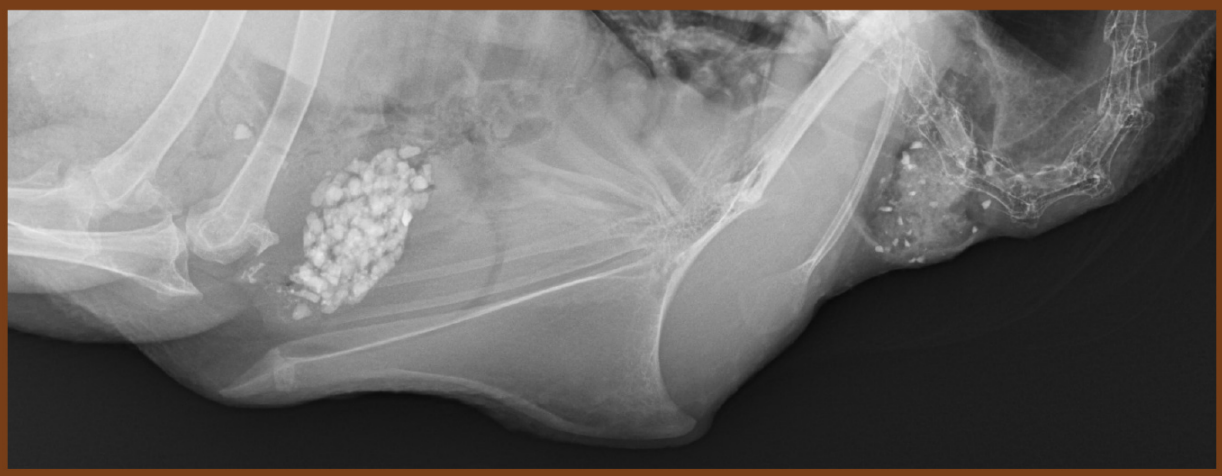
Aus dem Institut für Tierschutz, Tierverhalten und Versuchstierkunde
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin
und
dem Institut für Tierschutz und Tierhaltung
des Friedrich-Loeffler-Instituts



The Influence of Egg Production, Genetic Background, Age, and Housing System on Keel Bone Damage in Laying Hens

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Beryl Katharina Eusemann
Tierärztin aus Emmendingen

Berlin 2020
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To the Protagonists of this Work: The Laying Hens

Table of Contents

List of Abbreviations

BMD	**B**one **M**ineral **D**ensity
cGnRH	**C**hicken **G**o**n**adotropin-**R**eleasing **H**ormone
CT	**C**omputed **T**omography
DEXA	**D**ual **E**nergy **X**-ray **A**bsorptiometry
EU	**E**uropean **U**nion
FAO	**F**ood and **A**griculture **O**rganization of the United Nations
FSH	**F**ollicle-**S**timulating **H**ormone
GnIH	**G**o**n**adotropin-**I**nhibiting **H**ormone
GnRH	**G**o**n**adotropin-**R**eleasing **H**ormone
HPG axis	**H**ypothalamic-**P**ituitary-**G**onadal axis
KBD	**K**eel **B**one **D**amage
LB	**L**ohmann **B**rown
LSL	**L**ohmann **S**elected **L**eghorn
LH	**L**uteinizing **H**ormone
M.	***M****usculus* (= muscle)
PTH	**P**ara**t**hyroid **H**ormone

1 Introduction

Fractures and deviations of the keel bone, i.e., the ventral part of the sternum in birds, belong to the most severe animal welfare problems in laying hens (EFSA 2005; FAWC 2010; FAWC 2013). Both symptoms are often summarized to the term „keel bone damage“ (KBD) and show a very high prevalence of up to 97 % (keel bone fractures) (Rodenburg et al. 2008) or 83 % (keel bone deviations) (Käppeli et al. 2011a), respectively. There is strong evidence that keel bone fractures are painful and impair the mobility of affected hens (Nasr et al. 2012a; Nasr et al. 2012b; Richards et al. 2012; Nasr et al. 2013; Nasr et al. 2015; Riber et al. 2018). The etiology of KBD is not yet clear. Several factors such as the housing system (Keutgen et al. 1999; Wilkins et al. 2011; Petrik et al. 2015), genetic background (Wahlström et al. 2001; Hocking et al. 2003; Vits et al. 2005; Habig and Distl, 2013; Heerkens et al. 2016a; Candelotto et al. 2017), age (Habig and Distl 2013; Stratmann et al. 2015a; Heerkens et al. 2016a; Toscano et al. 2018), and nutrition (Toscano et al. 2015; Eusebio-Balcazar et al. 2018) seem to influence this disorder. However, the direction of these effects is not consistent throughout the studies. Furthermore, it has not been investigated into detail to which extent internal or external factors are responsible for the development of KBD. While some authors focus on the environment of the laying hens, i.e., on external factors such as the housing system, perch design, and ramps between different tiers (e.g., Wilkins et al. 2011; Stratmann et al. 2015a; Heerkens et al. 2016a), other authors put their focus on the laying hen itself, i.e., on internal factors such as genetics, age, bone composition or metabolic bone diseases (e.g., Fleming et al. 2004; Candelotto et al. 2017). Another internal factor that is closely linked to bone metabolism and may, thus, influence KBD is egg production. A lot of calcium is required to produce the eggshell (Romanoff and Romanoff 1949; Habig et al. 2017). The skeleton, especially the medullary bone, a special kind of woven bone which is unique to female birds and which is located in the medullary cavity of some bones (Bonucci and Gherardi 1975; Whitehead 2004), serves as a source of calcium (Urist and Deutsch 1960; Fleming et al. 1998b). It is suggested that at the onset of lay, osteoblasts change their function from forming structural bone to forming medullary bone which negatively influences bone strength (Whitehead 2004). It is further suggested that this is mediated by estrogens (Whitehead 2004). However, the influence of egg production and estrogens on keel bone health has not been studied into detail so far. The aim of this study was to get a better insight into the etiology of keel bone fractures and deviations. The role of the external factor housing system and the internal factors genetic background, age, egg production, and estradiol-17β in KBD were investigated. To that aim, a method to repeatedly assess the keel bone in living hens (chapter 3.1) and a method to obtain a model with non-egg laying hens (chapter 3.2) were developed and evaluated. With these methods, prevalence and severity of keel bone fractures and deviations were compared between two housing systems (chapter 3.1), different layer lines (chapters 3.1 and 3.3), and between egg laying and non-egg laying hens (chapters 3.2 and 3.3).

2 Literature Review

2.1 Keel Bone Damage in Laying Hens

2.1.1 Definition of Keel Bone Damage

The keel bone (*carina sterni*) is a very prominent part of the sternum in flying birds. It protrudes from the medial part of the *corpus sterni* and provides attachment surface for the flight muscles, namely the *M. pectoralis* and *M. supracoracoideus* (Zheng et al. 2012). The term “keel bone damage” (KBD) covers fractures and deviations of the keel bone (Figure 1). In some studies, especially the early ones about KBD, there is no differentiation between keel bone fractures and deviations and the definition of both also varies. This makes the comparison between results of different studies complicated. Depending on the assessment method, it can also be difficult to differentiate between both forms of damage. However, a publication by Casey-Trott et al. (2015) gave a definition for both fractures and deviations which helps to harmonize different studies. The authors defined a keel bone fracture as being “characterized by sharp bends, shearing, and/or fragmented sections of the keel bone. Fractures may extend from the ventral to the dorsal surface in the sagittal plane, though they can also be cranial to caudal, or a combination of both” (Casey-Trott et al. 2015). Keel bone deviations are also called keel bone deformations (O'Connor et al. 2015) and keel bone deformities (Fleming et al. 2004; Vits et al. 2005; Käppeli et al. 2011a). A deviated keel bone is defined as a “bone with an abnormally shaped structure that has not resulted from a fracture but contains section(s) that vary from a theoretically perfect 2-dimensional straight plane in either the transverse or sagittal planes. Additionally, indentations along the ventral surface can also be classified as a deviation” (Casey-Trott et al. 2015). Keel bone fractures and deviations can also be present in one keel bone at the same time.

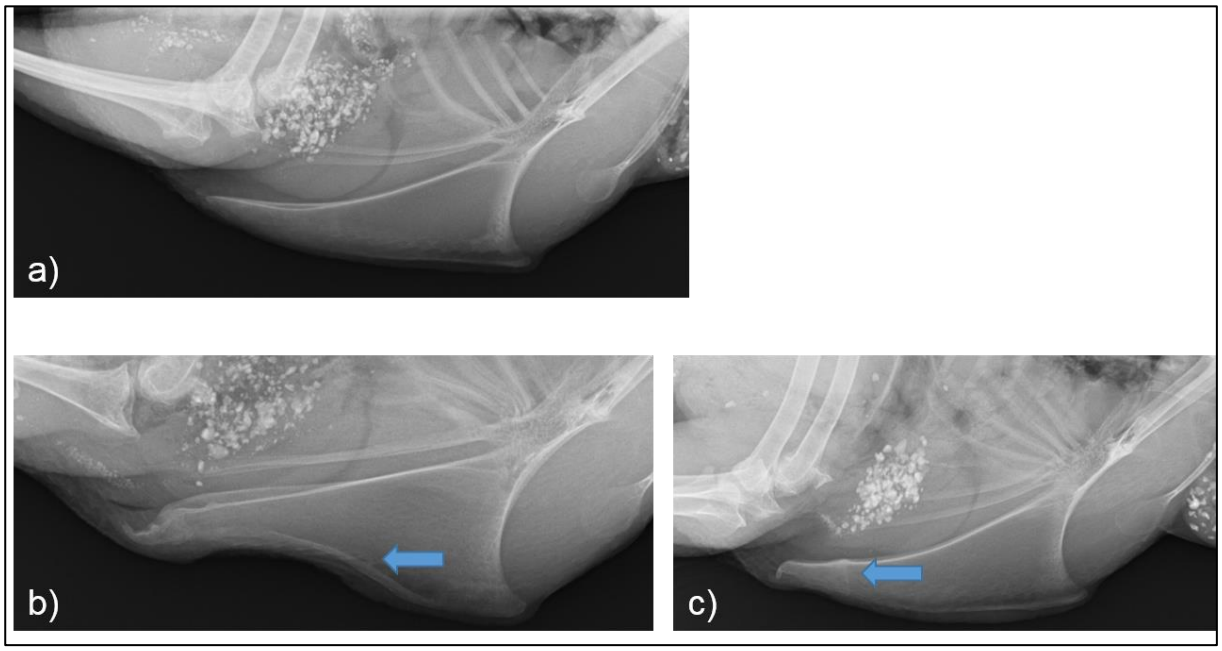

Figure 1: Radiographs of **a)** a healthy keel bone; **b)** a keel bone with a severe deviation (arrow), and **c)** a keel bone with a fracture (arrow).

2.1.2 Prevalence of Keel Bone Damage

The prevalence of keel bone fractures and deviations in laying hens is extremely high. While at the onset of lay, i.e., at about 20 weeks of age, around 5 % of the hens are affected by keel bone fractures, at the end of a production cycle, i.e., at about 72 weeks of age, prevalence reaches up to 97 % (Rodenburg et al. 2008; Wilkins et al. 2011; Richards et al. 2012; Petrik et al. 2015; Heerkens et al. 2016a). Prevalence of keel bone deviations ranges from 2.6 % to 83 % (Fleming et al. 2004; Käppeli et al. 2011a).
The Food and Agriculture Organization of the United Nations (FAO) estimated that in 2011, there were 6.6 billion laying hens worldwide, as reported in a compendium by the organization Compassion in World Farming (CIWF 2013). This means that possibly up to 6.4 billion laying hens may have been affected by keel bone fractures and up to 5.4 billion laying hens by keel bone deviations in 2011 alone. Although this is only a rough estimation and the number may be over- or underestimated due to differences in prevalence among housing systems and layer lines, it is clear that an alarmingly large number, i.e., several billions, of laying hens is affected by KBD every year.

2.1.3 Keel Bone Damage and Animal Welfare

Keel bone fractures may negatively influence animal welfare due to several reasons: First, as the “physiological, biochemical and anatomical mechanisms which are known to be correlated with painful experiences are very similar in both birds and humans” (Gentle 2011), bone fractures in laying hens may be as painful as bone fractures in humans (Gentle 2011). Second, as the flight muscles are attached to the keel bone, locomotive behavior may be impaired if this bone is damaged (Riber et al. 2018).
Several studies have observed the behavior of hens with keel bone fractures and compared it to the behavior of unaffected hens. An increased latency to descend perches at different heights to get a food reward was found in laying hens with keel bone fractures (Nasr et al. 2012a; Nasr et al. 2012b; Nasr et al. 2015). However, latency decreased significantly after subcutaneous administration of the analgesic butorphanol in hens with fractures while this treatment did not have any effect on hens without fractures (Nasr et al. 2012a). In a subsequent conditioned place preference experiment, the researchers found that hens with keel bone fractures preferred an environment of a specific color in which they had been given butorphanol compared to an environment of another color in which they had received saline. In contrast, hens without keel bone fractures showed no preference (Nasr et al. 2013). The authors concluded that hens with keel bone fractures experience pain which can be eased with butorphanol (Nasr et al. 2012a; Nasr et al. 2013). Furthermore, the same authors found that hens with keel bone fractures needed more time to finish a runway test with obstacles compared to hens without fractures and that the latency to fly from the ground to a perch at 100 cm height was positively correlated with fracture severity. Two possible explanations were given: movement could have been impaired physically or hens with fractures could be less motivated to move, perhaps because of associated pain (Nasr et al. 2012b). In addition, hens of the same study spent more time sleeping when affected by keel bone fractures and were less active compared to hens without keel bone fractures which indicates that “keel-bone fractures may prevent hens from performing normal behaviours or accessing resources, so keel fractures may be considered detrimental to hens’ welfare” (Nasr et al. 2012b). Similarly, hens with keel bone fractures were found to use pop holes to access free-range less frequently than hens without fractures, indicating that keel bone fractures “affect the birds’ ability or willingness to utilise the outdoor range provided by free-range housing systems, thereby reducing the potential welfare advantages of this type of housing” (Richards et al. 2012).

In contrast to keel bone fractures, the influence of keel bone deviations on animal welfare has not yet been investigated into detail. In general, it is assumed that deviations have a minor

impact on animal welfare compared to keel bone fractures (EFSA 2005). However, based on the presence of fracture callus in deviated keel bones found in two studies (Fleming et al. 2004; Scholz et al. 2008a), it is possible that deviations are caused by fractures and, thus, may be painful as well (Fleming et al. 2004; Scholz et al. 2008a).

All findings on altered behavior in affected hens together give strong evidence that keel bone fractures and possibly deviations are painful and have a negative impact on animal welfare. Furthermore, a keel bone fracture is less likely to be detected than a long bone fracture in a commercial farm, leading to prolonged suffering (Fleming et al. 2004). Due to the probable painfulness and the extremely high prevalence, keel bone fractures are considered as one of the most serious animal welfare problems in the egg production industry (EFSA 2005; FAWC 2010; FAWC 2013).

2.1.4 Current Knowledge About the Etiology and Influencing Factors of Keel Bone Damage

It is widely assumed that KBD is a multifactorial disorder (Heerkens et al. 2016b; Toscano et al. 2018). Some of the influencing factors seem to be the external factors housing system and nutrition and the internal factors genetic background and age of the laying hens.
The housing system has a large effect on the prevalence of keel bone fractures and deviations (Keutgen et al. 1999; Wilkins et al. 2011; Petrik et al. 2015). In the European Union (EU), conventional cages have been banned in 2012 (Appleby, 2003). Laying hens are now housed either in furnished cages, multi-tier, barn or free-range, including organic systems (Sandilands et al. 2009). On the one hand, the ban on conventional cages in the EU was a positive development for bone health of laying hens because lack of movement in conventional cages has been shown to impair mechanical and structural properties of bones (Fleming et al. 1994; Shipov et al. 2010). There is even a term for a disease characterized by poor bone quality in laying hens housed in cages: cage(d) layer fatigue (Urist and Deutsch 1960; Riddell et al. 1968; Fleming et al. 1998a; Whitehead and Fleming 2000; Jendral et al. 2008). This disease is understood as a severe form of osteoporosis, i.e., a loss of structural bone (Whitehead and Fleming 2000), that leads to spinal fractures and paralysis (Riddell et al. 1968). On the other hand, although bones are stronger in more active systems, the prevalence of keel bone fractures has been found to be higher in alternative housing systems compared to cages (Wilkins et al. 2011; Petrik et al. 2015). Also the prevalence of keel bone deviations has been found to be lower in hens housed in cages compared to floor housed hens in one study (Keutgen et al. 1999). Concerning keel bone fractures, it is assumed that these are caused by collisions with housing equipment such as perches or nest boxes and that this risk is especially high in housing systems which allow for increased activity (Sandilands et al. 2009; Wilkins et al. 2011). This assumption is supported by findings that ramps which facilitate to reach different tiers in aviaries help to decrease the prevalence of keel bone fractures (Heerkens et al. 2016a). Concerning both keel bone fractures and deviations, the presence of perches in alternative systems but not in conventional cages may explain differences in prevalence of KBD (Sandilands et al. 2009). Again, the risk of collisions and thus of keel bone fractures increases with the presence of perches and deviations may be caused by the pressure on the keel bone caused by perches while roosting (Sandilands et al. 2009). This possibly negative influence on the keel bone by perches, which contrariwise improve bone structure by load bearing opportunity (Sandilands et al. 2009), increase trabecular bone volume (Wilson et al. 1993) and bone mineral content (Donaldson et al. 2012), and enable hens to perform their natural roosting behavior (Sandilands et al. 2009), may be diminished by using different perch shapes and materials. For example, the prevalence of deviations has been found to be lower in pens with plastic perches compared to pens with metal perches (Käppeli et al. 2011b). In another study, covering metal perches with a soft cushion led to a reduced prevalence of fractures and deviations (Stratmann et al. 2015a). The authors assumed that the compressible cushion

absorbed part of the kinetic energy during collisions and thereby reduced the energy transferred to the keel bone, resulting in a lower risk to fracture (Stratmann et al. 2015a). The lower prevalence of deviations may be caused by a lower peak force and larger contact area in perches with a soft cushion which have been found by Pickel et al. (2011).

Also the nutrition of laying hens seems to influence the prevalence of KBD. In one experiment, Toscano et al. (2015) showed that keel bone fracture prevalence was lower in hens fed with a diet supplemented with omega-3 fatty acids compared to hens fed with a control diet. However, this was not the case in a second experiment where hens fed with a diet supplemented with omega-3 fatty acids showed even more keel bone fractures than hens fed with a control diet (Toscano et al. 2015). The authors assumed that these contrary findings might be explained by differences in the n3/n6 ratio and in the proportion of long chain fatty acids between both experiments (Toscano et al. 2015). Eusebio-Balcazar et al. (2018) found that feeding pullets with a diet containing a blend of fine and coarse limestone particles instead of a diet containing only fine limestone particles reduced the prevalence of keel bone deviations in the rearing and in the subsequent laying phase.

Another influencing factor on KBD is the genetic background of laying hens. KBD differs between white and brown layer lines. More keel bone deviations were found in Lohmann Brown (LB) compared to white Lohmann Selected Leghorn (LSL) hens (Vits et al. 2005; Habig and Distl 2013). Similarly, LSL hens were also less susceptible to keel bone deviations compared to a cross-breed of Leghorn and Rhode Island Red-line, called SLU-1329 (Wahlström et al. 2001). Furthermore, deviations were shown to be more severe in LB compared to LSL hens (Habig and Distl 2013). However, in another study, a higher prevalence of deviations was found in Dekalb White hens compared to ISA Brown hens, while fracture prevalence was higher in ISA Brown hens (Heerkens et al. 2016a). Taken together, it is neither clear whether white or brown layer lines are more susceptible to KBD nor which mechanisms are responsible for these differences. Another factor which differs between layer lines and may influence KBD is the laying performance. However, there is very limited knowledge about the possible relationship between selection for a high laying performance and KBD so far. Hocking et al. (2003) found a lower radiographic density of the keel bone and tibiotarsus in commercial layer lines that showed a high laying performance compared to traditional layer lines with a lower laying performance. Furthermore, breaking strength of humeri and tibiotarsi was also lower in commercial than in traditional breeds. Eggshell strength did not differ between the layer lines. The authors concluded that “eggshell quality is maintained in genetically selected lines at the expense of bone strength and bone radiographic density” (Hocking et al. 2003). Similarly, Candelotto et al. (2017) compared the probability of getting an experimental fracture between layer lines differing in, amongst others, laying performance and breeding goals. The authors found the lowest number of experimental fractures in one experimental line which descended from a dam line that had not been selected for any breeding goal for several years and a sire line that had been bred for dual egg and meat production. However, no difference was found between another experimental line with a low laying performance compared to the commercial lines that had been selected for high laying performance (Candelotto et al. 2017). Thus, there is evidence that selection for increased productivity may have led to poor keel bone health but further studies are required to strengthen this assumption.

Furthermore, the age of laying hens has an influence on KBD. Several authors found an increasing prevalence of keel bone fractures and deviations with age (Wahlström et al. 2001; Scholz et al. 2008b; Käppeli et al. 2011b; Habig and Distl 2013; Heerkens et al. 2016a) which is consistent with the finding that bone strength deteriorates with age (EFSA 2015). However, in some studies, prevalence did not increase steadily but peaked at about 50 weeks of age and then leveled off or even decreased (Petrik et al. 2015; Stratmann et al. 2015a; Toscano et al. 2018). Due to the age-dependence of KBD, longitudinal studies are indispensable when seeking for causes of this disorder.

Taken together, several internal factors such as age and genetic background as well as external factors such as housing system and nutrition have been found to be related to KBD in laying hens. However, the direction of the effects is not consistent throughout studies and it is not clear to which extent each of these factors contributes to the etiology of keel bone fractures and deviations.

2.1.5 Methods to Assess the Keel Bone in Laying Hens

Methods to assess KBD in laying hens can be divided into methods that are suitable for in vivo assessment and methods that are suitable for postmortem assessment.
Methods for in vivo assessment include palpation, radiographic examination, computed tomography scans, and ultrasonography of the keel bone. Palpation is the method that is mostly used to assess KBD (e.g., in Rodenburg et al. 2008; Richards et al. 2012; Bestman and Wagenaar, 2014; Petrik et al. 2015; Stratmann et al. 2015a; Toscano et al. 2015, amongst others). It is a cost-effective and quick method that is suitable for on-farm assessment and for large-scale studies (Wilkins et al. 2004; Petrik et al. 2013). However, results depend a lot on the training level of the individual person (Petrik et al. 2013; Buijs et al. 2019; Chargo et al. 2019b) and may differ between observers (Wilkins et al. 2004) as well as examinations (Buijs et al. 2019). Thus, in-depth training and the assessment of the inter- as well as intra-observer reliability are indispensable prior to any palpation of the keel bone (Casey-Trott et al. 2015; Buijs et al. 2019). Furthermore, highly variable values for the accuracy, sensitivity, and specificity of keel bone palpation have been found (Wilkins et al. 2004; Buijs et al. 2019). Especially, the prevalence of fractures has often been found to be overestimated because of unevenness of the keel bone which may feel like a fracture (Wilkins et al. 2004) and assessment of caudal tip fractures has been found to be inaccurate in one study (Buijs et al. 2019). Also, keel bone palpation is not sensitive enough to monitor the healing process and fresh and minor fractures cannot be detected due to missing swelling of the surrounding tissue (Richards et al. 2011). Radiography is a method which may overcome the disadvantages of palpation. However, there are only few studies on radiographic examination of the keel bone in laying hens. Richards et al. (2011) repeatedly radiographed the keel bone of 24 hens to observe fracture healing. They were able to display the whole keel bone, including the dorsal aspect which is not accessible by palpation, and to detect old and new keel bone fractures. Clark et al. (2008) radiographed the whole skeleton of laying hens and assessed, amongst others, indentations of the keel bone. Hens in both studies were either anaesthetized (Richards et al. 2011) or dead (Clark et al. 2008) when being radiographed. Širovnik and Toscano (2017) presented a method which allowed for taking radiographs of the keel bone without anesthesia by hanging the hens upside-down on a shackle and, thus, restraining them. Rufener et al. (2018) used the same method to develop a scoring system for keel bone fractures. However, none of these studies used radiography for the longitudinal assessment of KBD throughout the entire life of laying hens. Furthermore, most of them included only the detection of keel bone fractures, but not deviations, in their protocol. Studies about using computed tomography (CT) to examine the keel bone are even scarcer. So far, CT has been used to assess the presence and severity of KBD (Regmi et al. 2016; Chargo et al. 2019b;), keel bone morphology and bone mineral density (Regmi et al. 2016), as well as changes of the keel bone throughout the laying period (Chargo et al. 2019a). The advantage of CT scans is that they produce a three-dimensional image of the keel bone which allows for an even more precise description of fractures (Casey-Trott et al. 2015), including their exact location within the keel bone. Furthermore, they enable the assessment of bone mineral density in live birds. The disadvantage is that, in contrast to palpation and radiography, CT cannot be applied on-farm (Casey-Trott et al. 2015) and that the costs are high. The fourth method for in vivo assessment of the keel bone is ultrasonography. However, this method is very rarely used and has only been described once at a conference (Sandilands et al. 2010). Thus, detailed information about advantages and disadvantages of ultrasonography for the assessment of KBD is lacking.

The advantage of in vivo methods to assess KBD is that they allow for longitudinal studies, i.e., the keel bone of the same hen can be examined at different time points throughout the hen's life. As prevalence of KBD is age-dependent, these longitudinal studies are of utmost importance.

