, Germany.

[5] Animal Breeding and Genetics Group, Department of Animal Sciences, University of Göttingen, 37075 Göttingen, Germany.

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Abstract

Skeletal disorders, including fractures and osteoporosis, in laying hens cause major welfare and economic problems. Although genetics have been shown to play a key role in bone integrity, little is yet known about the underlying genetic architecture of the traits. This study aimed to identify genes associated with bone breaking strength and bone mineral density of the tibiotarsus and the humerus in laying hens. Potentially informative single nucleotide polymorphisms (SNP) were identified using both genome-wide association studies (GWAS) and Random Forests classification. We then searched for genes known to be related to bone stability in close proximity to the SNPs and identified 16 potential candidates. Some of them had human orthologues. Based on our findings, we can support the assumption that multiple genes determine bone strength, with each of them having a rather small effect, as illustrated by our SNP effect estimates. Furthermore, the enrichment analysis showed that some of these candidates are involved in metabolic pathways critical for bone integrity. In conclusion, the identified candidates represent genes that may play a role in the bone integrity of chickens. Although further studies are needed to determine causality, the genes reported here are promising in terms of alleviating bone disorders in laying hens.

Keywords: bone mineral density; bone breaking strength; gene set enrichment analysis; osteoporosis; Random Forests; single nucleotide polymorphism; skeletal integrity

1 Introduction

The very high incidence of skeletal disorders in laying hens, including brittle and fractured bones, is undoubtedly one of the most serious problems facing the egg production industry [1, 2]. Bone demineralisation associated with eggshell calcification favours the loss of structural bone tissue and ultimately predisposes the birds to osteoporosis in the course of the laying period [3, 4]. Besides dramatic effects on animal welfare [5–7], bone weakness also has an economic impact [4, 8]. According to a widespread assumption, the reduction in bone stability is primarily the result of selection for high laying performance [9–11]. However, the role of genetic selection on egg production is now seen in a more differentiated view, with recent studies pointing to factors other than egg number alone [12–14].

In the urgently needed improvement of the skeletal health of laying hens, genetics play an important role alongside husbandry and feeding of the birds [3, 15, 16]. To date, a number of quantitative trait loci (QTL) have been mapped to skeletal traits in chickens [17–22]. Dunn et al. [23] discovered a QTL on chromosome 1 that was recently fine-mapped leading to the identification of a promising region around the *cystathionine beta synthase* gene associated with osteoporosis [24]. The discovery of candidate positions for bone integrity is inevitably linked to technical advances in genotyping and bioinformatics. Today, testing hundreds of thousands of single nucleotide polymorphisms (SNP) by means of genome-wide association studies (GWAS) has become common practice [15, 25, 26].

Despite its widespread use, GWAS has some potential pitfalls. In addition to population stratification, these include the identification of gene loci with small effect sizes, which rarely

reach the statistical significance level due to their low strength of association [27]. At this point, machine learning algorithms represent a promising advance. Several studies have demonstrated their potential in identifying genes with small effect sizes [28, 29]. The Random Forests (RF) models in particular seem to have a great potential for analysing a large number of loci simultaneously and identifying corresponding associations [29–31]. Recently, this approach has been used to identify genes associated with eggshell strength [27].

The aim of the current study was to identify genomic positions associated with bone stability traits, i.e., breaking strength and mineral density of the tibiotarsus and the humerus, in laying hens. The animal model used comprised four layer lines that differed in their phylogenetic origin (brown-egg vs. white-egg layers) and their egg production level (high vs. moderately performing lines) [32]. Jansen et al. [14] have recently reported promising heritability estimates for bone traits in this set of populations, supporting the assumption of an inherited component of hens' susceptibility to osteoporosis. In the study reported here, we applied genome-wide association approaches to take a deeper look into the underlying genetic architecture of these hens. In addition to mixed linear model analysis, this also includes the adoption of RF-based feature selection in order to find potentially important SNPs. Subsequently, we performed a series of functional analyses including gene set enrichment analysis. Furthermore, SNP effects were estimated to confirm candidate genes known from the literature to be associated with bone metabolism.

2 Materials and Methods

2.1 Ethical Note

The present experiment was performed in accordance with the German Animal Welfare Law and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES) (33.19-42502-04-15/1988).

2.2 Population and Experimental Setup

The population consisted of four purebred chicken layer lines (*Gallus gallus domesticus*), which are phylogenetically distinct (brown vs. white-egg lines). Within each of these phylogenetic groups, the two lines differed in terms of egg-laying rate (high vs. moderate performing lines) [32, 33]. The set of populations was previously subjected to phenotypic analysis and the estimation of genetic parameters [14]. The data set only comprised hens whose total egg number was within the line specific threefold interquartile range and who laid at least one egg from 67 to 69 weeks of age [14]. For the statistical analyses done in this study, we combined the four chicken lines into one set as described below.

For the current research, we used the bone breaking strength (BBS) and bone mineral density (BMD) measurements previously reported by Jansen et al. [14]. Briefly, BBS and BMD of the tibiotarsus and humerus were determined by the three-point bending test and dual-energy X-ray absorptiometry, respectively, using dissected bones after the hens were sacrificed at 69 weeks of age.

The experimental setup is shown in Figure 4.1. We applied two alternate approaches to identify genomic positions potentially associated with the given phenotypes: (i) GWAS and

(ii) the machine learning-based approach of Random Forests. Subsequent functional analyses included gene set enrichment analysis and retrospective SNP effects analysis.

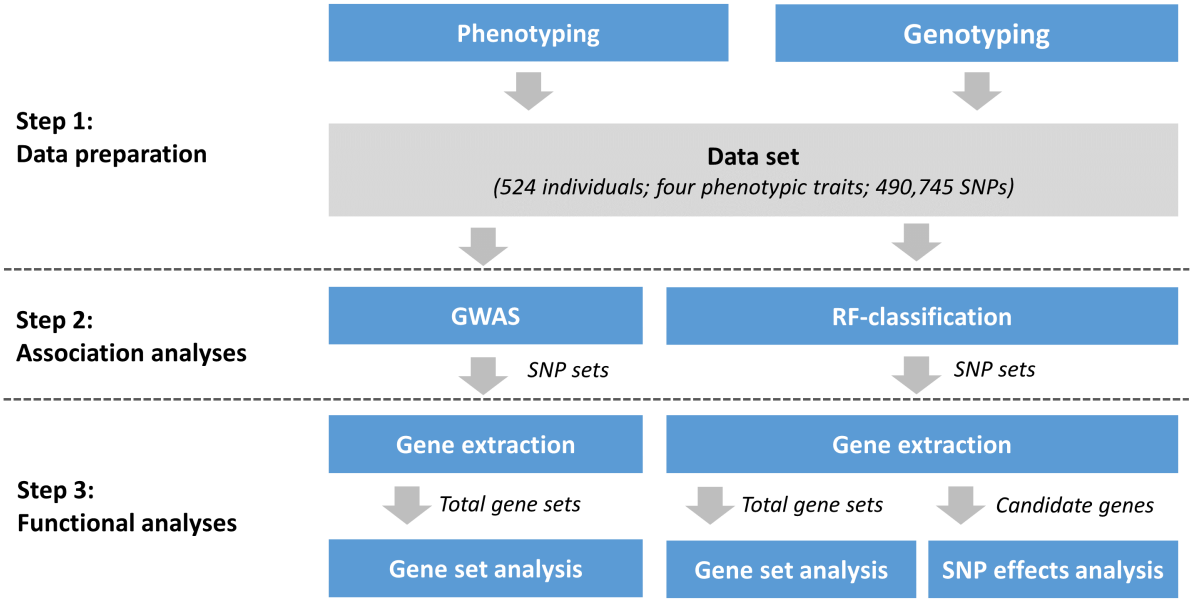

Figure 4.1: Schematic illustration of the study design. The data set included 524 laying hens phenotyped for bone stability traits. The corresponding genotypes included 490,745 SNP markers. Association analyses were performed applying both genome-wide association studies (GWAS) and Random Forests (RF) classification. Genes harbouring significant SNPs were extracted and screened for links to bone stability. Gene set analyses were performed considering all genes obtained from the GWAS or RF classification. Retrospectively, SNP effects were estimated for a subset of candidate genes identified in gene sets obtained from the RF classifier.

2.3 Genotyping

Initially deoxyribonucleic acid (DNA) samples from the hens and sires were extracted from blood samples. The hens were genotyped for 51,837 SNPs with a custom-made SNP array (Affymetrix Inc., Santa Clara, CA, USA). From the same chicken lines, in total 80 sires were genotyped for 580,961 SNP markers using the Affymetrix® Axiom® Genome-Wide Chicken Genotyping Array [34]. Quality control was applied to both data sets using the SNP & Variation Suite (SVS) v8.9 [35]. We only considered SNPs from autosomal chromosomes 1 to 28. The genotypes were filtered for a SNP call rate of $\geq$ 99% and an animal call rate of $\geq$ 95%. Furthermore, missing genotypes were imputed in a two-step procedure using BEAGLE 5.0 [36]. Initially, missing markers within the sire data set were imputed using the default settings. After this, the female genotypes were imputed from 37,606 SNPs left after quality control to 497,041 SNPs. Here, the sire genotypes served as a reference population and the effective population size was set to $ne = 5{,}000$. After imputation, 524 hens and 497,041 SNPs remained, of which 490,745 SNPs were finally annotated using the genome assembly GRCg6a (galGal6) [37], with duplicated SNPs and those with ambiguous chromosome annotation being removed.

2.4 Association Analyses

2.4.1 Genome-wide Association Study

For each of the phenotypic traits, i.e. BBS and BMD of the tibiotarsus and the humerus, a GWAS was performed in SVS applying a single-locus mixed linear model analysis. The statistical model was as follows:

$$\gamma = X\beta + Zu + \varepsilon \quad (1)$$

where γ is a $n \times 1$ vector of phenotypic values for n individuals; X is a $n \times f$ matrix of fixed effects; β is a $f \times 1$ vector of corresponding coefficients of the fixed effects; Z is a $n \times t$ matrix of random additive genetic effects; u is a $t \times 1$ vector of random additive genetic effects; and ε is a vector of random residuals. The model considered the identity by state (IBS) kinship matrix and included the generation as fixed effect. For the latter, a considerable influence on the bone properties has been previously shown [14]. Multiple testing correction was performed by applying the false discovery rate (FDR) approach [38]. For the latter, cut-off for significance was set at $p \leq 0.05$, so that less than or equal to 5% of the significant SNPs are false positives.

2.4.2 Random Forests Classification

As an alternative to the GWAS, we applied the machine learning algorithm of Random Forests (RF) to identify SNPs associated with bone characteristics. Briefly, the RF algorithm constructs a multitude of classifying decision trees assigning importance values to each SNP, thus determining those SNPs that explain variation in the response variable [29]. As shown by Ramzan et al. [27], we performed SNP selection by applying the Boruta algorithm, which works as a wrapper around the classification algorithm [39]. This algorithm is based on the idea that an unimportant attribute is not more useful for classification than a random one. Hence, if an attribute shows lower importance than a random attribute, it can be deemed irrelevant. The second idea is that importance measures get more accurate with less irrelevant attributes, such that iteratively removing unimportant attributes increases the accuracy of the importance measure. The procedure of the algorithm is as follows: The data set is first expanded by adding shuffled copies of the original values of each SNP, called shadow attributes. RF classification is then applied iteratively, assigning a value to each SNP, which is considered as the importance of the SNP. At each iteration, SNPs whose importance is less than the best of their shadow attributes are removed.

We used the Python (v3.8.3) [40] implementation from Homola [41] that specifies the proportion of the shadow attributes by which a SNP has to be better in order to be selected as important. Embedded in the Boruta algorithm, the RF classification itself was carried out with the 'RandomForestRegressor' from the Scikit-learn package [42] using default settings. The parameter *perc* was set to 99, representing a threshold of 99%, as no SNP has been confirmed as important at the 100% level. RF classification was performed separately for each bone trait. The input file consisted of the SNP genotypes, coded as '0' (AA), '1' (AB), or '2' (BB), and the phenotypic values of the respective bone trait. To account for possible confounding effects due to population stratification, residuals representing adjusted phenotypes were analysed instead

of the raw values [29]. The following model was used to estimate the residuals:

$$\gamma_{ijkl} = \mu + G_i + LL_j + S_k + \varepsilon_{ijkl} \quad (2)$$

where γ_{ijkl} is the observation for a bone trait; μ is the general mean; G_i is the fixed effect of generation (i = 1, 2); LL_j is the fixed effect of layer line (j = 1 to 4); S_k is the random effect of sire (k = 1 to 145); and ε_{ijkl} is the residual error. Tukey's HSD (honestly significant difference) test was performed for multiple comparisons of means. The model was computed using JMP v14.0 (SAS Institute Inc., Cary, NC, USA, 2018). Normal distribution of the residuals was assumed (Figure S4.1).

The output of the RF classification was a list of confirmed SNPs, i.e. markers that are more than coincidentally associated with a given bone trait.

2.5 Functional Analyses

2.5.1 Gene Extraction

All steps of the functional analyses were carried out using R v4.0.3 [43]. Extraction of genes associated with SNPs identified by the association analyses from the Ensembl database v102 [44] was performed using BioMart [45]. All protein-coding genes that are located within 5 kb upstream and downstream of the respective SNPs were considered for the gene lists. Information on the biological functions of these genes was obtained from both the NCBI [46] and Ensembl databases, as well as from the literature. The gene lists were then screened for genes known to be associated with bone stability traits. In this way, we identified a number of genes that were henceforth regarded as candidate genes.

2.5.2 SNP Effects Analysis

The genotypic effect was analysed for those SNPs located in intragenic or in flanking genomic regions of candidate genes, which have previously been shown to be significantly associated with a bone trait (see Table 4.1). SNP effects for each locus were analysed as described by Wiedemann et al. [47]. For this purpose, the actual SNP genotypes were coded as '0' (AA), '1' (AB), or '2' (BB), with the B allele representing the minor allele. The minor allele was considered the effect allele, whereas the major allele was termed 'other allele'. All models were computed with the R package lme4 [48].

A linear regression model adjusted for fixed factors was applied to estimate the allele substitution effects by single marker regression (SMR):

$$\gamma_{ijklm} = \mu + G_i + LL_j + b_1 SNP_k + S_l + \varepsilon_{ijklm} \quad (3)$$

where γ_{ijklm} is the observation for a bone trait; μ is the overall mean effect; G_i is the fixed effect of generation (i = 1, 2); LL_j is the fixed effect of layer line (j = 1 to 4); b_1 is the regression coefficient of the SNP genotype (SNP_k); S_l is the random effect of sire (l = 1 to 145); and ε_{ijklm} is the residual error. Standardised allele substitution effects were calculated according to model (3) after both the dependent variable and the SNP genotypes coded as '0', '1', or '2' were standardised to have a mean of 0 and a standard deviation of 1.

To calculate the additive and dominance effects, a dominant-recessive model (DRM) was applied considering the SNP genotype as a fixed class variable. The statistical model was as follows:

$$\gamma_{ijklm} = \mu + G_i + LL_j + SNP_k + S_l + \varepsilon_{ijklm} \quad (4)$$

where γ_{ijklm} is the observation for a bone trait; μ is the overall mean effect; G_i is the fixed effect of generation (i = 1, 2); LL_j is the fixed effect of layer line (j = 1 to 4); SNP_k is the fixed effect of SNP genotype (k = 1 to 3); S_l is the random effect of sire (l = 1 to 145); and ε_{ijklm} is the residual error. Least squares means (LSM) for the different genotypes were estimated with the emmeans package [49]. Significant differences between LSM were tested using a *t*-test and adjusted by the Bonferroni method. Additive and dominance effects were estimated by contrasting the respective genotypes according to the following formulas.

$$a = \frac{\mu_{AA} - \mu_{BB}}{2} \quad (5)$$

$$d = \mu_{AB} - \frac{\mu_{AA} + \mu_{BB}}{2} \quad (6)$$

where a is the additive effect; d is the dominance effect; μ_{AA} and μ_{BB} are the phenotypic mean values of the homozygous genotypes; and μ_{AB} is the phenotypic mean value of the heterozygous genotype.

2.5.3 Gene Set Analysis

With the gene sets including all genes extracted, we performed gene set analysis (GSA) using g:Profiler2 [50]. This involved the Gene Ontology (GO) (Ensembl v102) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) [51] (FTP release 2020-09-07) databases. The GSA was carried out considering all known genes obtained from Ensembl for the calculation of statistical significance and applying the default g:SCS algorithm [52] for computing the multiple testing correction. Only GO- and pathway terms with significant enrichment ($p < 0.05$) were considered for further analyses. Tree maps of the GO terms were generated using rrvgo [53].

3 Results

3.1 Association Analyses

3.1.1 Genome-wide Association Study

The GWAS results are illustrated as Manhattan plots representing the -$\log_{10}$ *p*-values on a genomic scale (Figure 4.2). The corresponding quantile-quantile (QQ) plots, also shown in Figure 4.2, revealed separation of the observed *p*-values from the distribution under the null hypothesis, when they are less than 0.01 (BBS) or 0.001 (BMD). Estimated genomic inflation factors (λ) ranged from λ = 0.96 to λ = 0.99, suggesting a negligible population stratification (Figure 4.2).

In the case of tibiotarsus BBS, 28 significant SNPs were identified. These markers were located on GGA (*Gallus gallus* chromosome) 1 (n = 12), 5 (n = 9), 6 (n = 3), 8 (n = 3), and 20 (n = 1) (Figure 4.2A). The strongest association signal, $p = 7.21 \times 10^{-8}$, was observed

for SNP *AX-76915891*, located on GGA 6 (3,171,707 bp). No significant association was identified for humerus BBS (Figure 4.2B). Analysis of tibiotarsus BMD resulted in 75 significant associations, with a peak of 41 SNPs found at GGA 1 (Figure 4.2C). The remaining loci were distributed across the whole genome with two additional signals at GGA 7 ($n = 11$) and 18 ($n = 9$). SNP *AX-75843394* located at GGA 15 (7,814,261 bp) showed the strongest association with tibiotarsus BMD ($p = 1.30 \times 10^{-11}$). Only one single locus, namely SNP *AX-75572283* located at GGA 10 (12,023,052 bp), was significantly associated with humerus BMD ($p = 6.81 \times 10^{-9}$) (Figure 4.2D).

3.1.2 Random Forests Classification

Lists of confirmed SNPs were obtained from the RF classifier for each of the phenotypic traits. For the tibiotarsus, 358 (BBS) and 374 (BMD) SNPs were confirmed as important, whereas for the humerus 188 (BBS) and 178 (BMD) markers were identified, respectively. There were no confirmed SNPs on GGA 16 for any of the four traits studied (Figure S4.2). In the case of the tibiotarsus, the majority of SNPs were located on GGA 1. In general, there were fewer markers for the humerus, with no markers found on GGA 28. Comparing the two bone types, more than twice as many SNPs were identified for the tibiotarsus, which is consistent with observations from GWAS.

3.2 Candidate Genes

3.2.1 Extracted Gene Sets

We identified 16 (BBS) and 39 (BMD) genes within an interval of 5 kb upstream and downstream of SNPs that were found to be significant for the tibiotarsus in GWAS. In contrast, gene sets for the humerus included no (BBS) or only one (BMD) gene. The gene lists obtained from the RF classifier contained a considerably higher number of genes. While 240 (BBS) and 220 (BMD) genes were assigned to the SNPs in the case of the tibiotarsus, 115 (BBS) and 113 (BMD) genes were mapped for the humerus.

Venn diagrams were used to find overlaps and differences between the genes identified for the BBS and BMD of the two bone types. Accordingly, Figure 4.3A and Figure 4.3B depict the genes that were unique or common among the traits within the lists obtained from GWAS and RF classifier analyses, respectively. The corresponding detailed gene lists are given in Table S4.1 (GWAS) and Table S4.2 (RF). It was found that within both methods, the overlaps of loci between the individual traits were rather small. In particular, within the GWAS only one gene in common for BBS and BMD was identified for the tibiotarsus, namely the *CNKSR2* gene. Despite the higher number of genes derived from the RF classifier, the relative proportion of overlaps was not substantially higher than for GWAS. It ranged from an overlap of 1.7% (six genes) between BBS of tibiotarsus and humerus up to an overlap of 6.7% (31 genes) between BBS and BMD within the tibiotarsus. In both methods of association analyses, no gene was found in all bone and trait combinations. Rather, they were mainly unique genes.

Based on the information on their biological functions and from the literature review, we reduced the gene lists to genes that are known to be related to bone stability. In the case of the gene sets obtained from the RF classifier, we found 16 genes with an already described association (Table 4.1). These genes are located on GGA 1 (*MCF2L*), GGA 2 (*MPP7, CALCR*,

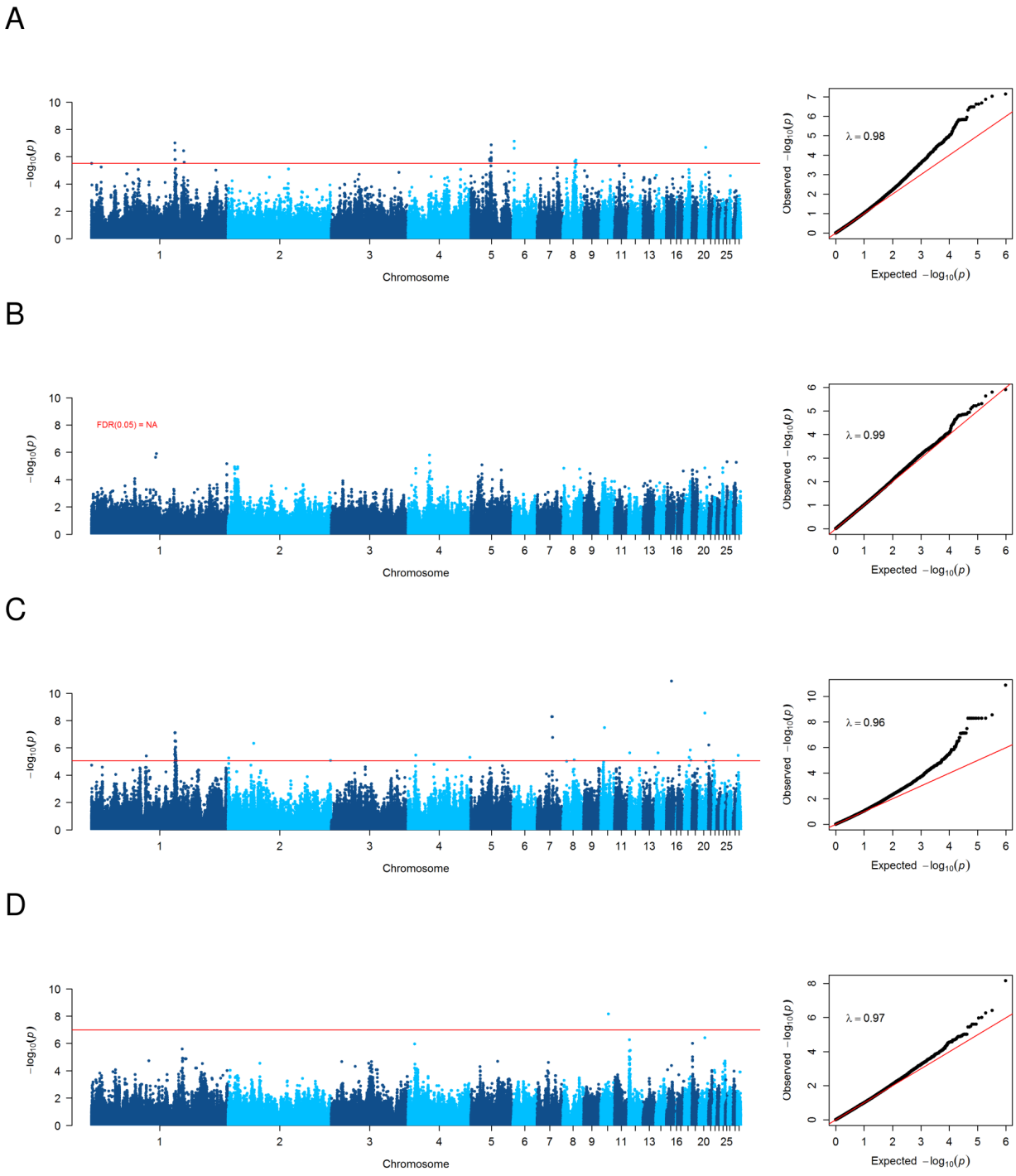

Figure 4.2: Manhattan plots (left) and quantile-quantile (QQ) plots (right) of the genome-wide association values for the bone breaking strengths of the tibiotarsus (**A**) and humerus (**B**), and the bone mineral densities of the tibiotarsus (**C**) and humerus (**D**). For the Manhattan plots, -$\log_{10}$ p-values for the markers (y-axis) are plotted against their genomic position on each chromosome. The red lines indicate a false discovery rate ≤ 0.05. For the QQ-plots, the y-axis shows the observed -$\log_{10}$ p-values and the x-axis represents the expected -$\log_{10}$ p-values. The corresponding genomic inflation factors (λ) are shown in the top left of the plots.