Methods for postmortem assessment of the keel bone include dissection, bone strength measurements, density measurements, histology, and chemical analyses. Dissection is often used in order to detect keel bone fractures and deviations and to assess the severity of KBD with a scoring system (e.g., in Fleming et al. 2004; Vits et al. 2005; Scholz et al. 2008a; Scholz et al. 2008b; Wilkins et al. 2011; Donaldson et al. 2012; Stratmann et al. 2015b; Toscano et al. 2015, amongst others). Furthermore, dissected keel bones can also be measured in order to get information about their morphology such as keel bone length, height, and surface area (Casey-Trott et al. 2017). In addition, keel bone dissection is used as a reference method to assess the accuracy of keel bone palpation (Wilkins et al. 2004; Wilkins et al. 2011; Heerkens et al. 2016a; Buijs et al. 2019). Keel bone strength can be assessed with a three-point compression test (Nicol et al. 2006; Rodenburg et al. 2008; Wilkins et al. 2011) or with a shear testing device (Fleming et al. 2004). In both tests, a certain load or force is applied to a specific point of the keel bone and the applied strength when the bone breaks is measured and defined as the breaking strength of the bone. There are several methods to assess the density of excised keel bones. Values for the radiographic density can be obtained by taking radiographs of the excised keel bone together with an aluminum step-wedge. Each radiograph is then calibrated from the step-wedge image and radiographic density given as millimeters of aluminum equivalent (Fleming et al. 1998a; Fleming et al. 2004). Bone mineral density (BMD) of the keel bone can be measured with dual energy X-ray absorptiometry (DEXA) as has been done by Enneking et al. (2012), Hester et al. (2013), and Stratmann et al. (2016). This method is "the gold standard for clinical use in diagnosing osteoporosis in human patients" (Kim et al. 2012) and determines BMD in mg/cm^2 (Kim et al. 2012). DEXA can also be used in live hens (Hester et al. 2013). Only a few authors have presented histological images of keel bones so far (Fleming et al. 2004; Scholz et al. 2008a). This method provides a detailed insight into keel bone structure, including the amount of cortical, trabecular, and medullary bone, the amount and distribution of different bone cells, and the detection of small amounts of callus material which are not detectable with other methods. Lastly, chemical analyses of the keel bone can give information about the composition of the bone, e.g., the amount of water, organic, and inorganic matter. However, this method has rarely been used with the keel bone to date and has been limited to bone ash analysis using a muffle furnace (Fleming et al. 2004). The advantage of postmortem assessment of the keel bone is that it gives a more detailed insight into the keel bone, including its structure and composition. However, the disadvantage is that it does not allow for longitudinal studies on the same hen.

2.2 Bone Characteristics and Calcium Metabolism in Laying Hens

2.2.1 Medullary Bone

Female birds possess different types of bone: structural bone, i.e., cortical and trabecular bone, which are also found in mammals and male birds, and, in addition, medullary bone, which is unique to female birds and crocodilians (Whitehead 2004). The medullary bone is a special kind of woven bone which is found in the medullary cavity (Bonucci and Gherardi 1975) (Figure 2) and that is formed shortly before the onset of lay in response to estrogens and androgens (Dacke et al. 1993). It is located at the endosteal surface of cortical bone as well as at the surface of trabecular bone and extends in spicules into the medullary cavity, towards the center of the bone (Bonucci and Gherardi 1975; Whitehead 2004). Medullary bone serves

as a source of calcium for eggshell calcification (Urist and Deutsch 1960; Fleming et al. 1998b) and differs from structural, i.e., cortical and trabecular bone in several aspects. Intrinsic strength of medullary bone is much lower compared to structural bone (Knott et al. 1995). This may be caused by the fact that collagen fibrils are irregularly arranged in medullary bone (Whitehead 2004), in contrast to the organization in osteons in cortical (Rho et al. 1998) and inline to their loading environment in trabecular bone (Davison et al. 2006) and by the fact that most of the spicules of medullary bone are not connected with each other (Whitehead 2004). Furthermore, a higher number of osteoclasts and osteoblasts is found in medullary compared to structural bone (Kim et al. 2012). Mineral particles are larger and thicker in cortical compared to medullary bone (Kerschnitzki et al. 2014). In addition, they are oriented with respect to the organic matrix in cortical bone, but randomly distributed in medullary bone (Dacke et al. 1993; Kerschnitzki et al. 2014). Lastly, medullary bone has a large surface area per bone volume and is well vascularized (Dacke et al. 1993; Kerschnitzki et al. 2014). All these characteristics of medullary bone together make it a very active bone remodeling system (Van de Velde et al. 1984) whose turnover rate is at least 10 – 15 times higher than that of cortical bone (Hurwitz 1965) and that serves the high metabolic calcium demand during lay (Kerschnitzki et al. 2014), contributing 35 to 40 % of the calcium for eggshell formation (Mueller et al. 1964; Kim et al. 2012). Medullary bone is usually found and investigated in the diaphysis of long bones, especially those of the legs, i.e., the tibiotarsus and the femur (Dacke et al. 1993; Whitehead 2004; Kerschnitzki et al. 2014). The humerus presents medullary bone in some hens but not in others, apparently due to varying degrees of pneumatization (Fleming et al. 1998b; Whitehead 2004). However, medullary bone has also been found in the keel bone (Fleming et al. 2004), indicating a possible contribution of the keel bone to calcium metabolism in the laying hen.

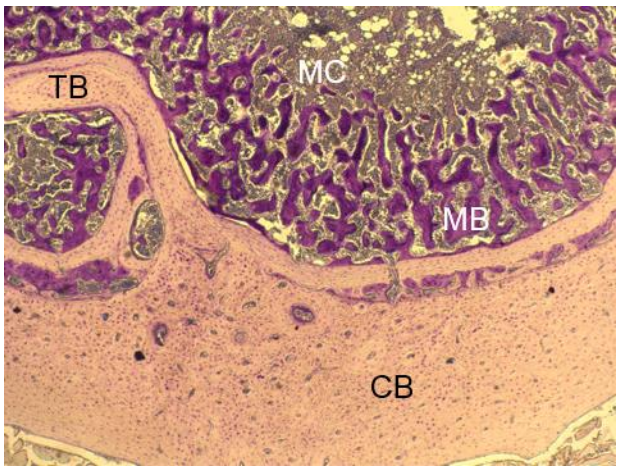

Figure 2: Histological slice of the right femur of an adult laying hen with medullary bone. H&E stain, 500x
CB = cortical bone, TB = trabecular bone,
MB = medullary bone, MC = medullary cavity.

2.2.2 Calcium Metabolism in Laying Hens

As commercial laying hens mostly lay one egg with a calcified eggshell each day, the calcium metabolism is extraordinarily intense (Kerschnitzki et al. 2014). Approximately 2 g of calcium are needed for each egg (Romanoff and Romanoff 1949; Habig et al. 2017) which represents about 10 % of the total body calcium (Kerschnitzki et al. 2014) and which has to be transported to the eggshell gland every day. 50 – 60 % of the calcium for eggshell formation is provided by dietary sources (Kerschnitzki et al. 2014; Habig et al. 2017). Calcium from dietary sources

is primarily absorbed in the duodenum and jejunum (Hurwitz and Bar 1965). This uptake is increased during eggshell deposition (Hurwitz and Bar 1965). Calcium which is not absorbed in the small intestine is excreted in feces (Etches 1987). The absorbed calcium moves into the vascular system which serves as a transitory storage and transport system for the calcium (Etches 1987). From the blood vessels, calcium is distributed to the different organ systems. Part of the calcium is continuously filtered in the kidneys and secreted with the urine. The amount of calcium that is secreted with the urine decreases during eggshell formation (Etches 1987). Large amounts of the calcium are transferred from the blood to the uterus where the eggshell is built. During eggshell formation, approximately 100 – 200 mg of calcium are transported from the blood to the uterus per hour (Etches 1987). As large part of the eggshell calcification takes place during the night, when the laying hen does not consume feed and, thus, calcium is not absorbed from the gut, a large amount of calcium for this purpose is provided by the skeleton, i.e., the medullary bone (Etches 1987; Kerschnitzki et al. 2014). This can be seen by increased collagenolytic activity in the medullary bone during periods of calcification, amongst others (Bannister and Candlish 1973). The calcium storage in the medullary bone is refilled when no eggshell is being produced, i.e., during the day (Dacke et al. 1993; Kerschnitzki et al. 2014). Thus, the medullary bone undergoes a recurring cycle of net gain and loss of calcium (Etches 1987). The role of cortical and trabecular bone in the calcium metabolism of the laying hen is less clear. Several researchers found that during a prolonged period of calcium deprivation in the diet, the degree of mineralization as well as the amount of cortical bone decreased, in contrast to the degree of mineralization and amount of medullary bone (Taylor and Moore 1954; Dacke et al. 1993). It is assumed that cortical and medullary bone differ in their ease of calcium mobilization and that during periods with adequate calcium levels in the diet, sufficient calcium for the eggshell can be mobilized from the medullary bone, while during prolonged periods of calcium deprivation, cortical bone is absorbed in order to provide sufficient calcium for the eggshell and, in addition, for refilling the medullary calcium reservoir (Dacke et al. 1993).
As in other vertebrates, the calcium metabolism is controlled by several hormones in laying hens. The main ones involved are parathyroid hormone (PTH), calcitonin, and 1,25-dihydroxycholecalciferol [l,25$(OH)_2D_3$], i.e., vitamin D_3 (Elaroussi et al. 1994). Also the gonadal steroid estradiol has an influence on the calcium metabolism (Beck and Hansen 2004; see chapter 2.4).

2.2.3 Bone Diseases in Laying Hens

In laying hens, different metabolic bone diseases have been described: osteomalacia (Wilson and Duff 1991; Whitehead et al. 2003), osteoporosis (Whitehead et al. 2003), and osteodystrophia fibrosa (Wilson and Duff 1991).
Osteomalacia is characterized by defective mineralization of bone tissue and mainly caused by nutritional deficiencies such as calcium, phosphorus or vitamin D deficiency (Whitehead et al. 2003). A typical finding in histological images of bones affected by osteomalacia are increased osteoid seams, i.e., immature, unmineralized bone matrix (Wilson and Duff 1991). Osteomalacia does not seem to have a genetic component (Whitehead et al. 2003).

Osteoporosis is more widespread in laying hens (Whitehead et al. 2003). It is defined as a “decrease in the amount of fully mineralized structural bone, leading to increased fragility and susceptibility to fracture” (Whitehead and Fleming 2000). Osteoporosis is a multifactorial disease (Whitehead et al. 2003). One important factor influencing the occurrence and severity of osteoporosis is the housing system and the permitted amount of movement within a system. In humans, there is a term for osteoporosis after prolonged lack of movement: disuse osteoporosis (Takata and Yasui 2001). Similarly, in laying hens housed in conventional cages with restricted possibilities to move, a severe form of osteoporosis, characterized by spinal fractures and paralysis (Riddell et al. 1968), has been found and named cage(d) layer fatigue (Urist and Deutsch, 1960; Whitehead and Fleming 2000). Osteoporosis also has a large

genetic component, indicating that selection for increased bone strength may help to alleviate this disorder (Whitehead and Fleming 2000). Concerning the role of nutrition on osteoporosis in laying hens, it is supposed that nutritional deficiencies of calcium, phosphorus or cholecalciferol may lead to greater severity of osteoporosis but that calcium deficiency does not seem to be a primary cause of osteoporosis (Whitehead and Fleming 2000).

Osteodystrophia fibrosa is characterized by resorption of bone material which is subsequently replaced by fibrous tissue (Wilson and Duff 1991). It has been found in laying hens after prolonged feeding on a diet deficient in vitamin D_3 (Wilson and Duff 1991).

2.3 Reproduction in Laying Hens

2.3.1 Anatomy of the Reproductive Tract and Egg Formation

In female avian embryos, a right and a left undifferentiated Müllerian duct are developed. However, the right one regresses before hatch. Thus, only the left reproductive tract is active in most female birds such as the laying hen (Johnson 2015). It begins to fully develop at about 16 weeks of age and achieves functionality shortly before the onset of lay (Johnson 2015). The left reproductive tract consists of the ovary and the oviduct. The latter consists of the infundibulum, magnum, isthmus, uterus or shell gland, and vagina (Johnson 2015).
The left ovary is located in the coelomic cavity, ventral to the caudal vena cava and close to the left kidney, lung, and adrenal gland and has a very short ligament (*mesovarium*) (Crosta et al. 2003; Johnson 2015). Oogenesis seems to be terminated by the time of hatching and the ovary of a one-day-old chick possesses around 480 000 oocytes (Hughes 1963). At this time, all oocytes are in the stage of primordial follicles (Johnson 2015). When the laying hen reaches sexual maturity, part of the primordial follicles develop into primary follicles, and, subsequently, into prehierarchal follicles (Johnson 2015). Of these, one follicle develops into a preovulatory follicle each day and undergoes a final growth phase which lasts for 4 – 6 days (Johnson 2015). Thus, the ovary of a laying hen is arranged with a typical hierarchy of follicles: there are numerous primordial follicles measuring up to 0.08 mm in diameter, numerous slow-growing primary follicles measuring approximately 0.08 – 1 mm, eight to twelve prehierarchal follicles measuring 1 – 8 mm, and four to six preovulatory follicles measuring 9 – 45 mm in diameter (Johnson and Woods 2007; Johnson 2015). A preovulatory follicle consists of the germinal vesicle, the germinal disc, large amounts of yellow yolk, a perivitelline membrane, granulosa cells, a basal lamina, a theca interna and externa layer, blood vessels, smooth muscles, and connective tissue (Johnson 2015). Each day, the largest preovulatory follicle ovulates, i.e., the follicle ruptures at a comparatively avascular region called stigma and releases the oocyte into the infundibulum (Johnson and Woods 2007; Johnson 2015). The granulosa and theca layers are left behind and build the postovulatory follicle (Johnson 2015). The infundibulum is not directly connected to the ovary. Thus, occasionally, the ovum is not picked up by the infundibulum but is released into the abdomen where it is usually reabsorbed within 48 – 72 hours (Johnson 2015). The ovum persists in the infundibulum for 15 – 30 minutes, is fertilized there (Johnson 2015), and a membrane which separates the ovum from the albumen, called vitelline membrane and containing antimicrobial peptides, is produced (Kaspers 2016). Subsequently, the ovum reaches the magnum, the largest part of the oviduct, where it remains for 2 – 3 hours and where the largest part of the albumen is produced (Johnson 2015). Afterwards, the ovum spends 1 – 2 hours in the isthmus where the inner and outer shell membranes are built (Johnson 2015). Lastly, the ovum reaches the uterus or shell gland and remains there for 18 – 26 hours (Johnson 2015). In the uterus, a fluid containing ions and different proteins is secreted into the albumen (Salevsky and Leach 1980). Furthermore, the eggshell formation and calcification as well as formation of the cuticula take place in the uterus (Johnson 2015; Kaspers 2016). Finally, the egg is laid by the hen. This process is also called oviposition and is initiated by contraction of the smooth muscles of the

uterus and relaxation of the uterine sphincter (Kaspers 2016) which separates the uterus from the vagina (Johnson 2015). During oviposition, the egg passes the vagina and the urodeum of the cloaca before being laid (Johnson 2015). The process of egg formation, i.e., from ovulation to oviposition, lasts 24 – 28 hours in total (Johnson 2015). 15 – 75 minutes after oviposition, the subsequent follicle ovulates (Johnson 2015).

2.3.2 Endocrine Regulation of Reproduction in Laying Hens

In both birds and mammals, reproduction is regulated by the hypothalamic-pituitary-gonadal (HPG) axis (Ottinger et al. 2002; Crosta et al. 2003). The hypothalamus produces the gonadotropin-releasing hormone (GnRH) and releases it into the hypothalamo-pituitary portal vasculature (Ottinger et al. 2002; Crosta et al. 2003) in a pulsatile manner (Carmel et al. 1976; Tsutsumi and Webster 2009). In the chicken, two forms of GnRH have been described: GnRH-I and GnRH-II (Johnson 2015) (also called chicken GnRH (cGnRH)-I (Joseph et al. 2009) and -II (Dunn and Millam 1998)). GnRH binds to receptors in the pituitary gland and stimulates the production and release of the two gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Ottinger et al. 2002). The difference between GnRH-I and GnRH-II in terms of their physiologic function is not yet fully understood but it is assumed that GnRH-I plays a predominant role in gonadotropin release while GnRH-II is more important for sexual behavior in female birds (Mans and Taylor 2008; Mans and Pilny 2014). The hypothalamus also produces the gonadotropin-inhibiting hormone (GnIH) which seems to be a counterpart of GnRH, possibly by inhibiting gonadotropin release (Mans and Taylor 2008) or by modulating the growth and differentiation of ovarian follicles via receptors in the ovary (Johnson 2015). Release of GnRH-I and -II as well as GnIH from the hypothalamus is influenced by environmental stimuli such as daylight exposure, food availability, and tactile stimuli, amongst others (Mans and Pilny 2014).
The anterior pituitary gland (also called hypophysis) produces FSH and LH (Mans and Taylor 2008). FSH is responsible for the maturation and differentiation of the ovarian follicles and stimulates the synthesis of progesterone and estrogens (Mans and Taylor 2008) in prehierarchel follicles (Johnson 2015). Thus, it has an influence on less mature, small follicles, but not on large, preovulatory follicles (Mans and Taylor 2008). In contrast, LH has an influence on preovulatory follicles. Its plasma concentration peaks 4 – 6 hours prior to ovulation (Johnson and van Tienhoven 1980) and this peak stimulates germinal vesicle breakdown and ovulation (Johnson 2015). Another peak of LH plasma concentration is observed at the onset of darkness and is assumed to initiate the subsequent preovulatory LH surge (Johnson and van Tienhoven 1980; Wilson et al. 1983; Johnson 2015).
The ovary produces and releases steroidal hormones (estrogens, androgens, and progesterone) as well as inhibin, prostaglandin, and growth factors (Mans and Taylor 2008). This process is mainly controlled by FSH and LH and depends on the stage of the follicles and on their sensitivity to FSH and LH, amongst others (Mans and Taylor 2008). Estrogens, namely estradiol-17β and estrone (Johnson 2015), are produced by theca interna and externa cells (Etches and Duke 1984) of small prehierarchal follicles and then again by large follicles shortly before ovulation (Mans and Taylor 2008). They play a role in the regulation of calcium metabolism, induce their own receptors in the oviduct and progesterone receptors in the ovary and oviduct, and induce the synthesis of different proteins in the oviduct and the liver such as ovalbumin, vitellogenin and transport proteins for estrogens, testosterone, cortisol, and thyroxin (Mans and Taylor 2008; Johnson 2015). Moreover, estrogens, together with progesterone, are responsible for priming the hypothalamus and pituitary gland for progesterone (Wilson and Sharp 1976) and influence secondary sex characteristics as well as sexual behavior (Johnson 2015). Androgens are also synthesized by the theca cells and regulate steroidogenesis via an androgen receptor on granulosa and theca cells of prehierarchal and hierarchal follicles (Yoshimura et al. 1993; Johnson 2015). Furthermore, androgens play an important role in the development of the comb and wattles and, together

with estrogens, in the ossification of medullary bone (Johnson 2015). Progesterone is produced by granulosa cells of the follicles and its production increases with follicle maturation, i.e., as a follicle approaches the time of ovulation (Johnson 2015). The concentration of progesterone in plasma peaks 4 – 6 hours prior to ovulation and it is supposed that this peak precedes and stimulates the LH peak via a positive feedback mechanism (Decuypere et al. 2002; Johnson 2015). Besides the hypothalamus and pituitary gland, progesterone receptors have also been found in the oviduct and in preovulatory follicles, suggesting that this hormone is involved in the production of avidin, contraction of the myometrium, and shell formation and has a direct effect on ovulation (Johnson 2015). Granulosa cells of the largest ovarian follicles also produce inhibin which is assumed to have a negative feedback on the release of gonadotropins, particularly FSH, and a potential influence on steroidogenesis of the ovary (Mans and Taylor 2008). Furthermore, ovarian follicles produce growth factors and prostaglandins. Growth factors play a role in steroidogenesis and follicular development (Mans and Taylor 2008), while prostaglandins seem to be involved in the process of ovulation and oviposition (Johnson 2015).

Besides the hormones that have been described above, prolactin from the pituitary gland, melatonin from the pineal gland, and corticosterone from the adrenal glands have been shown to influence reproductive functions in the laying hen (Johnson 2015; Mans and Taylor 2008).

2.3.3 Inhibition of Reproduction in Female Birds

The inhibition of reproduction can be necessary in female pet birds with reproductive disorders such as oviductal prolapse, chronic egg laying, egg binding, and ovarian neoplasia, amongst others (Cook and Riggs 2007; Keller et al. 2013; Mans and Pilny 2014). In theory, reproduction can be inhibited surgically or with medical treatment (Mans and Pilny 2014). However, due to the peculiar anatomical location of the ovary in birds, i.e., very close to the left kidney, lung, and adrenal gland, and due to its very short ligament (see chapter 2.3.1), it is impossible to completely remove this organ safely in these animals (Mans and Pilny 2014). Thus, the possibilities for surgical intervention in reproductive disorders are limited and carry a poor prognosis if not combined with environmental adjustment, for example (Mans and Pilny 2014). A possible complication of surgical intervention is egg-yolk coelomitis after removal of a part of the reproductive tract, e.g., hysterectomy (Mans and Pilny 2014). Therefore, medical treatment of reproductive disorders and medical inhibition of reproduction is usually the method of choice in the veterinary practice (Mans and Pilny 2014; Summa et al. 2017). The most popular agents for this purpose are GnRH agonists (Summa et al. 2017). In contrast to endogenous GnRH which is released in a pulsatile manner (Carmel et al. 1976; Tsutsumi and Webster 2009; see chapter 2.3.2), prolonged administration of GnRH agonists (either by repeated administration or by sustained release formulations (Mans and Pilny 2014)) leads to a continuously high concentration. This leads to a desensitization of the GnRH receptors in the pituitary gland and, consequently, a shutdown of the HPG axis (Belchetz et al. 1978; Rabin and McNeil 1980; Ottinger et al. 2002; Gobello 2007). The two GnRH agonists that are used in the veterinary practice and that have been shown to successfully inhibit reproduction in female birds are leuprolide acetate and deslorelin acetate (Ottinger et al. 2002; Mans and Pilny 2014). Leuprolide acetate is available as a depot formulation which has been developed for humans, while deslorelin acetate is available as a sustained-release subcutaneous implant which has been developed for chemical castration in male dogs and ferrets (Summa et al. 2017). Studies about the effect of GnRH agonists in laying hens are scarce. Leuprolide acetate has been used for induced molt and also reduced or completely inhibited egg production in these molted hens (Dickerman and Bahr 1989; Burke and Attia 1994;). Only one study that investigated the effect of deslorelin acetate implants in laying hens has been found and in this study, egg production was suppressed for 26 or 45.5 weeks, depending on the strength of the implant (Noonan et al. 2012). However, the hens in this study were already 2 years old when being treated and the study was not published in a peer-reviewed journal but as a scientific

abstract. Thus, detailed information about the study design and the analysis of the results is lacking and the results have to be interpreted with care. Taken together, knowledge about the possibilities to suppress egg production in laying hens is limited.

2.4 Reproduction and Keel Bone Damage

Due to the high calcium demand in egg laying hens which is in large parts covered by calcium from the skeleton, especially the medullary bone (Mueller et al. 1964; Kim et al. 2012; Kerschnitzki et al. 2014; see chapter 2.2.1), egg production and bone metabolism in laying hens are closely linked. This close relationship may contribute to a weak skeleton and to metabolic bone diseases, especially osteoporosis, in commercial laying hens. In particular, it is suggested that at the onset of sexual maturity of the hen, osteoblasts change their function from forming structural bone to forming medullary bone and that this change is total, i.e., that no structural bone is formed during lay while osteoclastic resorption of structural bone continues (Whitehead 2004). This assumption is supported by findings that no uptake of fluorochromes, which deposit at sites of bone mineralization, is observed in the cortical bone of adult laying hens and that cortical width and structural bone content decrease during the laying period (Hudson et al. 1993; Whitehead 2004). Only when the hen goes out of lay, structural bone formation recommences and medullary bone disappears (Whitehead 2004). It is supposed that this cycle of bone loss during periods with egg laying and regeneration during reproductively inactive periods allows a female bird that lays eggs in clutches followed by incubation to maintain good bone quality (Whitehead 2004). In contrast, as modern laying hens have been selected to remain in a continuously reproductive condition, their bones may not have time for regeneration which is assumed to make them susceptible to osteoporosis (Whitehead 2004).
It is further suggested that these processes are mediated by estrogens (Whitehead 2004). According to this, reduced bone strength, a thinner cortex, and cavity formation have been found in hens, roosters, and capons treated with exogenous estradiol (Urist and Deutsch 1960; Chen et al. 2014), indicating that this gonadal steroid has a negative influence on bone health in chickens. However, Beck and Hansen (2004) do not fully agree with this assumption. They review that estradiol has a positive effect on bone formation and plays an important role in calcium homeostasis both in mammals and birds: It enhances the calcium uptake from the diet and, thus, its plasma concentration because it increases the level of vitamin D_3 by activating the 25-hydroxyvitamin D_3-1α-hydroxylase in the kidney (Castillo et al. 1977) and increases the gene expression of vitamin D receptors in the gut (Schwartz et al. 2000). Furthermore, estrogen inhibits the formation of osteoclasts, i.e., the cells that resorb bone material (Kanatani et al. 1998). Therefore, estrogen has a protective effect on bones in humans and a decrease in its concentration can lead to postmenopausal osteoporosis in women (Miyamoto 2015). Thus, “it is […] difficult to imagine that a steroid hormone, particularly, would have such fundamentally different actions on similar tissues in 2 classes of vertebrates that in one class a decrease would cause osteoporosis and in another an increase would have the same effect” (Beck and Hansen 2004).

In conclusion, although there are theories about the influence of egg production and estrogens on bone health in laying hens, no detailed, evidence-based information about their relationship is available. Furthermore, knowledge about the influence of reproduction on the etiology of KBD, in specific, is lacking. The role of the keel bone in calcium metabolism is not yet clear and, thus, it is unknown to which extent the keel bone is affected by egg production. A study comparing egg laying hens with non-egg laying hens of the same age would help to investigate these questions into more detail. In contrast to studies comparing laying hens with premature hens or roosters, such a study design would facilitate the disentanglement of the possible effect of egg production on bone metabolism from other possibly influencing factors such as age and sex.

2.5 Aim of This Work

Although there is some knowledge about factors that influence keel bone health in laying hens, findings about the direction of the effects differ between studies and it is not clear to which extent each of the factors contribute to the etiology of KBD. In particular, information about the role of external factors such as housing system and management on the one hand and internal factors such as genetic background and age on the other hand is lacking.
The aim of this work was, thus, to get a better insight into the etiology of keel bone fractures and deviations, investigating both external and internal factors. Special attention was paid on the possible influence of the external factor housing system and the internal factors layer line, age, egg production, and estrogen on KBD because evidence that these factors contribute to the occurrence of KBD has been given in several studies but with highly variable outcome (compare chapter 2.1.4). While housing systems that allow for increased activity lead to higher bone strength, they also seem to lead to a higher prevalence of keel bone fractures due to collisions with housing equipment. However, it is not clear whether the increased mobility or the presence of perches in alternative housing systems but not in conventional cages are responsible for the higher keel bone fracture prevalence in cage-free systems. Concerning the influence of layer line on KBD, several studies found a higher prevalence of KBD in brown compared to white layer lines while the opposite was the case in other studies. Furthermore, there is evidence that selection for high laying performance may have led to weaker bones but detailed knowledge about this possible influencing factor on bone health in general and on KBD in specific is lacking. Age has been shown to influence the prevalence of KBD but contrary results have been found concerning the susceptibility to KBD beyond a certain age of about 50 weeks. Lastly, there are hypotheses about the possible negative impact that egg production may have on bone strength and constitution but no studies have been conducted to assess the role of egg production in the etiology of KBD. Furthermore, information about the role that estrogen may play in this context is also lacking. In addition, methods to assess the keel bone throughout the life of laying hens which allow for a reliable detection of fractures and deviations and for a clear differentiation between both forms of damage as well as methods to prevent egg laying in chickens in order to examine the possible influence of egg production on the keel bone are scarce (compare chapters 2.1.5 and 2.3.3).

To investigate the possible role of housing system, layer line, age, egg production, and estrogen in the etiology of keel bone fractures and deviations, three studies were conducted. The first study aimed at finding a radiographic method to assess the keel bone which would allow for a clear differentiation between keel bone fractures and deviations and for conducting longitudinal studies on the same hens (chapter 3.1). Furthermore, hens of layer lines differing in phylogenetic background and laying performance which were kept in two different housing systems (cages and floor housing) were compared in terms of prevalence and severity of KBD (chapter 3.1). The second study aimed at establishing an animal model with non-egg laying hens which would allow for clearly assessing the role of egg production in the etiology of KBD (chapter 3.2). The third study was planned and conducted according to the findings of the first two studies. Keel bone health of egg laying and non-egg laying hens of two different layer lines was assessed before and throughout the laying period using radiographic images (chapter 3.3).

3 Own Research Publications in Scientific Journals

3.1 Radiographic Examination of Keel Bone Damage in Living Laying Hens of Different Strains Kept in Two Housing Systems

Authors: Eusemann BK, Baulain U, Schrader L, Thöne-Reineke C, Patt A, Petow S

Year: 2018

Journal: PLOS ONE

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Declaration of own part in this research publication:

Contributions of BK Eusemann:

1. Conceptualization of the evaluation and interpretation of radiographs, together with S Petow.
2. Statistical analysis of the data, together with U Baulain.
3. Setup of the entire manuscript and writing the original draft.

Contributions of the other authors: Development of study design, conduct of investigations, conceptualization of methodology, administration, acquisition of resources, supervision, review of the manuscript.

Declaration on ethics: The experiment was performed in accordance with the German Animal Protection Law and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (No. 33.9-42502-05-10A079).

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RESEARCH ARTICLE

Radiographic examination of keel bone damage in living laying hens of different strains kept in two housing systems

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Abstract

A high prevalence of deviations and fractures of the keel bone is a widespread welfare problem in laying hens. The aim of this study was to experimentally investigate this multifactorial problem throughout the laying period and to compare the prevalence and severity in different layer lines and different housing systems. High performing white (WLA) and brown (BLA) pure bred layer lines and low performing white (R11, G11) and brown layer lines (L68) were kept in both single cages and a floor housing system. A total of 97 hens (19 or 20 from each line, respectively) were repeatedly radiographed in the 35th, 51st and 72nd week of age. Fracture prevalence increased with age ($p<0.001$). The proportion of deviated keel bone area increased only for caged BLA, WLA and R11 hens ($p<0.05$) and was significantly higher for caged WLA and R11 hens compared to floor-housed WLA and R11 hens in the 72nd week of age ($p<0.05$). In the 72nd week of age hens in the floor housing system showed significantly more fractures than hens kept in cages ($p<0.05$). Prevalence of keel bone deviations was significantly higher in the white layer line R11 but significantly lower in the white layer line G11 compared to both brown layer lines and WLA ($p<0.05$). Brown layers showed significantly more fractures than white layers ($p<0.05$) in the 51st and 72nd week of age. Within the brown layers there was a significantly lower prevalence of deviations ($p<0.05$) and fractures ($p<0.05$) in the low performing (L68) compared to the high performing line (BLA). Our results show a different development of keel bone damage in caged compared to floor-housed hens under experimental conditions. Additionally, they indicate genetic effects on keel bone damage.

Introduction

Keel bone fractures are one of the most serious animal welfare problems in the egg production industry [1–3]. The prevalence is very high: About 5.5% of hens at the onset of lay and up to

97% of the hens near the end of a production cycle are affected [4–8]. In several studies it has been shown that affected animals are likely to suffer pain [9, 10]. Beside broken keel bones there is also a high prevalence of deviated keel bones. These have been described by Casey-Trott et al. [11] as "bone[s] with an abnormally shaped structure that has not resulted from a fracture but contains section(s) that vary from a theoretically perfect 2-dimensional straight plane in either the transverse or sagittal planes. Additionally, indentations along the ventral surface can also be classified as a deviation." Reported percentages of keel bone deviations range from 2.6% to 82% [12, 13].

In addition to being a welfare problem, keel bone damage (KBD), i.e. deviations and fractures, might also be an economical problem due to reduced egg production, reduced egg weight and higher feed and water intake [14–16].

The etiology of both fractures and deviations is not yet clear but the causes seem to be numerous. One major factor seems to be the housing system. Wilkins et al. [7] found the lowest prevalence of keel bone fractures in furnished cages compared to all other investigated systems (free-range, organic static and barn), except organic mobile. Petrik et al. [4] found a higher keel bone fracture prevalence in floor-housed compared to cage housed flocks and Keutgen et al. [17] reported a higher prevalence of deviations in free-range and floor housing than in conventional cages. Several authors explain these findings by a higher risk of accidents and collisions in more extensive systems [7, 18] and by the presence of perches in alternative systems and furnished cages but not in conventional cages [18–20]. This explanation is supported by findings that shape and material of perches seem to have an influence on KBD. Käppeli et al. [21] found more deviations in pens with metal perches compared to pens with plastic perches. Stratmann et al. [22] were able to reduce the prevalence of fractures and deviations by covering metal perches with a soft cushion. In terms of fractures, the authors supposed that the compressible cushion absorbed part of the kinetic energy during collisions resulting in reduced energy transferred to the keel bone. In terms of deviations, their results can be explained by findings of a study by Pickel et al. [23] in which peak forces and contact area between perch and keel bone were measured. The authors found a lower peak force and larger contact area in perches with a soft cushion.

However, perches and the higher amount of movement in more extensive systems have also been shown to positively influence breaking strength and mineralization of long bones [24–27]. Movement, such as wing flapping, flying and load-bearing movements on perches and other structures, is important to preserve structural bone [27] which explains these findings.

The phylogenetic background of laying hens seems to affect the susceptibility of keel bones to fractures and deviations. Vits et al. [19] found more keel bone deviations in Lohmann Brown (LB) compared to Lohmann Selected Leghorn (LSL) hens. This finding was confirmed by Habig et al. [28] who added that keel bone deviations of LB hens were also more severe than those of LSL hens. In a study by Wahlström et al. [29] LSL hens were less susceptible to keel bone deviations when compared to a cross-breed of Leghorn and Rhode Island Red-line, called SLU-1329. However, Heerkens et al. [5] found a higher prevalence of keel bone deviations in Dekalb White hens compared to ISA Brown hens. The fracture incidence, in contrast, was higher for the ISA Brown hens. A detailed study about the influence of genetics on the prevalence of keel bone fractures has recently been published by Candelotto et al. [30]. The authors analyzed the susceptibility to keel bone fractures of different crossbred and pure lines with an impact test apparatus that minimized behavioral confounds. They found a reduced likelihood of experimental fractures in the crossbred line Experimental Brown (EB) compared to all other lines and a reduced likelihood of experimental fractures in the commercial line Dual Brown (DB) compared to the commercial line Dekalb White (DW).

Moreover, laying performance is likely to affect prevalence of keel bone damage as selection on high performance has led to a high demand for calcium to produce the egg shell. This might affect bone characteristics, since part of the calcium is mobilized from the bone, especially the medullary bone [31, 32]. However, little is known about the influence of laying performance on keel bone health. Hocking et al. [33] compared different commercial layer lines which showed a high laying performance to traditional lines which showed a lower laying performance. The authors found a significantly higher radiographic density of keel bones and tibiotarsi of traditional compared to commercial layer lines. Moreover, breaking strength of humeri and tibiotarsi was also higher in traditional than in commercial breeds while there was no difference in egg shell strength. The authors concluded that "eggshell quality is maintained in genetically selected lines at the expense of bone strength and bone radiographic density".

In order to better understand the etiology of KBD it is crucial to consider its development throughout the laying period. Several studies found an increasing prevalence of deviations and fractures with increasing age [5, 21, 26, 28, 29]. However, in some studies, the prevalence of KBD remained constant or even decreased after about the 50th week of age [4, 22]. The method which is mostly used to assess KBD throughout the laying period is the palpation of the keel bone [4, 5, 21, 22, 28]. An alternative method which would give more detailed information about the health status of the keel bone and allow conducting longitudinal studies is radiography. However, although a few studies have shown that it is a reliable tool to detect keel bone fractures [34–36] and deviations [35], radiography has not been used to assess keel bone damage throughout the laying period so far.

The aim of this study was to get a better insight into the influence of housing system, layer line and age on keel bone fractures and deviations within the same experimental design. Therefore, we established an experimental setup with five pure bred layer lines differing in their phylogenetic background (brown and white layer lines) and laying performance (high and low performing lines). Hens of all lines were kept in two different housing systems (single cages and floor group housing) within the same facility so that confounding factors such as different feed and management in the housing systems were minimized. To be able to investigate keel bone fractures and deviations throughout the laying period we took radiographs of all laying hens at three different weeks of age. With this method we also aimed to clearly differentiate between fractures and deviations and to provide an objective measure of the dimension, i.e. severity of deviations.

In view of findings of other studies we predicted that brown purebred lines would show more deviations and fractures compared to white purebred lines and that the high performing lines would show more keel bone damage than the low performing lines. We also predicted that prevalence of deviations would be higher in hens kept in cages enriched with perches compared to hens in the floor housing system and that deviations would be more severe in caged hens. In contrast, we hypothesized that hens in the floor housing system would show more fractures compared to hens kept in single cages. Lastly, we predicted that keel bone damage would increase with age.

Animals, materials and methods

Birds and housing conditions

This experiment was performed in accordance with the German Animal Protection Law and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (No. 33.9-42502-05-10A079).

We examined five different pure bred lines of laying hens (*Gallus gallus domesticus*): three closely related white layer lines (WLA, R11, G11) and two closely related brown layer lines

(BLA, L68). The two lines WLA and BLA (Lohmann Tierzucht GmbH, Cuxhaven, Germany) have been selected for laying performance and lay around 320 eggs per year. The other lines, R11, G11 and L68 (Friedrich-Loeffler-Institut, Institute of Farm Animal Genetics, Mariensee, Germany) are low performing lines with an average laying performance of 200 eggs per year (see also [37]). Laying maturity, defined as age at the first egg laid, was reached in the 20th week of age in WLA and BLA and in the 24th week of age in R11, G11 and L68 (see also [37]). Moreover, the layer lines differed in body mass: Both brown layer lines were heavier than the three white layer lines (mean body weight and standard deviation in the 72nd week of age: BLA: 1769 ± 205 g, L68: 2036 ± 212 g, WLA: 1564 ± 148 g, R11: 1440 ± 150 g, G11: 1456 ± 160 g).

All chicks (BLA: n = 162, L68: n = 163, WLA: n = 166, R11: n = 159, G11: n = 207) were hatched on the same day and the animals were raised in a floor housing system until 16 weeks of age. Birds of the different layer lines were kept in separate rearing compartments of 24 m^2 each that were littered with wood-shavings and straw. Perches were provided in the form of two wooden ladders each consisting of five rungs. Each rung was 92 cm in length and squared. The height of each rung was 5 cm and the width 4 cm. The distance between two rungs measured 40 cm and each ladder was 200 cm long. The ladders were leaned against the wall so that the highest rung was at a height of 150 cm. A standard light program was applied throughout the rearing period and a conventional complete feed for chicks (until 7 weeks of age; 12.97 MJ AMEn/kg DM, 189.61 g/kg crude protein, 31.38 g/kg crude fat, 9.14 g/kg Ca, 6.94 g/kg P) and pullets (from 8 to 16 weeks of age; 12.82 MJ AMEn/kg DM, 151.67 g/kg crude protein, 30.21 g/kg crude fat, 15.83 g/kg Ca, 8.11 g/kg P) as well as water were offered *ad libitum*.

At 16 weeks of age, a subset of the hens (BLA: n = 48, L68: n = 48, WLA: n = 49, R11: n = 48, G11: n = 47) was moved and housed individually in single cages (50 cm × 46 cm × 43 cm) for the remainder of the experiment. Each cage was equipped with a food trough, two drinking nipples and a perch. Siblings of the caged birds (n = 30 for each layer line) were moved to floor housing compartments and kept in groups of 15 hens of the same line, resulting in two compartments per line. All compartments measured 2 m x 2 m, were littered with wood-shavings and provided with perches and nests mounted on an elevated slatted floor 0.5 m above the littered area.

In both housing systems the duration of the light period increased gradually from 9 h (16th week of age) to 14 h light (20th week of age). All laying hens were fed *ad libitum* on a conventional laying hen diet (11.68 MJ AMEn/kg DM, 168.11 g/kg crude protein, 29.43 g/kg crude fat, 50.05 g/kg Ca, 5.06 g/kg P) and had *ad libitum* access to water.

X-ray examinations

Ten hens of each layer line and housing system were selected for X-ray examinations, resulting in 20 hens per layer line. From the lines WLA, R11 and G11 in the floor housing system only nine birds were X-rayed. This was due to a genetic study which was linked to the current study and which required the examination of one offspring each of ten different roosters. Due to losses in the layer lines WLA, R11 and G11 before the start of the experiment, offspring of only nine roosters were available in the floor housing system. Each of the selected hens was X-rayed in the 35th, 51st and 72nd week of age. These time points were chosen due to the following reasons: By the 35th week of age ossification of the keel bone is terminated [2] and egg production has reached its maximum. By the 51st week of age laying performance has started to decrease to some extent and the prevalence of keel bone fractures seems to have reached a maximum according to several studies [4, 22]. By the 72nd week of age laying performance has decreased considerably and commercial laying hens are often culled.

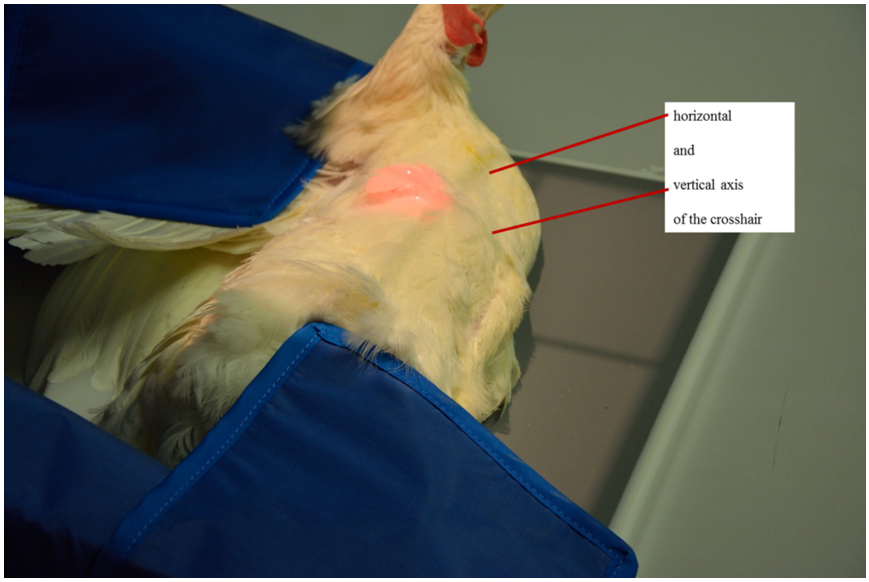

Fig 1. Positioning of the animal on the X-ray detector. The red lighted area indicates the radiation field center.

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Three hens in the floor housing system (2 WLA and 1 G11) died before the last X-ray examination resulting in fewer hens in the 72nd compared to the 35th and 51st week of age in these two groups.

Digital radiographs were taken using the X-ray generator WDT Blueline 1040 HF (Wirtschaftsgenossenschaft deutscher Tierärzte eG, Garbsen, Germany) and the X-ray suitcase Leonardo DR mini (Oehm und Rehbein GmbH, Rostock, Germany). The non-anaesthetized hen was gently placed on its left side on a digital flat panel detector (Thales Pixium 2430 EZ wireless, Thales Electron Devices S.A., Vélizy-Villacoublay, France). One person pulled the legs caudally and a second one fixed the wings above the back of the animal to make sure that the hen was safely fixated and that the limbs did not overlie the keel bone. The hen was positioned so that the keel bone was plane and at right angles to the x-rays. The vertical axis of the crosshair was located right in front of the cranial keel bone edge and the horizontal axis divided the hen's body into a ventral third and dorsal two-thirds. The radiation field center was located immediately above the crosshair center (Fig 1). Images were taken with 50.0 kV and at 2 mAs for each laying hen.

Initially, postero-anterior radiographs were taken as well. However, these X-rays could not be evaluated because the ventral surface of the keel bone is very small and because they resulted in projections in which other parts of the body such as the vertebral column inevitably overlay the keel bone. Consequently, we decided only to take lateral radiographs.

Evaluation of X-ray images

The images were evaluated using the image processing system AxioVision 4.8 (Carl Zeiss Microscopy GmbH, Jena, Germany). The same person blindly evaluated all images. Each radiograph was evaluated for deviations and fractures. According to suggestions by Casey-Trott et al. [11] fractures and deviations were addressed as two separate, mutually exclusive,

binary categories. This system allows an easy comparison across studies [11]. In addition, the size of the deviated keel bone area was also measured in order to get a value which indicates the severity of the deviation. Such approaches have also been encouraged by Casey-Trott et al. [11].

Deviations. In order to calculate the prevalence of deviations for each housing system, age and layer line, each radiograph was scored as 1 if a deviation was present and as 0 if no deviation was present.

In order to estimate the severity of a deviation, the proportion of deviated keel bone area (POD) was calculated as follows: The area of deviation was estimated by circumscribing the deformed outline and linking the start and end point of this outline by a straight line (Fig 2). The size of this area was calculated by AxioVision. Afterwards, the whole keel bone was circumscribed up to the insertion of the trabecula intermedia (Fig 2) and the size of its surface area was calculated by AxioVision. Again, the start and end point of the deformed outline were linked with a straight line (Fig 2) as an estimate for the size of the actual keel bone surface area. Finally, POD was calculated by dividing the area of deviation by the keel bone surface area.

Fractures. Fractures were defined as sections of the keel bone with thickened bone (callus) in the image (Fig 3) or as black, thin lines, indicating a fracture without callus formation (Fig 4). In order to calculate the fracture prevalence for each housing system, age and layer line, each radiograph was scored as 1 if any type of one or several fractures was present and as 0 if no fractures were present.

Statistical analysis

Data were analyzed using the statistical software packages JMP 11 (SAS Institute Inc., 2014) and R 3.3.3 (R Core Team, 2017). For statistical analysis of the prevalence of deviations, hens with a deviated keel bone were scored as 1 and those without any deviation as 0 at each time

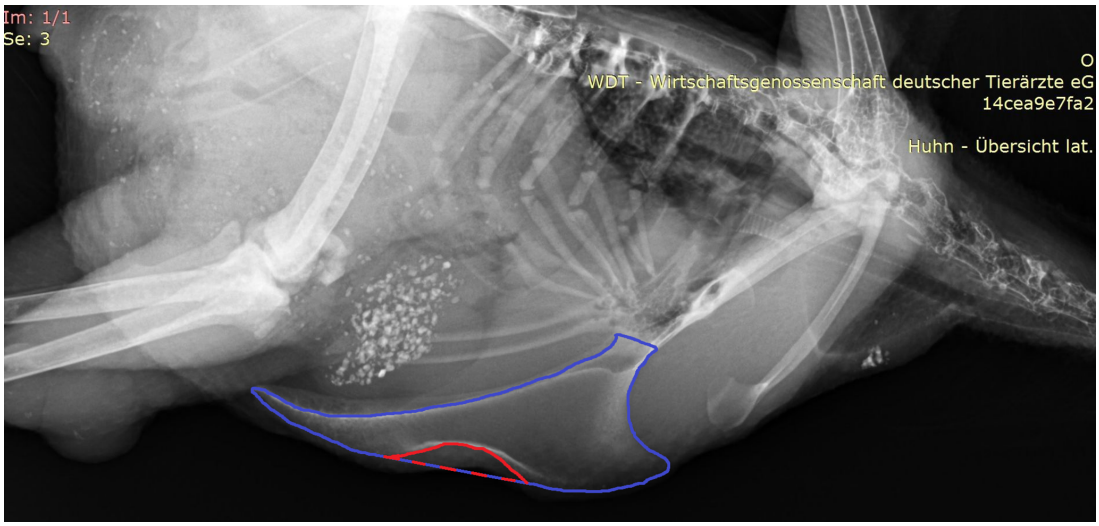

Fig 2. A deviated keel bone. The keel bone surface area is circumscribed with blue color; the area of deviation is circumscribed with red color. The blue-red line marks the straight line between the start and end point of thedeviated outline.

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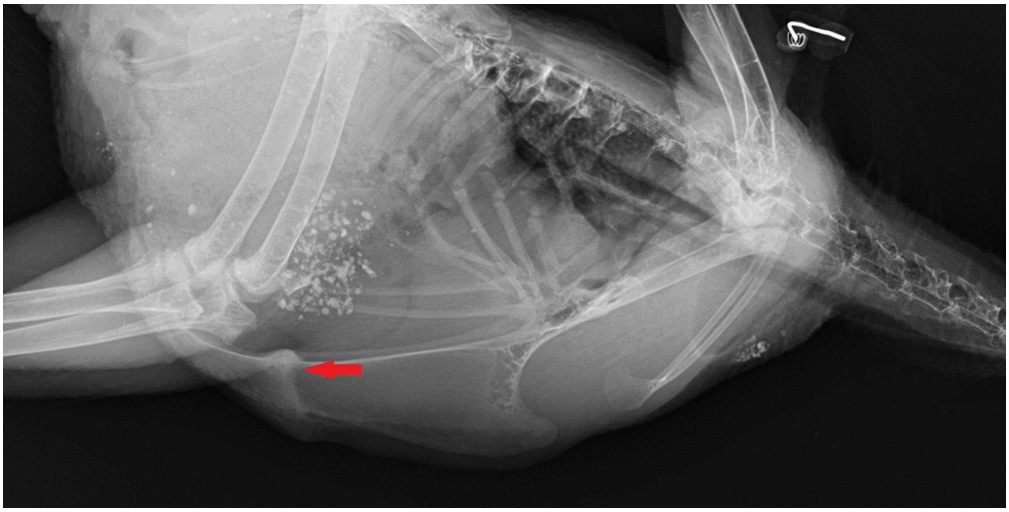

Fig 3. A fractured keel bone with callus formation. The arrow shows the fracture callus.