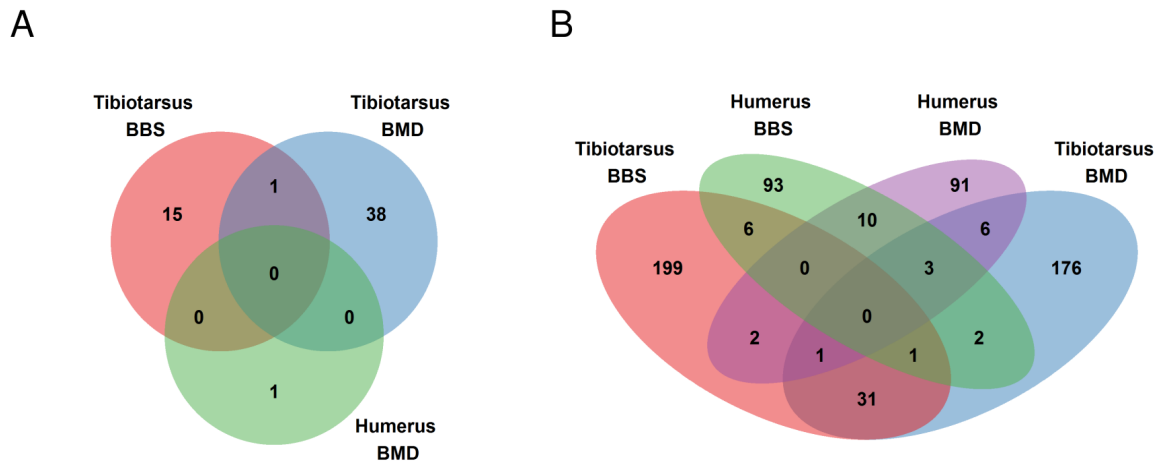

Figure 4.3: Venn diagrams showing the overlap of genes for the bone breaking strengths (BBS) and bone mineral densities (BMD) of the tibiotarsus and humerus derived from the genome-wide association study (**A**) and the Random Forests classifier (**B**).

and *SFRP4*), GGA 3 (*ACTR2*, *TGFB2*, and *CCDC170*), GGA 5 (*SOX6*), GGA 8 (*TMCO1*, *PODN*), GGA 10 (*SMAD6*), GGA 11 (*GPATCH1*), GGA 12 (*ASPN*), GGA 18 (*SOX9*), and GGA 27 (*WNT9B*, *WNT3*). In contrast, there was no evidence of an association to bone stability in the gene sets from the GWAS.

3.2.2 SNP Effects Analysis

To reveal the biological significance of the candidate genes, we analysed their associations with the corresponding phenotypic bone traits. To this end, we performed SNP effects analyses of all markers detected by the RF classifier and then assigned to genes (Table 4.1). Since the SNPs *AX-77091655* (*PODN*) and *AX-76772658* (*SOX6*) were associated with two traits each and, in addition, two further markers were assigned to the *WNT3* gene, SNP effects were estimated for 19 SNP and bone trait combinations.

Results from the SMR model are shown in Table 4.2. Analysis of variance revealed significant effects of SNP genotypes on the respective bone traits. Only the SNPs *AX-77276717* (*TGFB2*) and *AX-75711229* (*ASPN*) had no significant effect. Locus *AX-76099065* (*SFRP4*) had the greatest effect on tibiotarsus BMD, with the substitution of allele G for allele A leading to a reduction of 0.016 g/cm^2. In contrast, increasing the number of the copies of the effect alleles at loci *AX-76351898* and *AX-76351899*, both assigned to the *WNT3* gene, would yield an increase in humerus BMD of 0.016 g/cm^2. Of all loci significantly associated with BBS, the SNP *AX-76491534* (*CCDC170*) showed the largest effect, whereby substitution of one copy of allele A with allele G would result in a 15.63 N decrease of tibiotarsus BBS. The counterpart is the SNP *AX-76351785* (*WNT9B*) in which the T allele would presumably cause an increase of 11.51 N of humerus BBS. This is also the largest effect among all significant SNPs, with a change of 0.21 expressed in SD units.

Results obtained from the DRM are shown in Table 4.3. Comparison of the genotypic values (LSM) revealed significant differences among the genotypes. This applies to all loci studied, with exception of the SNPs *AX-77276717* (*TGFB2*) and *AX-80813610* (*CALCR*),

where only a tendency towards a higher value for the homozygote genotype of the effect allele was observed. For the other loci, the effects indicated by the direction of the beta coefficients (SMR) were also reflected in the genotypic values. A significantly higher LSM was found for the homozygote genotype of the effect allele of the SNP *AX-75711229* (*ASPN*). However, this estimate might be biased as the corresponding genotype had a frequency of only 0.01 (Table 4.1) and no significant allele substitution effect was detected for this locus (Table 4.2).

Significant additive effects of the respective other allele (major allele) were accounted for all loci with exception of the SNPs *AX-77276717* (*TGFB2*) and *AX-76772658* (*SOX6*) (Table 4.3). The estimates ranged from -0.02 to 0.017 g/cm^2 for the BMD-related SNPs and from -16.70 to 15.70 N for the markers associated with the BBS. Effects of complete dominance were observed for the SNPs *AX-76044166* (*MPP7*), *AX-75711229* (*ASPN*), *AX-75597497* (*SMAD6*) and *AX-76099065* (*SFRP4*), with one copy of the major allele masking the recessive allele, thus leading to full trait expression. In contrast, complete dominance in favour of the effect allele was seen for the SNP *AX-77113061* (*TMCO1*).

3.3 Gene Set Analysis

GSA was performed for all gene sets obtained from the RF classifier and for those of the tibiotarsus obtained from the GWAS. We restricted the results to the GO biological process (BP) category, as we sought to determine overarching biological objectives to which the gene products of the extracted genes contribute. Furthermore, the genes were grouped according to their KEGG pathways. Full lists of significantly enriched GO terms, including those from the cellular component and molecular function categories, are given in Table S4.3 (GWAS) and Table S4.4. The GWAS-derived genes for the tibiotarsal BBS were significantly associated with GO:0009266 (*response to temperature stimulus*), while in the case of BMD GO:0008150 (*biological_process*) and GO:0003674 (*molecular_function*) were enriched. However, these terms were not linked to bone stability in the GO hierarchy.

A large number of genes obtained from the RF classifier were involved in common processes. The analysis reported 81 (BBS) and 51 (BMD) significantly enriched BPs for the tibiotarsus and 33 (BBS) and 42 (BMD) BPs for the humerus, respectively (Table S4.4). Of these, Figure 4.4 (tibiotarsus) and Figure 4.5 (humerus) show the top 15 significantly enriched GO BP terms with the highest -$\log_{10}$ *p*-values and all significantly enriched KEGG pathways obtained from the RF classifier. Although certain BPs overlapped between the bone and trait combinations, no relation to the skeletal system was evident in the enriched BPs. Visualizing the results using tree maps to investigate redundancy based on semantic similarity between different GO terms also did not yield any biologically relevant findings (Figures S4.3, S4.4). However, the literature points to the involvement of Wnt- and MAPK signalling pathways in the pathogenesis of osteoporosis [25]. GSA revealed the *Wnt signaling pathway* (KEGG:04310) to be significantly enriched in both BMD gene sets (Figures 4.4B, 4.5B). In addition, significant enrichment for the *MAPK signaling pathway* (KEGG:04010) was identified in the genes for BMD of the tibiotarsus.

Table 4.1: General information for 17 loci associated with the bone breaking strengths (BBS) or bone mineral densities (BMD) of the tibiotarsus (Tib) and humerus (Hum) selected for the SNP effects analysis.

SNP	Trait	Location	GGA [1]	Position [2]	Genotypes	N individuals	Genotype frequencies	EA / OA [3]	EA frequency	Candidate gene	Reference [4]
AX-75268181	Tib_BMD	intragenic	1	139,001,157	CC / CT / TT	392 / 96 / 36	0.75 / 0.18 / 0.07	T / C	0.16	*MCF2L*	[54]
AX-76044166	Tib_BBS	intragenic	2	15,440,861	AA / AG / GG	421 / 63 / 40	0.80 / 0.12 / 0.08	G / A	0.14	*MPP7*	[55]
AX-80813610	Tib_BMD	downstream	2	23,056,581	CC / CG / GG	339 / 113 / 72	0.65 / 0.22 / 0.13	G / C	0.25	*CALCR*	[56]
AX-76099065	Tib_BMD	intragenic	2	46,101,680	GG / GA / AA	392 / 77 / 55	0.75 / 0.15 / 0.10	A / G	0.18	*SFRP4*	[57]
AX-76601713	Tib_BBS	intragenic	3	10,617,925	AA / AG / GG	265 / 102 / 157	0.51 / 0.19 / 0.30	G / A	0.40	*ACTR2*	[15]
AX-77276717	Tib_BBS	intragenic	3	19,498,104	GG / GA / AA	322 / 145 / 57	0.61 / 0.28 / 0.11	A / G	0.25	*TGFB2*	[58]
AX-76491534	Tib_BBS	intragenic	3	49,027,160	AA / AG / GG	432 / 62 / 30	0.82 / 0.12 / 0.06	G / A	0.12	*CCDC170*	[59]
AX-76772658	Tib_BBS / Hum_BBS	intragenic	5	11,438,677	TT / TC / CC	219 / 199 / 109	0.41 / 0.38 / 0.21	C / T	0.40	*SOX6*	[60]
AX-77113061	Tib_BMD	upstream	8	5,889,886	GG / AG / AA	202 / 156 /166	0.38 / 0.30 / 0.32	A / G	0.47	*TMCO1*	[61]
AX-77091655	Hum_BBS / Hum_BMD	upstream	8	24,931,025	CC / CA / AA	286 / 139 / 99	0.54 / 0.27 / 0.19	A / C	0.32	*PODN*	[15]
AX-75597497	Hum_BBS	downstream	10	19,108,829	AA / AG / GG	376 / 124 / 24	0.72 / 0.24 / 0.04	G / A	0.16	*SMAD6*	[62]
AX-75677174	Tib_BMD	intragenic	11	10,044,055	CC / CT / TT	377 / 107 / 40	0.72 / 020 / 0.08	T / C	0.18	*GPATCH1*	[55]
AX-75711229	Tib_BBS	intragenic	12	3,804,145	GG / AG / AA	459 / 58 / 7	0.88 / 0.11 / 0.01	A / G	0.07	*ASPN*	[63]
AX-75913642	Tib_BBS	upstream	18	8,793,585	GG / AG / AA	451 / 61 / 12	0.86 / 0.12 / 0.02	A / G	0.08	*SOX9*	[64]
AX-76351785	Hum_BBS	intragenic	27	3,497,444	CC / CT / TT	316 / 138 / 70	0.61 / 0.26 / 0.13	T / C	0.26	*WNT9B*	[65]
AX-76351898	Hum_BMD	downstream	27	3,518,924	GG / GA / AA	483 / 31 / 10	0.92 / 0.06 / 0.02	A / G	0.05	*WNT3*	[55]
AX-76351899	Hum_BMD	downstream	27	3,519,091	TT / TC / CC	483 / 31 / 10	0.92 / 0.06 / 0.02	C / T	0.05	*WNT3*	[55]

[1] GGA, Gallus gallus chromosome; [2] Physical position (bp) according to the GRCg6a (galGal6) genome assembly; [3] EA, effect allele (minor allele); OA, other allele (major allele); [4] References from the literature suggesting an association of the gene with bone stability traits.

Table 4.2: SNP effects analysis — Part 1: Analysis of variance table and allele substitution effect obtained from the single marker regression model.

SNP	Trait [1]	Candidate gene	Generation		Layer line		SNP genotype		Allele substitution effect [2]			
			F-statistics	*p*-value	F-statistics	*p*-value	F-statistics	*p*-value	Beta (SE [3])	Standardised beta [4] (SE [3])	*t*-value	*p*-value
AX-76044166	Tib_BBS	*MPP7*	80.92	<0.0001	46.34	<0.0001	4.05	0.0448	8.22 (4.09)	0.10 (0.05)	2.01	0.0448
AX-76601713	Tib_BBS	*ACTR2*	86.02	<0.0001	106.86	<0.0001	13.33	0.0003	-10.19 (2.79)	-0.18 (0.05)	-3.65	0.0003
AX-77276717	Tib_BBS	*TGFB2*	81.07	<0.0001	102.16	<0.0001	3.32	0.0696	4.67 (2.57)	0.06 (0.04)	1.82	0.0696
AX-76491534	Tib_BBS	*CCDC170*	91.49	<0.0001	84.86	<0.0001	12.58	0.0004	-15.63 (4.41)	-0.17 (0.05)	-3.55	0.0004
AX-76772658	Tib_BBS	*SOX6*	81.50	<0.0001	117.84	<0.0001	10.71	0.0012	7.63 (2.33)	0.12 (0.04)	3.27	0.0012
AX-75711229	Tib_BBS	*ASPN*	79.24	<0.0001	84.23	<0.0001	2.08	0.1503	6.66 (4.62)	0.05 (0.04)	1.44	0.1503
AX-75913642	Tib_BBS	*SOX9*	83.08	<0.0001	111.94	<0.0001	9.67	0.0019	-12.87 (4.14)	-0.11 (0.04)	-3.11	0.0019
AX-76772658	Hum_BBS	*SOX6*	36.26	<0.0001	52.59	<0.0001	5.67	0.0177	-5.32 (2.23)	-0.10 (0.04)	-2.38	0.0177
AX-77091655	Hum_BBS	*PODN*	39.91	<0.0001	41.64	<0.0001	8.35	0.0041	6.69 (2.31)	0.13 (0.04)	2.89	0.0041
AX-75597497	Hum_BBS	*SMAD6*	36.38	<0.0001	53.40	<0.0001	4.62	0.0321	-7.13 (3.32)	-0.10 (0.05)	-2.15	0.0321
AX-76351785	Hum_BBS	*WNT9B*	37.27	<0.0001	67.22	<0.0001	21.57	<0.0001	11.51 (2.48)	0.21 (0.04)	4.64	<0.0001
AX-75268181	Tib_BMD	*MCF2L*	4.30	0.0401	106.46	<0.0001	13.53	0.0003	-0.015 (0.004)	-0.15 (0.05)	-3.67	0.0003
AX-80813610	Tib_BMD	*CALCR*	4.24	0.0415	56.10	<0.0001	4.86	0.0298	0.008 (0.004)	0.10 (0.05)	2.21	0.028
AX-76099065	Tib_BMD	*SFRP4*	4.31	0.0400	65.23	<0.0001	8.55	0.0036	-0.016 (0.006)	-0.18 (0.06)	-2.92	0.0036
AX-77113061	Tib_BMD	*TMCO1*	4.45	0.0369	99.26	<0.0001	5.27	0.0221	0.008 (0.003)	0.11 (0.05)	2.30	0.0221
AX-75677174	Tib_BMD	*GPATCH1*	4.27	0.0406	61.13	<0.0001	10.84	0.0011	0.013 (0.004)	0.13(0.04)	3.29	0.0011
AX-77091655	Hum_BMD	*PODN*	20.70	<0.0001	51.56	<0.0001	11.53	0.0008	0.007 (0.002)	0.14 (0.04)	3.39	0.0008
AX-76351898	Hum_BMD	*WNT3*	19.82	<0.0001	77.58	<0.0001	13.81	0.0002	0.016 (0.004)	0.15 (0.04)	3.72	0.0002
AX-76351899	Hum_BMD	*WNT3*	19.82	<0.0001	77.58	<0.0001	13.81	0.0002	0.016 (0.004)	0.15 (0.04)	3.72	0.0002

[1] BBS, bone breaking strength; BMD, bone mineral density; Tib, tibiotarsus; Hum, humerus; [2] Allele substitution effect per copy of the effect allele (minor allele); [3] SE, standard error; [4] Standardised regression coefficients expressed in SD unit.

Table 4.3: SNP effects analysis — Part 2: Genotypic values (least squares means) and additive and dominance effects obtained from the dominant-recessive model.

SNP	Trait [1]	Candidate gene	Genotypic values			Homozygous additive allele effect [5]			Dominance effect [5]		
			AA [2,3] (SE [4])	AB [2,3] (SE [4])	BB [2,3] (SE [4])	Estimate (SE [4])	t-value	p-value	Estimate (SE)	*t*-value	*p*-value
AX-76044166	Tib_BBS	*MPP7*	155.33 (2.26) [a,b]	145.80 (5.85) [b]	172.76 (7.25) [a]	-8.71 (4.05)	-2.15	0.0320	-18.20 (5.45)	-3.35	0.0009
AX-76601713	Tib_BBS	*ACTR2*	162.77 (3.08) [a]	156.79 (3.81) [a]	143.10 (3.79) [b]	9.83 (2.82)	3.49	0.0005	3.86 (4.03)	0.96	0.3392
AX-77276717	Tib_BBS	*TGFB2*	153.42 (2.25) [a]	157.05 (3.06) [a]	163.72 (5.10) [a]	-5.15 (2.83)	-1.82	0.0694	-1.52 (3.73)	-0.41	0.6843
AX-76491534	Tib_BBS	*CCDC170*	159.13 (2.19) [a]	144.04 (6.28) [a,b]	127.83 (8.09) [b]	15.70 (4.42)	3.54	0.0004	0.56 (5.88)	0.096	0.9239
AX-76772658	Tib_BBS	*SOX6*	149.06 (2.65) [b]	158.53 (2.58) [a]	163.29 (3.90) [a]	-7.11 (2.43)	-2.93	0.0035	2.36 (3.13)	0.75	0.4520
AX-75711229	Tib_BBS	*ASPN*	155.14 (1.94) [b]	154.78 (5.29) [b]	188.53 (13.11) [a]	-16.70 (6.62)	-2.52	0.0120	-17.10 (8.02)	-2.13	0.0340
AX-75913642	Tib_BBS	*SOX9*	157.50 (1.93) [a]	148.13 (4.83) [a,b]	124.13 (10.37) [b]	16.70 (5.31)	3.14	0.0018	7.40 (6.44)	1.15	0.2506
AX-76772658	Hum_BBS	*SOX6*	127.04 (2.51) [a]	116.24 (2.46) [b]	119.38 (3.71) [a,b]	3.83 (2.31)	1.66	0.0984	-6.96 (3.02)	-2.31	0.0215
AX-77091655	Hum_BBS	*PODN*	118.01 (2.31) [b]	120.73 (3.04) [b]	132.21 (3.73) [a]	-7.10 (2.31)	-3.07	0.0023	-4.38 (3.44)	-1.27	0.2043
AX-75597497	Hum_BBS	*SMAD6*	122.16 (2.08) [a]	123.48 (3.64) [a]	98.16 (6.97) [b]	12.0 (3.71)	3.23	0.0013	13.30 (4.61)	2.88	0.0040
AX-76351785	Hum_BBS	*WNT9B*	115.73 (2.19) [c]	124.86 (3.05) [b]	139.61 (4.34) [a]	-11.90 (2.54)	-4.70	<0.0001	-2.81 (3.49)	-0.80	0.4215
AX-75268181	Tib_BMD	*MCF2L*	0.263 (0.003) [a]	0.253 (0.005) [a]	0.228 (0.008) [b]	0.017 (0.004)	3.92	0.0001	0.008 (0.006)	1.35	0.1768
AX-80813610	Tib_BMD	*CALCR*	0.256 (0.003) [a]	0.258 (0.005) [a]	0.273 (0.006) [a]	-0.009 (0.004)	-2.24	0.0257	-0.007 (0.005)	-1.27	0.2051
AX-76099065	Tib_BMD	*SFRP4*	0.261 (0.003) [a,b]	0.265 (0.008) [a]	0.235 (0.009) [b]	0.013 (0.006)	2.32	0.0206	0.018 (0.006)	2.71	0.0071
AX-77113061	Tib_BMD	*TMCO1*	0.246 (0.005) [a]	0.267 (0.004) [a]	0.266 (0.004) [a]	-0.01 (0.004)	-2.82	0.0050	0.011 (0.004)	2.51	0.0125
AX-75677174	Tib_BMD	*GPATCH1*	0.254 (0.003) [b]	0.269 (0.005) [a]	0.278 (0.007) [a]	-0.012 (0.004)	-3.05	0.0024	0.004 (0.005)	0.56	0.5739
AX-77091655	Hum_BMD	*PODN*	0.164 (0.002) [b]	0.167 (0.003) [b]	0.178 (0.003) [a]	-0.007 (0.002)	-3.53	0.0005	-0.004 (0.003)	-1.25	0.2117
AX-76351898	Hum_BMD	*WNT3*	0.166 (0.002) [b]	0.176 (0.006) [b]	0.206 (0.010) [a]	-0.02 (0.005)	-3.84	0.0001	-0.009 (0.007)	-1.29	0.1991
AX-76351899	Hum_BMD	*WNT3*	0.166 (0.002) [b]	0.176 (0.006) [b]	0.206 (0.010) [a]	-0.02 (0.005)	-3.84	0.0001	-0.009 (0.007)	-1.29	0.1991

[1] BBS, bone breaking strength; BMD, bone mineral density; Tib, tibiotarsus; Hum, humerus; [2] AA or BB represents the homozygote of the other allele or effect allele, respectively. AB denotes the heterozygote (see Table 4.1 for the actual genotypes); [3] Means with different letters within a row differ significantly at $p < 0.05$; [4] SE, standard error; [5] Effect of the other allele (major allele).

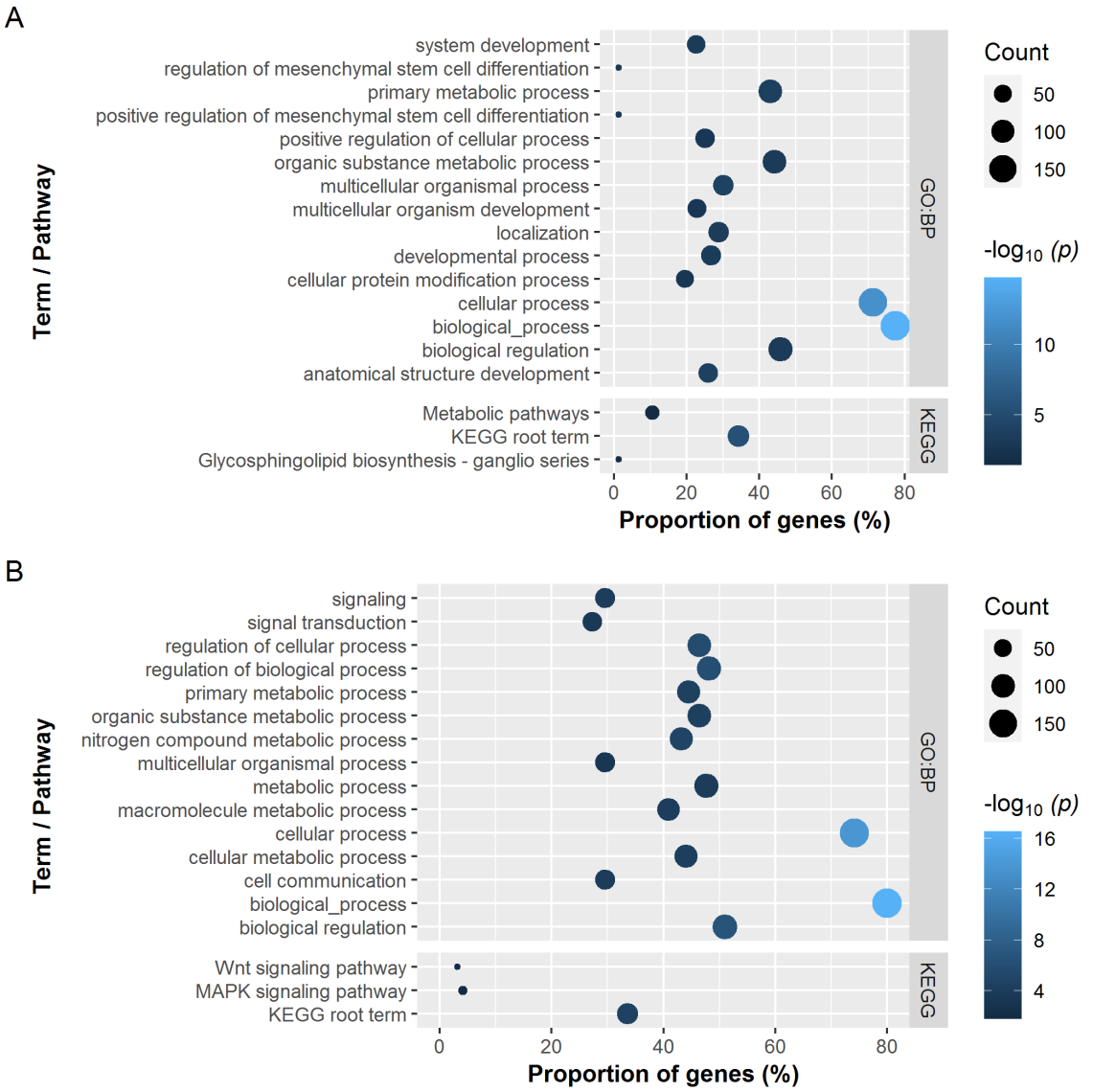

Figure 4.4: Significantly enriched Gene Ontology terms of the category biological processes (GO:BP; top 15 with the highest -$\log_{10}$ *p*-values) and KEGG pathways for the bone breaking strength (**A**) and bone mineral density (**B**) of the tibiotarsus. The dot size represents the absolute number of genes enriched in the term. The proportion of enriched genes in all queried genes is represented on the x-axis. The colour represents the -$\log_{10}$ transformed adjusted *p*-values.