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point. The effects of housing system (cages and floor housing system), layer line (BLA, L68, WLA, R11 and G11) and age (35th, 51st and 72nd week of age) on the binary outcome variable "deviation" were analyzed by means of Chi-square test and Fisher's exact test, respectively. For housing system and layer line a separate Chi-square or Fisher's exact test was performed for each of the three weeks of age. Data are presented as total numbers of affected animals. Hens without keel bone deviation were excluded from analysis of POD. A multifactorial ANOVA

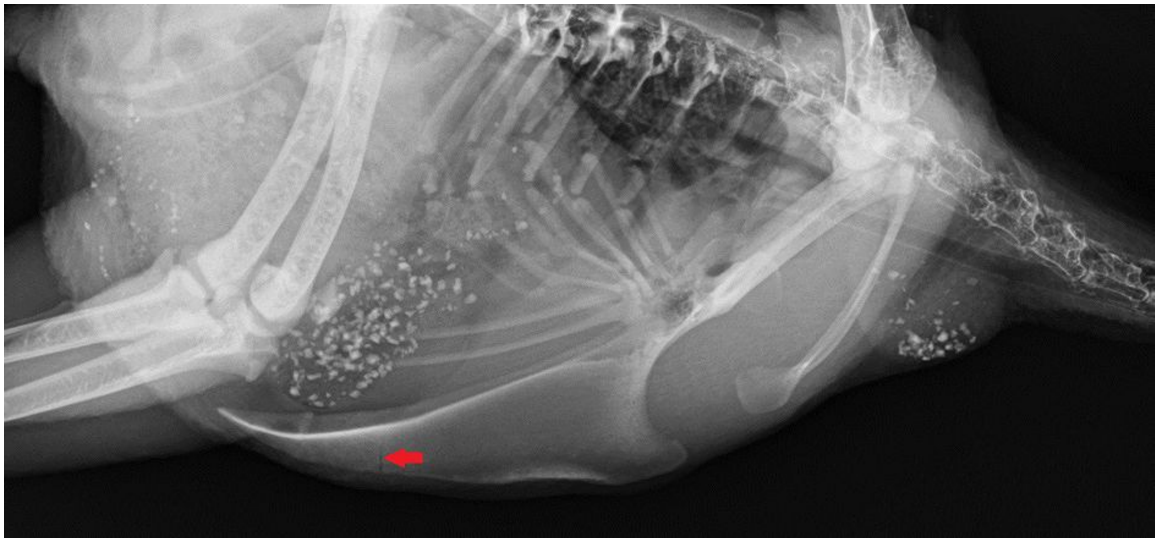

Fig 4. A fractured keel bone without callus formation. The arrow shows the fracture, a thin, black line.

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was applied to log-transformed POD. The mixed model contained the fixed effects housing system, layer line, age as a categorical variable and all interactions. To consider the repeated measures, animal was included as random effect. Multiple comparisons of means were performed using the Tukey's HSD test. Data for POD are presented as back-transformed LSM and upper and lower bounds of the 95% confidence interval. For statistical analysis of fractures, birds with one or multiple fractures were scored as 1 and those without any fracture as 0 at each time point. The effects of housing system (cages and floor housing system), layer line (BLA, L68, WLA, R11 and G11) and age (35th, 51st and 72nd week of age) on the binary outcome variable "fracture" were analyzed by means of Chi-square and Fisher's exact test, respectively. For housing system and layer line a separate Chi-square or Fisher's exact test was performed for each of the three weeks of age. If it was not possible to evaluate the keel bone for fractures or deviations because the legs of the hen overlay the keel bone in the radiograph, this image was excluded from analysis. The number of hens included in the analysis of prevalence of deviations and fractures is indicated in each figure.

Results

Deviations

The prevalence of keel bone deviations did not differ significantly between housing systems or weeks of age but between layer lines ($p<0.05$ for each age) (Fig 5). The highest prevalence was found in R11: all animals of this layer line had a deviated keel bone at all ages. The lowest

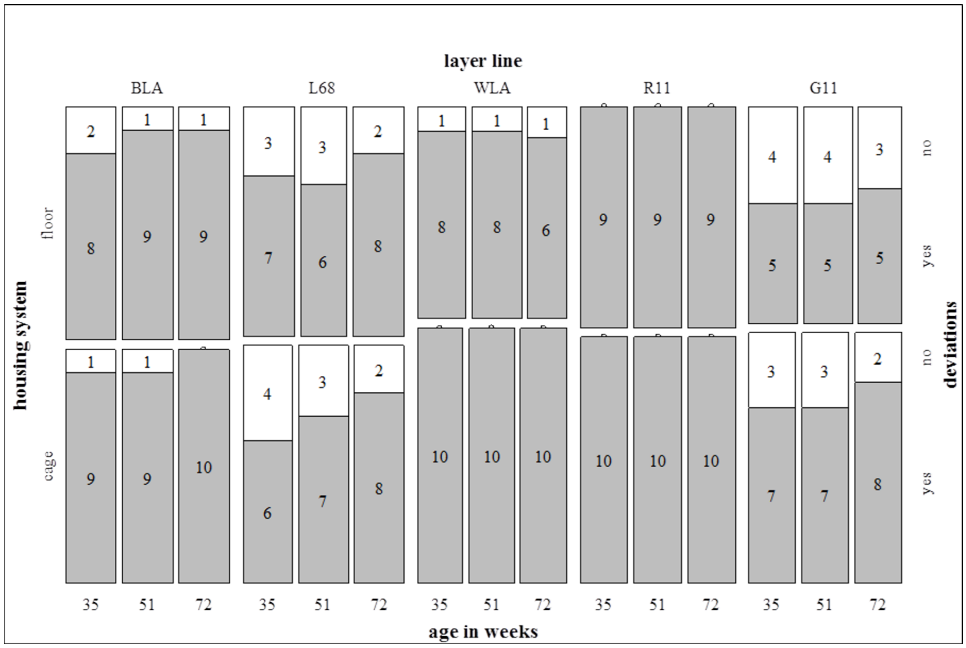

Fig 5. Prevalence of deviations. Each bar represents the total amount of radiographed and evaluated hens of one layer line within one housing system. The grey part of a bar shows the part of hens with a deviated keel bone, the white part those without any deviation. The numbers written in the bars show the total numbers of affected or non-affected hens, respectively. Prevalence of deviations was affected by layer line ($p<0.05$). There was no significant influence of housing system ($p>0.05$) or age ($p>0.05$) on prevalence of deviations.

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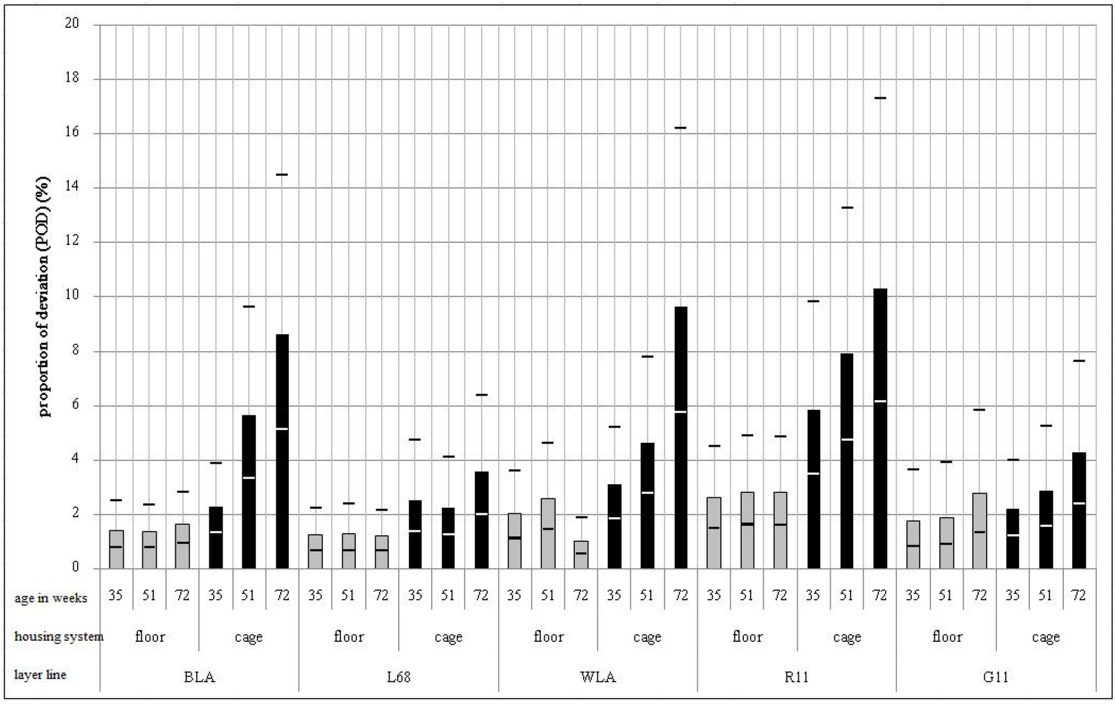

Fig 6. Proportion of deviated keel bone area (POD) (back-transformed LSM and upper and lower bounds of the 95% confidence interval) in %. POD was affected by the three-way interaction between housing system, layer line and age ($p<0.01$).

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prevalence of deviations was found in G11 (35th, 51st and 72nd week of age: 63.2%, 63.2% and 72.2%), followed by L68 (65%, 68.4% and 80%), BLA (85%, 95% and 95%) and WLA (94.7%, 94.7% and 94.1%).

The severity of deviations, measured as POD, was significantly affected by the three-way interaction between housing system, layer line and age ($p<0.01$) (Fig 6). In the floor housing system POD did not differ significantly between weeks of age in any of the five layer lines ($p>0.05$). In the cage system, in contrast, POD was significantly higher in the 72nd compared to the 35th week of age in BLA (back-transformed LSM and 95% confidence interval: 35th week of age: 1.34% < 2.28% < 3.88%; 72nd week of age: 5.14% < 8.63% < 14.47%; $p<0.05$), WLA (35th week of age: 1.84% < 3.09% < 5.19%; 72nd week of age: 5.75% < 9.65% < 16.19%; $p<0.05$) and R11 (35th week of age: 3.49% < 5.86% < 9.83%; 72nd week of age: 6.14% < 10.30% < 17.29%; $p<0.05$). This was not the case in L68 and G11 ($p>0.05$). POD was higher in cages compared to the floor housing system in the 72nd week of age in WLA and R11 (WLA floor housing: 0.55% < 1.02% < 1.89%; R11 floor housing: 1.63% < 2.81% < 4.85%; WLA and R11 in cages see above; $p<0.05$). In BLA, L68 and G11 POD did not differ significantly between housing systems in any week of age ($p>0.05$).

Fractures

Fracture prevalence increased with age (35th, 51st and 72nd week of age: 34.8%, 52.7% and 62.2% of all hens affected; $p<0.001$) (Fig 7).

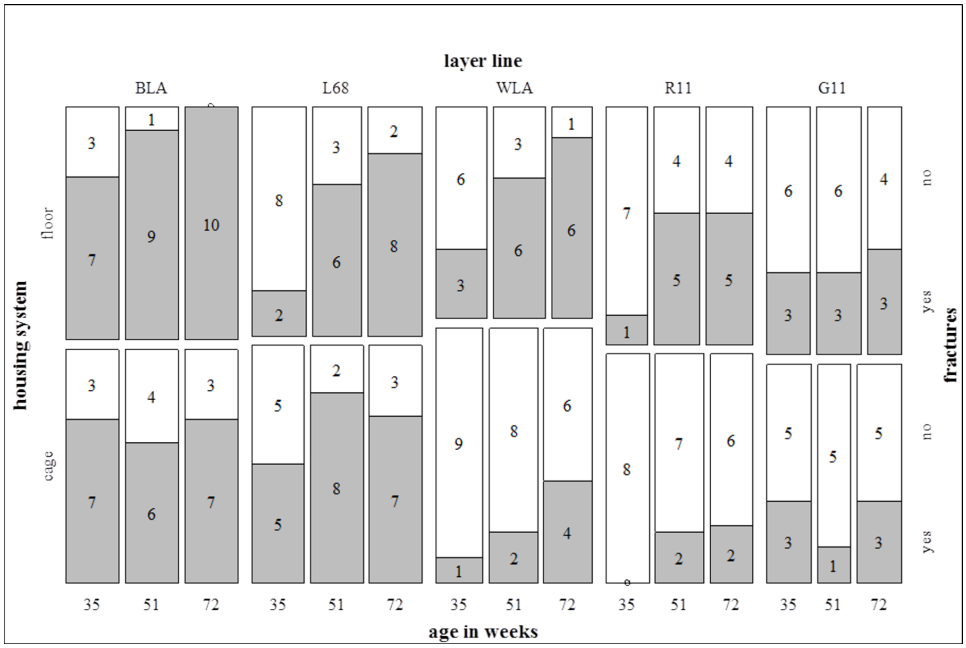

Fig 7. Fracture prevalence. Each bar represents the total amount of radiographed and evaluated hens of one layer line within one housing system. The grey part of a bar shows the part of hens with a fractured keel bone, the white part those without any fracture. The numbers written in the bars show the total numbers of affected or non-affected hens, respectively. Fracture prevalence was affected by layer line ($p<0.05$ at all three ages) and age ($p<0.001$). Housing system significantly influenced fracture prevalence in the 72nd week of age ($p<0.05$).

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There was no significant difference between housing systems in the 35th (floor housing: 34.8%, cages: 34.8% affected hens; $p>0.05$) and 51st week of age (floor housing: 61.7%, cages: 44.2% affected hens; $p>0.05$), but in the 72nd week of age fracture prevalence was significantly higher in the floor housing system compared to cages (floor housing: 73.3%, cages: 50%; $p<0.05$) (Fig 7).

Moreover, fracture prevalence differed significantly between layer lines at all three ages ($p<0.05$) (Fig 7). BLA and L68 showed a higher prevalence of fractures than WLA, R11 and G11 in the 51st and 72nd week of age (percentage of affected animals in the 35th, 51st and 72nd week of age: BLA: 70%, 78.9% and 85%; L68: 35%, 73.7% and 75%; WLA: 21%, 44.4% and 61.1%; R11: 6.3%, 38.9% and 41.2%; G11: 35.3%, 25% and 37.5%). Within the brown layer lines there were significantly more hens with fractures in the high performing line BLA compared to the low performing line L68 ($p<0.05$). Within the white layer lines there was no significant difference between high and low performing lines ($p>0.05$).

Discussion

The current experimental study confirmed an alarmingly high prevalence of keel bone fractures and deviations in laying hens.

34.8% of all radiographed laying hens had a keel bone fracture in the 35th week of age. In the 51st week of age the prevalence was 52.7% and in the 72nd week of age 62.2%. These numbers are comparable to findings by Petrik et al. [4] who found an overall keel bone fracture

prevalence of 36% at 35 and of 46.3% at 50 weeks of age in nine farms with conventional cages and eight farms with floor-housed flocks in Ontario, Canada. In other studies, fracture prevalence was even higher. Heerkens et al. [5] found an overall fracture prevalence of 60% at 29, 76% at 39 and 86.5% at 49 weeks of age in an experimental setup with 96 palpated hens that were housed in pens either with or without ramps. In a field study with 67 flocks in the UK, assigned to eight subcategories of housing system, Wilkins et al. [7] found 36% affected hens in furnished cages and 86% affected hens in a free-range system with aerial perches in the indoor house at the end of the production period.

The prevalence of deviations in the current study was 81% in the 35th, 83% in the 51st and 88% in the 72nd week of age and was higher compared to other studies. In the study by Heerkens et al. [5], which has been mentioned above, 31.2% of keels were found to be deviated with 39 weeks of age and 49% with 49 weeks of age via palpation. Käppeli et al. [21] reported a prevalence of 46.7% with 37, 64.6% with 43 and 72.9% with 62 weeks of age in one experiment in which 240 floor-housed hens were palpated at each time point. The higher prevalence of deviations in the current study might be explained by the fact that we used radiography to detect deviations which is more sensitive than palpation.

Due to the high prevalence and the probable painfulness of keel bone fractures [9, 10] it is crucial to investigate this multifactorial animal welfare problem into more detail.

Radiography as a tool to assess KBD throughout the laying period

In this study, we evaluated keel bone fractures and deviations throughout the laying period using radiography. For longitudinal studies which have investigated KBD at different ages, most research groups have used palpation [4, 5, 21, 22, 28]. This is an appropriate method, especially for a large number of animals and on-farm assessment, as it is quick, well-validated and cost-efficient [11, 38]. However, not all fractures, especially mild fractures and those at the dorsal aspect of the keel bone, can be detected by palpation [34]. Radiography has been shown to give a more detailed insight into the bone [34–36, 39]. Clark et al. [35] used radiography to detect fractures in the skeleton of laying hens, including the keel bone. They also detected and measured indentations of the keel bone. For their study, the hens had been euthanized before radiographs were taken. Richards et al. [34] took radiographs of live, sedated hens to detect keel bone fractures. The duration of the study was six weeks because the focus was on the healing process and not on the fracture prevalence at different ages. Our study is therefore the first published work in which radiographs have been used to assess keel bone fractures and deviations throughout the laying period. It was possible to get radiographs with a high resolution which showed the whole keel bone. Also small fractures without callus formation could be detected which might not have been the case with palpation. Furthermore, a clear differentiation between fractures and deviations was possible. Moreover, we presented a new method to measure keel bone deviations in relation to the whole keel bone. With this method we were able to measure the severity of deviations. Thus, no scoring system was necessary. Compared to a scoring system, calculating the proportion of deviated area allows assessing deviations objectively on a continuous scale. This increases the sensitivity of the measure and allows comparing keel bones with even very slight differences. Due to varying keel bone shape, it is not possible to accurately infer the intact outline of a keel bone once it is deviated. Consequently, we decided to draw a straight line between the start and end point of the deviated outline to get the most accurate estimation of the size of the deviated area. Even though this approach might underestimate the size of the deviated area, in our opinion this was the most accurate, objective and reproducible method to estimate the size of the deviated area.

In the current study the hens were not sedated for radiography. This required that two persons had to handle each hen. With our handling, all laying hens were immobilized and we did not have problems with hens moving. However, the safety of the two persons handling the animal is also an important factor. We measured the radiation dosage which was received by the two persons with dosimeters at the fingers and at the torso. The dosage was far below the maximal permitted dosage (data not shown) which was, most probable, because of the modern X-ray generator which does not emit a lot of radiation outside the radiation field. However, to avoid that persons have to be close to the generator, another method would be required. Sirovnik and Toscano [36] conducted a study on radiography in which they hung the hens upside down so that they remained still without further fixation. This seems to be a promising alternative to the handling of the animals.

In conclusion, radiography has been shown to be a good tool to assess keel bone fractures and deviations throughout the laying period. As the equipment which we used to take radiographs is portable, the presented method can also be used for on-farm assessment. It is, however, more expensive and more time-consuming compared to palpation which might therefore be more suitable for large-scale studies.

Housing system

Our hypothesis that laying hens in cages would show more keel bone deviations compared to hens in the floor housing system was not confirmed since prevalence of deviations did not differ between the systems at any age. However, as predicted, keel bone deviations were more severe in cages in the two layer lines WLA and R11 as can be seen by the fact that POD was larger compared to the floor housing system in the 72nd week of age in these two layer lines. Severity of deviations, i.e. POD, also increased with age in the cage system in the layer lines BLA, WLA and R11, but not in the floor housing system. Keutgen et al. [17] found a higher prevalence of deviations in free-range and deep litter stocks than in conventional cages. However, in our study cages were enriched with a perch which might explain the different findings because of the pressure on the keel bone caused by a perch. The impact of perches on the keel bone has been shown in different studies. Pickel et al. [23] showed that in laying hens sitting on a perch, the peak force induced by the perch was approximately 5 times higher on the keel bone compared to the peak force on a single foot pad. This indicates that hens have most of their weight on the keel bone during perching. Moreover, Stratmann et al. [22] showed that prevalence of deviations could be lowered by covering the perches with a soft material.

The present results of lower POD in the 72nd week of age in floor-housed compared to caged hens in some layer lines confirm the importance of movement for bone strength that has been shown by several authors [24, 26, 27]. The lack of movement, combined with the constant pressure on the keel bone caused by the perch, might have led to the high and increasing POD in caged laying hens.

As predicted, fracture prevalence was significantly higher for the laying hens in the floor housing system compared to the hens in cages in the 72nd week of age. The higher fracture prevalence in floor housing corresponds to findings of other studies [4, 7, 40]. These findings can be explained by the higher risk of collisions with perches, nests and other hens that birds in floor housing systems are exposed to compared to birds in single cages [7, 18]. The fact that fracture prevalence did not differ significantly between the housing systems in the 35th and 51st week of age might be explained by the general increase in fracture prevalence which was more marked for the hens kept in the floor housing system. Fracture prevalence was also high in cages, although these hens were at a lower risk of collisions. We assume that the fractures in cages were rather pathologic fractures due to bone weakness than of traumatic origin. In order to confirm this assumption, a histological examination of the keel bones would be required.

Our results concerning the differences between the two housing systems show that there seem to be different risks and causes for deviations on one hand and fractures on the other hand: Deviations are more severe in cages possibly due to lack of movement and a resulting deterioration of bone strength [24, 26, 27], combined with the constant pressure on the keel bone. Fractures are more common in the floor housing system probably due to a higher risk of accidents and collisions [7, 18].

Genetic background

We found significant differences between the five layer lines which differed in phylogenetic background (brown versus white layer lines) and in laying performance (high versus low performing lines).

In contrast to expectations and other studies [19, 28], we did not find more deviations in brown compared to white layer lines. In terms of prevalence of deviations there were differences between the layer lines but these could not clearly be classified by "white" versus "brown". Within the hens affected by keel bone deviations, POD tended to be higher in white layer lines. As body weight was lower in white layer lines which tended to show a higher POD and which, in total, did not show a lower prevalence of deviations compared to brown layer lines, we cannot confirm the assumption made by other authors that the prevalence and severity of keel bone deviations increase with body weight [19, 28].

Taken together, prevalence of deviations and POD might indicate that keel bone strength was higher in brown compared to white layer lines. This assumption is in accordance with other studies that showed that breaking strength of at least some of the long bones is higher in brown compared to white hybrids [19, 41]. Vits et al. found a higher humerus breaking strength in LB compared to LSL hens. No significant difference was found in tibia breaking strength [19]. Riczu et al. found a higher humerus and femur breaking strength in the brown-egg strain Shaver 579 compared to the white-egg strain Shaver 2000 [41].

As hypothesized, brown layer lines showed significantly more keel bone fractures than white layer lines in the 51st and 72nd week of age. This finding is consistent with a study by Heerkens et al. [5] who found a higher fracture prevalence in ISA Brown compared to Dekalb White hens. The higher fracture prevalence in brown layers might be explained by differences between white and brown layers with respect to motor skills. White layers have been found to show better flight and 3D-movement skills and, thus, their risk for collisions with housing equipment such as perches might be lower compared to brown layers [5, 42]. Another suggested explanation is that, as brown layers usually have a higher body weight, the impact of collisions could be higher [5, 22]. Both factors are likely to lead to higher bone fracture prevalence in brown than in white layers. Candelotto et al. [30] found fewer experimental fractures in two brown layer lines compared to other lines, including white layer lines. This is in contrast to our findings. However, they investigated the susceptibility to fractures in recently euthanized hens using an impact test apparatus. Thus, behavioral confounds were eliminated. Their results therefore give a further hint that higher fracture prevalence in the brown layer lines in our study was probably mainly due to differences in flight abilities as mentioned above and not due to non-behavioral factors like bone strength.

Our hypothesis that high performing layer lines would show more keel bone damage could neither be rejected nor supported. On one hand, findings within the brown layer lines confirmed this hypothesis because the low performing line L68 had fewer deviations and fractures and the severity of deviations increased with age for the high performing line BLA but not for L68. On the other hand, within the white layer lines there was no clear difference between the high performing line WLA and both low performing lines R11 and G11. Whereas G11 had a

better keel bone health than WLA, this was not the case for R11. This shows that other differences between the white layer lines might have played a more important role than the difference in laying performance. Possible differences might be endocrine or behavioral factors.

In a comparative study of commercial breeds with a high laying performance and traditional breeds with a significantly lower laying performance Hocking et al. [33] found differences of bone parameters between the breeds. Traditional breeds had a higher breaking strength of humeri and tibiotarsi and also a higher radiographic density of keel bones and tibiotarsi compared to commercial breeds. The results of that study and of our study together indicate that selection on high productivity may have led to poor bone quality and that further research should be done in this field.

Altogether, the observed differences regarding genetic background confirm that the incidence of keel bone damage is influenced by layer line. However, susceptibility to deviations on one hand and fractures on the other hand do not seem to be directly linked.

Age

In contrast to expectations and to findings by Habig et al. [28], age did not have any significant effect on prevalence of keel bone deviations and POD only increased for some layer lines in cages.

As predicted, fracture prevalence increased with age. This corresponds to other studies [26, 28] and is consistent with findings that bone strength deteriorates with age [43]. However, some studies showed an increase of fractures only until a certain age of about 50 weeks [4, 22]. This was not confirmed in the present study where a higher fracture prevalence was found in the 72nd compared to the 51st week of age.

Conclusions

This experimental study has confirmed the relevance of keel bone damage in laying hens and the influence of the housing system and phylogenetic background on prevalence of KBD. Furthermore, indicators that selection on high performance might influence the prevalence of KBD have been found. Lastly, we have presented a new method to evaluate keel bone fractures and deviations throughout the life of a laying hen with radiography.

Supporting information

S1 Data. Complete data set.
(XLSX)

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3.2 Influence of a Sustained Release Deslorelin Acetate Implant on Reproductive Physiology and Associated Traits in Laying Hens

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Declaration of own part in this research publication:

Contributions of BK Eusemann:

1. Drafting and development of the study design including animal test proposal.
2. Preparation and conduct of the experiment, together with A-K Reinhard.
3. Statistical analysis and interpretation of the data, together with AR Sharifi.
4. Visualization of the data.
5. Setup of the entire manuscript except for the section on statistical methods.

Contributions of the other authors: Assistance with data analysis, writing of the section on statistical methods, review of the manuscript, supervision.