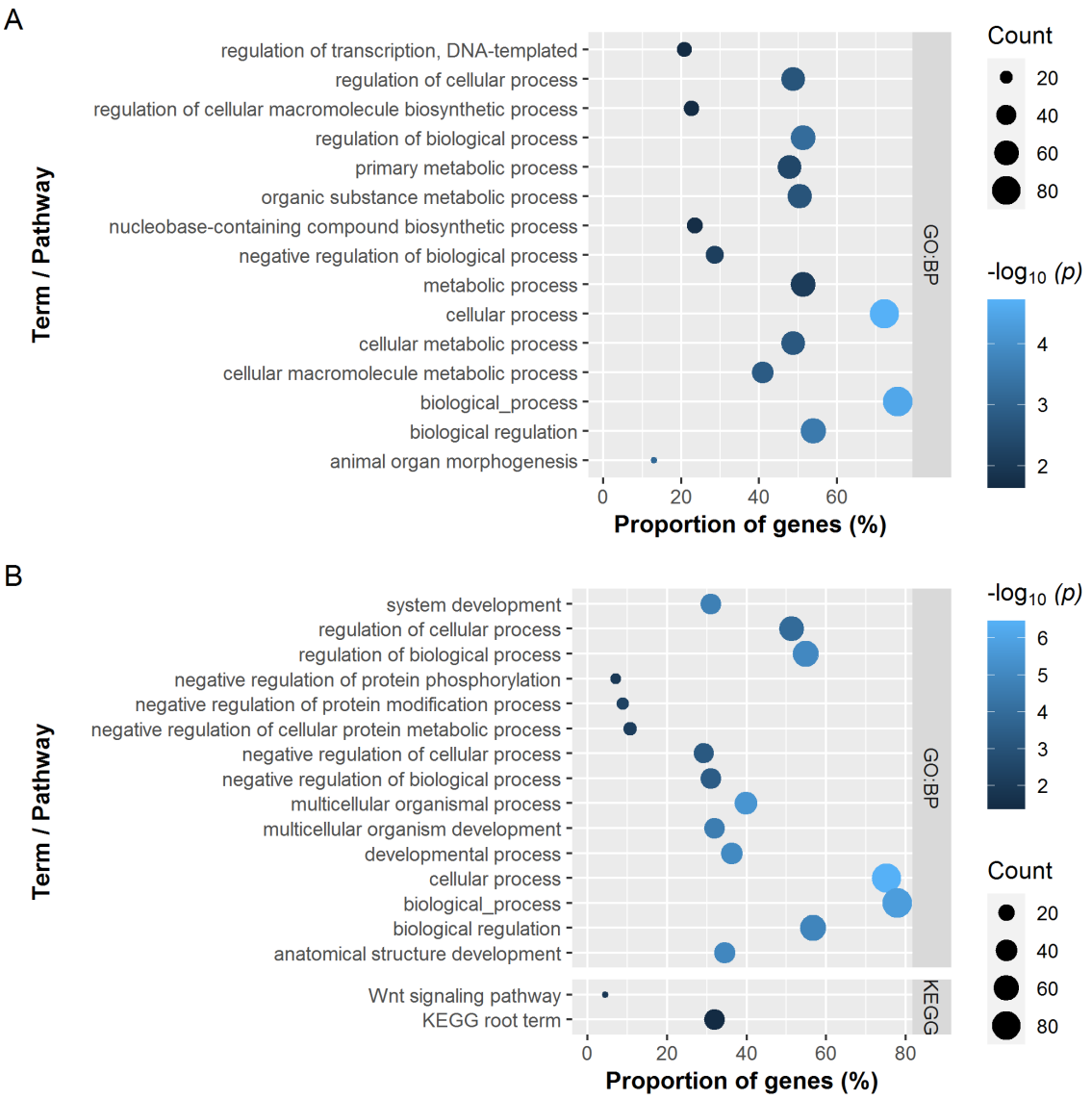

Figure 4.5: Significantly enriched Gene Ontology terms of the category biological processes (GO:BP; top 15 with the highest -$\log_{10}$ *p*-values) and KEGG pathways for the bone breaking strength (**A**) and bone mineral density (**B**) of the humerus. The dot size represents the absolute number of genes enriched in the term. The proportion of enriched genes in all queried genes is represented on the x-axis. The colour represents the -$\log_{10}$ transformed adjusted *p*-values.

4 Discussion

The objective of the present study was to identify genomic positions potentially associated with skeletal integrity in a laying hen population. There is solid evidence that osteoporosis is a polygenic disorder, i.e. determined by multiple functional genes acting conjointly rather than a few major genes [15, 25]. For this reason, in addition to GWAS, we applied RF classification, an approach known to be able to detect genes with modest effects [29, 30]. To our knowledge, this is the first study applying a machine learning approach to bone data in chickens. As both methods have advantages and promising candidate genes have so far been identified by means of GWAS [15, 25], we ran both in parallel rather than considering them complementary and

only examining loci that both methods have in common [29]. RF classification led to the identification of a higher number of SNPs compared to GWAS. Consequently, the methods also differed considerably in the number of genes extracted, with only very little overlap. No genes functionally related to the skeleton were found in GWAS. Although a large number of genes were adjacent to the SNPs from the RF classifier, only 16 candidate genes related to skeletal disorders were identified; of these, many had human orthologues. However, for the vast majority of genes, no involvement in bone metabolism has been suspected so far, which is in line with previous reports [15, 66]. From the 16 identified candidates discussed below, we first focus on genes that have previously been linked to BBS or BMD ($n = 10$), followed by genes for which an association with osteoarthritis is suggested ($n = 3$). Finally, genes are discussed that are functionally related to the Wnt signalling pathway ($n = 3$).

Ten of our candidate genes can be grouped as having previously been associated with BBS or BMD traits in the literature. Of these, the *membrane palmitoylated protein 7* gene (*MPP7*) was associated with vertebral BMD in humans [55]. Its strong functional role in osteoblast biology was demonstrated by means of in vivo and in vitro studies [67]. Based on these reports, we consider *MPP7* to be a good candidate for bone disorders in chickens. In our study, the *calcitonin receptor* gene (*CALCR*) was identified as a strong candidate for BMD. Calcitonin plays a role in calcium homeostasis and is primarily an inhibitor of bone resorption [68]. Our observations are in line with previous reports, as *CALCR* polymorphisms were associated with site-specific BMD in humans [56, 69], and alpha-calcitonin gene-related peptide deficient mice were shown to have a lower bone mass [70]. One of the candidates for BBS located on GGA 3 is the *actin related protein 2* gene (*ACTR2*), which was recently identified by Raymond et al. [15] as being associated with BBS in laying hens. *ACTR2* is functionally linked to bone via its importance for cilia formation, as cilia are known to play an integral role in skeletal development [15, 71]. Although no significant effect of the variant corresponding to the *transforming growth factor beta 2* gene (*TGFB2*) was observed in our study, *TGFB2* is considered a very promising candidate for skeletal integrity in the chicken. As a cytokine, the protein encoded by *TGFB2* has important functions in many biological processes related to bone remodelling [19, 58]. Analyses in different chicken populations including broilers and layers suggest *TGFB2* to be associated with various bone characteristics [19, 21, 58]. In this context, the *SMAD family member 6* gene (*SMAD6*) has to be mentioned, which we identified as a candidate for BBS. Its protein acts as a regulator of the TGF-beta family and inhibits bone morphogenetic protein pathways, which are integral parts of osteoblast and chondrocyte differentiation [72, 73]. A study on mice revealed their essential role in bone formation, as *SMAD3* knockout resulted in osteopenia [62]. The *coiled coil domain containing 170* gene (*CCDC170*) is our third candidate for BBS located on GGA 3. The region around this locus has been linked to BMD in humans [59, 65].However, since the function of the protein is yet unclear, it has been speculated whether associations attributed to *CCDC170* do not rather belong to the adjacent *estrogen receptor 1* gene [59]. In a follow-up study, *CCDC170* polymorphisms were in turn associated with osteoporosis-relevant phenotypes [74]. Only one of our candidates was located on GGA 5. The corresponding variant is located in the intron of the *SRY-box 6* gene (*SOX6*), which encodes a transcription factor known to affect developmental processes and skeletal formation in humans [60, 65]. In addition, the gene was linked with BMD of the femoral neck [75], and skeletal abnormalities have previously been observed in *SOX6* knockout mice, suggesting an integral role in cartilage formation [76]. We identified the *transmembrane and coiled-coil domains 1* gene (*TMCO1*), located on GGA 8, as a candidate for BMD. *TMCO1* plays an important role in bone formation mediating calcium homeostasis within the endoplasmic reticulum [61]. Disruption of the endoplasmic reticulum of an osteoblast can lead to severe bone disorders [77]. Recently, Li et al. [61] demonstrated that *TMCO1* deficiency

leads to reduced bone formation and osteoblast differentiation in humans and mice. In addition to *SOX6*, the *podocan* gene (*PODN*) is another candidate that was associated with two traits, namely BBS and BMD of the humerus. *PODN* encodes a proteoglycan that was shown to bind type 1 collagen, suggesting a potential role in growth regulation [78]. At this point, the great influence of collagen on mechanical properties of bones should be mentioned, which is assumed to apply equally to humans [79] and chickens [80]. That *PODN* could be a promising candidate for bone integrity in laying hens is supported by findings of Raymond et al. [15]. Although the *G-patch domain containing 1* gene (*GPATCH1*), identified as BMD candidate, is considered a candidate gene for osteoporosis in humans [55], functional information is limited and its role in skeletal pathophysiology is not yet clear.

For a group of three candidates, the literature suggests a functional relationship with osteoarthritis, a pathological condition of cartilage degradation [81]. Osteoarthritis and osteoporosis are closely related and characterised by subchondral bone loss and excessive bone resorption [20, 81, 82]. It is assumed that both diseases are partly determined by common genes [83]. One of the candidates found in our study is the *MCF.2 cell line derived transforming sequence like* gene (*MCF2L*), shown to be expressed in cartilage tissue, and linked to joint osteoarthritis in humans [54, 84]. In addition, Mao et al. [85] recently pointed out the relevance of *MCF2L* for osteoporosis, which underlines the link between both disorders. The *asporin* gene (*ASPN*), also known as *biglycan* (*BGN*), is assumed to regulate chondrogenesis. While the results of Mishra et al. [63] point to a functional role of *ASPN* in osteoarthritis, other studies reported only a marginal relationship or contradict such an association [86, 87]. Given these contradictory results and the fact that the association with *ASPN* was not significant in our study, we consider *ASPN* a suggestive candidate that requires further investigation. The *SRY-box 9* gene (*SOX9*) is our third candidate linked to osteoarthritis [88]. *SOX9* is considered a pivotal player in chondrogenesis, as its protein, the transcription factor SOX9, was shown to stimulate chondrocyte differentiation [64, 89]. In addition, *SOX9* mediates the Wnt signalling pathway, abnormalities of which are correlated with cartilage degradation [64].

The remaining candidates, i.e. the *SFRP4*, *WNT3*, and *WNT9B* genes, are functionally linked to the Wnt signalling pathway, which plays a key role in various basic developmental processes [90]. The *secreted frizzled related protein 4* gene (*SFRP4*) encodes a protein that primarily antagonizes Wnt polypeptides [90] and is one of the BMD candidates. A mutation in *SFRP4* was shown to cause pathological reduction of cortical bone tissue in mice and humans [57]. The Wnt signalling pathway is crucial for bone metabolism and to date, several Wnt genes are known to be associated with traits such as bone mass and BMD [55, 91]. This also includes the *Wnt family member 3* gene (*WNT3*), which was identified in this study [55, 88]. The *Wnt family member 9B* gene (*WNT9B*), located adjacent to *WNT3* on GGA 27, was identified as a candidate for BBS. Although its role in skeletal biology is less explored than that of other Wnt genes, we consider *WNT9B* a susceptibility gene for bone strength due to its association with femur BMD [65]. The high importance of the Wnt signalling pathway for bone strength is supported by the significant enrichment that was shown in the GSA for this functional pathway. Furthermore, the *mitogen-activated protein kinase* (*MAPK*) signalling pathway was enriched, which is also very important for skeletal development and, in particular, for chondrogenesis [92]. These observations are in accordance with recent results from pathway analyses [25, 93].

Taken together, we identified a number of genetic loci associated with the bone traits studied. Based on these findings, we can confirm the assumption that bone stability is determined by multiple genes, each of which has a rather small effect size. The genes presented here represent suggestive susceptibility genes of bone integrity in chickens, some of which are nonetheless very promising based on what is known so far. Follow-up studies will

be required to determine causalities and further uncover the biological significance of these genes. Here, the use of an F2 mapping population for high-resolution mapping of loci is recommended [94]. Considering the animal model, a follow-up study should also investigate the influence of phylogenetic origin on bone phenotypic plasticity, which was not done here, as we focused on finding loci that are significant for laying hens across phylogenetically divergent layer lines.

5 Conclusions

In this study, association analyses were performed to identify loci related with bone integrity in laying hens. In the subsequent functional analyses, a set of 16 promising candidate genes was identified, although in some cases rather small SNP effect estimates were observed. Some of the genes were shown to be involved in pivotal pathways that regulate bone metabolism. Our results strongly support genetics as a crucial factor that contributes significantly to the regulation of bone strength and thus offers great opportunities to improve bone health in laying hens. Further functional analyses on the candidate genes identified at a suggestive level have to follow in order to confirm their biological significance.

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Supplementary Materials

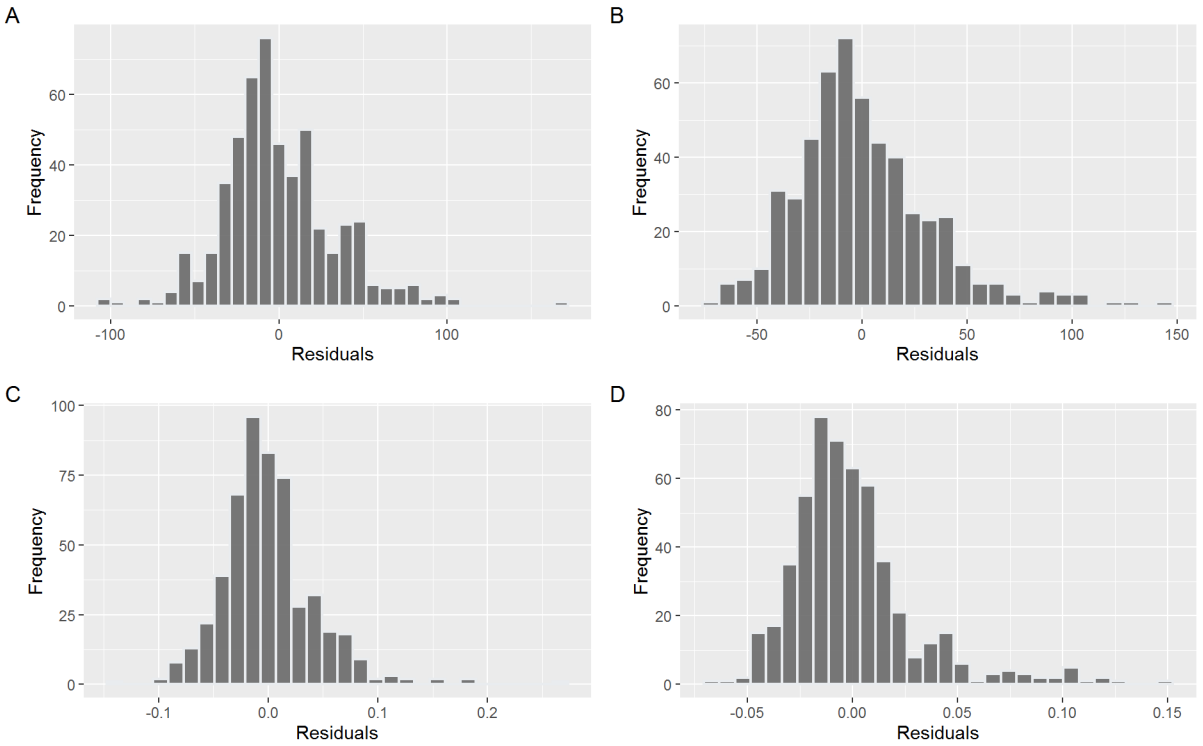


Figure S4.1: Histograms of the residuals for the bone breaking strengths of the tibiotarsus (**A**) and humerus (**B**), and the bone mineral densities of the tibiotarsus (**C**) and humerus (**D**).

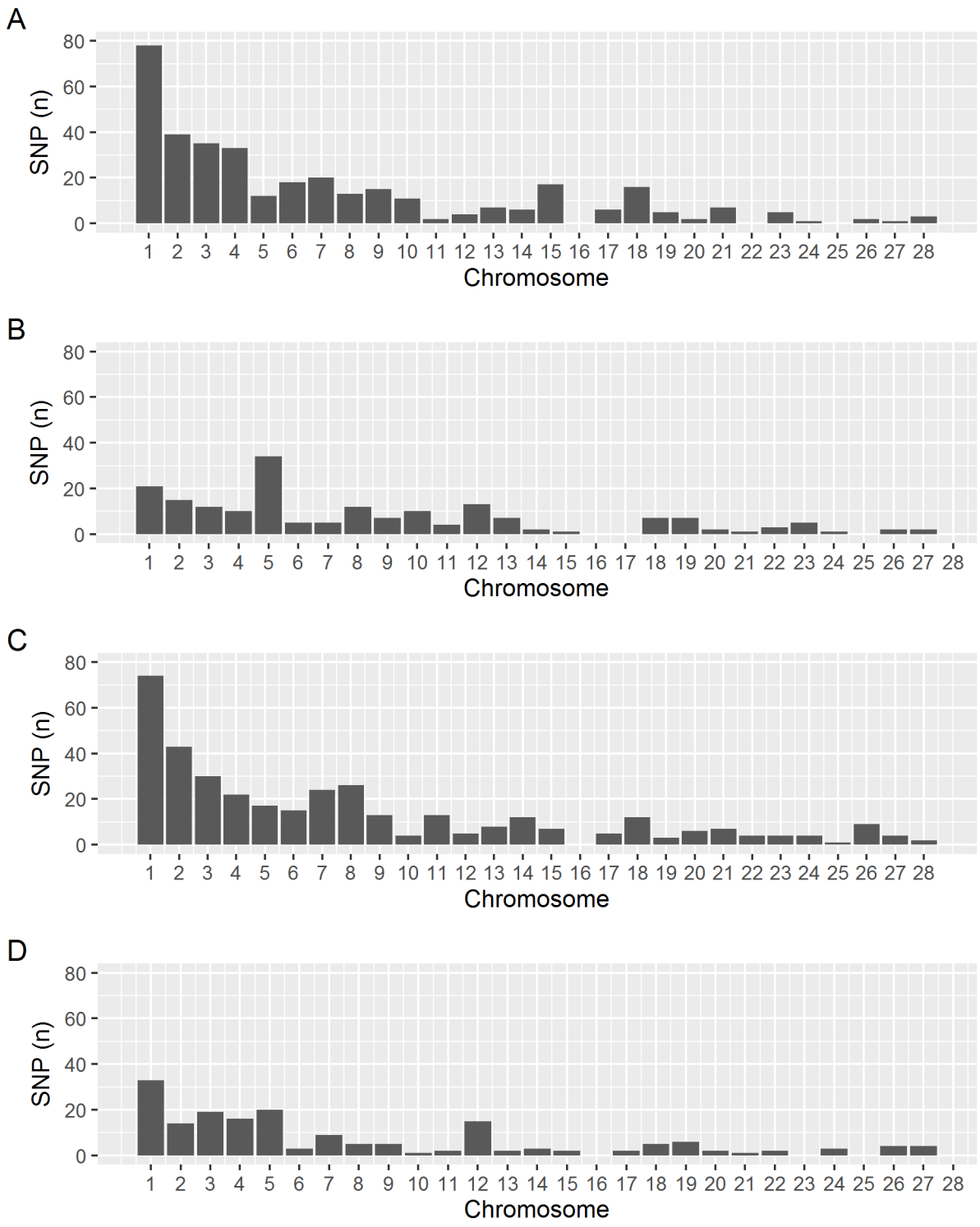
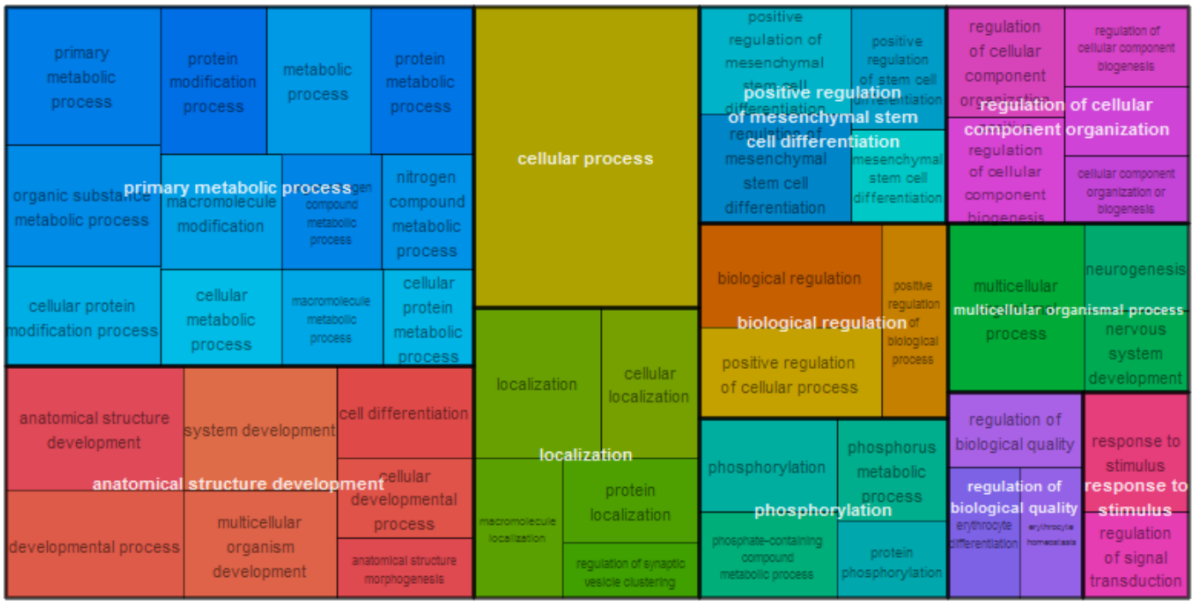


Figure S4.2: Results from the Random Forests classification: Number of annotated SNPs per chromosome for the bone breaking strengths of the tibiotarsus (**A**) and humerus (**B**), and the bone mineral densities of the tibiotarsus (**C**) and humerus (**D**).

A



B

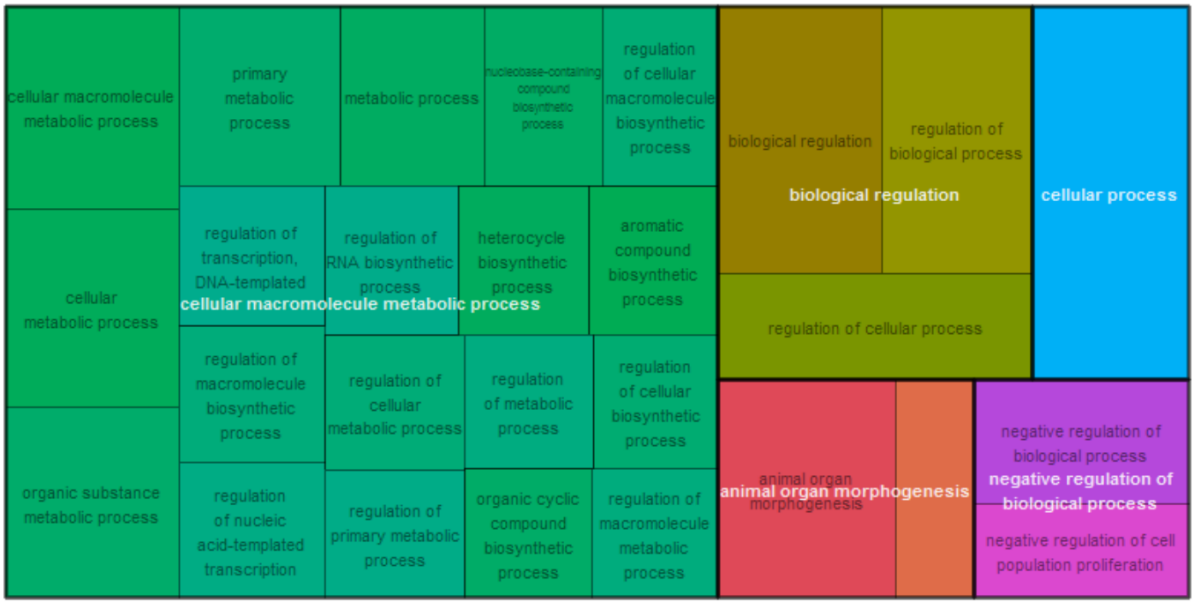
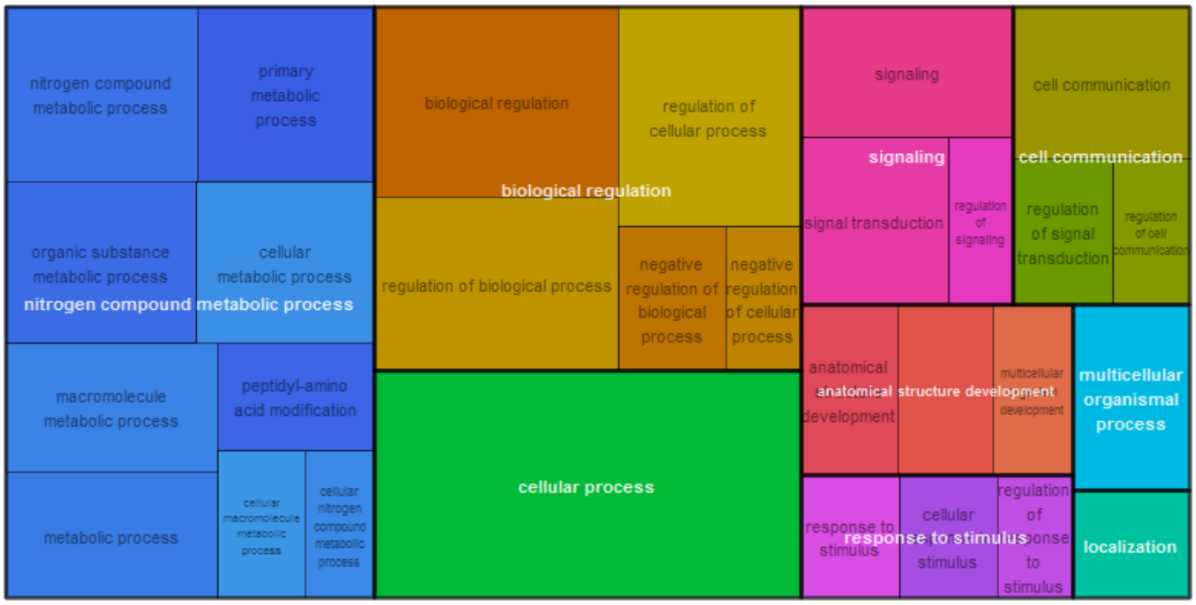


Figure S4.3: Tree maps of significantly enriched Gene Ontology (GO) terms of the category biological processes for genes associated with the bone breaking strengths of the tibiotarsus (**A**) and humerus (**B**). Each colour indicates a parent GO term drawn as a box in which the lower-level terms are plotted. The space filled by the terms is proportional to their -$\log_{10}$ *p*-values.

A



B

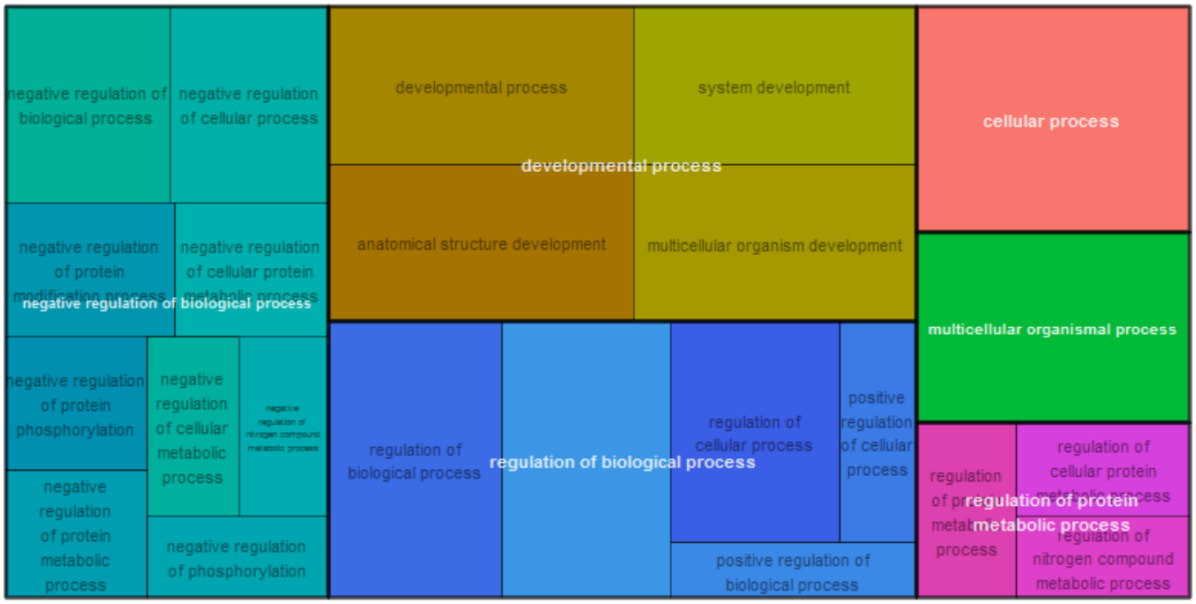


Figure S4.4: Tree maps of significantly enriched Gene Ontology (GO) terms of the category biological processes for genes associated with the bone mineral densities of the tibiotarsus (**A**) and humerus (**B**). Each colour indicates a parent GO term drawn as a box in which the lower-level terms are plotted. The space filled by the terms is proportional to their -$\log_{10}$ p-values.

Table S4.1: List of all genes extracted for the bone breaking strengths (BBS) or bone mineral densities (BMD) of the tibiotarsus (Tib) and humerus (Hum) obtained from the genome-wide association studies.

Ensembl gene id	Chr.	Start	End	Gene name
Unique in Tib_BBS				
ENSGALG00000053594	1	133320865	133325014	—
ENSGALG00000016767	1	133973567	134294259	AFF3
ENSGALG00000037326	5	27522846	27570512	MAP3K9
ENSGALG00000009387	5	27699720	27751679	SYNJ2BP
ENSGALG00000009421	5	28087093	28091730	SRSF5
ENSGALG00000009587	5	29263703	29530569	GPHN
ENSGALG00000009611	5	29687623	29720743	EIF2AK4
ENSGALG00000009619	5	29722191	29753143	GPR176
ENSGALG00000001907	6	2968233	3091445	WAPL
ENSGALG00000001934	6	3059913	3082527	OPN4
ENSGALG00000001977	6	3104861	3175701	LDB3
ENSGALG00000008939	8	19243036	19264136	FUBP1
ENSGALG00000008945	8	19269326	19285928	NEXN
ENSGALG00000008951	8	19297753	19332508	MIGA1
ENSGALG00000006194	20	9910901	9915465	SLC52A3
Unique in Tib_BMD				
ENSGALG00000050866	1	79311694	79327367	HSD3B1
ENSGALG00000014766	1	79328749	79338994	HAO2
ENSGALG00000016379	1	120425336	120468539	SMS
ENSGALG00000016382	1	120491889	120522883	MBTPS2
ENSGALG00000019157	1	120533946	120578001	SMPX
ENSGALG00000016406	1	121157720	121225454	RPS6KA3
ENSGALG00000016420	1	121385060	121606919	SH3KBP1
ENSGALG00000016426	1	121619269	121702537	MAP3K15
ENSGALG00000016430	1	121691899	121704059	PDHA2
ENSGALG00000016511	1	121777765	121819130	ADGRG2
ENSGALG00000016518	1	121822368	121859658	PHKA2
ENSGALG00000016522	1	121861691	121891693	PPEF1
ENSGALG00000016523	1	121900872	121912941	RS1
ENSGALG00000016529	1	121923761	122022946	CDKL5
ENSGALG00000016537	1	122078244	122134183	SCML2
ENSGALG00000016538	1	122146582	122148228	RAI2
ENSGALG00000045584	4	10959282	10973769	VSIG10L
ENSGALG00000028222	4	10977328	10983991	LOC422295
ENSGALG00000016034	4	89176735	89316390	ATRN
ENSGALG00000038543	7	20946474	21036251	KCNH7
ENSGALG00000041192	7	21044187	21071090	IFIH1
ENSGALG00000011099	7	21075293	21113292	FAP
ENSGALG00000011132	7	21626535	21764185	RBMS1
ENSGALG00000011149	7	21828489	21864267	PLA2R1
ENSGALG00000011172	7	21930914	21996451	MYO10L
ENSGALG00000053241	7	22082731	22084314	LOC424199
ENSGALG00000011250	7	22086695	22105857	ASIC4
ENSGALG00000011252	7	22129021	22132591	LOC429032
ENSGALG00000008733	8	16810670	16822126	LPAR3
ENSGALG00000038242	12	1874442	2001509	CACNA2D2
ENSGALG00000052837	14	3778529	3801415	—
ENSGALG00000026119	15	7779109	7810799	MN1
ENSGALG00000005654	15	7819084	7836307	PITPNB
ENSGALG00000003476	18	6755211	6787645	ERN1
ENSGALG00000003496	18	6791861	6805476	TEX2
ENSGALG00000033569	21	508552	539804	—
ENSGALG00000039187	22	405357	409427	GKN2
ENSGALG00000001812	28	1582029	1594481	ATCAY
Unique in Hum_BMD				
ENSGALG00000053663	10	12005808	12033052	—
Common in Tib_BBS and Tib_BMD				
ENSGALG00000016391	1	120600351	120804008	CNKSR2

Table S4.2: List of all genes extracted for the bone breaking strengths (BBS) or bone mineral densities (BMD) of the tibiotarsus (Tib) and humerus (Hum) obtained from the Random Forests classification.

Ensembl gene id	Chr.	Start	End	Gene name
Unique in Tib_BBS				
ENSGALG00000031975	1	1491840	1533752	UBE2H
ENSGALG00000008048	1	1584921	1594275	CPA2
ENSGALG00000027296	1	1595635	1599967	CPA5
ENSGALG00000008087	1	1600797	1604593	CPA1
ENSGALG00000038834	1	11833981	12556826	MAGI2
ENSGALG00000008167	1	13903212	13955809	KMT2E
ENSGALG00000008154	1	13958935	14006452	SRPK2
ENSGALG00000043872	1	15584706	15614988	—
ENSGALG00000004897	1	15836984	15848465	GTSE1
ENSGALG00000009596	1	30234476	30430729	TMEM117
ENSGALG00000009601	1	30463434	30606675	NELL2
ENSGALG00000011325	1	45339203	45346941	NDUFA12
ENSGALG00000011327	1	45354985	45399450	NR2C1
ENSGALG00000012488	1	51511486	51518776	TST
ENSGALG00000012490	1	51519368	51524961	—
ENSGALG00000012530	1	51716683	51725962	FOXRED2
ENSGALG00000012532	1	51727168	51734903	TXN2
ENSGALG00000032768	1	65833774	66173568	SOX5
ENSGALG00000047534	1	67946668	67956166	—
ENSGALG00000014106	1	68191397	68291071	PPFIBP1
ENSGALG00000014178	1	68564109	68765156	SCUBE1
ENSGALG00000014561	1	77994636	78014698	PTPN6
ENSGALG00000014567	1	78015335	78020461	PHB2
ENSGALG00000014568	1	78020566	78022842	EMG1
ENSGALG00000014687	1	78162527	78195976	EPHA1
ENSGALG00000015367	1	89486875	89545180	NECTIN3
ENSGALG00000015378	1	89812172	89817466	ABHD10
ENSGALG00000019157	1	120533946	120578001	SMPX
ENSGALG00000016410	1	121229301	121238473	EIF1AX
ENSGALG00000016415	1	121300649	121335637	MAP7D2
ENSGALG00000016522	1	121861691	121891693	PPEF1
ENSGALG00000016545	1	122667268	122766511	REPS2
ENSGALG00000053594	1	133320865	133325014	—
ENSGALG00000016832	1	138904758	138912133	PROZ
ENSGALG00000054981	1	138914225	138918510	—
ENSGALG00000016941	1	164781979	164862248	PCDH17
ENSGALG00000013489	1	186228663	186241876	CCDC82
ENSGALG00000006173	2	6172986	6255970	XYLB
ENSGALG00000007408	2	15436178	15577480	MPP7
ENSGALG00000040028	2	44367312	44413014	GLB1
ENSGALG00000050380	2	44408018	44413032	TMPPE
ENSGALG00000032647	2	44615726	44749055	CLASP2
ENSGALG00000037940	2	54930529	54947966	IGFBP3
ENSGALG00000013073	2	79940291	79950017	UPP1
ENSGALG00000044675	2	127306456	127363344	MATN2
ENSGALG00000031700	2	134839746	135006696	TRPS1
ENSGALG00000029596	2	137356485	137374399	HAS2
ENSGALG00000036085	2	148823679	148841111	—
ENSGALG00000008779	3	10612572	10632536	ACTR2
ENSGALG00000008734	3	12111551	12286838	PCSK2
ENSGALG00000009612	3	19449227	19512091	TGFB2
ENSGALG00000009652	3	20458579	20827409	USH2A
ENSGALG00000010538	3	31481892	31584930	TTC27
ENSGALG00000032440	3	33691214	33705352	QPCT
ENSGALG00000010635	3	33929665	33952049	—
ENSGALG00000010641	3	33951857	33961883	SCCPDH
ENSGALG00000011560	3	44391258	44584053	PACRG
ENSGALG00000011562	3	44583493	45261117	PRKN
ENSGALG00000012971	3	49020942	49053025	CCDC170
ENSGALG00000013754	3	52353589	52378280	PLAGL1
ENSGALG00000013756	3	52378552	52388126	ZC2HC1B
ENSGALG00000013803	3	52959497	53067360	ADGRG6
ENSGALG00000015465	3	70672876	70939143	ASCC3
ENSGALG00000015517	3	72530453	72641635	KLHL32
ENSGALG00000015808	3	76451007	76467565	SLC35A1
ENSGALG00000016174	3	83099425	83166464	LMBRD1
ENSGALG00000035016	3	95853920	95856597	ID2
ENSGALG00000041900	4	2475257	2530889	DLG3
ENSGALG00000010061	4	32846926	33218988	LRBA
ENSGALG00000030065	4	40053454	40498128	TENM3
ENSGALG00000012015	4	56092083	56195330	NDST4
ENSGALG00000033286	4	56443111	56482183	ARSJ
ENSGALG00000012222	4	59688409	59777549	RAP1GDS1
ENSGALG00000041121	4	74981753	75225786	SLIT2
ENSGALG00000014425	4	75726455	75920712	NCAPG
ENSGALG00000054173	4	75879121	75894354	—
ENSGALG00000015623	4	81930559	81966939	HGFAC
ENSGALG00000050742	4	83238778	83290229	WHSC1
ENSGALG00000006192	5	12148432	12193954	USH1C
ENSGALG00000009387	5	27699720	27751679	—
ENSGALG00000009587	5	29263703	29530569	GPHN
ENSGALG00000009801	5	31651198	31766696	C5H15orf41
ENSGALG00000010117	5	36951399	37178495	SLC25A21
ENSGALG00000020402	5	43821392	43876997	EFCAB11
ENSGALG00000011811	5	53231805	53388473	SYNE2
ENSGALG00000011889	5	54228533	54333650	PRKCH
ENSGALG00000012017	5	55009316	55090253	DAAM1
ENSGALG00000053215	6	2282853	2284631	—
ENSGALG00000001907	6	2968233	3091445	WAPL
ENSGALG00000001934	6	3059913	3082527	OPN4
ENSGALG00000001977	6	3104861	3175701	LDB3
ENSGALG00000004222	6	11585402	11612168	HK1
ENSGALG00000004257	6	11622065	11633629	TSPAN15
ENSGALG00000051554	6	11674438	11711662	—
ENSGALG00000004325	6	11904936	11935569	P4HA1
ENSGALG00000004372	6	12061891	12152583	MICU1
ENSGALG00000005893	6	18594248	18614513	ZFAND4
ENSGALG00000009445	6	31268250	31304876	INPP5F
ENSGALG00000043307	7	187764	224770	HIBCH
ENSGALG00000003812	7	4582880	4684385	UBE2F
ENSGALG00000003821	7	4685059	4695434	RAMP1
ENSGALG00000003836	7	4695620	4701337	—
ENSGALG00000003862	7	4706293	4793733	LRRFIP1
ENSGALG00000023419	7	16275972	16277643	HOXD4
ENSGALG00000011515	7	23626750	23643336	ERCC3
ENSGALG00000011697	7	26927568	26942616	SEC22A
ENSGALG00000011708	7	27172896	27356465	MYLK
ENSGALG00000012156	7	29190336	29372124	DPP10
ENSGALG00000012382	7	31584693	31720416	—
ENSGALG00000012526	7	35550399	35568418	PRPF40A
ENSGALG00000021047	8	5375820	5440117	KIFAP3
ENSGALG00000036264	8	5441379	5454440	SCYL3
ENSGALG00000005647	8	13947647	13974017	ABCD3
ENSGALG00000005850	8	14265560	14318955	FNBP1L
ENSGALG00000029582	8	14456259	14525294	EVI5
ENSGALG00000043650	8	14533300	14542952	GFI1
ENSGALG00000031652	8	19432096	19518959	AK5
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ENSGALG00000006655	9	3763611	3895836	MAP3K13
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ENSGALG00000005788	9	5174155	5238789	FARP2
ENSGALG00000007253	9	14006895	14047894	IL1RAP
ENSGALG00000026862	9	14097822	14108342	CLDN1
ENSGALG00000040866	9	14139404	14193800	P3H2
ENSGALG00000007337	9	14499435	14783213	LPP
ENSGALG00000007595	9	15206056	15256069	LRCH3
ENSGALG00000040938	9	18880997	19157283	NLGN1
ENSGALG00000050897	9	19725004	19825188	TNIK
ENSGALG00000044085	9	22492741	22501127	IQCJ
ENSGALG00000043506	10	1476303	1479577	NMB
ENSGALG00000001362	10	2929279	2937676	CLK3
ENSGALG00000026674	10	4240576	4273507	CIB2
ENSGALG00000004087	10	6784258	7036593	THSD4
ENSGALG00000047105	10	13308949	13342922	—
ENSGALG00000032073	10	13322631	13341683	—
ENSGALG00000046779	10	14523385	14545340	—
ENSGALG00000001945	12	1593635	1640711	RFT1
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ENSGALG00000004760	15	6543749	6557011	TMEM116
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ENSGALG00000041821	15	6563515	6588861	NAA25
ENSGALG00000043208	15	6590284	6599691	TRAFD1
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ENSGALG00000004840	15	6743586	6745464	—
ENSGALG00000043053	15	6902659	6911582	DAO
ENSGALG00000034278	15	6912999	6930693	SVOP
ENSGALG00000013848	15	7068522	7079367	MVK
ENSGALG00000005885	15	8150942	8161390	ESS2
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ENSGALG00000006648	15	8803892	8857762	SPECC1L
ENSGALG00000007147	15	9560589	9561338	—
ENSGALG00000040869	15	9561568	9563173	—
ENSGALG00000007153	15	9563293	9565829	DYL1
ENSGALG00000046877	15	9563746	9566046	—
ENSGALG00000007185	15	9565975	9572193	COQ5
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ENSGALG00000005066	17	5233790	5239994	DPM2
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ENSGALG00000005063	17	5241787	5243598	ST6GALNAC4
ENSGALG00000043394	17	5692033	5729186	NUP188
ENSGALG00000053552	17	5837944	5838933	IER5L
ENSGALG00000017357	18	4241336	4245766	SRSF2
ENSGALG00000001790	18	4245590	4247698	METTL23
ENSGALG00000001802	18	4247802	4258928	JMJD6
ENSGALG00000002993	18	5153057	5313472	CA10
ENSGALG00000003011	18	5812118	5833003	TOM1L1
ENSGALG00000027190	18	5833851	5836668	COX11
ENSGALG00000004386	18	8785940	8789042	SOX9
ENSGALG00000004583	18	9145624	9173263	NPLOC4
ENSGALG00000034210	18	9342248	9461097	RPTOR
ENSGALG00000007364	18	10077443	10079309	TOB1
ENSGALG00000001031	19	769653	792925	—
ENSGALG00000002312	19	4900519	4918418	TMEM132E
ENSGALG00000006062	20	9569513	9605598	ZBTB46

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Table S4.2: (*continued*)