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Influence of a Sustained Release Deslorelin Acetate Implant on Reproductive Physiology and Associated Traits in Laying Hens

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The aim of this study was to develop an animal model with non-laying hens which would allow for investigation of the relationship between egg production and common diseases in hens. A total of 40 Lohmann Selected Leghorn hens were kept for 20 weeks in a floor housing system in two groups: group "Adult" (21 weeks of age) and group "Juvenile" (14 weeks of age). In each group, 10 hens were administered a 4.7 mg sustained release deslorelin acetate implant subcutaneously; in group "Adult" after, in group "Juvenile" before the onset of lay. In both groups, the remaining hens served as control hens. An examination of each hen was performed weekly, including ultrasonography to check for ovarian follicles, analysis of estradiol-17ß plasma concentration, and assessment of comb size. Digital radiographs of the keel bone were taken in experimental weeks 7 and 15. No follicles were detected on the ovary of treated hens for a certain time period which varied between individuals (between 8 weeks and until the end of the experiment). Estradiol-17ß concentrations were significantly higher in control hens. The comb was significantly smaller in treated hens. A lower prevalence of keel bone damage (group "Adult") and foot pad dermatitis (FPD) (both groups) was found in treated compared to control hens. These results show that a model with laying and non-laying hens can be achieved by administering a deslorelin acetate implant. Furthermore, they indicate a relationship between egg production and keel bone damage as well as FPD.

Keywords: laying hen, egg, follicle, gonadotropin-releasing hormone, deslorelin acetate, estradiol, keel bone, foot pad dermatitis

INTRODUCTION

Laying hens often suffer from a variety of diseases such as osteoporosis (FAWC, 2010), keel bone fractures and deviations (Fleming et al., 2004; Rodenburg et al., 2008; Käppeli et al., 2011b; Wilkins et al., 2011; Petrik et al., 2015), and fatty liver hemorrhagic syndrome (Shini and Bryden, 2009). The high laying performance might be a related factor to these medical conditions. Concerning bone diseases, it is known that there is a high calcium demand for the egg shell and thus high laying performance may lead to weaker bones (Whitehead et al., 2003; FAWC, 2010). Concerning the fatty liver hemorrhagic syndrome, high performing hens may be more susceptible because of the

stimulation of lipogenesis in the liver during egg production (Butler, 1976; Aydin, 2005; Deng et al., 2012).

A promising way to investigate possible relationships between egg production and these common diseases in laying hens as well as the underlying mechanisms may be the comparison of laying and non-laying hens of the same breed and age. Therefore, the aim of the current study was to develop an animal model in which egg production was selectively suppressed in hens to allow comparisons of traits with laying control hens.

An agent which is often used for reproductive management in several species is the synthetic gonadotropin-releasing hormone (GnRH) agonist deslorelin acetate. Naturally produced GnRH is secreted by the hypothalamus in a pulsatile manner (Carmel et al., 1976; Tsutsumi and Webster, 2009) and acts at pituitary receptors to induce the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Ottinger et al., 2002). FSH and LH act at the gonads where they induce gametogenesis, gonadal steroidogenesis and, in females, ovulation. In contrast to pulsatile secretion, continuous presence of GnRH or its agonist results in a desensitization of the GnRH receptors and, consequently, a shutdown of the reproductive cascade (Belchetz et al., 1978; Rabin and McNeil, 1980; Ottinger et al., 2002; Gobello, 2007). This can be achieved by the use of slow-release deslorelin acetate implants, for example Suprelorin® (Virbac, Carros, France). This implant has been developed for chemical castration in male dogs and is available in two different strengths: 4.7 mg deslorelin acetate and 9.4 mg deslorelin acetate. In dogs, the duration of effectiveness, i.e., the time period during which reproductive function is suppressed, is six (4.7 mg) and twelve (9.4 mg) months, respectively (Ponglowhapan, 2011). In pet birds, deslorelin acetate implants are frequently used to suppress undesired reproductive activity (Keller et al., 2013; Mans and Pilny, 2014). In adult female cockatiels, one 4.7 mg deslorelin acetate implant has been shown to significantly prevent egg laying for at least 180 days (Summa et al., 2017). Investigating the effect of deslorelin acetate on egg production in poultry, most studies have been conducted using Japanese quail. Petritz et al. (2013) found a complete suppression of egg production by a 4.7 mg deslorelin acetate implant in six out of ten hens for 10 weeks. Plasma concentration of estradiol-17ß was significantly lower in non-laying compared to laying quail. However, the implant did not show any effect on the laying activity in the remaining hens (Petritz et al., 2013). In a subsequent study, the authors found seven out of ten Japanese quail without oviposition in two treatment groups: one group was simultaneously treated with two deslorelin acetate 4.7 mg implants; the other group was treated with a single deslorelin acetate 9.4 mg implant. Egg production was decreased for approximately 14 weeks in the group with two deslorelin acetate 4.7 mg implants. In the group with one deslorelin acetate 9.4 mg implant, egg production was decreased for at least 14 weeks but there was a large variation between the birds (Petritz et al., 2015). In another study with Japanese quail, seven out of nine hens stopped laying eggs after the administration of a deslorelin acetate implant, the majority of them for more than 14 weeks (Schmidt et al., 2013). There is almost no knowledge about the effect of deslorelin acetate implants in chicken. Noonan et al. (2012) tested deslorelin acetate 4.7 mg and deslorelin acetate 9.4 mg in laying hens (*Gallus gallus*). Two weeks after implantation, all treated hens stopped laying eggs, regardless of the deslorelin acetate concentration. Suppression of egg production lasted almost 26 weeks in hens treated with deslorelin acetate 4.7 mg and 45.5 weeks in hens treated with deslorelin acetate 9.4 mg (Noonan et al., 2012). However, these results have to be interpreted with care because the hens were already 2 years old when being treated and the study was not published in a peer-reviewed journal but as a scientific abstract. Thus, detailed information about the study design and the analysis of the results is lacking.

Based on the results of these studies, we chose to use deslorelin acetate for the development of the desired animal model with non-laying and laying control hens in the current study. We tested whether a deslorelin acetate implant could suppress egg production in hens if implanted before or after the onset of lay. Furthermore, to characterize the animal model, we assessed whether the implant would lead to undesirable side effects and whether it would have any influence on other traits such as body weight, organ weight, general health, sexual hormone concentrations, and comb size.

MATERIALS AND METHODS

Birds and Housing Conditions

The current experiment was performed in accordance with the German Animal Protection Law and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (No. 33.19-42502-04-15/1966).

A total of 40 Lohmann Selected Leghorn (LSL) hens (*Gallus gallus domesticus)* were housed in two different age groups: one group ("Adult", 20 hens) was 21 weeks at the beginning of the experiment whereas the other group ("Juvenile", 20 hens) was 14 weeks old.

Table 1 details the age, management, and all experimental procedures for both groups in each experimental week.

All birds were obtained from a conventional breeding company (Zahrte, Wrestedt, Germany) at 14 weeks of age. In the breeding company, the animals had been reared under conventional conditions. After being brought to the experimental site, birds were kept in a floor housing system. During the first three experimental weeks, group "Adult" and group "Juvenile" were kept in two pens within the same poultry house that were separated by a solid wall. Each pen measured 11 m^2, was littered with wood-shavings and straw and equipped with perches and a nest box measuring 0.54 m^2. The light programs of both pens were independent from each other. The duration of the light period increased gradually from 10 h/day to 14 h/day. The 14 h light period was reached in the 24th week of age (group "Ault") or in the 17th week of age (group "Juvenile"), respectively, and kept constant until the end of the experiment. Since hens of group "Juvenile" were exposed to 14 h light earlier than hens of group "Adult", the two age groups were analyzed separately (see section Statistical Analysis). Light intensity was 20 lux at bird level.

TABLE 1 | Management, experimental procedures, and age as well as equivalent implant week of the laying hens of group "Adult" and "Juvenile" in each experimental week.

Experimental week	Management	Experimental procedures	Group "Adult" (20 hens)		Group "Juvenile" (20 hens)	
			Week of age	Implant week	Week of age	Implant week
1	Arrival at experimental site		21	−3	14	−1
2		B+E	22	−2	15	0
3		B+E	23	−1	16	1
4	Relocation	B+E+U	24	0	17	2
5		B+E+U	25	1	18	3
6		B+E+U	26	2	19	4
7		B+E+U+R	27	3	20	5
8		B+E+U	28	4	21	6
9		B+E+U	29	5	22	7
10		B+E+U	30	6	23	8
11		B+E+U	31	7	24	9
12		B+E+U	32	8	25	10
13		B+E+U+CM	33	9	26	11
14		B+E+U+CM	34	10	27	12
15		B+E+U+CM+R	35	11	28	13
16		B+E+U+CM	36	12	29	14
17		B+E+U+CM	37	13	30	15
18		B+E+U+CM	38	14	31	16
19		B+E+U+CM	39	15	32	17
20		B+E+U+CM+D	40	16	33	18

B, blood withdrawal for analysis of estradiol-17ß concentrations; E, health examination; U, ultrasonography of ovaries; CM, comb size measurement; R, radiography of keel bones; D, dissection; implant week 0, administration of deslorelin acetate implant; from this time point on, there were 10 treated and 10 control hens per group.

In experimental week 4, all hens were relocated to another poultry house. Hens of both groups were housed in two pens which were separated by a fence, resulting in both groups being exposed to the same light program. Each pen measured 9 m^2, was littered with wood-shavings and straw and equipped with perches and a nest box measuring 0.24 m^2. The hens were offered water and a conventional complete feed for pullets (until the 18th week of age) and laying hens (from the 19th week of age on) *ad libitum*.

Implantation of Deslorelin Acetate Implants

Ten hens of each group were given a 4.7 mg deslorelin acetate implant (Suprelorin®, Virbac, Carros, France). In group "Adult", the implant was administered when all hens started laying (24th week of age; **Table 1**), whereas in group "Juvenile", the implant was administered before the onset of lay (15th week of age; **Table 1**). The remaining ten hens of each group were kept as control hens within the same pen. The implant was administered subcutaneously between the scapulae. Hens were anaesthetized with 2–3% isoflurane (CP-Pharma Handelsgesellschaft mbH, Burgdorf, Germany) in compressed air with a flow rate of 500 ml/min delivered via face mask. Before application, the skin was aseptically prepared. After application, the implantation site was sealed with a tissue adhesive (Surgibond®, SMI, St. Vith, Belgium). Control hens did not receive any treatment.

Weekly Health Examination of the Hens

The general health of all hens was checked daily and assessed weekly (**Table 1**) and body weight was measured weekly. Moreover, the implantation site was checked to confirm that the implant was still present and to check for any signs of inflammation or irritation. As some hens were observed to develop foot pad dermatitis (FPD) over the course of the experiment, special attention was paid to this disease and it was noted down weekly whether a hen was affected or not. Assessment of FPD was always performed by the same person who was blinded to the treatment. Affected hens were treated with antiseptics.

Ultrasonography of Ovaries

Each hen was examined via ultrasonography weekly (**Table 1**) to check for ovarian follicles. The examination was conducted with the ultrasound system DUS 60 vet and the microconvex transducer C611-2 (both Edan Instruments GmbH, Shenzhen, China). The transducer was placed on the area between the vertebral column and the caudal rib (**Figure 1**). A frequency of 9.4 MHz was used and penetration depth varied between 39 and 58 mm. If present, follicles were visible as a round, anechoic zone with a smaller, round, hyperechoic zone in the middle (**Figure 2**). For each hen, the duration of effectiveness of the implant was assessed based on the number of weeks in which no follicles were detected by ultrasonography.

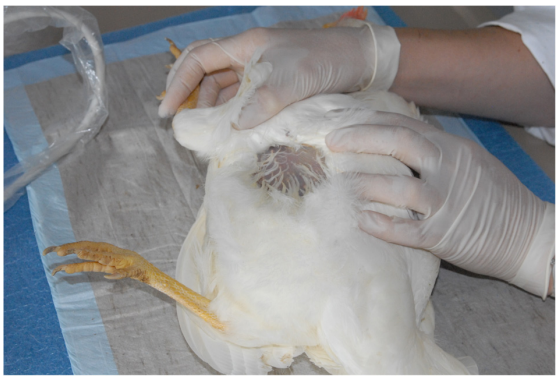

FIGURE 1 | Area between the caudal rib and the dorsal column where the transducer was placed.

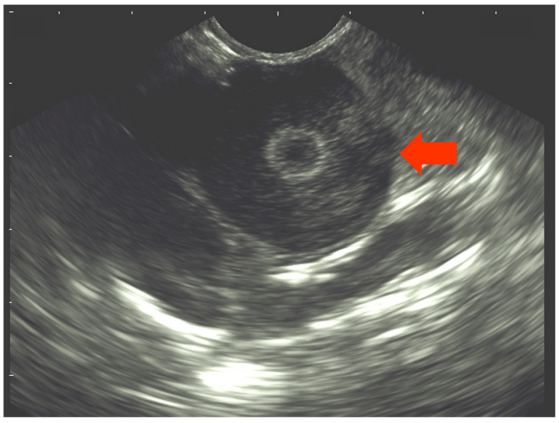

FIGURE 2 | Sonogram of the ovary of a hen. The arrow marks an ovarian follicle.

Egg Yolk Staining and Egg Collection

As an additional control of egg production, egg yolks of treated and of control hens were stained in two different colors, based on a method described by Appleby and McRae (1983). Two different liposoluble dyes were used. Hens treated with deslorelin acetate were given Sudan Black B; control hens were given Oil Red O (both Sigma-Aldrich, St. Louis, United States). The dyes were administered weekly in gelatin capsules (Capsler, Stuhr, Germany) which contained 30 mg sugar and 30 mg dye. The liposoluble dye accumulates in the outer layer of all oocytes and can be seen in the egg yolk from the 2nd to the 10th day after administration.

Eggs were collected daily at 10 a.m. and cracked. The color of each egg yolk was noted and each egg could be related to control (red egg yolk) or treated hens (black egg yolk).

Measurement of Estradiol-17ß Concentration in Plasma

Blood samples were taken weekly (**Table 1**) with twenty animals (five treated and five control hens of both "Adult" and "Juvenile") being sampled each Tuesday, the remaining twenty animals each Wednesday. All blood samples were taken between 8 and 11 a.m. A maximum of 2 ml blood was taken from the ulnar vein. Immediately after sampling, blood samples were centrifuged at 3500 rpm at 4°C for 10 min. Plasma was stored at -20°C until further analysis.

Estradiol-17ß was measured in pg/ml using a commercial enzyme-linked immunosorbent assay (ELISA) kit (IBL International GmbH, Hamburg, Germany). A pool plasma sample was included on each kit together with the individual samples to calculate the inter-assay coefficient of variation which was 0.18. Each blood sample was measured in duplicate to calculate the intra-assay coefficient of variation. If the intra-assay coefficient was higher than ten, the measurement was repeated.

Measurement of the Comb Size

From experimental week 13 on, the comb of each hen was photographed weekly with a digital reflex camera (**Table 1**). The hen was gently laid down on one side and the comb was placed on a small box (13.5 cm × 13.5 cm × 3.5 cm) to ensure it was plane. A ruler was placed beside the comb for scaling purposes.

The same person blindly evaluated all comb photos, using the image processing system AxioVision 4.8 (Carl Zeiss Microscopy GmbH, Jena, Germany). For each photo, a scale was generated using the ruler. Afterward, the outline of the comb was circumscribed and the size of its surface area was calculated by Axio Vision.

Radiographic Examination of the Keel Bone

In order to detect any differences in keel bone damage between the treatments, all hens were radiographed in experimental weeks 7 and 15 (**Table 1**).

Digital, lateral radiographs were taken and evaluated as described previously (Eusemann et al., 2018) with 50.0 kV and at 2 mAs. The evaluation of all images was performed blindly by the same person and included the presence or absence of fractures and the measurement of deviations. The latter was used to calculate the proportion of deviated keel bone area (POD):

$$POD\ (\%) = deviated\ area / keel\ bone\ surface\ area^{*}100.$$

Dissection

At the end of the experiment (experimental week 20), all hens were euthanized. Hens were stunned electronically and death was provoked by severing the jugular veins and carotid arteries. The hens of group "Adult" were 40 weeks old and the hens of group "Juvenile" were 33 weeks old at the time point of euthanasia (**Table 1**). The ovary, oviduct, heart, liver, spleen, intestine, gizzard, proventriculus, thyroid

glands, lungs, kidneys, and brain were weighed and the relative organ weights were calculated by dividing organ weight by body weight. The length of the oviduct was measured. Before weighing the ovary, all large follicles were removed. Moreover, the implant was removed and the implantation site was checked once again for any signs of inflammation or irritation.

Statistical Analysis

Differences between treated and control hens were analyzed separately for group "Adult" and group "Juvenile" due to different light programs and different experimental time periods between the groups. Data were analyzed using commercially available software (SAS 9.3, SAS Institute Inc., 2011).

For statistical analysis of the binary variables "Presence of follicles" and "Presence of FPD," a linear logistic mixed model for repeated measurements (Littel et al., 2006) was applied. An analysis of covariance was performed for predicting the effect of age on "Presence of follicles" or "Presence of FPD." Regression curves were fitted by considering age as a covariate term up to degree 4 of polynomials and the fixed effect of treatment ("treated" and "control") as well as significant interactions between the main factor treatment and the covariate (age) up to degree 4 of polynomials. Least squares means were estimated on the logit scale to fulfill model assumptions and then back-transformed using the inverse link function to the original scale (probability to have follicles or FPD, respectively). *Post hoc* multiple comparisons of least squares means were performed using Tukey's test.

Data of the numerical variables "Estradiol-17ß concentration," "Body weight," and "Comb size" were analyzed with a linear mixed model using the same factors as above but with underlying normal distribution. For analysis of estradiol-17ß concentrations, the fixed effects of treatment and age up to degree 4 of polynomials, their interaction up to degree 4 of polynomials, and the pre-treatment estradiol-17ß plasma concentrations were considered. For body weight development of the hens of group "Juvenile", the interaction between treatment and age was only considered up to degree 2 of polynomials and for body weight development of group "Adult", a second-degree polynomial was selected for the fixed regression term and the interaction as only these effects were shown to be significant. *Post hoc* multiple comparisons of least squares means were performed using Tukey's test.

For analysis of POD in both groups and at both time points of data recording (experimental weeks 7 and 15), the fixed effect of treatment, the time point and its interaction were considered in the general linear model.

For statistical analysis of keel bone fractures, birds with one or multiple fractures were scored as 1 and those without any fracture as 0 at both time points. The effect of treatment on the binary outcome variable "fracture" was analyzed by means of one Chi-square test for each time point and group.

The relative weight to the body weight of the various organs at the end of the experiment was analyzed using a general linear model, considering only the fixed effect of the treatment in the statistical model as described above.

RESULTS

General Health and Body Weight

No detectable adverse effects were found in any of the hens treated with a deslorelin acetate implant. Neither were there any signs of inflammation or irritation at the implantation site nor did treated hens show any negative alterations in health or behavior compared to control hens.

Group "Adult"

Control hens of group "Adult" were heavier compared to treated hens throughout the experiment (treatment*age: $p < 0.0001$; **Figure 3C**).

Group "Juvenile"

In group "Juvenile", no significant difference was found between body weight of control and treated hens ($p = 0.22$; **Figure 4C**).

Follicles, Duration of Effectiveness, and Egg Production

Group "Adult"

In group "Adult", proportion of hens with follicles was significantly higher in control compared to treated hens from implant week 2 to implant week 13 (treatment*age: $p < 0.05$; **Figure 3A**). Follicles were detectable in all control hens from implant week 1 onwards. Only in implant week 8, one control hen did not show any detectable follicles. In all treated hens of group "Adult", follicles were detectable prior to implantation. After implantation, the percentage of treated hens with follicles decreased continuously and between implant week 5 and implant week 10, none of the treated hens had follicles. From implant week 11 onwards, 4/10 treated hens developed follicles again. From implant week 14 onwards, treated and control hens of group "Adult" did not differ significantly in proportion of hens with follicles anymore ($p > 0.05$). The duration of effectiveness of the deslorelin implant showed interindividual differences. The shortest duration of effectiveness was 8 weeks in two treated hens while it was at least 16 weeks in one of the hens remaining without detectable follicles until the end of the experiment.

Group "Juvenile"

In none of the hens of group "Juvenile" follicles were detectable at the beginning of the study (**Figure 4A**). In control hens, ovarian follicles were first detected at implant week 6. Between implant week 7 and the end of the experiment (implant week 18), ovarian follicles were detectable in all control hens. 5/10 treated hens developed follicles between implant week 9 and the end of the study. The remaining five treated hens did not show follicles throughout the study. In group "Juvenile", treated and control hens differed significantly in proportion of hens with follicles until the end of the study (treatment*age: $p < 0.0001$).

Egg Yolk Staining and Egg Collection

The results of egg yolk staining and egg collection confirmed the findings of the ultrasonography in both groups ("Adult" and "Juvenile"): The number of eggs laid by control or treated hens

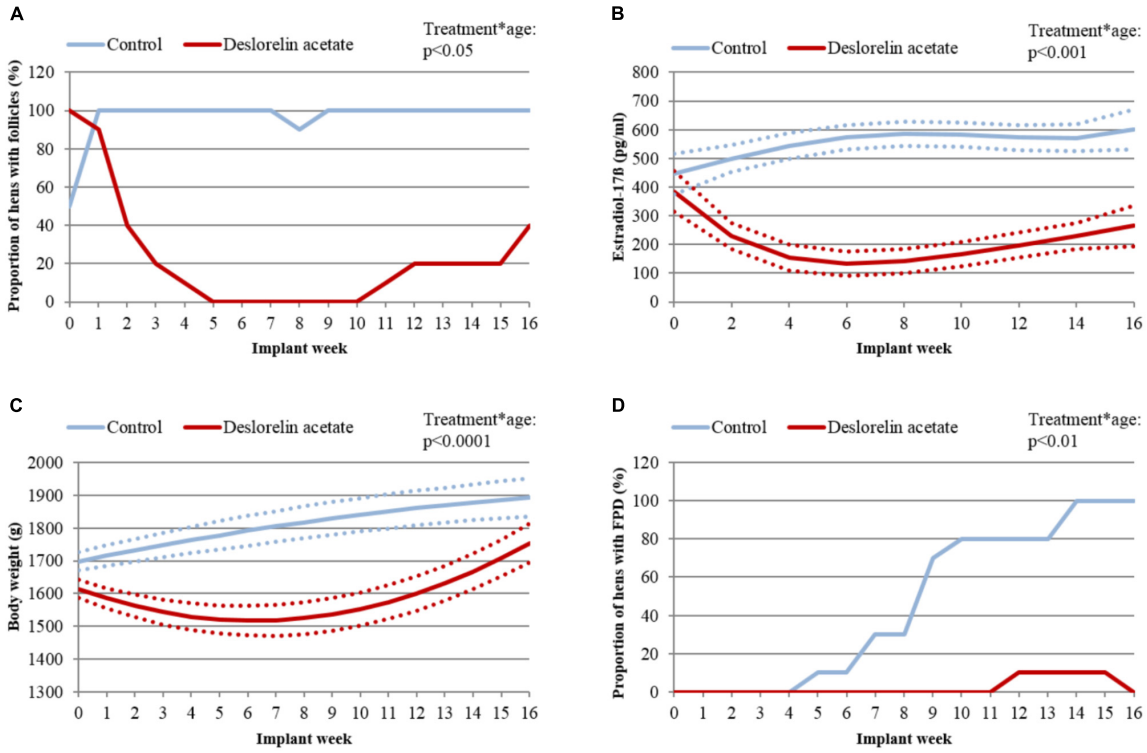

FIGURE 3 | Hens with follicles, estradiol-17ß concentrations, body weight and hens with foot pad dermatitis (FPD) of group "Adult". **(A)** Proportion of control (blue line) and treated hens (red line) with follicles on the ovary. **(B)** Least squares means (LSM; continuous lines) and upper and lower bounds of the 95% confidence interval (dotted lines) of estradiol-17ß in plasma in control (blue) and treated hens (red). Missing overlap of lines indicates a significant difference ($p < 0.05$) between groups. **(C)** LSM (continuous lines) and upper and lower bounds of the 95% confidence interval (dotted lines) of body weight of control (blue) and treated hens (red). Missing overlap of lines indicates a significant difference ($p < 0.01$) between groups. **(D)** Proportion of hens with FPD of control (blue) and treated hens (red).

corresponded to the number of hens in which follicles were detected via ultrasonography.

Estradiol-17ß Concentration in Plasma

Group "Adult"

Estradiol-17ß plasma concentration was significantly higher in control compared to treated hens of group "Adult" from implant week 2 until the end of the experiment (treatment*age: $p < 0.001$; **Figure 3B**). Both treated and control hens started with a high estradiol-17ß plasma concentration. While it remained at a high level, reaching more than 500 pg/ml estradiol-17ß in control hens, it decreased below 200 pg/ml in implant week 3 in treated hens. By implant week 6, estradiol-17ß concentration started to increase in treated hens but did not reach more than 300 pg/ml until the end of the experiment (implant week 16). Pre-treatment estradiol-17ß plasma concentrations did not affect estradiol-17ß concentrations after implantation ($p = 0.71$).

Group "Juvenile"

Both treated and control hens of group "Juvenile" started with a low concentration of estradiol-17ß (<100 pg/ml; **Figure 4B**). The concentration increased throughout the experiment in all hens. This increase in concentration was more pronounced and started earlier in control compared to treated hens. Consequently, higher concentrations of estradiol-17ß were found in control hens compared to treated hens from implant week 1 until the end of the experiment (treatment*age: $p < 0.01$). Pre-treatment estradiol-17ß plasma concentrations did not affect estradiol-17ß concentrations after implantation ($p = 0.75$).