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ENSGALG00000041708	21	6313309	6325046	WNT4
ENSGALG00000001329	23	2743037	2816538	EPB41
ENSGALG00000029653	23	2842969	2885597	—
ENSGALG00000032763	23	2898792	2905063	MECR
ENSGALG00000002499	23	4494059	4529122	—
ENSGALG00000006969	24	4453214	4474869	USP28
ENSGALG00000001512	26	3298602	3392361	KCND3
ENSGALG00000040017	27	7106164	7164078	THRA
ENSGALG00000029142	27	7109348	7115931	—
ENSGALG00000053742	28	1160094	1165845	—
ENSGALG00000002085	28	1169318	1181306	FZR1
ENSGALG00000032469	28	3019600	3031885	ARHGAP45
ENSGALG00000002591	28	3032188	3034572	POLR2E
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ENSGALG00000033051	1	6924409	7137720	CAMK1D
ENSGALG00000010177	1	36454512	36492133	ZFC3H1
ENSGALG00000033589	1	53021721	53223751	SYN3
ENSGALG00000037807	1	77649727	77654873	—
ENSGALG00000050127	1	77655239	77658122	—
ENSGALG00000046776	1	77658563	77661179	—
ENSGALG00000050866	1	79311694	79327367	HSD3B1
ENSGALG00000014766	1	79328749	79338994	HAO2
ENSGALG00000040620	1	82041116	83002376	LSAMP
ENSGALG00000015230	1	84810190	84899752	NME7
ENSGALG00000015511	1	97569877	97995865	ROBO1
ENSGALG00000040926	1	99493728	99545821	SAMSN1
ENSGALG00000016755	1	133708781	133728300	—
ENSGALG00000040621	1	135205160	135223617	IL18R1
ENSGALG00000016788	1	135229034	135244807	IL18RAP
ENSGALG00000016834	1	138951176	139094938	MCF2L
ENSGALG00000016872	1	145418480	145689429	PCCA
ENSGALG00000042339	1	175449309	175545587	—
ENSGALG00000025748	1	179686487	179717720	FGF9
ENSGALG00000019042	1	194292601	194303042	ALG8
ENSGALG00000029172	1	194303527	194307071	NDUFC2
ENSGALG00000052452	1	194308712	194309570	—
ENSGALG00000000681	1	194484628	194551701	PAK1
ENSGALG00000040478	1	194700308	194747859	CAPN5
ENSGALG00000000755	1	194764118	194819371	ACER3
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ENSGALG00000006233	2	6578374	6618231	GALNT11
ENSGALG00000041791	2	7565007	7718788	DPP6
ENSGALG00000009500	2	22944848	23062879	VPS50
ENSGALG00000009509	2	23060807	23197789	CALCR
ENSGALG00000030455	2	32987174	33160334	JAZF1
ENSGALG00000039092	2	45701178	45771546	STAC
ENSGALG00000031997	2	46095977	46105425	SFRP4
ENSGALG00000039139	2	55050336	55235133	TNS3
ENSGALG00000012681	2	59566193	59625203	MBOAT1
ENSGALG00000012702	2	61065775	61271210	JARID2
ENSGALG00000013081	2	80493427	80551989	VWC2
ENSGALG00000043570	2	115207256	115264028	SGK3
ENSGALG00000031131	2	126048980	126075321	VIRMA
ENSGALG00000031076	2	126080168	126109221	ESRP1
ENSGALG00000037955	2	126250048	126274919	INTS8
ENSGALG00000031282	2	128985552	129064019	UBR5
ENSGALG00000035199	2	129063965	129086910	ODF1
ENSGALG00000034099	2	129172101	129193565	ATP6V1C1
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ENSGALG00000026600	3	40608347	40628562	C1orf198
ENSGALG00000032780	3	40629526	40666062	TTC13
ENSGALG00000034030	3	57964558	58039567	L3MBTL3
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ENSGALG00000015605	3	75307454	75404609	BACH2
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ENSGALG00000028709	3	95000776	95059703	RNF144A
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ENSGALG00000007151	4	9426824	9460455	—
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ENSGALG00000043106	4	44309648	44347593	WDR17
ENSGALG00000010932	4	45696109	45702330	NUDT9
ENSGALG00000010963	4	45702438	45709564	—
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ENSGALG00000038269	5	514262	515368	—
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ENSGALG00000037195	5	712496	717693	TALDO1
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ENSGALG00000008553	5	24663248	24738505	INO80
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ENSGALG00000011861	5	54047342	54104268	SYT16
ENSGALG00000002942	6	8516356	8663452	JMJD1C
ENSGALG00000031534	6	8972573	9078848	ARID5B
ENSGALG00000003526	6	10033025	10057694	TUBGCP2
ENSGALG00000003533	6	10057707	10062489	ZNF511
ENSGALG00000030677	6	18374424	18392107	ABCG2
ENSGALG00000008281	6	24677212	24753757	NEURL1
ENSGALG00000008883	6	28006744	28178462	TCF7L2
ENSGALG00000009152	6	29096845	29512936	ATRNL1
ENSGALG00000010461	6	35112704	35230061	EBF3
ENSGALG00000004288	7	6215668	6433721	HDAC4
ENSGALG00000008621	7	12666450	12744588	NRP2
ENSGALG00000014209	7	16882459	16902727	GPR155
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ENSGALG00000014276	7	16914151	16935072	CIR1
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ENSGALG00000038543	7	20946474	21036251	KCNH7
ENSGALG00000041192	7	21044187	21071090	IFIH1
ENSGALG00000011132	7	21626535	21764185	RBMS1
ENSGALG00000011149	7	21828489	21864267	PLA2R1
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ENSGALG00000003510	8	5888634	5903888	UCK2
ENSGALG00000026263	8	5960767	5974556	RGS8
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ENSGALG00000003933	8	6368170	6376220	QSOX1
ENSGALG00000004273	8	6682209	6791752	RALGPS2
ENSGALG00000004290	8	6716174	6733176	ANGPTL1
ENSGALG00000001863	8	16869042	16889747	VTG2
ENSGALG00000010440	8	22150522	22174621	MKNK1
ENSGALG00000025946	8	22179851	22194222	MOB3C
ENSGALG00000040113	9	1566800	1567411	SFT2D3
ENSGALG00000053889	9	1580200	1582884	—
ENSGALG00000002155	9	1733842	1899914	HS6ST1
ENSGALG00000005550	9	5412062	5444046	ST6GAL1
ENSGALG00000007767	9	15555615	15719509	DIS3L2
ENSGALG00000030420	9	15727596	15733606	ECEL1
ENSGALG00000042612	9	15737715	15748501	—
ENSGALG00000009355	9	19935349	19943732	CLDN11
ENSGALG00000004004	11	7505176	7578698	PHKB
ENSGALG00000004839	11	10018024	10138369	GPATCH1
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ENSGALG00000012978	11	15268801	15320700	CDYL2
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ENSGALG00000024379	13	3147680	3166499	ECSCR
ENSGALG00000002457	13	3246934	3338390	SIL1
ENSGALG00000006057	13	14827875	14836883	CLK4
ENSGALG00000003596	14	1723283	1795839	TRRAP
ENSGALG00000043806	15	5158427	5211083	DNAH10
ENSGALG00000026119	15	7779109	7810799	MN1
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ENSGALG00000028810	15	8254366	8255372	CHCHD10
ENSGALG00000007247	15	9679685	9721617	TGFB1I1
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ENSGALG00000050837	18	2441277	2442356	—
ENSGALG00000001375	18	2594690	2691529	COX10
ENSGALG00000001503	18	3231950	3341251	TBCD
ENSGALG00000040244	18	3765314	3832983	SEPTIN9
ENSGALG00000001892	18	4342538	4349605	CYGB
ENSGALG00000003015	19	5554574	5561459	SERPINF1
ENSGALG00000003031	19	5561964	5568555	SMYD4
ENSGALG00000028210	19	5945913	5947011	—
ENSGALG00000026315	19	5946988	5949553	RAB34
ENSGALG00000003966	19	5949676	5952152	RPL23A
ENSGALG00000003973	19	5955265	5957173	TLCD1
ENSGALG00000039155	20	4936185	4966601	ZHX3
ENSGALG00000055078	20	4966750	4971884	—
ENSGALG00000003815	20	5019515	5049331	RPN2
ENSGALG00000003842	20	5042743	5050245	GHRH
ENSGALG00000004028	20	5162754	5167745	MATN4
ENSGALG00000004098	20	5185964	5224532	STK4
ENSGALG00000004110	20	5224028	5228641	—
ENSGALG00000033569	21	508552	539804	—
ENSGALG00000002222	21	2886877	2919910	NOC2L
ENSGALG00000001608	22	2087959	2217683	UNC5D
ENSGALG00000001283	23	2679438	2696177	SRRM1
ENSGALG00000030900	23	2696846	2700720	NCMAP
ENSGALG00000001310	23	2708287	2723179	TRNAU1AP
ENSGALG00000040857	24	3565031	3587794	TECTA
ENSGALG00000007794	24	5740654	5762035	DRD2
ENSGALG00000043021	25	3671693	3678692	HORMAD1
ENSGALG00000014640	25	3675357	3682115	GOLPH3L
ENSGALG00000037480	25	3682741	3686986	ENSA
ENSGALG00000000142	26	1041982	1065980	CEPT1
ENSGALG00000000583	26	1695658	1714634	SOX13
ENSGALG00000000856	26	2507117	2516979	EIF2D
ENSGALG00000000863	26	2516995	2523234	DYRK3
ENSGALG00000001091	26	2605514	2611145	—
ENSGALG00000037597	26	2615310	2619004	YOD1
ENSGALG00000001117	26	2618673	2632975	PFKFB2
ENSGALG00000023953	26	2635048	2641917	—
ENSGALG00000001264	26	2758582	2845137	PLXNA2
ENSGALG00000000367	27	4187017	4266979	ASIC2
ENSGALG00000034204	27	7412912	7417642	—
ENSGALG00000000470	28	729616	757180	LMNA
ENSGALG00000042900	28	3557584	3601481	GATAD2A

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Table S4.2: (*continued*)

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ENSGALG00000008144	1	1619330	1638626	COPG2
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ENSGALG00000013069	1	62167702	62186545	BPGM
ENSGALG00000015379	1	89826120	89837361	TAGLN3
ENSGALG00000017047	1	173658067	173691648	SUPT20H
ENSGALG00000017165	1	181692016	181714409	CUL5
ENSGALG00000042992	1	190815780	191167322	—
ENSGALG00000006443	1	195587654	195590524	MADPRT1
ENSGALG00000000868	1	195591204	195602075	RNF121
ENSGALG00000017493	1	195602594	195605798	TRPC2L
ENSGALG00000005710	2	4568785	4637109	CTDSPL
ENSGALG00000041154	2	8624610	8627626	MNX1
ENSGALG00000006652	2	9990437	10275976	DIP2C
ENSGALG00000007351	2	14995874	15011632	MTPAP
ENSGALG00000037769	2	18124237	18368109	NEBL
ENSGALG00000029235	2	41858803	42075903	CPNE4
ENSGALG00000035505	2	46604093	46670393	AOAH
ENSGALG00000008725	3	5496073	5630444	KIF16B
ENSGALG00000001661	3	16081562	16095818	SLC22A7
ENSGALG00000008614	3	16096879	16112242	TTL
ENSGALG00000045514	3	16135916	16149641	PEX6
ENSGALG00000037576	3	16155290	16157946	VSX1
ENSGALG00000010050	3	28348991	28495437	LRFN2
ENSGALG00000011571	3	45283852	45353246	AGPAT4
ENSGALG00000038782	3	48204075	48212692	ZC3H12D
ENSGALG00000053107	3	58190054	58231715	—
ENSGALG00000013651	3	60217079	60270213	RNF217
ENSGALG00000039756	3	60319457	60834072	NKAIN2
ENSGALG00000009830	4	29651474	29681776	MGAT4D
ENSGALG00000028643	4	30838745	31010530	ANAPC10
ENSGALG00000009948	4	30847852	30924570	HHIP
ENSGALG00000035482	4	37267897	37308644	SEC24B
ENSGALG00000005716	5	9248870	9478933	SBF2
ENSGALG00000005986	5	10457958	10515004	TUB
ENSGALG00000006035	5	10693066	10768567	PDE3B
ENSGALG00000032558	5	10855015	10936272	INSC
ENSGALG00000006873	5	16064998	16084574	—
ENSGALG00000029260	5	16217236	16252397	H-RAS
ENSGALG00000035023	5	56658775	56665015	CNIH1
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ENSGALG00000009495	6	31834704	31906976	FGFR2
ENSGALG00000042374	7	15549638	15673377	—
ENSGALG00000011630	7	25779538	25958144	GLI2
ENSGALG00000012470	7	34907319	34912403	LYPD6
ENSGALG00000002182	8	1987324	2069077	NR5A2
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ENSGALG00000005203	8	11872299	11926941	OLFM3
ENSGALG00000005580	8	13738852	13781167	TMEM56
ENSGALG00000008835	8	17017291	17029086	THAP10
ENSGALG00000036909	8	23047528	23885562	BEND5
ENSGALG00000010540	8	24366673	24408000	TTC39A
ENSGALG00000010543	8	24411559	24454049	EPS15
ENSGALG00000010570	8	24612084	24622313	BTF3L4
ENSGALG00000010805	8	25639131	25696695	USP24
ENSGALG00000006395	9	4871958	4876064	WDR53
ENSGALG00000006392	9	4879911	4889610	RNF168
ENSGALG00000006317	9	4943378	4951651	ING5
ENSGALG00000006305	9	4951779	4957263	DTYMK
ENSGALG00000033618	10	1858212	2010750	MYO9A
ENSGALG00000002883	10	3662745	3799455	SCAPER
ENSGALG00000002925	10	3810178	3840160	ETFA
ENSGALG00000006516	10	13083181	13104916	MTHFS
ENSGALG00000044204	10	13568712	13577891	ISG20
ENSGALG00000025898	10	19113380	19141564	SMAD6
ENSGALG00000002798	11	1901047	1949555	WDR59
ENSGALG00000005377	11	14060228	14531588	WWOX
ENSGALG00000002335	12	2360795	2623950	DOCK3
ENSGALG00000005909	12	9596109	9612130	GATA2
ENSGALG00000038684	12	9789679	9901939	EEFSEC
ENSGALG00000006526	12	11683446	11738536	PLXNB3
ENSGALG00000007168	12	12285419	12348377	CFAP20DC
ENSGALG00000002203	13	3919090	4012490	FGF18
ENSGALG00000002191	13	4053522	4055527	TLX3
ENSGALG00000028602	13	10211261	10285881	HMP19
ENSGALG00000038848	13	10514513	10519859	MSX2
ENSGALG00000004398	14	3830739	3870033	CARD11
ENSGALG00000009205	14	14438034	14559559	—
ENSGALG00000008201	15	11611346	11627560	FBXO21
ENSGALG00000008206	15	11628671	11631426	TESC
ENSGALG00000004333	18	7950536	7974012	ABCA5
ENSGALG00000004413	18	8903444	8963934	SLC39A11
ENSGALG00000001042	19	801748	844553	MTMR4
ENSGALG00000035131	19	859638	866958	—
ENSGALG00000050734	19	866383	869249	METTL27
ENSGALG00000005084	19	7313633	7334393	TRIM37
ENSGALG00000005103	19	7353284	7364909	GDPD1
ENSGALG00000028801	19	7369597	7400205	YPEL2
ENSGALG00000005295	19	7797174	8098869	BCAS3
ENSGALG00000005774	20	8729917	8777657	NKAIN4
ENSGALG00000003398	22	2737380	2757692	ADAM9
ENSGALG00000001604	24	2597820	2619876	—
ENSGALG00000001097	27	3494643	3509799	WNT9B

Ensembl gene id	Chr.	Start	End	Gene name
Unique in Hum_BMD				
ENSGALG00000054793	1	1291724	1300691	NCAPH2
ENSGALG00000046885	1	1300935	1301690	SCO2
ENSGALG00000039982	1	1303775	1313361	IRF5
ENSGALG00000008312	1	12815093	12861596	GSAP
ENSGALG00000046379	1	12862932	12874644	—
ENSGALG00000031340	1	38375611	38391308	E2F7
ENSGALG00000013143	1	64882883	65118721	PDE3A
ENSGALG00000016128	1	109397569	109410287	B3GALT5
ENSGALG00000016702	1	131249780	131299962	PPP2R3B
ENSGALG00000016736	1	131681532	131744672	CYFIP1
ENSGALG00000052027	1	131722616	131727023	—
ENSGALG00000032090	1	133848484	133859438	TXNDC9
ENSGALG00000016764	1	133860171	133890836	EIF5B
ENSGALG00000016843	1	140248816	140390143	COL4A2
ENSGALG00000016926	1	156971305	157226206	KLF12
ENSGALG00000016929	1	157476165	157591528	PIBF1
ENSGALG00000037015	1	158037290	158403765	DACH1
ENSGALG00000033671	1	172661162	172776030	FREM2
ENSGALG00000041263	1	174079522	174312779	DCLK1
ENSGALG00000045052	1	174520441	174521798	MAB21L1
ENSGALG00000019077	1	178515945	178724914	—
ENSGALG00000054619	1	193942684	194025658	GAB2
ENSGALG00000000830	1	195145039	195161469	—
ENSGALG00000000845	1	195484990	195565945	UVRAG
ENSGALG00000005401	2	2665702	2717022	WNT9A
ENSGALG00000007417	2	15599282	15670561	ARMC4
ENSGALG00000030866	2	126884462	127048750	CPQ
ENSGALG00000039587	3	15796600	15900738	SYNDIG1
ENSGALG00000010618	3	33708793	33721721	SLC30A6
ENSGALG00000010620	3	33727602	33759099	SPAST
ENSGALG00000010653	3	34048590	34412338	SMYD3
ENSGALG00000010664	3	34279395	34717416	KIF26B
ENSGALG00000028017	3	34734303	34757372	EFCAB2
ENSGALG00000010713	3	35221764	35325431	SDCCAG8
ENSGALG00000010737	3	35569976	35737294	PLD5
ENSGALG00000027036	3	61297796	61352281	PKIB
ENSGALG00000005936	4	2997857	3013197	AGTR2
ENSGALG00000008076	4	13698638	13736813	TMEM164
ENSGALG00000008404	4	14216213	14240493	LOC772071
ENSGALG00000008408	4	14256173	14306628	GAB3
ENSGALG00000039942	4	84541204	84762592	CTBP1
ENSGALG00000006904	5	16327023	16335748	RNH1
ENSGALG00000009415	5	27925918	28047170	SMOC1
ENSGALG00000010372	5	39066319	39104510	ANGEL1
ENSGALG00000043036	5	39074525	39084657	VASH1
ENSGALG00000048497	5	39103652	39113949	—
ENSGALG00000027255	5	39775061	40667438	NRXN3
ENSGALG00000034898	5	45101764	45230310	ITPK1
ENSGALG00000012145	5	56361766	56369688	TBPL2
ENSGALG00000012148	5	56371803	56387589	ATG14
ENSGALG00000012165	5	56433608	56454798	DLGAP5
ENSGALG00000008135	7	10794292	10924557	SATB2
ENSGALG00000009031	7	15017686	15069511	SESTD1
ENSGALG00000002246	8	2721709	2868051	DENND1B
ENSGALG00000003955	8	6384431	6436044	CEP350
ENSGALG00000008988	8	19375099	19428986	ZZZ3
ENSGALG00000011019	8	28332890	28375114	ROR1
ENSGALG00000006566	9	4522384	4530759	TBCCD1
ENSGALG00000006564	9	4531072	4544764	DNAJB11
ENSGALG00000023440	10	11304508	11323918	GABPB1
ENSGALG00000043672	12	1023267	1074387	STAB1
ENSGALG00000002016	12	1720584	1784240	PRKCD
ENSGALG00000002099	12	1786656	1827828	—
ENSGALG00000036263	12	2849850	2853236	—
ENSGALG00000050845	12	2853257	2855018	—
ENSGALG00000004932	12	4788083	4877625	ATG7
ENSGALG00000005001	12	5392472	5461851	CNBP
ENSGALG00000048543	12	12187651	12189390	RPP14
ENSGALG00000048013	12	12188216	12190575	HTD2
ENSGALG00000007052	12	12191823	12223412	PXK
ENSGALG00000007152	12	12271572	12275706	FAM3D
ENSGALG00000007172	12	12565610	12938688	FHIT
ENSGALG00000007927	12	17945860	18046961	CNTN6
ENSGALG00000008351	12	19676683	19680120	CAV3
ENSGALG00000004420	14	3925849	4284907	SDK1
ENSGALG00000007762	14	12891875	12969501	CREBBP
ENSGALG00000009217	14	14270201	14295203	TBC1D24
ENSGALG00000006728	15	9054070	9080058	SLC5A1
ENSGALG00000029893	18	8976176	8984896	COG1
ENSGALG00000026401	18	8983486	8992851	FAM104A
ENSGALG00000036742	19	2690575	2780557	GATSL2
ENSGALG00000013642	21	4968293	5020062	KAZN
ENSGALG00000000476	22	4410028	4620737	LRRTM4
ENSGALG00000038948	26	544801	573605	KDM5B
ENSGALG00000000397	26	574234	594203	IPO9
ENSGALG00000031175	26	595820	614752	LZTR1
ENSGALG00000037370	26	629113	668761	NAV1
ENSGALG00000047476	27	3245893	3246618	—
ENSGALG00000001079	27	3523220	3544732	WNT3
ENSGALG00000009943	27	5717774	5744311	FAM117A
ENSGALG00000035057	27	5745950	5749615	SLC35B1

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Table S4.2: (*continued*)

Ensembl gene id	Chr.	Start	End	Gene name
Common in Tib_BBS and Tib_BMD				
ENSGALG00000019361	1	32828666	32879723	TAFA2
ENSGALG00000016391	1	120600351	120804008	CNKSR2
ENSGALG00000016420	1	121385060	121606919	SH3KBP1
ENSGALG00000043118	1	132986041	133096377	ATP10A
ENSGALG00000016767	1	133973567	134294259	AFF3
ENSGALG00000008574	1	194436252	194446714	CLNS1A
ENSGALG00000031450	1	194597369	194675601	MYO7A
ENSGALG00000006153	2	5782448	5801847	EXOG
ENSGALG00000049653	2	6621972	6760289	KMT2C
ENSGALG00000038265	2	33231086	33475464	CREB5
ENSGALG00000013086	2	80659236	80730292	IKZF1
ENSGALG00000037014	2	146916969	147397126	TSNARE1
ENSGALG00000011473	3	42691591	42928349	RPS6KA2
ENSGALG00000016451	3	97177532	97270178	ROCK2
ENSGALG00000007028	4	8732666	8906442	—
ENSGALG00000011078	4	45807324	45891224	PTPN13
ENSGALG00000011109	4	45932534	46032124	MAPK10
ENSGALG00000052375	4	57098783	57108321	—
ENSGALG00000009172	7	15434552	15516341	OSBPL6
ENSGALG00000030350	9	5719637	5755791	—
ENSGALG00000003767	11	6241053	6411630	NKD1
ENSGALG00000002229	13	3572457	3637749	FBXW11
ENSGALG00000003548	14	1552022	1586667	BAIAP2L1
ENSGALG00000051488	15	9238928	9263621	SFI1
ENSGALG00000027897	15	11471568	11477474	DTX1
ENSGALG00000008150	15	11476814	11485534	RASAL1
ENSGALG00000003476	18	6755211	6787645	ERN1
ENSGALG00000003496	18	6791861	6805476	TEX2
ENSGALG00000021636	19	4027300	4254453	CUX1
ENSGALG00000004878	21	6417028	6426299	—
ENSGALG00000052799	21	6426944	6443949	—
Common in Tib_BBS and Hum_BBS				
ENSGALG00000007125	2	13147629	13581750	PARD3
ENSGALG00000014485	4	76025150	76382215	LDB2
ENSGALG00000006074	5	11366630	11601652	SOX6
ENSGALG00000011801	5	53184725	53226447	ESR2
ENSGALG00000004741	21	5956010	6053651	EPHB2
ENSGALG00000031440	23	2911566	3132209	PTPRU

Ensembl gene id	Chr.	Start	End	Gene name
Common in Tib_BBS and Hum_BMD				
ENSGALG00000034989	18	7450622	7589963	CEP112
ENSGALG00000001122	19	1102760	1225316	CALN1
Common in Tib_BMD and Hum_BMD				
ENSGALG00000020292	4	10827137	10859217	GABRE
ENSGALG00000043448	4	80649049	80786524	ABLIM2
ENSGALG00000039221	5	16259485	16322745	LOC423110
ENSGALG00000011677	7	26448702	26481703	HSPBAP1
ENSGALG00000008456	17	2470501	2727279	CACNA1B
ENSGALG00000041473	17	6893819	6935800	DDX31
Common in Tib_BMD and Hum_BBS				
ENSGALG00000016312	1	119273945	119465002	POLA1
ENSGALG00000021395	18	7924961	7946282	ABCA9
Common in Hum_BBS and Hum_BMD				
ENSGALG00000043771	1	1110497	1128266	DENND6B
ENSGALG00000013095	1	63109353	63163425	DERA
ENSGALG00000017062	1	174375172	174843962	NBEA
ENSGALG00000027853	4	87427085	87823826	CTNNA2
ENSGALG00000006608	5	14310133	14525237	—
ENSGALG00000006647	5	14360355	14381768	DUSP8
ENSGALG00000002015	6	3338993	3346509	SNCG
ENSGALG00000021869	8	24907072	24927370	PODN
ENSGALG00000003699	13	11388767	11649631	EBF1
ENSGALG00000001153	19	1512994	2173032	AUTS2
Common in Tib_BBS, Tib_BMD and Hum_BMD				
ENSGALG00000009400	5	27839047	27917728	SLC8A3
Common in Tib_BBS, Tib_BMD and Hum_BBS				
ENSGALG00000007025	1	15633800	15729892	CPNE8
Common in Tib_BMD, Hum_BMD and Hum_BBS				
ENSGALG00000015768	3	75547294	75651254	ANKRD6
ENSGALG00000026258	11	14531508	14691257	MAF
ENSGALG00000001433	18	3121259	3232611	B3GNTL1

Table S4.3: List of all enriched terms / pathways obtained from the genome-wide association studies.

Source	Term ID	Term name	*p*-value	Term size	Query size	Intersection
Enrichend in Tib_BBS						
GO:BP	GO:0009266	response to temperature stimulus	2.62×10^{-6}	88	16	3
Enrichend in Tib_BMD						
GO:BP	GO:0008150	biological_process	4.46×10^{-2}	12167	39	32
GO:MF	GO:0003674	molecular_function	3.46×10^{-3}	12467	39	32

Table S4.4: List of all enriched terms / pathways obtained from the Random Forests classification.

Source	Term ID	Term name	*p*-value	Term size	Query size	Intersection
Enriched in Tib_BBS						
GO:BP	GO:0008150	biological_process	7.08×10^{-20}	13579	240	205
GO:BP	GO:0009987	cellular process	1.24×10^{-15}	12832	240	192
GO:BP	GO:0048856	anatomical structure development	1.31×10^{-6}	3647	240	73
GO:BP	GO:0051179	localization	2.96×10^{-6}	4500	240	83
GO:BP	GO:0071704	organic substance metabolic process	8.04×10^{-6}	8088	240	123
GO:BP	GO:0008152	metabolic process	1.09×10^{-5}	8596	240	128
GO:BP	GO:0032501	multicellular organismal process	1.11×10^{-5}	4463	240	81
GO:BP	GO:0044238	primary metabolic process	1.23×10^{-5}	7676	240	118
GO:BP	GO:0065007	biological regulation	1.24×10^{-5}	8517	240	127
GO:BP	GO:0032502	developmental process	1.61×10^{-5}	3940	240	74
GO:BP	GO:0033036	macromolecule localization	3.97×10^{-5}	2024	240	47
GO:BP	GO:0048731	system development	4.20×10^{-5}	3027	240	61
GO:BP	GO:0007275	multicellular organism development	5.80×10^{-5}	3280	240	64
GO:BP	GO:0071840	cellular component organization or biogenesis	8.50×10^{-5}	4748	240	82
GO:BP	GO:0016043	cellular component organization	9.26×10^{-5}	4592	240	80
GO:BP	GO:2000574	regulation of microtubule motor activity	1.01×10^{-5}	12	240	5
GO:BP	GO:0022008	neurogenesis	1.14×10^{-4}	1126	240	32
GO:BP	GO:0051641	cellular localization	1.78×10^{-4}	1989	240	45
GO:BP	GO:0007399	nervous system development	1.88×10^{-4}	1526	240	38
GO:BP	GO:0030154	cell differentiation	1.97×10^{-4}	2563	240	53
GO:BP	GO:0043170	macromolecule metabolic process	2.38×10^{-4}	6949	240	106
GO:BP	GO:0048522	positive regulation of cellular process	2.89×10^{-4}	3818	240	69
GO:BP	GO:0008104	protein localization	2.91×10^{-4}	1751	240	41
GO:BP	GO:0048869	cellular developmental process	3.98×10^{-4}	2620	240	53
GO:BP	GO:0044237	cellular metabolic process	5.48×10^{-4}	7793	240	114
GO:BP	GO:0006807	nitrogen compound metabolic process	5.88×10^{-4}	7249	240	108
GO:BP	GO:0048518	positive regulation of biological process	7.90×10^{-4}	4163	240	72
GO:BP	GO:2000580	regulation of ATP-dependent microtubule motor activity	9.45×10^{-4}	8	240	4
GO:BP	GO:2000576	positive regulation of microtubule motor activity	9.45×10^{-4}	8	240	4
GO:BP	GO:2000582	positive regulation of ATP-dependent microtubule motor activity	9.45×10^{-4}	8	240	4
GO:BP	GO:2000741	positive regulation of mesenchymal stem cell differentiation	1.43×10^{-3}	3	240	3
GO:BP	GO:2000739	regulation of mesenchymal stem cell differentiation	1.43×10^{-3}	3	240	3
GO:BP	GO:0051128	regulation of cellular component organization	2.36×10^{-3}	1555	240	36
GO:BP	GO:0034613	cellular protein localization	2.77×10^{-3}	1302	240	32
GO:BP	GO:1901564	organonitrogen compound metabolic process	2.88×10^{-3}	4809	240	78
GO:BP	GO:0070727	cellular macromolecule localization	3.16×10^{-3}	1310	240	32
GO:BP	GO:0019538	protein metabolic process	3.51×10^{-3}	4164	240	70
GO:BP	GO:0030182	neuron differentiation	3.80×10^{-3}	945	240	26
GO:BP	GO:0050896	response to stimulus	3.95×10^{-3}	5711	240	88
GO:BP	GO:0048468	cell development	3.96×10^{-3}	1456	240	34
GO:BP	GO:0006796	phosphate-containing compound metabolic process	4.27×10^{-3}	2305	240	46
GO:BP	GO:0051093	negative regulation of developmental process	5.16×10^{-3}	615	240	20
GO:BP	GO:0006793	phosphorus metabolic process	5.38×10^{-3}	2325	240	46
GO:BP	GO:0065009	regulation of molecular function	5.52×10^{-3}	1964	240	41
GO:BP	GO:0043412	macromolecule modification	5.63×10^{-3}	3012	240	55
GO:BP	GO:0048513	animal organ development	5.69×10^{-3}	2183	240	44
GO:BP	GO:0006464	cellular protein modification process	5.73×10^{-3}	2859	240	53
GO:BP	GO:0036211	protein modification process	5.73×10^{-3}	2859	240	53
GO:BP	GO:0050790	regulation of catalytic activity	6.55×10^{-3}	1558	240	35
GO:BP	GO:0009653	anatomical structure morphogenesis	7.99×10^{-3}	1851	240	39
GO:BP	GO:0044093	positive regulation of molecular function	8.16×10^{-3}	1175	240	29
GO:BP	GO:0010563	negative regulation of phosphorus metabolic process	8.25×10^{-3}	376	240	15
GO:BP	GO:0045936	negative regulation of phosphate metabolic process	8.25×10^{-3}	376	240	15
GO:BP	GO:0048699	generation of neurons	8.57×10^{-3}	1051	240	27
GO:BP	GO:0050789	regulation of biological process	1.21×10^{-2}	7967	240	111
GO:BP	GO:0050793	regulation of developmental process	1.25×10^{-2}	1675	240	36
GO:BP	GO:0044087	regulation of cellular component biogenesis	1.28×10^{-2}	654	240	20
GO:BP	GO:0030029	actin filament-based process	1.53×10^{-2}	606	240	19
GO:BP	GO:0044260	cellular macromolecule metabolic process	1.58×10^{-2}	5902	240	88
GO:BP	GO:0008544	epidermis development	1.82×10^{-2}	179	240	10
GO:BP	GO:0022603	regulation of anatomical structure morphogenesis	1.98×10^{-2}	674	240	20
GO:BP	GO:0006928	movement of cell or subcellular component	2.01×10^{-2}	1366	240	31
GO:BP	GO:2000738	positive regulation of stem cell differentiation	2.31×10^{-2}	16	240	4
GO:BP	GO:0030218	erythrocyte differentiation	2.34×10^{-2}	81	240	7
GO:BP	GO:0016310	phosphorylation	2.37×10^{-2}	1654	240	35
GO:BP	GO:0051130	positive regulation of cellular component organization	2.53×10^{-2}	744	240	21
GO:BP	GO:0048666	neuron development	2.63×10^{-2}	746	240	21
GO:BP	GO:0072497	mesenchymal stem cell differentiation	2.80×10^{-2}	6	240	3
GO:BP	GO:0060429	epithelium development	2.85×10^{-2}	750	240	21
GO:BP	GO:0007155	cell adhesion	3.18×10^{-2}	877	240	23
GO:BP	GO:0045595	regulation of cell differentiation	3.42×10^{-2}	1136	240	27
GO:BP	GO:0022610	biological adhesion	3.48×10^{-2}	882	240	23
GO:BP	GO:0072657	protein localization to membrane	3.55×10^{-2}	375	240	14
GO:BP	GO:0048523	negative regulation of cellular process	3.57×10^{-2}	3208	240	55
GO:BP	GO:0044089	positive regulation of cellular component biogenesis	3.66×10^{-2}	376	240	14
GO:BP	GO:0065008	regulation of biological quality	3.74×10^{-2}	2583	240	47
GO:BP	GO:0034101	erythrocyte homeostasis	4.34×10^{-2}	89	240	7
GO:BP	GO:0043113	receptor clustering	4.54×10^{-2}	37	240	5
GO:BP	GO:0043085	positive regulation of catalytic activity	4.60×10^{-2}	961	240	24
GO:BP	GO:0050794	regulation of cellular process	4.70×10^{-2}	7623	240	105
GO:BP	GO:2000807	regulation of synaptic vesicle clustering	4.86×10^{-2}	7	240	3
GO:CC	GO:0110165	cellular anatomical entity	8.09×10^{-23}	13827	240	210
GO:CC	GO:0005575	cellular_component	2.98×10^{-22}	13932	240	210

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Table S4.4: (*continued*)

Source	Term ID	Term name	*p*-value	Term size	Query size	Intersection
GO:CC	GO:0043226	organelle	1.24×10^{-14}	9149	240	154
GO:CC	GO:0005622	intracellular	1.49×10^{-14}	10264	240	165
GO:CC	GO:0043229	intracellular organelle	8.04×10^{-13}	8849	240	147
GO:CC	GO:0005737	cytoplasm	3.01×10^{-9}	7503	240	124
GO:CC	GO:0043227	membrane-bounded organelle	1.20×10^{-8}	7921	240	127
GO:CC	GO:0043231	intracellular membrane-bounded organelle	2.91×10^{-8}	7375	240	120
GO:CC	GO:0032991	protein-containing complex	7.20×10^{-6}	4028	240	74
GO:CC	GO:0016020	membrane	5.30×10^{-5}	6623	240	102
GO:CC	GO:0005634	nucleus	1.15×10^{-4}	4975	240	82
GO:CC	GO:0005856	cytoskeleton	1.40×10^{-4}	1685	240	39
GO:CC	GO:0043228	non-membrane-bounded organelle	2.96×10^{-4}	3206	240	59
GO:CC	GO:0043232	intracellular non-membrane-bounded organelle	2.96×10^{-4}	3206	240	59
GO:CC	GO:0070161	anchoring junction	3.32×10^{-4}	426	240	17
GO:CC	GO:0030054	cell junction	5.87×10^{-4}	1250	240	31
GO:CC	GO:0015630	microtubule cytoskeleton	2.46×10^{-3}	894	240	24
GO:CC	GO:0005886	plasma membrane	2.53×10^{-3}	3349	240	58
GO:CC	GO:0098590	plasma membrane region	2.94×10^{-3}	667	240	20
GO:CC	GO:0071944	cell periphery	3.57×10^{-3}	3468	240	59
GO:CC	GO:0005911	cell-cell junction	1.94×10^{-2}	315	240	12
GO:CC	GO:0005829	cytosol	2.01×10^{-2}	2391	240	43
GO:CC	GO:0043005	neuron projection	3.16×10^{-2}	791	240	20
GO:CC	GO:0042995	cell projection	3.35×10^{-2}	1399	240	29
GO:CC	GO:0032391	photoreceptor connecting cilium	4.09×10^{-2}	28	240	4
GO:CC	GO:0120025	plasma membrane bounded cell projection	4.32×10^{-2}	1349	240	28
GO:CC	GO:0070013	intracellular organelle lumen	4.49×10^{-2}	3121	240	51
GO:CC	GO:0043233	organelle lumen	4.49×10^{-2}	3121	240	51
GO:CC	GO:0031974	membrane-enclosed lumen	4.49×10^{-2}	3121	240	51
GO:MF	GO:0003674	molecular_function	6.54×10^{-21}	13343	240	204
GO:MF	GO:0005488	binding	8.39×10^{-19}	10509	240	176
GO:MF	GO:0005515	protein binding	5.77×10^{-14}	6570	240	125
GO:MF	GO:0043167	ion binding	6.88×10^{-8}	4187	240	82
GO:MF	GO:0046872	metal ion binding	5.68×10^{-7}	2430	240	56
GO:MF	GO:0043169	cation binding	1.53×10^{-6}	2497	240	56
GO:MF	GO:0003824	catalytic activity	8.16×10^{-5}	4943	240	83
GO:MF	GO:0140096	catalytic activity, acting on a protein	2.16×10^{-4}	1872	240	42
GO:MF	GO:0016740	transferase activity	3.11×10^{-3}	1933	240	40
GO:MF	GO:0005509	calcium ion binding	5.43×10^{-3}	553	240	18
GO:MF	GO:0042802	identical protein binding	7.55×10^{-3}	1239	240	29
GO:MF	GO:0005524	ATP binding	8.01×10^{-3}	1177	240	28
GO:MF	GO:0032559	adenyl ribonucleotide binding	1.39×10^{-2}	1214	240	28
GO:MF	GO:0030554	adenyl nucleotide binding	1.49×10^{-2}	1219	240	28
GO:MF	GO:0016773	phosphotransferase activity, alcohol group as acceptor	1.90×10^{-2}	608	240	18
GO:MF	GO:0016301	kinase activity	2.02×10^{-2}	669	240	19
GO:MF	GO:0016772	transferase activity, transferring phosphorus-containing groups	3.96×10^{-2}	827	240	21
GO:MF	GO:0019899	enzyme binding	4.69×10^{-2}	1513	240	31
KEGG	KEGG:00000	KEGG root term	2.85×10^{-6}	4773	240	82
KEGG	KEGG:00604	Glycosphingolipid biosynthesis - ganglio series	1.57×10^{-2}	15	240	3
KEGG	KEGG:01100	Metabolic pathways	3.60×10^{-2}	1280	240	25
Enriched in Tib_BMD						
GO:BP	GO:0008150	biological_process	1.12×10^{-21}	13579	220	193
GO:BP	GO:0009987	cellular process	6.60×10^{-16}	12832	220	179
GO:BP	GO:0065007	biological regulation	1.19×10^{-10}	8517	220	131
GO:BP	GO:0050794	regulation of cellular process	7.11×10^{-9}	7623	220	118
GO:BP	GO:0050789	regulation of biological process	1.08×10^{-8}	7967	220	121
GO:BP	GO:0007154	cell communication	1.86×10^{-5}	4259	220	73
GO:BP	GO:0008152	metabolic process	2.80×10^{-5}	8596	220	118
GO:BP	GO:0023052	signaling	3.47×10^{-5}	4234	220	72
GO:BP	GO:0032501	multicellular organismal process	5.87×10^{-5}	4463	220	74
GO:BP	GO:0044238	primary metabolic process	5.91×10^{-5}	7676	220	108
GO:BP	GO:0071704	organic substance metabolic process	6.25×10^{-5}	8088	220	112
GO:BP	GO:0006807	nitrogen compound metabolic process	1.02×10^{-4}	7249	220	103
GO:BP	GO:0007165	signal transduction	1.73×10^{-4}	3878	220	66
GO:BP	GO:0051179	localization	1.89×10^{-4}	4500	220	73
GO:BP	GO:0043170	macromolecule metabolic process	4.35×10^{-4}	6949	220	98
GO:BP	GO:0044237	cellular metabolic process	6.01×10^{-4}	7793	220	106
GO:BP	GO:0032502	developmental process	1.58×10^{-3}	3940	220	64
GO:BP	GO:0050896	response to stimulus	2.09×10^{-3}	5711	220	83
GO:BP	GO:0060255	regulation of macromolecule metabolic process	2.18×10^{-3}	4242	220	67
GO:BP	GO:0010467	gene expression	2.44×10^{-3}	3901	220	63
GO:BP	GO:0048856	anatomical structure development	2.61×10^{-3}	3647	220	60
GO:BP	GO:0051716	cellular response to stimulus	3.52×10^{-3}	4937	220	74
GO:BP	GO:0048519	negative regulation of biological process	3.56×10^{-3}	3508	220	58
GO:BP	GO:0019222	regulation of metabolic process	3.75×10^{-3}	4578	220	70
GO:BP	GO:0007275	multicellular organism development	4.67×10^{-3}	3280	220	55
GO:BP	GO:0034641	cellular nitrogen compound metabolic process	5.19×10^{-3}	4437	220	68
GO:BP	GO:0051171	regulation of nitrogen compound metabolic process	5.33×10^{-3}	3903	220	62
GO:BP	GO:0080090	regulation of primary metabolic process	5.44×10^{-3}	3994	220	63
GO:BP	GO:0006810	transport	7.19×10^{-3}	3327	220	55
GO:BP	GO:0010468	regulation of gene expression	7.37×10^{-3}	3074	220	52
GO:BP	GO:0051234	establishment of localization	8.22×10^{-3}	3428	220	56
GO:BP	GO:0048523	negative regulation of cellular process	1.15×10^{-2}	3208	220	53
GO:BP	GO:0048869	cellular developmental process	1.16×10^{-2}	2620	220	46
GO:BP	GO:0050793	regulation of developmental process	1.19×10^{-2}	1675	220	34

(continued on next page)

Table S4.4: (*continued*)

Source	Term ID	Term name	*p*-value	Term size	Query size	Intersection
GO:BP	GO:0018193	peptidyl-amino acid modification	1.36×10^{-2}	907	220	23
GO:BP	GO:0071840	cellular component organization or biogenesis	1.39×10^{-2}	4748	220	70
GO:BP	GO:0030154	cell differentiation	1.49×10^{-2}	2563	220	45
GO:BP	GO:0006725	cellular aromatic compound metabolic process	1.59×10^{-2}	4036	220	62
GO:BP	GO:0016043	cellular component organization	1.72×10^{-2}	4592	220	68
GO:BP	GO:0051239	regulation of multicellular organismal process	1.92×10^{-2}	1945	220	37
GO:BP	GO:1901360	organic cyclic compound metabolic process	1.96×10^{-2}	4153	220	63
GO:BP	GO:0010646	regulation of cell communication	2.21×10^{-2}	2195	220	40
GO:BP	GO:0048731	system development	2.28×10^{-2}	3027	220	50
GO:BP	GO:0046483	heterocycle metabolic process	2.41×10^{-2}	3999	220	61
GO:BP	GO:0006139	nucleobase-containing compound metabolic process	2.43×10^{-2}	3910	220	60
GO:BP	GO:0023051	regulation of signaling	2.61×10^{-2}	2211	220	40
GO:BP	GO:2000026	regulation of multicellular organismal development	3.01×10^{-2}	1303	220	28
GO:BP	GO:0044260	cellular macromolecule metabolic process	3.02×10^{-2}	5902	220	81
GO:BP	GO:0048468	cell development	3.29×10^{-2}	1456	220	30
GO:BP	GO:0016070	RNA metabolic process	3.85×10^{-2}	3173	220	51
GO:BP	GO:0031323	regulation of cellular metabolic process	3.92×10^{-2}	4153	220	62
GO:CC	GO:0110165	cellular anatomical entity	1.68×10^{-23}	13827	220	196
GO:CC	GO:0005575	cellular_component	5.91×10^{-23}	13932	220	196
GO:CC	GO:0005622	intracellular	3.80×10^{-13}	10264	220	151
GO:CC	GO:0043226	organelle	1.72×10^{-8}	9149	220	130
GO:CC	GO:0043229	intracellular organelle	1.12×10^{-7}	8849	220	125
GO:CC	GO:0005737	cytoplasm	4.96×10^{-7}	7503	220	110
GO:CC	GO:0016020	membrane	1.25×10^{-5}	6623	220	97
GO:CC	GO:0043227	membrane-bounded organelle	1.34×10^{-5}	7921	220	110
GO:CC	GO:0043231	intracellular membrane-bounded organelle	4.29×10^{-5}	7375	220	103
GO:CC	GO:0005634	nucleus	1.81×10^{-4}	4975	220	76
GO:CC	GO:0005886	plasma membrane	2.93×10^{-4}	3349	220	57
GO:CC	GO:0071944	cell periphery	4.04×10^{-4}	3468	220	58
GO:CC	GO:0043025	neuronal cell body	3.34×10^{-3}	197	220	10
GO:CC	GO:0005829	cytosol	1.14×10^{-2}	2391	220	41
GO:CC	GO:0031224	intrinsic component of membrane	1.19×10^{-2}	4422	220	64
GO:CC	GO:0044297	cell body	1.35×10^{-2}	232	220	10
GO:CC	GO:0043232	intracellular non-membrane-bounded organelle	1.71×10^{-2}	3206	220	50
GO:CC	GO:0043228	non-membrane-bounded organelle	1.71×10^{-2}	3206	220	50
GO:CC	GO:0016604	nuclear body	1.97×10^{-2}	516	220	15
GO:CC	GO:0045092	interleukin-18 receptor complex	2.08×10^{-2}	2	220	2
GO:CC	GO:0016021	integral component of membrane	2.64×10^{-2}	4352	220	62
GO:CC	GO:0031981	nuclear lumen	3.50×10^{-2}	2859	220	45
GO:CC	GO:0000118	histone deacetylase complex	3.86×10^{-2}	56	220	5
GO:CC	GO:0017053	transcription repressor complex	3.86×10^{-2}	56	220	5
GO:CC	GO:0016581	NuRD complex	4.98×10^{-2}	13	220	3
GO:CC	GO:0090545	CHD-type complex	4.98×10^{-2}	13	220	3
GO:MF	GO:0003674	molecular_function	2.32×10^{-20}	13343	220	189
GO:MF	GO:0005488	binding	3.35×10^{-12}	10509	220	152
GO:MF	GO:0005515	protein binding	1.93×10^{-5}	6570	220	97
GO:MF	GO:0043167	ion binding	2.41×10^{-4}	4187	220	68
GO:MF	GO:0003824	catalytic activity	6.97×10^{-4}	4943	220	75
GO:MF	GO:0043168	anion binding	1.47×10^{-3}	2194	220	42
GO:MF	GO:0097159	organic cyclic compound binding	1.51×10^{-3}	4401	220	68
GO:MF	GO:0005524	ATP binding	1.78×10^{-3}	1177	220	28
GO:MF	GO:0016740	transferase activity	2.63×10^{-3}	1933	220	38
GO:MF	GO:0032559	adenyl ribonucleotide binding	3.21×10^{-3}	1214	220	28
GO:MF	GO:0030554	adenyl nucleotide binding	3.46×10^{-3}	1219	220	28
GO:MF	GO:1901363	heterocyclic compound binding	4.02×10^{-3}	4342	220	66
GO:MF	GO:0036094	small molecule binding	4.04×10^{-3}	1969	220	38
GO:MF	GO:0017076	purine nucleotide binding	4.56×10^{-3}	1525	220	32
GO:MF	GO:0032553	ribonucleotide binding	4.56×10^{-3}	1525	220	32
GO:MF	GO:0035639	purine ribonucleoside triphosphate binding	5.61×10^{-3}	1467	220	31
GO:MF	GO:1901265	nucleoside phosphate binding	8.30×10^{-3}	1722	220	34
GO:MF	GO:0000166	nucleotide binding	8.30×10^{-3}	1722	220	34
GO:MF	GO:0004674	protein serine/threonine kinase activity	9.36×10^{-3}	342	220	13
GO:MF	GO:0032555	purine ribonucleotide binding	1.01×10^{-2}	1512	220	31
GO:MF	GO:0097367	carbohydrate derivative binding	1.80×10^{-2}	1711	220	33
GO:MF	GO:0016301	kinase activity	2.46×10^{-2}	669	220	18
GO:MF	GO:0016772	transferase activity, transferring phosphorus-containing groups	4.06×10^{-2}	827	220	20
GO:MF	GO:0140096	catalytic activity, acting on a protein	4.48×10^{-2}	1872	220	34
GO:MF	GO:0042008	interleukin-18 receptor activity	4.95×10^{-2}	2	220	2
KEGG	KEGG:00000	KEGG root term	2.33×10^{-5}	4773	220	74
KEGG	KEGG:04310	Wnt signaling pathway	9.00×10^{-4}	135	220	7
KEGG	KEGG:04010	MAPK signaling pathway	1.78×10^{-2}	249	220	9
Enriched in Hum_BBS						
GO:BP	GO:0008150	biological_process	6.01×10^{-9}	13579	115	99
GO:BP	GO:0009987	cellular process	5.59×10^{-7}	12832	115	93
GO:BP	GO:0065007	biological regulation	7.94×10^{-5}	8517	115	69
GO:BP	GO:0050789	regulation of biological process	9.47×10^{-5}	7967	115	66
GO:BP	GO:0044260	cellular macromolecule metabolic process	1.96×10^{-4}	5902	115	54
GO:BP	GO:0071704	organic substance metabolic process	4.91×10^{-4}	8088	115	65
GO:BP	GO:0044237	cellular metabolic process	7.73×10^{-4}	7793	115	63
GO:BP	GO:0044238	primary metabolic process	1.12×10^{-3}	7676	115	62
GO:BP	GO:0048519	negative regulation of biological process	2.26×10^{-3}	3508	115	37
GO:BP	GO:0009887	animal organ morphogenesis	3.77×10^{-3}	727	115	15
GO:BP	GO:0060255	regulation of macromolecule metabolic process	4.55×10^{-3}	4242	115	41

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Table S4.4: (*continued*)