Comb Size

The comb was significantly larger in control compared to treated hens in group "Adult" (LSM ± SE: 26.7 ± 1.52 cm^2 vs. 6.66 ± 1.52 cm^2; $p < 0.0001$) as well as in group "Juvenile" (27.05 ± 1.52 cm^2 vs. 7.23 ± 1.52 cm^2; $p < 0.0001$).

Foot Pad Dermatitis

Group "Adult"

In group "Adult", prevalence of FPD was significantly higher in control compared to treated hens from implant week 6 until the end of the experiment (treatment*age: $p < 0.01$; **Figure 3D**). In implant week 5, 1/10 control hens was affected by FPD and, at

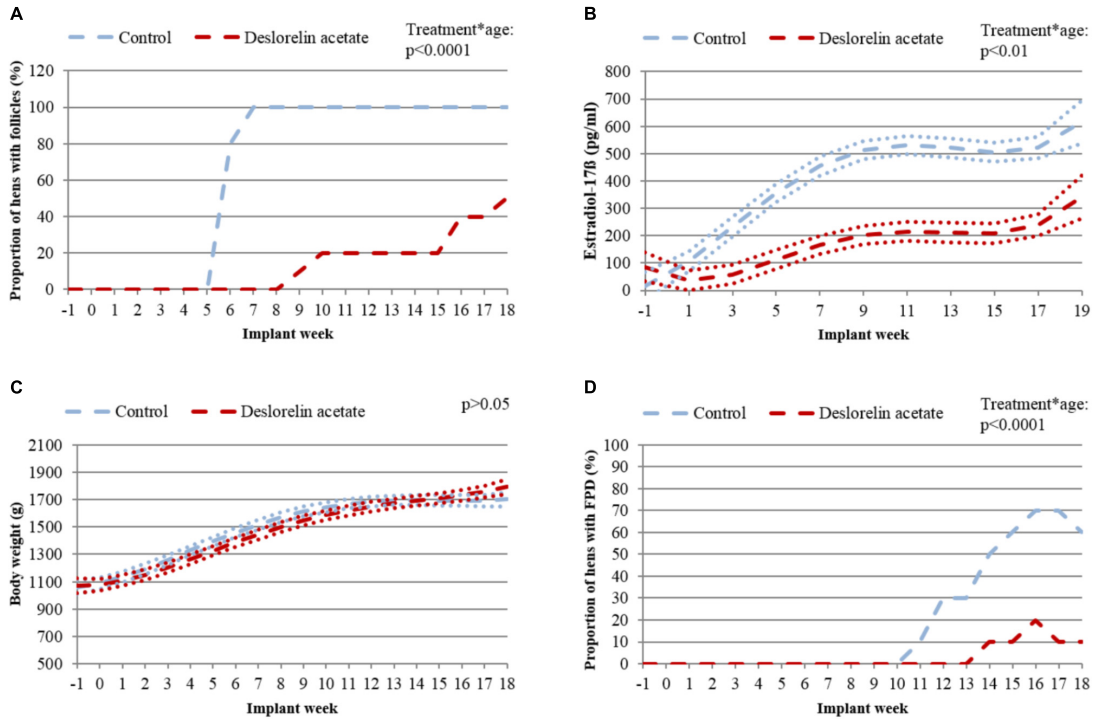

FIGURE 4 | Hens with follicles, estradiol-17ß concentrations, body weight and hens with FPD of group "Juvenile". **(A)** Proportion of control (blue dashed line) and treated hens (red dashed line) with follicles on the ovary. **(B)** LSM (dashed lines) and upper and lower bounds of the 95% confidence interval (dotted lines) of estradiol-17ß in plasma in control (blue) and treated hens (red). Missing overlap of lines indicates a significant difference ($p < 0.05$) between groups. **(C)** LSM (dashed lines) and upper and lower bounds of the 95 % confidence interval (dotted lines) of body weight of control (blue) and treated hens (red). Overlap of lines indicates no significant difference ($p > 0.05$) between groups. **(D)** Proportion of hens with FPD of control (blue dashed line) and treated hens (red dashed line).

the end of the experiment, all ten control hens were affected. In contrast, only 2/10 treated hens were affected by FPD throughout the entire experimental period. One of these two treated hens started laying eggs again 3 weeks prior to being affected by FPD.

Group "Juvenile"

In group "Juvenile", prevalence of FPD was significantly higher in control compared to treated hens from implant week 11 until the end of the experiment (treatment*age: $p < 0.0001$; **Figure 4D**). In implant week 11, 1/10 control hens was affected by FPD and the prevalence increased up to 7/10 control hens in implant weeks 16 and 17 before decreasing to 6/10 control hens in implant week 18. In contrast, only 2/10 treated hens were affected by FPD throughout the entire experimental period. Both of these two treated hens started laying eggs 5 weeks prior to being affected by FPD.

Keel Bone Health

Group "Adult"

In group "Adult", POD significantly increased from experimental week 7 (mean and standard error: 3.49 ± 0.5%) to experimental week 15 (4.44 ± 0.4%; $p < 0.01$). Moreover, deviated keel bone area was significantly larger in control (5.57 ± 0.6%) compared to treated hens (2.36 ± 0.6%; $p < 0.01$). None of the hens had a keel bone fracture in experimental week 7. In experimental week 15, the prevalence of keel bone fractures was significantly higher in control (4/10 hens) compared to treated hens (0/10 hens; $p < 0.05$).

Group "Juvenile"

In group "Juvenile", no significant influence on POD was found for age (experimental week 7: 3.13 ± 0.53%, experimental week 15: 3.28 ± 0.4%; $p = 0.76$), treatment (control: 3.35 ± 0.57%, treated: 3.06 ± 0.57%; $p = 0.72$) or their interaction ($p = 0.12$). Similarly, no significant influence on prevalence of keel bone fractures was found for treatment (experimental week 7: $p = 1$; experimental week 15: $p = 0.3$). In experimental week 7, none of the hens had a keel bone fracture and in experimental week 15, one of the control hens but none of the treated hens had a keel bone fracture.

Dissection

Group "Adult"

In group "Adult", the relative weight of the following organs was significantly higher in control compared to treated hens ($p < 0.01$; **Table 2**): ovary, oviduct, liver, intestine, and kidneys. The

TABLE 2 | Relative weight (% of body weight) of the organs of group "Adult" (left part) and group "Juvenile" (right part).

	Group "Adult": Relative organ weight (% of body weight) (Least squares means ± Standard Error)		*P*-value	Group "Juvenile": Relative organ weight (% of body weight) (Least squares means ± Standard Error)		*P*-value
	Control hens	Treated hens		Control hens	Treated hens	
Ovary	0.66 ± 0.05	0.26 ± 0.05	< 0.0001	0.60 ± 0.07	0.38 ± 0.07	< 0.05
Oviduct	3.55 ± 0.34	1.18 ± 0.32	< 0.0001	3.43 ± 0.36	1.96 ± 0.38	< 0.05
Liver	2.42 ± 0.07	1.80 ± 0.08	< 0.0001	2.22 ± 0.12	1.76 ± 0.11	< 0.05
Spleen	0.10 ± 0.01	0.14 ± 0.01	< 0.05	0.11 ± 0.01	0.12 ± 0.01	0.26
Intestine	5.30 ± 0.14	4.73 ± 0.14	< 0.01	5.30 ± 0.23	4.67 ± 0.23	0.06
Gizzard	1.77 ± 0.10	1.59 ± 0.10	0.21	1.85 ± 0.09	1.51 ± 0.09	< 0.05
Proventriculus	0.36 ± 0.01	0.37 ± 0.01	0.6	0.37 ± 0.02	0.31 ± 0.02	< 0.01
Thyroid glands	0.07 ± 0.03	0.01 ± 0.03	0.17	0.01 ± 0.001	0.01 ± 0.001	0.61
Lungs	0.40 ± 0.02	0.40 ± 0.02	0.77	0.38 ± 0.01	0.35 ± 0.01	0.12
Kidneys	0.72 ± 0.02	0.59 ± 0.02	< 0.001	0.73 ± 0.03	0.54 ± 0.03	< 0.001
Brain	0.18 ± 0.01	0.19 ± 0.01	0.38	0.18 ± 0.01	0.18 ± 0.01	0.57
Heart	0.37 ± 0.02	0.33 ± 0.02	0.15	0.37 ± 0.02	0.28 ± 0.02	< 0.01

P < 0.05 represent a significant difference between the least squares means of treated and control hens within one group ("Adult" or "Juvenile", respectively).

relative weight of the spleen, in contrast, was significantly higher in treated compared to control hens ($p < 0.05$). No significant difference ($p > 0.05$) was found in the relative weight of the heart, gizzard, proventriculus, thyroid glands, lungs, and brain. The oviduct was significantly longer in control compared to treated hens (LSM ± SE: 61.9 ± 3.7 cm vs. 37.9 ± 3.7 cm; $p < 0.001$).

Group "Juvenile"

In group "Juvenile", the relative weight of the following organs was significantly higher in control compared to treated hens ($p < 0.05$; **Table 2**): ovary, oviduct, liver, gizzard, proventriculus, kidneys, and heart. No significant difference ($p > 0.05$) was found in the relative weight of the spleen, intestine ($p < 0.1$), thyroid glands, lungs, and brain. The oviduct was significantly longer in control compared to treated hens (LSM ± SE: 62.4 ± 5.1 cm vs. 39.4 ± 5.1 cm; $p < 0.01$).

DISCUSSION

With the current study we have successfully established an animal model with non-laying and laying control hens which can be used in further studies to investigate the relationship between egg production and common diseases in laying hens as well as the underlying mechanisms.

One deslorelin acetate 4.7 mg implant has been shown to inhibit egg production both if implanted before and shortly after the onset of lay. Based on the aim of the study in which this animal model is intended to be used, researchers can decide whether to apply the implant before or after the onset of lay. In case of implantation after the onset of lay, laying hens have already developed all reproductive functions and thus have the same conditions as control hens. Implantation before the onset of lay might ensure that treated hens never lay any eggs throughout their lives. Further, this method can also be used to protract the onset of lay to investigate the relationship between the early onset of lay and different traits or diseases in laying hens. This could especially be interesting for studies concerning keel bone damage. Gebhardt-Henrich and Fröhlich (2015) found a negative correlation between the age of hens when laying their first egg and the probability of keel bone fracture presence at depopulation. The keel bone ossifies at about 35 weeks of age (FAWC, 2010) when laying hens have already been laying eggs for several weeks. Therefore, ossification might be disturbed by the competing demand for calcium to produce the egg shell, leading to a weak keel bone.

A very important finding of the current study is the relatively short duration of effectiveness of deslorelin acetate in laying hens. In dogs, the species the implant has been developed for, it is declared that Suprelorin® 4.7 mg suppresses reproduction for 6 months, i.e., 24 weeks. However, in group "Adult" of the current study, the shortest duration of effectiveness was only 8 weeks in two treated hens and at only 14 weeks after implantation, treated and control hens did not differ significantly anymore in proportion of hens with follicles. These findings imply that when comparing non-laying and laying hens, implant application would have to be repeated the latest 14 weeks after initial implantation. However, these findings are not consistent with the study by Noonan et al. (2012) in which the same implant inhibited egg production for almost 26 weeks in laying hens. However, hens in the study by Noonan et al. were older than the hens in the present study which may explain this discrepancy. Moreover, the use of different layer lines may also result in different findings. However, we have no information which line Noonan et al. used in their study since this study was not published in a peer-reviewed journal, but as a scientific abstract (Noonan et al., 2012). Duration of effectiveness of deslorelin acetate, when implanted before the onset of lay, seemed to be increased since a statistically significant difference in proportion of hens with follicles between treated and control hens was still present at the end of the experiment, i.e., 18 weeks after implantation in group "Juvenile". Due to different results between

groups and studies and due to large interindividual differences in duration of effectiveness, it seems to be impossible to give a general recommendation concerning the interval after which a new deslorelin acetate implant should be administered in laying hens. In order to do so, long-term studies with hens of different layer lines that are treated at different ages are required.

In accordance to findings by Noonan et al. (2012), the deslorelin acetate implant was effective in all treated hens. In Japanese quail, however, deslorelin acetate did not inhibit egg production in some individuals (Petritz et al., 2013; Schmidt et al., 2013). Even after administration of two 4.7 mg implants or one 9.4 mg implant, some of the treated quail continued to lay eggs (Petritz et al., 2015). This indicates that the effect of deslorelin acetate largely differs between species and thus, results from one bird species cannot be readily transferred to another species.

In the current study, no placebo implant was given to control hens. This is in contrast to other studies which investigated the effect of sustained release deslorelin acetate implants on reproduction in different species (Trigg et al., 2001; Petritz et al., 2013, 2015; Schmidt et al., 2013; Summa et al., 2017). It is therefore possible that different findings in treated compared to control hens may be additionally due to the treatment procedure itself and not due to deslorelin acetate alone. However, as our results are very similar to results of studies with Japanese quail in which placebo implants were used (Petritz et al., 2013, 2015; Schmidt et al., 2013), it is more probable that differences were caused by deslorelin acetate. Nevertheless, a study investigating the effect of deslorelin acetate implants on egg production in laying hens including control hens receiving a placebo implant would help to strengthen our conclusions.

In both groups, "Adult" and "Juvenile", no observable adverse effects of the implant were found. The injection site was devoid of signs of irritation or inflammation which indicates that the use of the implant is safe in laying hens. This is consistent with findings of other studies where deslorelin acetate implants were proven to be safe in different bird species (Cook and Riggs, 2007; Petritz et al., 2013). The only finding of the current study which may point toward an adverse effect was the lower body weight in treated compared to control hens of group "Adult". However, this may also be explained by the weight of an inactive oviduct and ovary being much lower than the weight of an active oviduct and ovary. The difference in weight of these two organs alone is sufficient to explain the different body weight. Moreover, the relative weight of the digestive tract, the liver, and the kidneys was also higher in control compared to treated hens which may be explained by an increased activity of these organs in control hens, leading to the difference in body weight.

Some of our results on differences between treated and control hens in other traits may facilitate the time point determination of the implant's effectiveness wearing off and its need to be replaced. Control hens displayed larger combs compared to treated hens. Thus, this characteristic may facilitate a quick estimation whether a hen lays eggs or not. Furthermore, estradiol-17ß plasma concentrations were lower in treated compared to control hens. Hence, repeated hormone concentration measurements may also serve as an indicator of implant effectiveness. However, in order to ensure if a hen is laying or not, ultrasonography of the ovary as well as egg yolk staining can be applied.

The findings on lower estradiol-17ß plasma concentrations in treated hens, which are in accordance with findings in other species (ferrets: Wagner et al., 2005; Japanese quail: Petritz et al., 2013), also have implications on further studies aiming at comparing laying with non-laying hens. As estradiol-17ß has an influence on several organs and mechanisms, it may be necessary to substitute this hormone in an animal model with deslorelin acetate depending on the hypothesis. For example, estradiol-17ß plays an important role in bone metabolism and bone diseases in laying hens as has been reviewed by Beck and Hansen (2004). In order to investigate the influence of egg production on bone health, it is therefore recommendable to substitute estradiol-17ß at least in a subgroup of the treated hens in order to compare laying and non-laying hens which have a similar estradiol-17ß plasma concentration.

The decreased keel bone deviations and fractures in treated compared to control hens of group "Adult" as well as the incidental finding of decreased FPD prevalence in treated compared to control hens of both groups indicate that egg production may be related to these diseases. Concerning keel bone damage, our findings in group "Adult" support the hypothesis that the high demand for calcium to produce the egg shell leads to weaker bones. In contrast, we did not find any differences between treated and control hens in group "Juvenile". This may be explained by the relatively young age in which these hens were radiographed (20th and 28th week of age). Keel bone damage has been shown to increase with age (Käppeli et al., 2011a; Heerkens et al., 2016; Eusemann et al., 2018). Consequently, we may have detected differences between control (= laying) and treated (= non-laying) juvenile hens later in life, i.e., if we had kept and radiographed the hens at later ages. In order to gain more insight into the etiology of keel bone fractures and deviations and the role of egg production, a study comparing keel bones of laying and non-laying hens throughout the entire laying period would be required. Concerning FPD, almost all affected hens were control hens or treated hens which had started laying eggs again. This finding suggests that non-laying hens may display a more active immune system which is in accordance with the higher relative weight of the spleen, an important organ of the immune system, in treated compared to control hens. Differences in the immune system between laying and non-laying hens could be explained by different plasma concentrations of estradiol-17ß and possibly other gonadal steroids as these have been shown to regulate the immune system (reviewed by Grossman, 1985). However, to fully understand this incidental finding, the relationship between egg production, estradiol-17ß plasma concentrations, FPD, and the immune system needs to be investigated in more detail.

CONCLUSION

We present a valid animal model with non-laying and laying control hens which can be used to investigate the relationship between egg production and different diseases in laying hens.

This model has been achieved by administration of one 4.7 mg deslorelin acetate implant per hen. However, based on our results, duration of effectiveness in laying hens seems to be much shorter than previously reported.

Furthermore, we have shown differences between treated and control hens in other traits such as comb size and estradiol-17ß concentrations. Lastly, our findings indicate that keel bone fractures and deviations as well as FPD may be related to egg production in laying hens.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

BE, LS, and SP conceived and designed the experiments. BE and A-KR performed the experiments. AS, BE, and AP analyzed the data. BE wrote the original draft of the manuscript. AS wrote the section on statistical methods. AP, SP, LS, CT-R, and AS reviewed and edited the original draft of the manuscript. BE visualized the data. All authors read and approved of the final manuscript.

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3.3 The Role of Egg Production in the Etiology of Keel Bone Damage in Laying Hens

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Declaration of own part in this research publication:

Contributions of BK Eusemann:

1. Drafting and development of the study design including animal test proposal.
2. Preparation and conduct of the experiment.
3. Statistical analysis and interpretation of the data, together with A Patt.
4. Visualization of the data.
5. Setup of the entire manuscript and writing the original draft.

Contributions of the other authors: Assistance with data analysis, review of the manuscript, supervision.

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The Role of Egg Production in the Etiology of Keel Bone Damage in Laying Hens

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Keel bone fractures and deviations belong to the most severe animal welfare problems in laying hens and are influenced by several factors such as husbandry system and genetic background. It is likely that egg production also influences keel bone health due to the high demand of calcium for the eggshell, which is, in part, taken from the skeleton. The high estrogen plasma concentration, which is linked to the high laying performance, may also affect the keel bone as sexual steroids have been shown to influence bone health. The aim of this study was to investigate the relationship between egg production, genetically determined high laying performance, estradiol-17ß concentration, and keel bone characteristics. Two hundred hens of two layer lines differing in laying performance (WLA: high performing; G11: low performing) were divided into four treatment groups: Group S received an implant containing a GnRH agonist that suppressed egg production, group E received an implant containing the sexual steroid estradiol-17ß, group SE received both implants, and group C were kept as control hens. Between the 12th and the 62nd weeks of age, the keel bone of all hens was radiographed and estradiol-17ß plasma concentration was assessed at regular intervals. Non-egg laying hens showed a lower risk of keel bone fracture and a higher radiographic density compared to egg laying hens. Exogenous estradiol-17ß was associated with a moderately higher risk of fracture within egg laying but with a lower risk of fracture and a higher radiographic density within non-egg laying hens. The high performing layer line WLA showed a significantly higher fracture risk but also a higher radiographic density compared to the low performing layer line G11. In contrast, neither the risk nor the severity of deviations were unambiguously influenced by egg production or layer line. We assume that within a layer line, there is a strong association between egg production and keel bone fractures, and, possibly, bone mineral density, but not between egg production and deviations. Moreover, our results confirm that genetic background influences fracture prevalence and indicate that the selection for high laying performance may negatively influence keel bone health.

Keywords: hen, keel bone, fracture, deviation, radiographic density, laying performance, egg production

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INTRODUCTION

Keel bone damage (KBD) is one of the most serious animal welfare problems in laying hens (1–3). The term comprises fractures and deviations of the keel bone, i.e., the ventral part of the sternum in birds. Keel bone fractures can affect up to 97% of hens within one flock (4–8), and it is likely that these fractures are painful (9, 10). The prevalence of deviated keel bones, being defined as "bone[s] with an abnormally shaped structure that has not resulted from a fracture but contains section(s) that vary from a theoretically perfect 2-dimensional straight plane in either the transverse or sagittal planes. Additionally, indentations along the ventral surface can also be classified as a deviation" (11), can reach up to 82% (12, 13). Age (4, 5, 14–18), different housing systems (4, 7, 19), nutrition (20–22), and genetic background (5, 17–19, 23, 24) have been shown to influence KBD. Egg production and the high laying performance of modern laying strains may also favor the occurrence of keel bone damage. There is a high calcium demand for the eggshell. The skeleton is an important source of calcium, especially the medullary bone, a special kind of woven bone which is found in the medullary cavity of female birds and which serves as a labile source of calcium (25, 26). It is suggested that the osteoblasts switch from building structural, i.e., cortical and trabecular bone, to building medullary bone when hens reach sexual maturity and that, thus, no formation of structural bone occurs during lay while resorption of structural bone continues, leading to a progressive bone loss (27). However, no detailed studies about the role of egg production in keel bone damage are available so far. In a previous work that aimed at testing the influence of the GnRH agonist deslorelin acetate on reproductive physiology in laying hens, we found a lower prevalence of keel bone fractures and a smaller proportion of deviated keel bone area in hens that were prevented from egg laying by a sustained release deslorelin acetate implant compared to control hens (28). However, the effect of treatment with deslorelin acetate on keel bone health could also have been mediated via other pathways that are not dependent on egg production. For example, treated hens in the previous study showed a decreased estradiol-17ß plasma concentration compared to control hens. This hormone plays an important role in bone structure, bone metabolism, and bone diseases in chickens (29, 30). It is assumed that it is the rise in estrogen plasma concentration at the onset of lay that stimulates the osteoblasts to form medullary rather than structural bone, leading to depression in structural bone formation and to osteoporosis (31). Furthermore, weaker bones with a thinner cortex and large defects within the cortical bone were found in hens treated with exogenous estradiol compared to untreated hens (32). Reduced bone strength and cavity formation in the cortical bone were also found after treatment with exogenous estradiol in roosters (32) and capons (30). Thus, differences between treated and control hens in our previous study may have additionally been caused by different estradiol concentrations and possibly other factors that were influenced by deslorelin acetate, and not by egg production alone.

There are also only few studies on the high laying performance that modern laying strains have been selected for and its possible role in KBD. Hocking et al. (33) compared commercial breeds with a high laying performance and traditional breeds with a significantly lower laying performance and found a higher radiographic density of keel bones and tibiotarsi and also a higher breaking strength of humeri and tibiotarsi in traditional compared to commercial breeds. Furthermore, Candelotto et al. (24) compared the risk of experimental keel bone fractures in an experimental line that descended from a dam line which had not been selected for any breeding goal for several years and a sire line which had been bred for dual egg and meat production to the risk in layer lines that had been selected for high productivity. They found a lower number of experimental fractures in the experimental line (24). Similarly, we found a higher prevalence of keel bone fractures and more severe keel bone deviations in a high compared to a low performing brown layer line in a previous study (19). However, in the same study, no such clear differences between high and low performing layer lines were found within the white layers (19).

When investigating keel bone damage in laying hens, it is crucial to observe the same hens over a longer period of time because prevalence of both keel bone fractures and deviations has been shown to increase with age (5, 14, 16–19). However, in some studies, prevalence only increased until about the 50th week of age and then leveled off or even decreased (4, 15, 21). Although it is possible that this peak in prevalence of fractures and deviations may partly be explained by the decreasing pool of hens that are still fracture and deviation free, it is also possible that the keel bone is less susceptible to damage after a certain age. Thus, the exact influence of age on keel bone damage and the mechanism behind it still remain to be examined.

The aim of the current study was to experimentally investigate the potential influence of egg production, selection for high laying performance, estradiol-17ß, and age on keel bone fractures and deviations as well as radiographic density of the keel bone.

We additionally addressed the locomotor activity of the hens because this may influence the prevalence of KBD as well. Physical activity has been shown to lead to a higher radiographic density, a higher amount of cortical and cancellous bone, and a higher breaking strength of different bones (16, 34–36). However, keel bone fracture prevalence was found to be higher in more active housing systems such as floor housing and aviaries compared to cage systems (4, 7, 19, 37). This phenomenon is usually explained by the higher risk of collisions with housing equipment that may lead to fractures (7, 38). Thus, we aimed at investigating whether egg laying and non-egg laying hens as well as high and low performing layer lines differed in their level of locomotor activity in order to account for this potential confounding factor in prevalence of KBD. Locomotor activity could differ between egg laying and non-egg laying hens in two opposite directions: On the one hand, egg-laying hens could show decreased locomotor activity levels compared to non-egg laying hens in order to compensate for the energy costs of egg production. This has been suggested for zebra finches by Williams and Ternan (39) who found lower locomotor activity levels in breeding compared to non-breeding pairs. On the other hand, administration of gonadal steroids such as testosterone or estradiol has been found to increase locomotor activity in

castrated male Japanese quail (40) and in ovariectomized rats (41), indicating that these hormones have a large effect on locomotor activity. Since, based on results of a previous study (28), egg laying hens were supposed to show higher estradiol-17ß plasma concentrations compared to non-egg laying hens, they could also show higher, estrogen-mediated locomotor activity levels. Furthermore, we suspected egg laying hens but not non-egg laying hens to show nesting behavior which has been shown to be mediated by estrogens and progesterone (42). As nesting behavior includes increased activity levels prior to oviposition (43, 44), this could also result in an increase in general activity throughout the day in egg laying compared to non-egg laying hens. Thus, we hypothesized that general locomotor activity would differ between treatment groups but did not speculate about the direction of this difference as both possibilities, i.e., decreased or increased locomotor activity levels in egg laying compared to non-egg laying hens, are plausible.