Source	Term ID	Term name	*p*-value	Term size	Query size	Intersection
GO:BP	GO:0018130	heterocycle biosynthetic process	4.77×10^{-3}	2721	115	31
GO:BP	GO:0019438	aromatic compound biosynthetic process	5.00×10^{-3}	2727	115	31
GO:BP	GO:0050794	regulation of cellular process	5.53×10^{-3}	7623	115	60
GO:BP	GO:0006807	nitrogen compound metabolic process	5.62×10^{-3}	7249	115	58
GO:BP	GO:0008152	metabolic process	5.65×10^{-3}	8596	115	65
GO:BP	GO:0080090	regulation of primary metabolic process	7.16×10^{-3}	3994	115	39
GO:BP	GO:1901362	organic cyclic compound biosynthetic process	8.69×10^{-3}	2800	115	31
GO:BP	GO:0034654	nucleobase-containing compound biosynthetic process	9.73×10^{-3}	2670	115	30
GO:BP	GO:0019222	regulation of metabolic process	1.26×10^{-2}	4578	115	42
GO:BP	GO:0031323	regulation of cellular metabolic process	1.87×10^{-2}	4153	115	39
GO:BP	GO:0006355	regulation of transcription, DNA-templated	1.90×10^{-2}	2190	115	26
GO:BP	GO:0006357	regulation of transcription by RNA polymerase II	2.25×10^{-2}	1546	115	21
GO:BP	GO:2000112	regulation of cellular macromolecule biosynthetic process	2.52×10^{-2}	2509	115	28
GO:BP	GO:1903506	regulation of nucleic acid-templated transcription	2.72×10^{-2}	2235	115	26
GO:BP	GO:2001141	regulation of RNA biosynthetic process	2.72×10^{-2}	2235	115	26
GO:BP	GO:0051171	regulation of nitrogen compound metabolic process	2.87×10^{-2}	3903	115	37
GO:BP	GO:0006366	transcription by RNA polymerase II	3.22×10^{-2}	1583	115	21
GO:BP	GO:0008285	negative regulation of cell population proliferation	3.44×10^{-2}	388	115	10
GO:BP	GO:0006351	transcription, DNA-templated	3.69×10^{-2}	2274	115	26
GO:BP	GO:0010556	regulation of macromolecule biosynthetic process	4.12×10^{-2}	2576	115	28
GO:BP	GO:0043170	macromolecule metabolic process	4.45×10^{-2}	6949	115	54
GO:BP	GO:0044271	cellular nitrogen compound biosynthetic process	4.52×10^{-2}	3190	115	32
GO:CC	GO:0005575	cellular_component	8.98×10^{-8}	13932	115	97
GO:CC	GO:0110165	cellular anatomical entity	2.04×10^{-7}	13827	115	96
GO:CC	GO:0005622	intracellular	5.15×10^{-6}	10264	115	78
GO:CC	GO:0043226	organelle	2.77×10^{-5}	9149	115	71
GO:CC	GO:0043229	intracellular organelle	4.59×10^{-5}	8849	115	69
GO:CC	GO:0043227	membrane-bounded organelle	5.88×10^{-5}	7921	115	64
GO:CC	GO:0043231	intracellular membrane-bounded organelle	6.61×10^{-5}	7375	115	61
GO:CC	GO:0005737	cytoplasm	3.26×10^{-4}	7503	115	60
GO:CC	GO:0005634	nucleus	1.07×10^{-2}	4975	115	42
GO:CC	GO:0005794	Golgi apparatus	4.12×10^{-2}	963	115	14
GO:MF	GO:0003674	molecular_function	2.14×10^{-13}	13343	115	103
GO:MF	GO:0005488	binding	4.88×10^{-10}	10509	115	87
GO:MF	GO:0043167	ion binding	7.49×10^{-6}	4187	115	45
GO:MF	GO:0005515	protein binding	7.71×10^{-5}	6570	115	57
GO:MF	GO:0046872	metal ion binding	2.46×10^{-4}	2430	115	30
GO:MF	GO:0003824	catalytic activity	3.91×10^{-4}	4943	115	46
GO:MF	GO:0043169	cation binding	4.37×10^{-4}	2497	115	30
GO:MF	GO:0003682	chromatin binding	2.37×10^{-3}	429	115	11
GO:MF	GO:0042578	phosphoric ester hydrolase activity	8.18×10^{-3}	319	115	9
GO:MF	GO:0019103	pyrimidine nucleotide binding	8.56×10^{-3}	2	115	2
GO:MF	GO:0016787	hydrolase activity	2.80×10^{-2}	2184	115	24
Enriched in Hum_BMD						
GO:BP	GO:0008150	biological_process	3.04×10^{-7}	13579	113	95
GO:BP	GO:0009987	cellular process	1.39×10^{-6}	12832	113	91
GO:BP	GO:0032502	developmental process	3.80×10^{-5}	3940	113	43
GO:BP	GO:0048856	anatomical structure development	4.20×10^{-5}	3647	113	41
GO:BP	GO:0032501	multicellular organismal process	5.47×10^{-5}	4463	113	46
GO:BP	GO:0007275	multicellular organism development	8.04×10^{-5}	3280	113	38
GO:BP	GO:0065007	biological regulation	8.81×10^{-5}	8517	113	68
GO:BP	GO:0048731	system development	1.10×10^{-4}	3027	113	36
GO:BP	GO:0050789	regulation of biological process	1.10×10^{-4}	7967	113	65
GO:BP	GO:0050794	regulation of cellular process	1.39×10^{-4}	7623	113	63
GO:BP	GO:0048519	negative regulation of biological process	1.45×10^{-3}	3508	113	37
GO:BP	GO:0048523	negative regulation of cellular process	4.51×10^{-3}	3208	113	34
GO:BP	GO:0051246	regulation of protein metabolic process	2.87×10^{-2}	1861	113	23
GO:BP	GO:0051179	localization	3.36×10^{-2}	4500	113	40
GO:BP	GO:0032268	regulation of cellular protein metabolic process	3.62×10^{-2}	1753	113	22
GO:BP	GO:0031400	negative regulation of protein modification process	4.09×10^{-2}	401	113	10
GO:BP	GO:0098885	modification of postsynaptic actin cytoskeleton	4.84×10^{-2}	2	113	2
GO:CC	GO:0005575	cellular_component	3.96×10^{-14}	13932	113	104
GO:CC	GO:0110165	cellular anatomical entity	1.59×10^{-13}	13827	113	103
GO:CC	GO:0005622	intracellular	3.21×10^{-9}	10264	113	83
GO:CC	GO:0005737	cytoplasm	2.56×10^{-6}	7503	113	64
GO:CC	GO:0043226	organelle	3.53×10^{-6}	9149	113	72
GO:CC	GO:0043229	intracellular organelle	6.28×10^{-6}	8849	113	70
GO:CC	GO:0005813	centrosome	1.99×10^{-3}	452	113	11
GO:CC	GO:0005815	microtubule organizing center	1.06×10^{-2}	542	113	11
GO:CC	GO:0098793	presynapse	1.17×10^{-2}	280	113	8
GO:CC	GO:0043227	membrane-bounded organelle	1.36×10^{-2}	7921	113	57
GO:CC	GO:0005856	cytoskeleton	2.11×10^{-2}	1685	113	20
GO:CC	GO:0043231	intracellular membrane-bounded organelle	3.40×10^{-2}	7375	113	53
GO:MF	GO:0003674	molecular_function	6.12×10^{-8}	13343	113	94
GO:MF	GO:0005488	binding	3.00×10^{-6}	10509	113	79
GO:MF	GO:0005515	protein binding	6.97×10^{-4}	6570	113	54
GO:MF	GO:0008641	ubiquitin-like modifier activating enzyme activity	6.14×10^{-3}	11	113	3
KEGG	KEGG:04310	Wnt signaling pathway	1.29×10^{-2}	135	113	5
KEGG	KEGG:00000	KEGG root term	4.06×10^{-2}	4773	113	36

5 General Discussion

The aim of this work was to characterise the influence of genetics on the differentiation of bone stability and to evaluate its potential for improving bone health in laying hens. Studies were carried out at the phenotypic and genomic level. After the general introduction in Chapter 1, the two following chapters of this thesis focused on the possible effects of the hens' phylogenetic background and laying performance on skeletal traits. At this, Chapter 2 examined the relationship between bone strength and laying performance in hens in a normal metabolic state and performed genetic parameter estimations, while Chapter 3 focused on the influence of the two factors mentioned above on bone properties in a state of nutritive calcium deficiency. Finally, Chapter 4 dealt with the identification and functional annotation of candidate genes associated with bone quality measures.

Taking into account the results of the second and third chapters, this general discussion will first address the question of whether the phylogenetic origin and egg production level are two main determinants of bone stability. Subsequently, the second part of the discussion will focus on the potential of genetics to improve skeletal health in laying hens. To this end, the genetic parameter estimations from the second chapter and the outcomes from the genomic analyses presented in the fourth chapter are discussed in a broader context. In addition to the overarching discussion of the main results achieved, important methodological aspects are addressed in the following and an outlook for future research is given. At the end, the main conclusions of this thesis are drawn.

5.1 Phylogenetic Origin and Egg Production Level — Two Main Determinants?

The Four-Line Animal Model

As shown in the literature review, the two factors of phylogenetic origin and performance level are generally considered to have a high influence on hens' bone stability. However, our understanding of the interaction of these two factors is sparse, and this is where this work ties in. The experiments of this thesis were carried out applying the four-line animal model, which is characterised by a two-dimensional divergence with respect to these factors (see Figure 1.2). The model was developed as a part of a multidisciplinary collaboration at the Friedrich-Loeffler-Institut that aimed to investigate the adaptability of laying hens to changing environmental conditions. It was first introduced by Lieboldt et al. [1] describing the growth and performance of the four chicken lines. Since then, a number of studies have been conducted applying this model to investigate behavioural [2–4], nutritional [5, 6], parasitological [7] and immunological objectives [8, 9]. Another group of studies dealt with bone characteristics [10, 11] and keel bone damages [12–14]. The experiments underlying this thesis complement these studies on skeletal integrity.

The most striking advantage of the four-line animal model is that it allows the two factors of phylogenetic origin and performance level to be addressed within one study. This enables to investigate potential influences of both factors on a trait simultaneously. Moreover, it covers a broad spectrum of laying hen genetics as the lines cluster with regard to their phylogenetic origin [15, 16]. However, considering the studies mentioned above, it becomes clear that disentangling these factors can be difficult. It seems that the most distinct demarcation of the lines with regard to one of the two dimensions can be observed when examining performance parameters [1, 10]. In contrast, the results for other traits are sometimes contradictory or ambiguous, which could indicate, for example, the effect of behavioural differences not accounted for by this model [10–12, 14].

Another critical point is that despite the phylogenetic relationships [15, 16], all lines in the model represent distinct strains which have evolved separately. To study selection effects in the true sense, the two lines of a phylogenetic group would have to have descended from the same founder and have been divergently selected over several generations. One such long-term study is that of Dunnington and Siegel [17], in which White Plymouth Rock chickens were divergently selected for eight-week body weight over 38 generations. In laying hens, we are only aware of shorter experiments, such as that of Bishop et al. [18], in which White Leghorn chickens were divergently selected for bone quality over five generations. However, given the clear divergence in productivity between WLA and R11 or BLA and L68 shown in Chapter 2, it seems justified to infer indications of different levels of productivity.

In summary, the four-line animal model is a very effective way of accounting for phylogenetic and performance effects within the same experiment, which is advantageous given the often limited experimental capacity.

Effects of Egg Production

One of the most important issues in relation to bone health in laying hens concerns the influence of egg production level on skeletal integrity. There is an ongoing discussion about whether and to what extent bone stability is influenced by hens' productivity. A common hypothesis is that the susceptibility to osteoporosis occurs because of physiological adaptations that have evolved for egg production in modern laying hens [19]. Accordingly, bone weakness is thought to be the result of gradual but persistent structural bone resorption to meet the calcium requirements for eggshell formation [14, 20]. In this context, it is frequently claimed that there is a negative correlation between bone stability and egg number and that this is the result of intensive selection for laying performance [19, 21].

There are indeed results suggesting this conclusion and given the large number of studies in which traditional or low performing lines were inferior to modern laying hybrids in terms of bone health, this presumed negative correlation seems conclusive [22–26]. However, most of these studies drew their conclusions from comparisons of mean values obtained for different strains kept under similar conditions. Provided we only contrast differentially productive lines, our results from Chapter 2 also strongly suggest that bone fragility is mainly caused by high laying rates. This is supported by previous studies on the same four lines, where a correlation between poor bone quality and high performance was suggested based on line comparisons [10, 11]. One difficulty with this approach is that possible effects of the lines genetic background

remain unconsidered, which can lead to an oversimplification of the differences in skeletal traits between genotypes [22].

Another argument pointing to a negative influence of high egg production relates to non-reproductive hens. Compared to birds in reproductive state, hens that do not lay eggs showed higher amounts of cortical and medullary tissue in the long bones [27] as well as an icreased keel bone density and a significantly lower risk of keel bone fractures [13, 28]. Furthermore, a link between laying activity and fracture susceptibility is suspected because males usually have higher bone quality than females and are virtually non-osteoporotic [19, 29, 30]. However, increased bone strength can also be expected in hens with very low egg production [31, 32], as structural bone formation resumes when laying activity ceases [22]. Failure to account for non-laying and very low performing individuals during data analysis can lead to bias [18]. In the present study (Chapter 2), this was taken into account by excluding hens from the data set that either did not show continuous laying activity in the last three weeks before slaughter or whose total egg count did not exceed a certain line specific threshold. In retrospect, this approach seems to have been justified because, as shown in Figure 5.1, the inclusion of those birds that did not meet the specified criteria would inevitably have led to a distortion of the results. Figure 5.1 depicts the phenotypic correlation between total egg number and bone characteristics of the tibiotarsus, calculated either based on the full data set containing all hens sampled ($n = 576$) or based on the truncated one containing only those meeting the egg-laying criteria ($n = 524$). It can be clearly seen that in the case of the complete data set there is partial evidence of phenotypic correlations, apparently due to the effect of a few individuals that laid poorly and at the same time had high bone quality. These data points have a kind of leverage effect that can lead to a strong shift in the correlation. Our observations are supported by findings from [33], who also observed that a few chickens with particularly low laying performance and high bone index values cause a strong negative correlation between these traits. By excluding hens with less than 230 and 250 eggs laid, the phenotypic correlation declined from -0.36 to -0.06 and -0.05, respectively. It has been argued that in these chickens, as the number of eggs decreases, the periods during which bone formation could potentially take place increase, finally resulting in higher bone strength [33].

Discarding outliers seems to be a crucial step in calculating valid correlations from which biological inferences can be drawn. Exclusion of non-layers can simply be done visually at dissection based on the presence of active ovarian follicles, however, data curation as in Chapter 2 necessarily requires individual performance records, which can be difficult to collect. In addition to cage housing, transponder-based nest boxes offer a way of recording these data [34, 35]. Such a system was used in our second study presented in Chapter 3. However, as some hens did not use these nests at all or did not use them consistently, the manually recorded laying performance at pen level was used at the end for reasons of data validity.

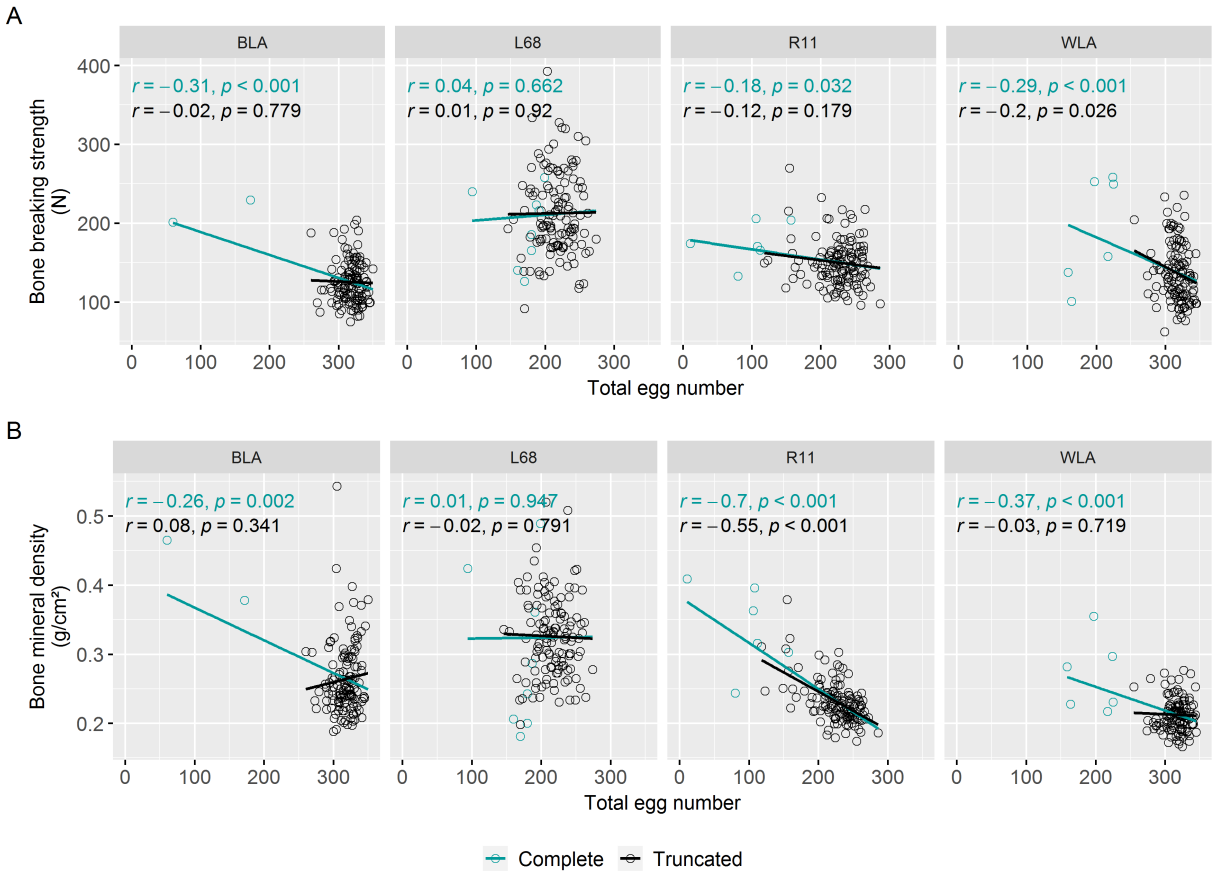

Figure 5.1: Linear relationship between total egg number and bone breaking strength (**A**) or bone mineral density (**B**) of the tibiotarsus in four different chicken layer lines (BLA, L68, R11, WLA). Two phenotypic correlations are given for each trait × line combination, either based on the complete dataset ($n = 576$) (cyan) or on the truncated one in which non-layers and very low performing hens were excluded ($n = 524$) (black). Pearson's correlation coefficients; correlations are considered significantly different from zero at $p < 0.05$.

Even when considering these methodological aspects, i.e. testing within lines and excluding low producing hens, there is no evidence in the literature that validly support the hypothesis of a direct relationship between egg number and bone stability [19, 21]. Rather, it has been repeatedly observed that high laying performance or sufficient eggshell strength and bone stability can coexist [19, 36–40]. Furthermore, skeletal problems were observed long before intensive selection for laying performance began [21, 41]. Certainly, the hypothesis is most likely to be questioned given the lack of phenotypic relationship. Based on the phenotypic correlations between egg production and bone strength, which were 0.0 and -0.16, respectively, Rennie et al. [22] concluded that there could be at most a minimal link between these traits. Bishop et al. [18], who also observed no significant difference in mean egg number between the lines divergently selected for high and low bone stability, supported this. Furthermore, both, Jendral et al. [42] and Alfonso-Carrillo et al. [43] reported an absence of correlation between bone quality traits and egg production. Gebhardt-Henrich and Fröhlich [44] focused on keel bone damages and observed no relationship between the total egg number and fractures. Recently, Dunn et al. [19] investigated two phylogenetically unrelated lines and negated any phenotypic correlations between bone quality and post-peak egg production. These results are

particularly interesting because the layer lines constituting the four-line animal model originate from the same breeds that were studied by Dunn et al. [19], namely White Leghorn and Rhode Island Red. In fact, their observations are very much in line with our findings from Chapter 2. Within the layer lines, we observed no significant effect of total eggshell production on either bone breaking strength or bone mineral density. Here our results could be even more meaningful, as we refer to the trait of eggshell production, which might be biologically closer to the pivot point of calcium supply than the egg number without simultaneous consideration of shell quality. Regression analyses showed a solitary effect of eggshell production on bone mineral density in line R11, which was recently confirmed for keel bone mineral density [14]. However, the effect size was rather small in both studies and was not significant at all in the other layer lines.

Taken together, it can be concluded that there is no strong correlation between the number of eggs laid or, given our own results, the amount of eggshell produced and bone stability. However, according to Toscano et al. [20] obviously there is a link of some kind between bone health and physiological adaptations to laying activity, so other factors than egg number, either causative or predisposing, should be considered. One of these factors is the laying persistency, as osteoporosis is caused by prolonged structural bone loss, so the duration of continuous laying could be a decisive factor [22, 33, 45]. Another promising factor is the development of medullary bone content, which can prevent cortical bone resorption by ensuring adequate calcium supply for eggshell formation [19, 43]. Recently, indications for a positive genetic correlation between late egg production and medullary bone mineralisation have been found [19]. Furthermore, the age at the onset of laying seems to be of particular importance for bone stability [20]. In this respect, the earlier switch to medullary bone formation associated with selection of hens for early sexual maturity and high peak production may have prevented both, the accumulation of sufficient amounts of medullary bone [43] and complete ossification of the skeleton [19, 20]. A number of studies has observed effects of age at first egg suggesting a negative impact of early puberty on skeletal integrity [19, 29, 44, 46, 47]. As puberty is a trait with clear genetic determination [20], age at onset of laying seems to be most promising in terms of identifying possible links between laying activity and bone health. However, it is beyond the scope of the present study to investigate this in more detail. Therefore, follow-up studies are needed to further characterise the implications of this factor.

Effects of Phylogeny

In addition to the influence of productivity on laying hen skeleton, the question of the role of phylogenetic origin on bone health is also of particular importance. As reviewed in Chapter 1.4, the literature points to a strong phylogenetic effect on bone stability. Our results are supportive of this assumption in that marked phenotypic differences in production and bone characteristics were found between the groups of brown and white-egg layer lines in the present analyses (Chapter 2).

So far, however, little is known about what causes the differences in bone integrity, leaving the white-egg lines more susceptible to bone weakness [13, 48, 49]. Results from Habig et al. [49], who studied the same four lines, suggested a higher bone turnover in the high performing brown-egg line, while white-egg layers were assumed to have depleted bone calcium reserves and therefore rely more on dietary calcium intake. According to Dudde et al. [11] phylogenetic

effects on bone stability may also reflect the brown-egg strains genetic heritage, as they are partly originating from Malay chickens, which are typical game birds in which robust individuals may have been favoured. Overall, it seems that brown-egg layers are basically in a more favourable situation, i.e. due to their physical constitution they are better equipped to cope with the physiological adaptations that have evolved for high egg laying activity. This constitutional aspect includes the tendency of the brown-egg laying hens to have a higher body weight, as observed in Chapter 2 and Chapter 3, which is associated with a higher mechanical load on the bones likely leading to a higher bone stability [13, 50, 51].

Constitutional advantages of the brown-egg lines seem to be particularly rewarding in metabolic challenging situations. In the case of the present work, such a situation was induced by repeated transient periods of nutritional calcium deprivation (Chapter 3), which are known to trigger adaptive response [52]. The results showed that the hens reacted differently depending on their phylogenetic origin, whereas the genetic selection for increased egg production only played a minor role. White-egg lines showed a higher drop of egg production and quality as well as a higher level of bone degradation, suggesting that medullary reserves were unable to buffer temporary calcium fluctuations in these lines. The brown-egg strains, in contrast, showed a higher tolerance to the calcium deprivation, likely reflecting advantageous physical constitution in which the skeletal system was able to provide a higher amount of calcium without severe bone health restrictions.

In summary, phylogeny is a crucial factor for bone stability. That was confirmed by the results of this thesis. However, compared to the question of productivity effects, which has been extensively studied so that quite valid statements can be made on this, there is still a large gap in knowledge regarding phylogenetic influences on bone health. Currently, the phylogenetic component in bone stability seems to be primarily based on a more favourable physical constitution of the brown-egg chicken lines, but further studies addressing the exact mechanisms are needed.

5.2 Potential of Genetics to Improve Bone Stability

Quantitative Genetic Properties of Skeletal Traits

Given the considerable variation within and between different chicken breeds or lines, genetic determination of bone stability is considered high [25, 53, 54]. Hence, enormous potential is seen in animal breeding to improve the bone health of laying hens [18, 55].

The heredity of a desired trait is a critical factor and, in principle, bone quality traits in chickens are assumed to be weakly to moderately heritable [47, 53, 56]. Our results of the genetic parameters estimated in Chapter 2 are consistent with this range of inheritance. Average values of $h^2 = 0.38$ and $h^2 = 0.40$ were observed across the lines for bone breaking strength of the tibiotarsus and humerus, respectively. For bone mineral density, heritability estimates were $h^2 = 0.60$ for the tibiotarsus and $h^2 = 0.48$ for the humerus. These values are in line with those reported by others [18, 19] and indicate a moderate heritability of these traits. However, as shown in Table 2.4, we found a relatively large variation in the estimators among the four lines. This could be due to methodological reasons, as our sample size was rather small with an average of $n = 131$ observations per line. Small sample size may also explain why in two cases no heritability estimation was possible due to a lack of convergence

of the model. On the other hand, the variation in these estimates may reflect distinct breeding history of the four lines and/or diverse genetic composition [16, 19, 57]. It is probably a combination of biological and methodological effects.

The close phenotypic relationship between bone breaking strength and bone mineral density found in Chapter 2, was corresponding with a close and even more pronounced genetic relation as reflected in mean genetic correlations of $r_g = 0.61$ (tibiotarsus) and $r_g = 0.71$ (humerus), respectively. In this work, the genetic correlations were limited to bone traits. However, since the main goal of layer breeding will remain the number of saleable eggs, estimates of the genetic correlation between bone and performance traits in these four lines must follow to complete the picture. In view of the large number of studies reporting the absence or at most weak correlations [19, 22, 43, 53, 58, 59], it can be assumed that the two areas of skeletal properties and egg production and quality are relatively independent of each other. This would allow genetic selection for improved bone stability without unfavourable effects on hen productivity [19, 56]. Furthermore, when selecting for improved bone strength, attention should be paid to body weight. An increase would have a negative effect on feed efficiency, but the body weight is positively correlated with bone stability at both phenotypic and genotypic levels [32, 43, 50, 60, 61]. Nevertheless, body weight independent selection for bone strength is possible [18, 53].

In order to select for a desired trait, it is important to accurately phenotype this trait in a large number of animals in addition to heritability [59]. In experimental settings, extensive and elaborate dissections and post mortem analyses are acceptable in order to capture the biology of the bone most precisely [19, 56, 62]. This includes virtually all the methods we have employed throughout the present studies, including the dual energy X-ray absorptiometry and three-point bending test measurement. However, other requirements are placed on phenotyping in commercial settings, as bone traits of interest must be assessable on large scale, i.e. populations of thousands of birds, rapidly and at low cost [63]. Furthermore, as post mortem analyses always require the rearing of a high number of possible selection candidates from each individual, the aim is to measure the trait on the selection candidate themselves [56, 64]. In this regard, the keel bone palpation and the radiography of the humerus are two traits under consideration that show reasonable heritabilities and can be assessed with sufficient accuracy on the living bird [47, 65]. According to Preisinger [64], both traits will be included in the balanced selection approach of commercial layer breeding programs.

Application of Genomic Methods

The introduction and continuous development of DNA marker technology has led to major advances in assessing genetic variability and identifying the genetic basis of diseases and traits [56, 65, 66]. After replacing microsatellites with single nucleotide polymorphisms (SNP) as genetic markers, the development of high-density SNP arrays followed, which are now used as a state-of-the-art technique for genetic analysis in animal breeding [66]. As described in Chapter 1.4 and Chapter 4, a number of potential genomic regions that may be related with bone health in laying hens have been identified through genome-wide analyses using microsatellite or SNP markers.

In the third study, presented in Chapter 4, we performed genome-wide association analyses, which aimed at identifying genomic regions associated with the bone phenotypes we previously

collected in Chapter 2. Since bone characteristics are assumed to be polygenic, i.e. regulated by multiple functional genes, each of which has a rather small effect [32, 67], Random Forests (RF) classification was used for SNP identification in parallel to the traditional single-locus mixed linear model analysis. With an average of 275 identified SNPs, a considerably higher number of markers were classified as potentially informative in the RF classification compared to the mixed linear model analysis, in which an average of only 26 SNPs were identified across all bone and trait combinations. This reflects the great potential of RF classification to identify loci with modest effects [68–70], many of which probably did not reach the significance threshold in the traditional approach and thus remained unnoticed given many small signals in the corresponding Mahattan plots (Figure 4.2). RF classification seems to be more robust in terms of population stratification bias, as least in our case. Although the four-line animal model has advantages because it includes a broad diversity of different lines, population stratification is a challenge. The RF algorithm includes random shuffling of loci and phenotypes and irratic exclusion of SNPs, which seems to have solved this problem better than single marker regression. For future studies on these bone phenotypes, the combination of RF classification with other methods should be considered in order to increase the power to detect relevant genotype-phenotype associations [71]. Recently, Ramzan et al. [72] successfully performed a two-step analysis of egg quality data in which the detection of quantitative trait loci (QTL) was followed by RF classification to prioritise SNPs within these QTLs.

The need for follow-up studies also arises from the fact that the four layer lines were combined for the present analysis in order to obtain a sufficient sample size. Hence, the focus was on finding loci that might be significant for laying hens in general. However, considering the four-line animal model (see Chapter 1.6), a subsequent study should investigate the influence of phylogenetic origin and egg production level on bone phenotypic plasticity. Here, for example, F2 mapping populations could be generated within the white and brown-egg lines, which show higher variability for the purpose of high-resolution mapping of loci. In addition, genomic regions of high monomorphism could be compared within phylogenetic groups, with such regions occurring only in highly selected lines possibly representing selection signatures. Subsequently, such an investigation for runs of homozygosity could be performed on phylogenetically divergent lines. A higher rate of homozygosity is to be expected especially in white-egg layers due to their overall lower genetic diversity [16].

Following the association analyses, sixteen candidate genes known from the literature to be functionally related to bone metabolism were identified in close proximity to significant SNPs. Their biological importance is suspected because they were partly involved in the *Wnt signaling* and *MAPK signaling* pathways, which are critical for bone stability. Although some of these genes are quite promising according to the current state of knowledge, they are all still suggestive genes for bone integrity in laying hens, whose biological significance and, most importantly, causality have yet to be proven in follow-up studies. Given the polygenic nature of complex traits such as bone stability, identification of causative genes is generally a challenging and tedious endeavour [56, 61, 73]. However, according to Johnsson [56], new interventions for increased bone health can be derived from biological insights drawn from causal genes. At this point, functional genomics approaches such as RNA sequencing could be used to gain deeper insights into bone plasticity, e.g. of phylogenetically divergent lines.

Practical application of genomic information in poultry breeding takes place in marker-assisted selection (MAS), i.e. selection based on phenotypic information combined with genetic

information obtained from SNP markers associated with QTLs [66]. It was initially thought that by identifying QTLs associated with genes related to bone properties, MAS for resistance to osteoporosis could be established [53]. However, unlike for major genes, conventional MAS does not seem feasible for complex traits such as bone strength [56]. Genomic selection, on the other hand, which is an advanced form of MAS that takes into account all markers across the entire genome [66, 74], appears promising in terms of improving bone health [56, 65, 75]. Various advantages led to the rapid adoption of genomic selection by poultry breeders [76]. This includes that by applying genomic selection, males and females can be selected for any traits soon after hatch thus enabling increasing selection pressure and making difficult measurements more practical [21, 59, 65]. Fulton [76] recently concluded that genomic selection is a valuable instrument that can significantly improve the efficiency and accuracy of breeding programs when combined with genetic selection methods.

5.3 General Conclusions

In this dissertation, the influence of genetics on the differentiation of bone stability traits in purebred chicken layer lines was characterised, focusing on effects of phylogenetic origin and egg laying performance. Furthermore, the potential of genetics for improving bone health was addressed. The main conclusions of this thesis can be summarised as follows:

i. No significant effect of total eggshell production on either bone breaking strength or bone mineral density was observed within the layer lines, suggesting that a high egg laying rate in itself does not necessarily pose a risk for bone weakness. Instead, a causal or predisposing effect of the age at onset of laying in combination with medullary bone quality is suspected.

ii. There were considerable differences in performance and bone characteristics between the phylogenetic groups, pointing to a strong effect of phylogenetic origin on bone stability. Our results suggest that the white-egg lines are more susceptible to bone weakness.

iii. The phylogenetic effect was particularly evident in the state of metabolic challenge, in which brown-egg lines proved more resilient and showed a less pronounced response to calcium deficiency. Our findings support the hypothesis of a more favourable physical constitution of brown-egg hens, possibly making them better adapted to high laying activity and more stable with regard to a varying calcium supply.

iv. The quantitive genetic analyses revealed a moderate inheritance of bone breaking strength and bone mineral density, suggesting that bone health could be improved through genetic selection. As there was no correlation between egg production and bone strength, selection for higher skeletal integrity seems to be possible, but effects of selection for extended laying persistence need to be monitored with regard to bone stability.

v. Genomic analyses identified a number of promising candidate genes for bone stability, all of which have a rather small effect confirming the assumption that skeletal traits are influenced by multiple genes. Functional analyses consolidated the evidence for a biological significance of these genomic regions for the chicken. To our knowledge, this is the first study applying a machine learning approach to bone data in chickens.

5.4 References

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6 Appendix

6.1 Corrigendum

animals

MDPI

Correction

Correction: Jansen, S., et al. Relationship between Bone Stability and Egg Production in Genetically Divergent Chicken Layer Lines. *Animals* 2020, *10*, 850

Simon Jansen [1,*], Ulrich Baulain [1], Christin Habig [1], Annett Weigend [1], Ingrid Halle [2], Armin Manfred Scholz [3], Henner Simianer [4,5], Ahmad Reza Sharifi [4,5] and Steffen Weigend [1,5]

1 Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut, 31535 Neustadt, Germany; ulrich.baulain@fli.de (U.B.); christin.habig@fli.de (C.H.); annett.weigend@fli.de (A.W.); steffen.weigend@fli.de (S.W.)
2 Institute of Animal Nutrition, Friedrich-Loeffler-Institut, 38116 Braunschweig, Germany; ingrid.halle@fli.de
3 Livestock Center of the Faculty of Veterinary Medicine, Ludwig-Maximilians-University Munich, 85764 Oberschleissheim, Germany; armin.scholz@lvg.vetmed.uni-muenchen.de
4 Animal Breeding and Genetics Group, Department of Animal Sciences, University of Göttingen, 37075 Göttingen, Germany; hsimian@gwdg.de (H.S.); rsharif@gwdg.de (A.R.S.)
5 Center for Integrated Breeding Research, University of Göttingen, 37075 Göttingen, Germany
* Correspondence: simon.jansen@fli.de

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The authors wish to make the following corrections to this paper [1]:

The body weight given as the weight of the 35th week of age is in fact the weight of the 49th week of age. However, the data presented are correct and the changes do not alter their interpretation.

Main Body Paragraphs Correction

There was an error in the original article. On page 4, Section *2.3. Experimental Procedure*, 1st paragraph, the sentence:

"Body weight (g) was measured at hatch and during the experimental period (at week 21, 25, 35 and 69) using a digital table scale (CPA 16001S, Sartorius, Göttingen, Germany) with a weighing accuracy of 0.1 g."

should be

"Body weight (g) was measured at hatch and during the experimental period (at week 21, 25, 49 and 69) using a digital table scale (CPA 16001S, Sartorius, Göttingen, Germany) with a weighing accuracy of 0.1 g."

Figures/Tables Correction

Due to the mistake mentioned above, we need to make the following changes to figures and tables:

Replace Figure 1:

Animals **2020**, *10*, 2355

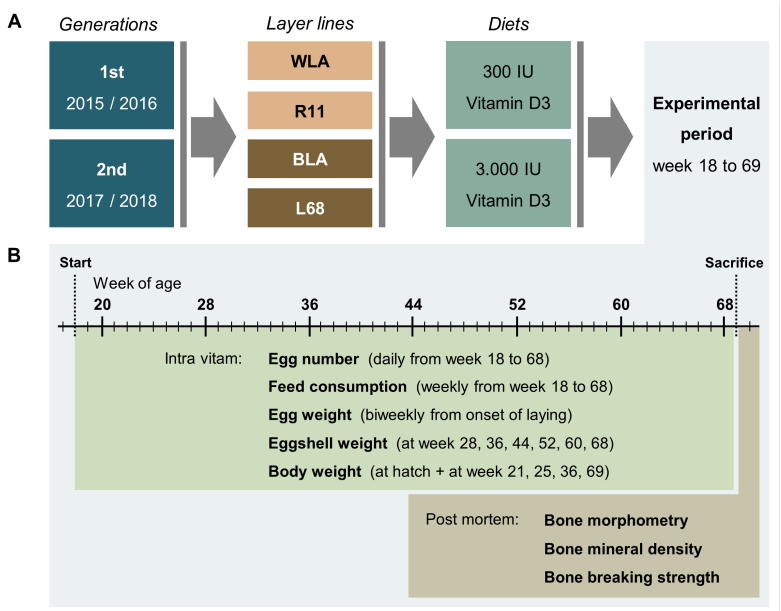

Figure 1. Schematic illustration of the experimental setup (**A**) and related data collection (**B**). In two consecutive generations, four chicken layer lines were allocated to a diet containing either 300 or 3000 IU of vitamin D3. During the experimental period, data on egg number, egg quality, feed consumption, and body weight were collected as indicated. Post mortem, bone morphometry, bone mineral density, and bone breaking strength were assessed.

With new Figure 1 below:

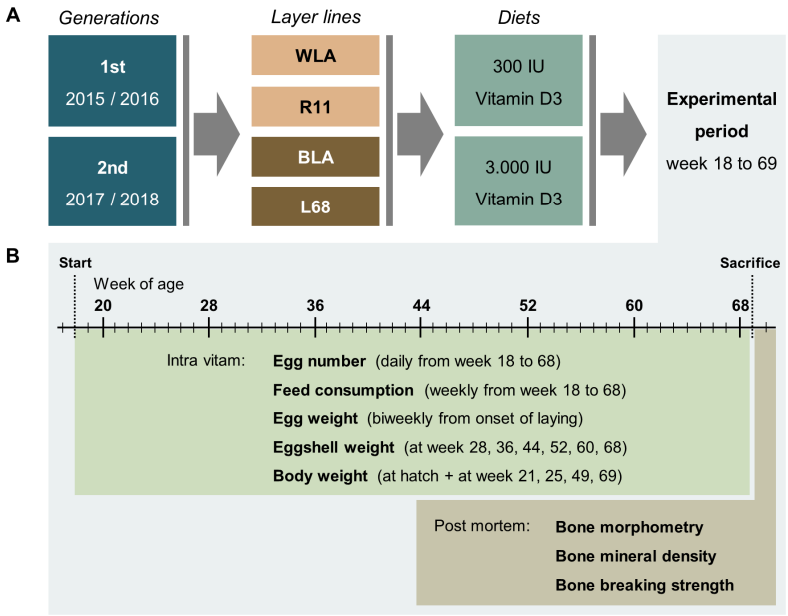

Figure 1. Schematic illustration of the experimental setup (**A**) and related data collection (**B**). In two consecutive generations, four chicken layer lines were allocated to a diet containing either 300 or 3000 IU of vitamin D3. During the experimental period, data on egg number, egg quality, feed consumption, and body weight were collected as indicated. Post mortem, bone morphometry, bone mineral density, and bone breaking strength were assessed.

Change in Supplementary File

Replace Table S5:

Table S5. Sample sizes for the analysis.

Variable	Total	Layer Line			
		WLA	R11	BLA	L68
Laying maturity	524	129	134	133	128
Total number of eggs	524	129	134	133	128
Egg weight	524	129	134	133	128
Eggshell weight	524	129	134	133	128
Eggshell proportion	524	129	134	133	128
Total eggshell production	524	129	134	133	128
Daily feed consumption	513	128	131	129	125
Feed-to-egg-conversion rate	513	128	131	129	125
Feed-to-eggshell conversion rate	513	128	131	129	125
Bone breaking strength Tibiotarsus	518	126	134	131	127
Bone mineral density Tibiotarsus	524	129	134	133	128
Weight Tibiotarsus	524	129	134	133	128
Length Tibiotarsus	524	129	134	133	128
Thickness Tibiotarsus	524	129	134	133	128
Bone breaking strength Humerus	516	128	131	132	125
Bone mineral density Humerus	519	129	134	128	128
Weight Humerus	521	127	134	132	128
Length Humerus	523	129	134	132	128
Thickness Humerus	523	129	134	132	128
Body weight at hatch	523	129	133	133	128
Body weight at week 21	524	129	134	133	128
Body weight at week 25	524	129	134	133	128
Body weight at week 35	524	129	134	133	128
Body weight at week 69	524	129	134	133	128

With new Table S5:

Table S5. Sample sizes for the analysis.

Variable	Total	Layer Line			
		WLA	R11	BLA	L68
Laying maturity	524	129	134	133	128
Total number of eggs	524	129	134	133	128
Egg weight	524	129	134	133	128
Eggshell weight	524	129	134	133	128
Eggshell proportion	524	129	134	133	128
Total eggshell production	524	129	134	133	128
Daily feed consumption	513	128	131	129	125
Feed-to-egg-conversion rate	513	128	131	129	125
Feed-to-eggshell conversion rate	513	128	131	129	125
Bone breaking strength Tibiotarsus	518	126	134	131	127
Bone mineral density Tibiotarsus	524	129	134	133	128
Weight Tibiotarsus	524	129	134	133	128
Length Tibiotarsus	524	129	134	133	128
Thickness Tibiotarsus	524	129	134	133	128
Bone breaking strength Humerus	516	128	131	132	125
Bone mineral density Humerus	519	129	134	128	128
Weight Humerus	521	127	134	132	128
Length Humerus	523	129	134	132	128
Thickness Humerus	523	129	134	132	128
Body weight at hatch	523	129	133	133	128
Body weight at week 21	524	129	134	133	128
Body weight at week 25	524	129	134	133	128
Body weight at week 49	524	129	134	133	128
Body weight at week 69	524	129	134	133	128

And replace Table S6:

Table S6. Least squares means ± standard errors and level of significance for body weight measured at hatching, and different weeks of age under the effect of layer line (LL), generation (Gen), and their interaction.

Effect	Body Weight (g)				
	Hatch	Week 21	Week 25	Week 35	Week 69
Layer line (LL)					
WLA	38.35 ± 0.37 [a]	1420.02 ± 15.79 [b]	1468.38 ± 16.19 [b]	1497.54 ± 20.46 [b]	1504.23 ± 22.26 [c]
R11	33.17 ± 0.36 [c]	1040.84 ± 15.60 [c]	1236.40 ± 15.99 [c]	1309.28 ± 20.21 [c]	1362.79 ± 21.99 [d]
BLA	39.35 ± 0.37 [a]	1584.15 ± 15.71 [a]	1663.55 ± 16.11 [a]	1821.81 ± 20.34 [a]	1838.10 ± 22.13 [b]
L68	34.84 ± 0.37 [b]	1568.91 ± 15.81 [a]	1714.92 ± 16.21 [a]	1837.91 ± 20.48 [a]	1923.44 ± 22.29 [a]
Generation (Gen)					
Gen 1	35.86 ± 0.26	1379.01 ± 11.17	1485.67 ± 11.45	1567.49 ± 14.47	1616.21 ± 15.76
Gen 2	37.00 ± 0.26	1427.95 ± 11.07	1555.96 ± 11.36	1665.79 ± 14.34	1698.07 ± 15.59
LL × Gen					
WLA × Gen1	37.77 ± 0.52	1376.72 ± 22.36	1415.84 ± 22.91	1460.33 ± 28.96	1443.48 ± 31.51
WLA × Gen2	38.93 ± 0.52	1463.31 ± 22.32	1520.93 ± 22.88	1534.75 ± 28.91	1564.98 ± 31.45
R11 × Gen1	32.64 ± 0.51	1027.33 ± 21.99	1222.77 ± 22.53	1284.20 ± 28.49	1338.12 ± 31.02
R11 × Gen2	33.69 ± 0.52	1054.36 ± 22.14	1250.04 ± 22.71	1334.37 ± 28.66	1387.45 ± 31.17
BLA × Gen1	38.84 ± 0.52	1549.66 ± 22.41	1627.91 ± 22.97	1767.90 ± 29.03	1804.33 ± 31.60
BLA × Gen2	39.87 ± 0.51	1618.63 ± 22.02	1699.19 ± 22.59	1875.73 ± 28.50	1871.86 ± 30.98
L68 × Gen1	34.18 ± 0.52	1562.32 ± 22.61	1676.16 ± 23.16	1757.53 ± 29.31	1878.89 ± 31.92
L68 × Gen2	35.51 ± 0.51	1575.50 ± 22.11	1753.67 ± 22.68	1918.30 ± 28.62	1968.00 ± 31.12
	ANOVA significance level (p value)				
	Layer line		Generation		LL × Gen
Hatch	<0.0001		0.0019		0.9908
Week 21	<0.0001		0.0020		0.3097
Week 25	<0.0001		<0.0001		0.3907
Week 35	<0.0001		<0.0001		0.2486
Week 69	0.0003		<0.0001		0.6892

Means within a column with different letters differ significantly (Tukey's HSD-Test, $p < 0.05$).

With new Table S6:

Table S6. Least squares means ± standard errors and level of significance for body weight measured at hatching, and different weeks of age under the effect of layer line (LL), generation (Gen), and their interaction.

Effect	Body Weight (g)				
	Hatch	Week 21	Week 25	Week 49	Week 69
Layer line (LL)					
WLA	38.35 ± 0.37 [a]	1420.02 ± 15.79 [b]	1468.38 ± 16.19 [b]	1497.54 ± 20.46 [b]	1504.23 ± 22.26 [c]
R11	33.17 ± 0.36 [c]	1040.84 ± 15.60 [c]	1236.40 ± 15.99 [c]	1309.28 ± 20.21 [c]	1362.79 ± 21.99 [d]
BLA	39.35 ± 0.37 [a]	1584.15 ± 15.71 [a]	1663.55 ± 16.11 [a]	1821.81 ± 20.34 [a]	1838.10 ± 22.13 [b]
L68	34.84 ± 0.37 [b]	1568.91 ± 15.81 [a]	1714.92 ± 16.21 [a]	1837.91 ± 20.48 [a]	1923.44 ± 22.29 [a]
Generation (Gen)					
Gen 1	35.86 ± 0.26	1379.01 ± 11.17	1485.67 ± 11.45	1567.49 ± 14.47	1616.21 ± 15.76
Gen 2	37.00 ± 0.26	1427.95 ± 11.07	1555.96 ± 11.36	1665.79 ± 14.34	1698.07 ± 15.59
LL × Gen					
WLA × Gen1	37.77 ± 0.52	1376.72 ± 22.36	1415.84 ± 22.91	1460.33 ± 28.96	1443.48 ± 31.51
WLA × Gen2	38.93 ± 0.52	1463.31 ± 22.32	1520.93 ± 22.88	1534.75 ± 28.91	1564.98 ± 31.45
R11 × Gen1	32.64 ± 0.51	1027.33 ± 21.99	1222.77 ± 22.53	1284.20 ± 28.49	1338.12 ± 31.02
R11 × Gen2	33.69 ± 0.52	1054.36 ± 22.14	1250.04 ± 22.71	1334.37 ± 28.66	1387.45 ± 31.17
BLA × Gen1	38.84 ± 0.52	1549.66 ± 22.41	1627.91 ± 22.97	1767.90 ± 29.03	1804.33 ± 31.60
BLA × Gen2	39.87 ± 0.51	1618.63 ± 22.02	1699.19 ± 22.59	1875.73 ± 28.50	1871.86 ± 30.98
L68 × Gen1	34.18 ± 0.52	1562.32 ± 22.61	1676.16 ± 23.16	1757.53 ± 29.31	1878.89 ± 31.92
L68 × Gen2	35.51 ± 0.51	1575.50 ± 22.11	1753.67 ± 22.68	1918.30 ± 28.62	1968.00 ± 31.12
	ANOVA significance level (p value)				
	Layer line		Generation		LL × Gen
Hatch	<0.0001		0.0019		0.9908
Week 21	<0.0001		0.0020		0.3097
Week 25	<0.0001		<0.0001		0.3907
Week 49	<0.0001		<0.0001		0.2486
Week 69	0.0003		<0.0001		0.6892

Means within a column with different letters differ significantly (Tukey's HSD-Test, $p < 0.05$).

The authors would like to apologize for any inconvenience caused to the readers by these changes.

Reference

1. Jansen, S.; Baulain, U.; Habig, C.; Weigend, A.; Halle, I.; Scholz, A.M.; Simianer, H.; Sharifi, A.R.; Weigend, S. Relationship between Bone Stability and Egg Production in Genetically Divergent Chicken Layer Lines. *Animals* **2020**, *10*, 850. [CrossRef] [PubMed]

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Finally, I would like to thank my family and in particular Hannes for their invaluable support on the way to my doctorate. My deep and sincere gratitude to my parents who have enabled me to go this way. I dedicate this thesis to them.

6.3 Declarations

1. Hereby, I declare that this doctoral thesis has not been presented to any other examination body either in its present or a similar form. Furthermore, I also affirm that I have not applied for a doctoral degree at any other university.

2. Hereby, I declare that this dissertation was undertaken independently and without any unauthorised aid.

Göttingen, 18 June 2021

Simon Jansen