Taken together, we hypothesized that

1) non-egg laying hens would show a lower risk of keel bone fracture and deviation, less severe keel bone deviations, and a higher radiographic density of the keel bone compared to egg laying hens.
2) exogenous estradiol-17ß would increase the risk of keel bone fracture and deviation as well as the severity of deviations in egg laying and non-egg laying hens.
3) hens of a low performing layer line would show a lower risk of keel bone fracture and deviation, less severe keel bone deviations, and a higher radiographic density of the keel bone compared to hens of a high performing layer line.
4) the prevalence of keel bone fractures and deviations as well as the severity of keel bone deviations would increase with age.
5) locomotor activity would differ between treatment groups.

MATERIALS AND METHODS

Birds and Housing Conditions

The experiment was performed in accordance with the German Animal Protection Law and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (No. 33.19-42502-04-15/1966).

We examined two different but genetically closely related purebred White Leghorn lines of laying hens (*Gallus gallus domesticus*) which differ in laying performance: Layer line WLA originates from a breeding line of Lohmann Tierzucht GmbH, Cuxhaven, Germany selected for laying performance. The line has been maintained without selection since 2012 at the Friedrich-Loeffler-Institut, Institute of Farm Animal Genetics, Mariensee, Germany. Hens of this line lay around 320 eggs per year. The other line, G11, kept at the institute since 1965 as a conservation flock, is a low performing layer line with an average laying performance of 200 eggs per year.

All chicks (WLA: $n = 256$, G11: $n = 235$) were hatched on the same day and raised in a floor housing system. Birds of the different layer lines were kept in two separate rearing compartments of 23 m² each that were littered with wood-shavings and straw. Perches were provided from the 4th week of age onwards. A standard light program was applied throughout the rearing period and a conventional complete feed for chicks (until 7 weeks of age; 12.97 MJ AMEn/kg DM, 189.61 g/kg crude protein, 31.38 g/kg crude fat, 9.14 g/kg Ca, 6.94 g/kg P) and pullets (from 8 to 19 weeks of age; 12.82 MJ AMEn/kg DM, 151.67 g/kg crude protein, 30.21 g/kg crude fat, 15.83 g/kg Ca, 8.11 g/kg P) as well as water were offered *ad libitum*.

At 11 weeks of age, males were separated from the group and 100 female pullets per layer line were relocated to the experimental site where they were kept for the remainder of the experiment. There were two pens per layer line resulting in 50 hens per pen. All four pens were located in the same poultry house and were set up in the same way: Each pen measured 11 m², was littered with wood-shavings and straw and equipped with perches and a nest box. There were four mushroom-shaped plastic perches per pen. Each perch measured 205 cm × 7 cm × 5 cm (length × height × width at the top, i.e., where the feet are in contact with the perch). All perches were installed at a height of 60 cm and the distance between two perches measured 25 cm while the distance between the wall and the first perch measured 20 cm. There was one wooden nest box per pen which measured 92 cm × 76 cm × 60 cm (length × height × width) and which was installed at a height of 70 cm. For alleviated access to the nest box, two squared wooden perches (92 cm × 3 cm × 5 cm) were installed in front of the nest box and at the same height. The distance between both perches as well as the distance between the first perch and the nest box measured 10 cm. Duration of the light period increased gradually from 10 h/d (until the18th week of age) to 14 h/d (from the 24th week of age onwards). All laying hens were fed *ad libitum* on a conventional laying hen diet (11.68 MJ AMEn/kg DM, 168.11 g/kg crude protein, 29.43 g/kg crude fat, 50.05 g/kg Ca, 5.06 g/kg P) and had *ad libitum* access to water.

Treatment

There were four different treatment groups per layer line. Thirty-eight hens per layer line (group S) were administered an implant containing 4.7 mg of the gonadotropin-releasing hormone (GnRH) agonist deslorelin acetate (Suprelorin®, Virbac, Carros, France). Twelve hens per layer line (group E) were administered an implant containing 75 mg of the gonadal steroid estradiol-17ß (Innovative Research of America, Sarasota, Florida, USA). Twelve hens per layer line were administered both implants (group SE) and thirty-eight hens per layer line were kept as control hens (group C) and did not receive any implant or sham handling. Unbalanced sample sizes were due to the high costs of the estradiol-17ß implant, which only allowed for a comparably low number of hens treated with this implant. Within the two pens per layer line, the four treatment groups were equally allocated, resulting in 19 S, 6 E, 6 SE, and 19 C hens per pen. Both implants, deslorelin acetate and estradiol-17ß, are sustained release implants which continuously emit their active component. The deslorelin acetate 4.7 mg implants have been shown to inhibit follicle maturation and thereby egg production in laying hens for about 12 weeks in a previous study (28). The estradiol-17ß implants are declared by the manufacturer to emit the steroid hormone for 90 days. Based on the results of a

previous study (28), hens were first implanted shortly after the onset of lay. As age at onset of lay differed between the layer lines, WLA received the first implant in the 25th week of age while G11 received the first implant in the 27th week of age. Throughout the experimental period, administration was repeated every 90 days (three successive implants in total).

All implants were administered subcutaneously. Hens were anesthetized with 2–3% isoflurane (CP-Pharma Handelsgesellschaft mbH, Burgdorf, Germany) in compressed air with a flow rate of 500 ml/min delivered via face mask. Before application, the application site (first implantation: between vertebral column and left scapula; second implantation: between vertebral column and right scapula; third implantation: left knee fold) was aseptically prepared. In case of administration of a deslorelin acetate implant, the implant was administered with the aid of an applicator that had been delivered together with the implants and the implantation site was sealed with a tissue adhesive (Surgibond®, SMI, St. Vith, Belgium). In case of application of an estradiol-17ß or both implants, the skin was cut with surgical scissors at a length of about 2 cm and the implant was administered subcutaneously with forceps. The implantation site was then sealed with two to three simple interrupted stitches.

Ultrasonography of Ovaries

To verify that hens treated with deslorelin acetate or both implants (deslorelin acetate and estradiol-17ß) did not lay eggs, each hen was examined via ultrasonography every 3 weeks to check for ovarian follicles. The examination was conducted with the ultrasound system DUS 60 vet and the microconvex transducer C611-2 (both Edan Instruments GmbH, Shenzhen, China) as previously described (28).

Periodical Sampling

During the experimental period of a total of 50 weeks, seven sampling periods of 4 weeks each (sampling period 7 = 3 weeks only) were defined (**Table 1**). In each of these sampling periods, each hen was radiographed and 2–3 blood samples were collected (see below). Additionally, the activity of the hens was measured during the last 2 weeks of each sampling period.

A subgroup of hens (6 S and 6 C hens both of each layer line) was euthanized for another project after sampling period 6. Hence, and due to animal losses as well as parameters that could not be assessed for some hens in some sampling periods (e.g., fractures could not certainly be detected or excluded when the legs were overlapping with the keel bone in the radiograph), the number of hens varied between sampling periods and parameters. The numbers of hens of each layer line and treatment group that were included in the analysis of each parameter within a certain sampling period are given in the results section.

Weighing of the Hens

Each hen was weighed before each blood withdrawal (= 2 to 3 times per sampling period) and the mean between all values within one sampling period was calculated to get the mean body weight of each hen per sampling period.

Radiographic Examination of the Keel Bone

All hens were radiographed seven times throughout the experiment (= once per sampling period).

Digital radiographs were taken and evaluated as previously described (19). The non-anesthetized hen was gently placed on its left side on the digital flat panel detector Thales Pixium 2430 EZ wireless (Thales Electron Devices S.A., Vélizy-Villacoublay, France) and lateral radiographs were taken with 50.0 kV and at 2 mAs using the X-ray apparatus WDT Blueline 1040 HF (Wirtschaftsgenossenschaft deutscher Tierärzte eG, Garbsen, Germany) and the X-ray suitcase Leonardo DR mini (Oehm und Rehbein GmbH, Rostock, Germany). To deduce the radiographic density of the keel bone (see below), an aluminum step-wedge was radiographed together with each hen for calibration purposes. One person (SP) blindly evaluated all images for the presence of fractures, using the image processing system AxioVision 4.8 (Carl Zeiss Microscopy GmbH, Jena, Germany). Another person (BE) blindly evaluated all images for the presence and dimension of deviations and for radiographic density, using the image processing program ImageJ (National Institutes of Health, Bethesda, Maryland, USA).

Fractures

Each radiograph was evaluated for the presence of one or several keel bone fractures. These were either seen as areas of the bone with callus formation (old fractures) or as black thin lines without callus formation (new fractures). A radiograph was scored 1 if

TABLE 1 | Schedule of the experiment with the seven sampling periods, the corresponding weeks of age, and the experimental procedures that were carried out in the respective week of age.

Week of age	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Procedure		B	B R	A	A				B	B B R	A	A				B	B B R	A	A			B	B B R	A	A	
Sampling period		1							2							3						4				
Week of age	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62
Procedure			B	B B R	A	A							B	B B R	A	A								B	B B R A	A
Sampling period			5										6											7		

B, blood sampling (two B in one cell mean that blood samples were collected twice within the respective week); R, radiography of the keel bone; A, locomotor activity assessment.

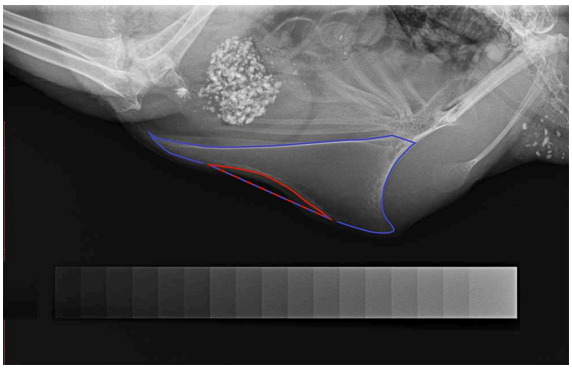

FIGURE 1 | Radiograph of a deviated keel bone. The keel bone surface area is circumscribed with blue color; the area of deviation is circumscribed with red color. The blue-red line marks the straight line between both extremes of the deviated outline. The aluminum step-wedge can be seen at the bottom of the figure.

one or multiple fractures were visible (regardless whether it was an old or a new fracture) and 0 if no fractures were visible.

Deviations

Each radiograph was evaluated for the presence of a deviation and scored 1 if the keel bone was deviated and 0 if the keel bone was not deviated. Further, the severity of a deviation was estimated by calculating the proportion of the deviated keel bone area (POD). The deviated area was circumscribed along the deformed outline and both extremes of this outline were linked by a straight line as an estimate for the size of the deviated keel bone area (**Figure 1**). The size of this area was calculated by ImageJ. Afterwards, the whole keel bone was circumscribed up to the insertion of the trabecula intermedia and the size of its surface area was calculated by ImageJ. Again, both extremes of the deformed outline were linked with a straight line as an estimate for the size of the assumed total keel bone surface area (**Figure 1**). Finally, POD was calculated as the proportion of deviated keel bone area in relation to the assumed total keel bone surface area in percent.

Radiographic Density

The method to assess radiographic density of the keel bone was similar to a method described by Fleming et al. (45) who assessed radiographic density of the humerus in laying hens. In the present study, an aluminum step-wedge was radiographed together with each hen for calibration purposes (**Figure 1**). The step-wedge consisted of 17 steps with thickness ranging from 0.5 to 4.5 mm (in 0.25 mm-increments). The gray value of the background and of all 17 steps was measured after which a calibration curve was generated with a 3rd degree polynomial function. Based on this calibration curve, keel bone radiographic density was assessed, circumscribing the whole keel bone up to the insertion of the trabecula intermedia. The mean gray value was given as millimeters of aluminum equivalent (mm Al eq). Callus formation and legs overlapping with parts of the keel bone resulted in increased, non-representative density measures. Thus, such areas were excluded from radiographic density assessment.

Measurement of Estradiol-17ß Concentration in Plasma

Blood samples of all 200 hens were collected on 3 days within one sampling period. In sampling period 1, blood samples were only taken twice because the animals were young and blood volume was still low. All blood samplings took place between 8 a.m. and 1 p.m. A maximum of 2 ml blood was taken from the ulnar vein and collected in a test tube coated with Ethylenediaminetetraacetic acid (EDTA) as an anticoagulant (VACUETTE® EDTA tubes, Greiner Bio-One GmbH, Frickenhausen, Germany). Immediately after sampling, blood samples were centrifuged at 3,500 rpm at 4°C for 10 min (Centrifuge Z 300 K, HERMLE Labortechnik GmbH, Wehingen, Germany) and stored at −20°C until further analysis.

Estradiol-17ß concentration was measured in pg/ml using a commercial enzyme-linked immunosorbent assay (ELISA) kit (IBL International GmbH, Hamburg, Germany). The analysis was performed following the instructions of the kit. A pool plasma sample was included on each kit together with the individual samples to calculate the inter-assay coefficient of variation, which was 6.72%. Each blood sample was measured in duplicate. Thereby, the intra-assay coefficient of variation was calculated (mean of all intra-assay coefficients of variation: 1.96%). The mean of both values of the duplicate was defined as the estradiol-17ß concentration of the hen for the specific day. The mean of the two (sampling period 1) or three (sampling periods 2–7) day concentrations within one sampling period was then calculated and defined as the estradiol-17ß plasma concentration of the hen within the given sampling period.

Locomotor Activity Assessment

Locomotor activity was recorded at the end of each sampling period by the use of an electronic transponder system (Gantner Pigeon Systems GmbH, Schruns, Austria) as described by others (46–48). All hens were fitted with an electronic transponder that was attached to the right leg with a plastic case and cable straps and which was individually identifiable by the antennas. Two antennas (76 × 29.5 × 3 cm) were placed on the floor of each pen (**Figure 2**). One antenna was placed between the perches and one of the two feeding troughs (distance to the closest perch: 55 cm, distance to the feeding trough: 40 cm). The other antenna was placed at right angles with the first one between the same feeding trough and the drinking trough (distance to the feeding trough: 50 cm, distance to the drinking trough: 40 cm). Within a 15 cm range of an antenna, transponders and thereby hens were individually registered and hen identity, antenna location, date and time of registration were recorded and stored as ASCII files. Analysis (SAS® 9.4, SAS Institute Inc., Cary, NC) included the lighting period of 10 (sampling period 7), 13 (sampling period 6), or 14 (sampling periods 1 to 5) days, respectively. Since lighting period differed between sampling periods, locomotor activity was measured as the mean number of antenna crossings/h for each sampling period resulting in one value per hen and sampling period.

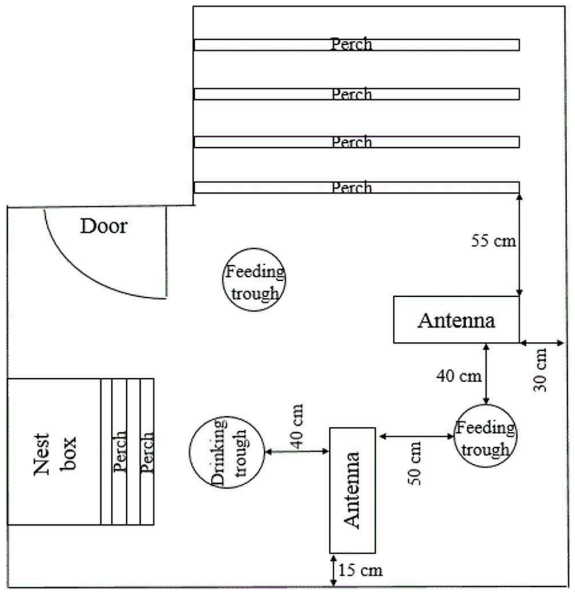

FIGURE 2 | Antennae location. Schematic, not to scale representation of the pen, demonstrating the location of both antennae in relation to the elements of the housing equipment.

Statistical Analysis

Statistical analysis was performed in R 3.5.2 (49). In order to adequately reflect dependencies in the experimental design (nesting, repeated measurements), generalized linear mixed-effects models were used to evaluate the numerical outcome variables body weight, POD (log transformed), radiographic density, estradiol-17ß plasma concentration (log transformed), and locomotor activity (log transformed) with the lme method from the nlme package (50). In each of these models, layer line (factor with two levels: G11 and WLA), treatment (factor with four levels: C, E, S, and SE), sampling period (factor with seven levels: 1–7), and all two-way interactions as well as the three-way interaction were included as fixed effects. Hen nested in pen was included as random effect. To avoid multiple hypothesis testing, no model simplification was performed (51). Significant *p*-values were obtained from examination of the full model and the test statistic for the full model is presented for each outcome variable in the results section. For analysis of POD, only hens with a keel bone deviation were included in the analysis. Since the model including all seven levels of the fixed effect sampling period was overspecified due to only a few hens having deviations in sampling periods 1 (4 hens) and 2 (31 hens), only sampling periods 3–7 were included in the analysis of POD. Results of all numerical outcome variables are described using the model estimates.

The effect of layer line and treatment on the occurrence of fractures and deviations (yes/no) was assessed with a survival analysis using the coxph method from the survival package (52, 53). Consequently, "survival" was equivalent with an intact keel bone, i.e., no occurrence of fractures or deviations, respectively. Only hens that lived until the end of the experiment and whose keel bone could consistently be assessed for the occurrence of fractures or deviations were included in the respective survival analysis. Some radiographs could be assessed for occurrence of deviations but not for occurrence of fractures because the legs of the hen overlapped with the caudal part of the keel bone where most of the fractures occurred while deviations were usually present in the middle or cranial part of the keel bone. Thus, number of animals varied between analysis of deviations on the one hand and fractures on the other hand. In both models, layer line (factor with two levels: G11 and WLA) and treatment (factor with four levels: C, E, S, and SE) were included as fixed effects. Pen was included as random effect. For analysis of keel bone deviations, the interaction between layer line and treatment group was included in the model, which was not possible for analysis of keel bone fractures due to model overspecification.

All model assumptions were verified using graphical analysis of residuals.

RESULTS

Ultrasonography of Ovaries

All control hens (group C) and hens treated only with estradiol-17ß (group E) showed ovarian follicles in all examinations. One of the hens treated with deslorelin acetate (group S) of layer line WLA still showed ovarian follicles after implantation and, thus, was excluded from statistical analysis. None of the hens treated with both implants (group SE) showed ovarian follicles after implantation.

Body Weight

Body weight was significantly influenced by the three-way interaction between treatment, layer line, and sampling period [$F_{(18,\ 1021)} = 7.1$, $p < 0.0001$; **Figure 3**]. In all treatment groups of both layer lines, body weight increased until sampling period 3. In contrast to the other treatment groups, body weight of group S of both layer lines decreased between sampling periods 3 and 4 and increased after sampling period 4 again. Body weight was lower in layer line G11 compared to WLA throughout the entire experimental period (groups E, S, and SE) or until sampling period 6 (group C), respectively.

Radiographic Examination of the Keel Bone

Fractures

The proportion of hens whose keel bone was not fractured throughout the experimental period was higher in group S (hazard ratio: 0.20, 95% confidence interval (CI) [0.11; 0.35]; i.e., risk of keel bone fracture reduced by 80%) and in group SE [hazard ratio: 0.06, CI [0.006; 0.69]; i.e., risk reduced by 94%] compared to group C. In contrast, the proportion of hens without fractured keel bone was lower in group E [hazard ratio: 1.17, CI [0.52; 2.60]; i.e., risk increased by 17%) compared to group C (**Figure 4A**). In layer line WLA, the proportion of hens without fractured keel bone was lower compared to layer line G11 [hazard

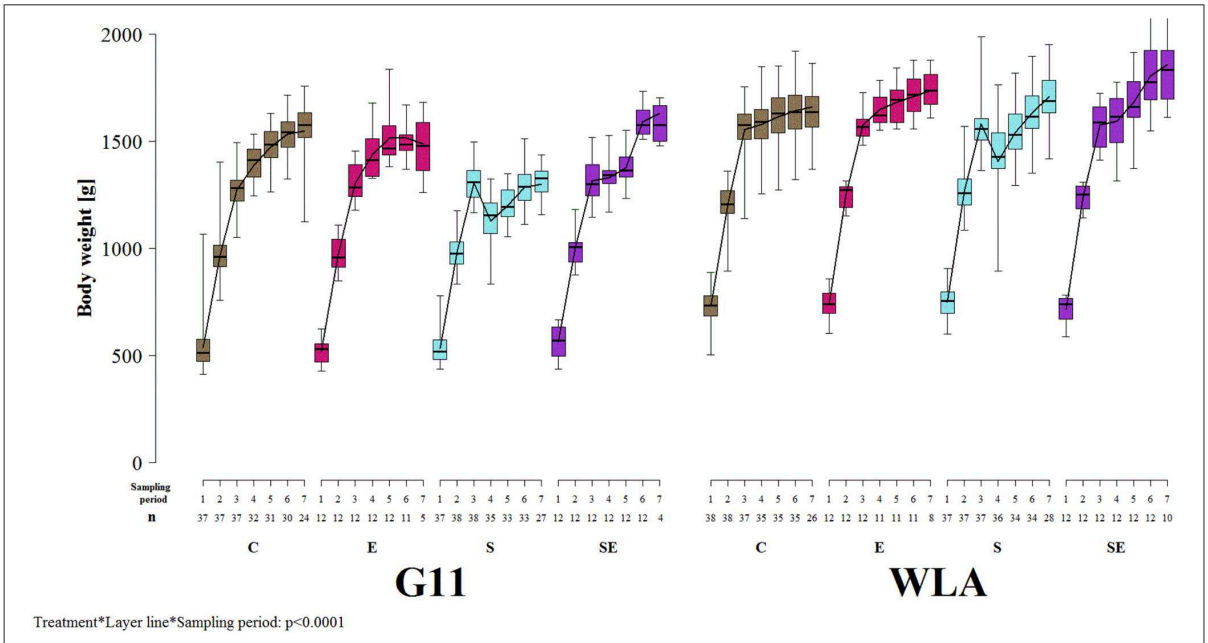

FIGURE 3 | Body weight. Each boxplot represents the median, first and third quartile, and minimum and maximum of the body weight of a specific treatment group and layer line within a certain sampling period. The black lines represent the model estimates. Numbers of hens that were included in the analysis are given under each sampling period. C, control hens (egg laying); E, hens treated with estradiol-17ß (egg laying); S, hens treated with deslorelin acetate (non-egg laying); SE, hens treated with deslorelin acetate and estradiol-17ß (non-egg laying); G11, low performing layer line; WLA, high performing layer line; Sampling period 1 = 12th–13th week of age, 2 = 19th–20th week of age, 3 = 26th–27th week of age, 4 = 32nd–33rd week of age, 5 = 39th–40th week of age, 6 = 49th–50th week of age, 7 = 60th–61st week of age.

ratio: 1.66, CI [1.32; 2.10], i.e., risk of keel bone fracture increased by 66%; **Figure 4B**; treatment + layer line: Wald test: $\chi^2_4 = 134$; $p < 0.0001$]. This analysis only included hens that lived until the end of the study and in which all radiographs could be assessed for the presence of fractures.

In addition, **Table 2** presents the prevalence of keel bone fractures for each sampling period including all hens that were radiographed and whose radiograph could be evaluated in the respective sampling period.

Deviations

The proportion of hens whose keel bone was not deviated throughout the experimental period was lower in group E [hazard ratio: 1.03, CI [0.58; 1.84]; i.e., risk of keel bone deviation increased by 3%] and group S [hazard ratio: 1.37, CI [1.26; 1.49]; i.e., risk increased by 37%] of layer line G11 compared to group C of layer line G11 (= reference group / intercept). In contrast, the proportion of hens without deviated keel bone was higher in group SE of layer line G11 compared to group C of layer line G11 [hazard ratio: 0.78, CI [0.53; 1.16]; i.e., risk reduced by 22%]. Proportion of hens without deviation was higher in groups C (hazard ratio: 0.98, CI [0.67; 1.43]; i.e., risk reduced by 2%) and S [hazard ratio: 0.75, CI [0.65; 0.87]; i.e., risk reduced by 25%] of layer line WLA but lower in groups E [hazard ratio: 1.25, CI [0.67; 2.34]; i.e., risk increased by 25%] and SE [hazard ratio: 1.65, CI [0.70; 3.89]; i.e., risk increased by 65%] of layer line WLA compared to group C of layer line G11 (treatment*layer line: Wald test: $\chi^2_7 = 990.5$; $p < 0.0001$; **Figures 5A,B**). This analysis only included hens that lived until the end of the study. For purposes of clarity, survival analysis is presented in two separate graphs for the layer lines in spite of the significant interaction between layer line and treatment. The reference group (intercept), i.e., treatment group C of layer line G11, has been added to the graph of layer line WLA so that comparisons can be made between the groups of WLA and the reference group.

The proportion of the deviated keel bone area (POD) was not significantly affected by the three-way interaction between layer line, treatment, and sampling period ($F_{(12,\ 3486)} = 1.06$, $p = 0.39$] but by the two-way interaction between layer line and treatment [$F_{(3,\ 168)} = 3.3$, $p = 0.0219$; **Figure 6**]. Within layer line G11, POD was higher in group S compared to all other treatment groups. In contrast, within layer line WLA, POD was lower in groups E and S compared to groups C and SE. Within groups C, E, and SE, POD was slightly higher in WLA compared to G11. Within group S, POD was higher in layer line G11 compared to WLA.

Radiographic Density

Radiographic density of the keel bone was significantly influenced by the three-way interaction between treatment,

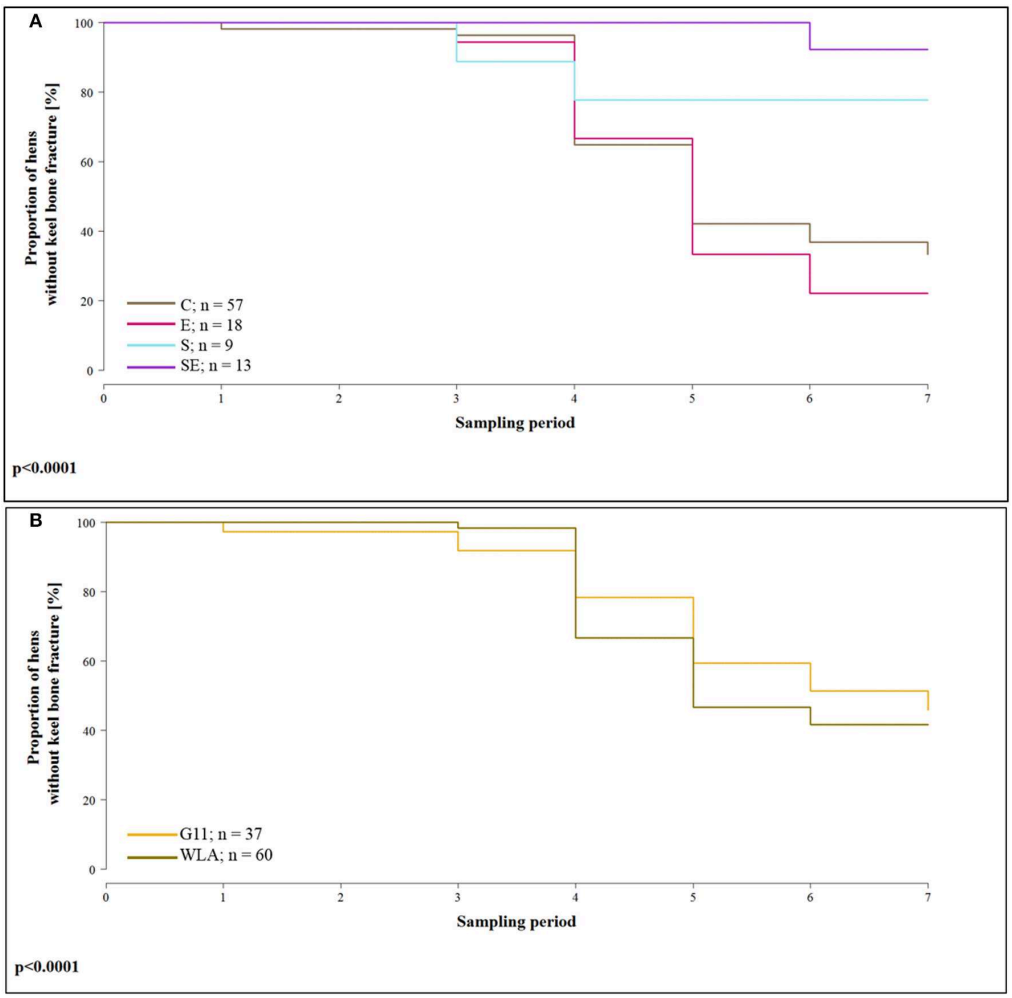

FIGURE 4 | Survival analysis of keel bone fractures. Note that only hens that lived until the end of the study and of which all seven radiographs could be evaluated for fractures were included in the analysis. **(A)** Proportion of hens without keel bone fractures of the four different treatment groups for all seven sampling periods. **(B)** Proportion of hens without keel bone fractures of the two different layer lines for all seven sampling periods. These data include all treatment groups. C, control hens (egg laying); E, hens treated with estradiol-17ß (egg laying); S, hens treated with deslorelin acetate (non-egg laying)l; SE, hens treated with deslorelin acetate and estradiol-17ß (non-egg laying); G11, low performing layer line; WLA, high performing layer line; Sampling period 1 = 13th week of age, 2 = 20th week of age, 3 = 27th week of age, 4 = 33rd week of age, 5 = 40th week of age, 6 = 50th week of age, 7 = 61st week of age.

layer line, and sampling period ($F_{(18,\ 1019)} = 1.91$, $p = 0.0123$; **Figure 7**]. Radiographic density increased at the beginning of the study in all treatment groups of both layer lines. This increase leveled off in groups C and E of both layer lines after sampling period 4. In contrast, radiographic density decreased between sampling periods 3 and 4 but markedly increased thereafter in group S of both layer lines while it steadily increased throughout the study in group SE of both layer lines. Thus, treatment groups S and SE reached higher radiographic density values compared to groups C and E in both layer lines toward the end of the study. Furthermore, group SE showed a higher radiographic density compared to group S from sampling period 4 onwards, which was more pronounced in layer line G11. WLA showed a higher radiographic density compared to G11 until sampling period 4 within groups C and E and throughout the study within groups S and SE, respectively.

Estradiol-17ß Plasma Concentration

Estradiol-17ß plasma concentration was significantly influenced by the three-way interaction between layer line, treatment, and sampling period ($F_{8,\ 1018} = 15.3$, $p < 0.0001$; **Figure 8**). In all treatment groups of both layer lines, estradiol-17ß concentration increased until sampling period 3. In group C of layer line

TABLE 2 | Prevalence of keel bone fractures of all radiographed hens.

Treatment group	Sampling period 1 13th woa	Sampling period 2 20th woa	Sampling period 3 27th woa	Sampling period 4 33rd woa	Sampling period 5 40th woa	Sampling period 6 50th woa	Sampling period 7 61st woa
Layer line G11							
C	0% (0/35)	0% (0 /33)	0% (0/24)	12.9% (4/31)	28.57% (8/28)	35.71% (10/28)	43.48% (10/23)
E	0% (0/11)	0% (0/12)	10% (1/10)	27.27% (3/11)	50% (6/12)	60% (6/10)	33.33% (1/3)
S	0% (0/37)	0% (0/27)	0% (0/28)	0% (0/3)	0% (0/3)	n.a. (0/0)	n.a. (0/0)
SE	0% (0/12)	0% (0/10)	0% (0/11)	0% (0/5)	0% (0/2)	25% (1/4)	0% (0/3)
Layer line WLA							
C	0% (0/38)	0% (0/36)	0% (0/36)	45.45% (15/33)	63.89% (23/36)	74.29% (26/35)	76.92% (20/26)
E	0% (0/12)	0% (0/12)	0% (0/12)	27.27% (3/11)	54.55% (6/11)	63.64% (7/11)	62.5% (5/8)
S	0% (0/36)	0% (0/30)	2.86% (1/35)	8.33% (1/12)	0% (0/9)	0% (0/9)	0% (0/8)
SE	0% (0/11)	0% (0/12)	0% (0/11)	0% (0/10)	0% (0/10)	0% (0/8)	0% (0/9)

Each cell indicates the prevalence of keel bone fractures for a specific layer line and treatment group in a specific sampling period. Note that in contrast to ***Figure 3****, also hens that died before the end of the study or in which only part of the radiographs could be evaluated for the presence of fractures were included in the calculation of fracture prevalence. The numbers in brackets in each cell indicate the number of hens with a keel bone fracture against the number of hens that were radiographed and whose radiograph could be evaluated for a specific treatment group and sampling period. woa, week of age; n.a., No radiograph of this group could be evaluated in this sampling period; C, control hens (egg laying); E, hens treated with estradiol-17ß (egg laying); S, hens treated with deslorelin acetate (non-egg laying); SE, hens treated with deslorelin acetate and estradiol-17ß (non-egg laying); G11, low performing layer line; WLA, high performing layer line.*

G11, it did not increase or decrease beyond sampling period 3, while it increased throughout the entire experimental period in group C of layer line WLA. Thus, control hens of layer line WLA reached higher estradiol-17ß plasma concentrations than control hens of layer line G11. In group E of both layer lines, estradiol-17ß plasma concentration increased throughout the experiment and reached higher values compared to group C, although concentration varied a lot between sampling periods in group E of layer line WLA which was not the case in group E of layer line G11. In group S of both layer lines, estradiol-17ß concentration decreased between sampling periods 3 and 4 and stayed at a low level, although increasing again toward the end of the experiment, especially in group S of layer line WLA. In group SE of both layer lines, estradiol-17ß plasma concentrations varied a lot between sampling periods but reached higher values compared to group S. The variation between sampling periods was more pronounced in group SE of layer line WLA compared to group SE of layer line G11. Within layer line G11, estradiol-17ß plasma concentration was highest in group E, followed by group SE, C, and S from sampling period 4 onwards. Within layer line WLA, estradiol-17ß plasma concentration was highest in group E, followed by groups C and SE which showed comparable values, and lowest in group S from sampling period 3 onwards.

Locomotor Activity

Locomotor activity was not significantly affected by the three-way interaction between layer line, treatment, and sampling period [$F_{(18,\ 984)} = 1.30$, $p = 0.18$] but by two two-way interactions.

The treatment groups showed very different patterns of locomotor activity over time. In groups C and E, locomotor activity did not vary a lot between sampling periods. In contrast, groups S and SE showed a steady decrease in locomotor activity from sampling period 2 (group S) or sampling period 3 (group SE) onwards, respectively. Thus, toward the end of the study, locomotor activity was higher in groups C and E compared to groups S and SE [sampling period*treatment: $F_{(18,\ 984)} = 4.16$, $p < 0.0001$; **Figure 9A**].

The patterns of locomotor activity over time also differed between layer lines. In G11, locomotor activity was higher in the first three sampling periods compared to the last four. In WLA, locomotor activity was lower in sampling periods 1, 5, and 7 compared to the other sampling periods. Locomotor activity differed between layer lines at the beginning of the study (sampling periods 1–3) with G11 being more active compared to WLA. However, this difference leveled off from sampling period 4 onwards [sampling period*layer line: $F_{(6,984)} = 9.45$, $p < 0.0001$; **Figure 9B**].

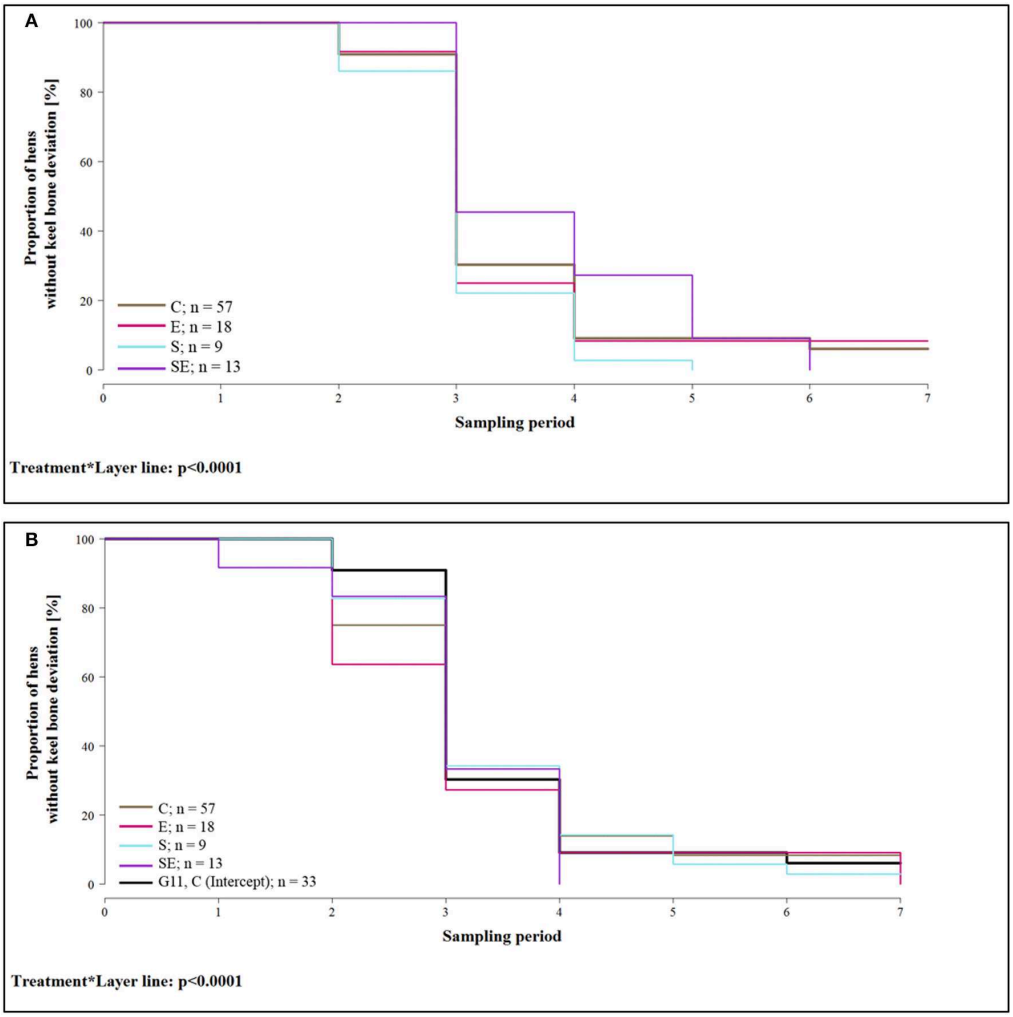

FIGURE 5 | Survival analysis of keel bone deviations. Note that only hens that lived until the end of the study were included in the analysis. **(A)** Proportion of hens without keel bone deviations of layer line G11 for the four different treatment groups and all seven sampling periods. **(B)** Proportion of hens without keel bone deviations of layer line WLA for the four different treatment groups and all seven sampling periods. For comparison, the intercept (control hens of layer line G11) is also included in the graph (black, bold line). C, control hens (egg laying); E, hens treated with estradiol-17ß (egg laying); S, hens treated with deslorelin acetate (non-egg laying); SE, hens treated with deslorelin acetate and estradiol-17ß (non-egg laying); G11, low performing layer line; WLA, high performing layer line; Sampling period 1 = 13th week of age, 2 = 20th week of age, 3 = 27th week of age, 4 = 33rd week of age, 5 = 40th week of age, 6 = 50th week of age, 7 = 61st week of age.

DISCUSSION

Egg Production and Keel Bone Damage

Our results clearly indicate that there is an association between egg production and keel bone fractures as well as radiographic density of the keel bone but that no association seems to exist between egg production and keel bone deviations.

As hypothesized, the risk of keel bone fracture was markedly lower in non-egg laying compared to egg laying hens. In comparison to group C, fracture risk was decreased by 80% in hens of group S and by 94% in hens of group SE. This is consistent with a previous study in which egg laying hens showed a significantly higher prevalence of keel bone fractures (up to 40%) compared to non-egg laying hens (0% throughout) (28). These findings suggest that egg production makes the keel bone very susceptible to fractures. It is crucial to find out which are the mechanisms behind the different risk of keel bone fracture in non-egg laying and egg laying hens. It is known that fracture risk and bone strength are influenced by material properties such as degree of mineralization, mineral composition, crystallinity, collagen characteristics, and osteocyte viability and by structural properties such as thickness and porosity of the cortex as well

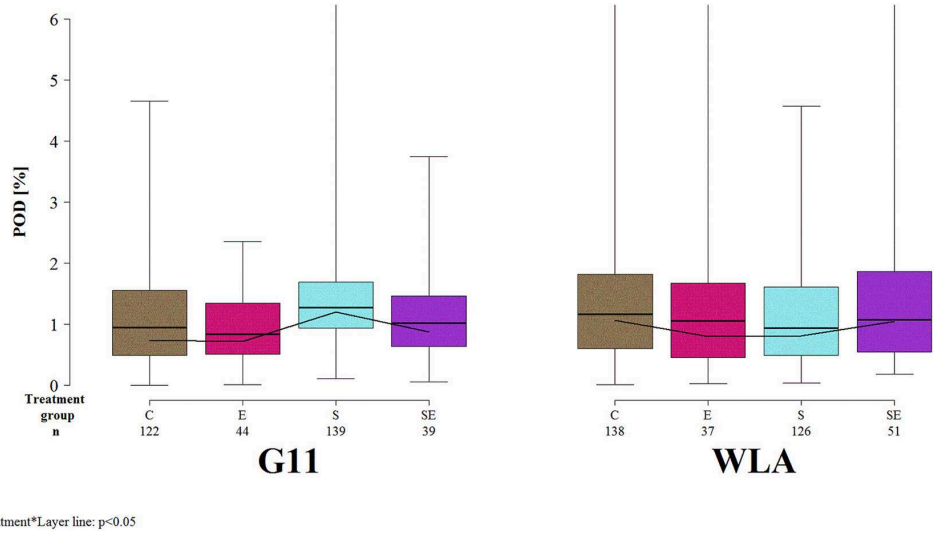

FIGURE 6 | Severity of keel bone deviations. Each boxplot represents the median, first and third quartile, and minimum and maximum of the proportion of the deviated keel bone area (POD) of a specific treatment group and layer line within a certain sampling period. The black lines represent the model estimates. Numbers of hens that were included in the analysis are given under each treatment group. C, control hens (egg laying); E, hens treated with estradiol-17ß (egg laying); S, hens treated with deslorelin acetate (non-egg laying); SE, hens treated with deslorelin acetate and estradiol-17ß (non-egg laying); G11, low performing layer line; WLA, high performing layer line; Sampling period 1 = 13th week of age, 2 = 20th week of age, 3 = 27th week of age, 4 = 33rd week of age, 5 = 40th week of age, 6 = 50th week of age, 7 = 61st week of age.

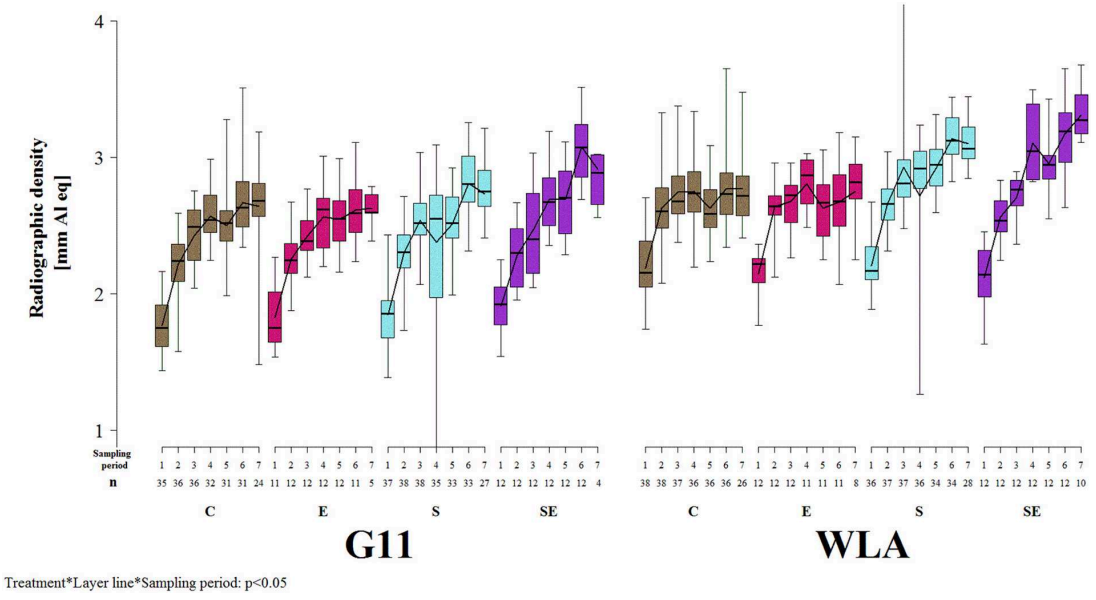

FIGURE 7 | Radiographic density of the keel bone. Each boxplot represents the median, first and third quartile, and minimum and maximum of the radiographic density of a specific treatment group and layer line within a certain sampling period. The black lines represent the model estimates. Numbers of hens that were included in the analysis are given under each sampling period. C, control hens (egg laying); E, hens treated with estradiol-17ß (egg laying); S, hens treated with deslorelin acetate (non-egg laying); SE, hens treated with deslorelin acetate and estradiol-17ß (non-egg laying); G11, low performing layer line; WLA, high performing layer line; Sampling period 1 = 13th week of age, 2 = 20th week of age, 3 = 27th week of age, 4 = 33rd week of age, 5 = 40th week of age, 6 = 50th week of age, 7 = 61st week of age.

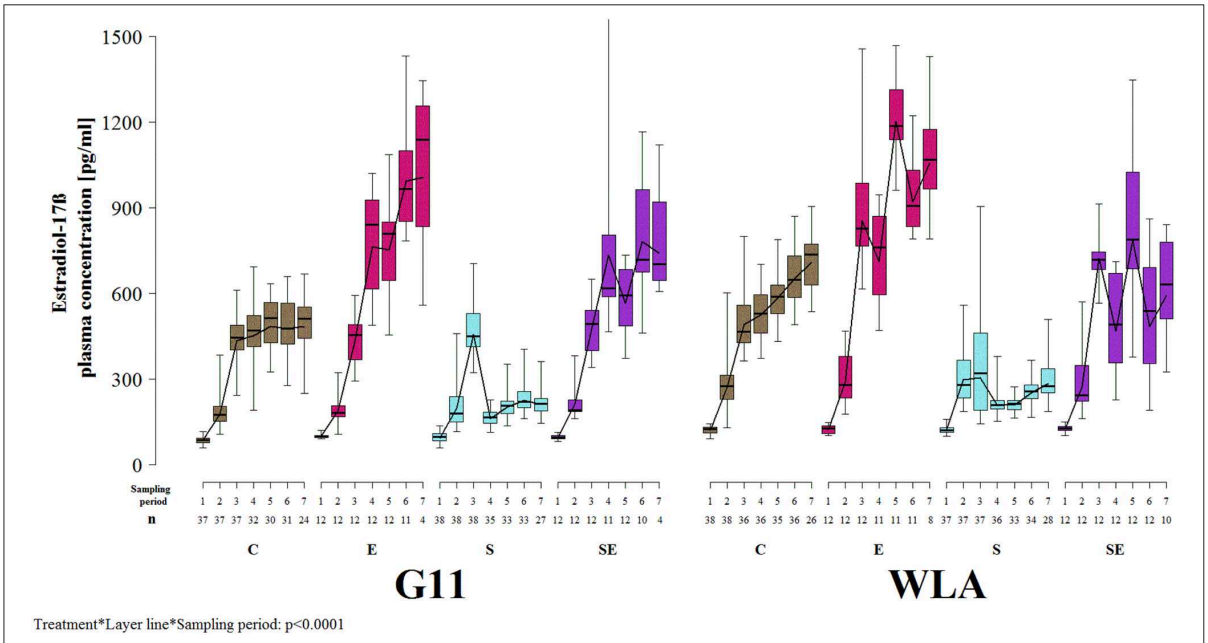

FIGURE 8 | Estradiol-17ß plasma concentration. Each boxplot represents the median, first and third quartile, and minimum and maximum of the estradiol-17ß plasma concentration of a specific treatment group and layer line within a certain sampling period. The black lines represent the model estimates. Numbers of hens that were included in the analysis are given under each sampling period. C, control hens (egg laying); E, hens treated with estradiol-17ß (egg laying); S, hens treated with deslorelin acetate (non-egg laying); SE, hens treated with deslorelin acetate and estradiol-17ß (non-egg laying); G11, low performing layer line; WLA = high performing layer line; Sampling period 1 = 12th–13th week of age, 2 = 19th–20th week of age, 3 = 26th–27th week of age, 4 = 32nd–33rd week of age, 5 = 39th–40th week of age, 6 = 49th–50th week of age, 7 = 60th–61st week of age.

as thickness and connectivity of the trabeculae (54, 55). In order to find solutions against keel bone fractures, it seems essential to find out whether the keel bone of egg laying hens differs from that of non-egg laying hens in any of these characteristics. On the one hand, it is possible that egg laying hens resorbed more calcium from the bones for the egg shell, leading to poorer bone quality, or that energy balance differed between egg laying and non-egg laying hens, resulting in increased resource availability for anabolic processes, including bone growth, in non-egg laying hens. On the other hand, it is also possible that deslorelin acetate had a direct effect on bone characteristics, independently of egg production. However, the potential direct effect of this drug on bone health does not seem to be likely to be positive as studies investigating the effect of GnRH agonists on bone traits in humans found lower bone densities in treated compared to non-treated persons (56, 57). Nevertheless, further studies comparing egg-laying with non-egg laying hens but suppressing egg production by other means would be helpful to further assess the role of egg production in keel bone damage.

One characteristic of the keel bone that differed between egg laying and non-egg laying hens in the present study was the radiographic density. At the end of the study, it reached higher values in non-egg laying hens (groups S and SE) compared to egg laying hens (groups C and E). The radiographic density reflects bone mineral density (BMD) (45, 58). Thus, BMD of the keel bone seemed to be higher in non-egg laying compared to egg laying hens toward the end of the study. This may be an underlying cause of the higher fracture prevalence in egg laying hens. A relationship between radiographic density or BMD and bone strength in laying hens has been shown in other studies (35, 45). Furthermore, Toscano et al. (59) found that increased BMD of the keel bone decreased the likelihood of an experimental keel bone fracture. In humans, it has been shown that a model including the change in BMD is more suitable to estimate the risk of fractures compared to a model that only includes baseline BMD (60). In the current study, radiographic density decreased between sampling periods 4 and 5, i.e., between the 33rd and the 40th week of age, in groups C and E of layer line WLA and, less pronounced, of layer line G11. This was also the time when fracture prevalence increased the most. Thus, the results concerning radiographic density and fracture risk in egg laying compared to non-egg laying hens indicate that radiographic density of the keel bone as assessed in the current study seems to be a suitable approach to predict keel bone strength and that keel bone fractures seem indeed to be associated with changes in BMD throughout the laying cycle. However, this is in contrast to findings about radiographic density and fracture risk between the different layer lines where the high performing layer line showed a higher radiographic density but also a higher risk of fracture compared to the low performing layer line as discussed

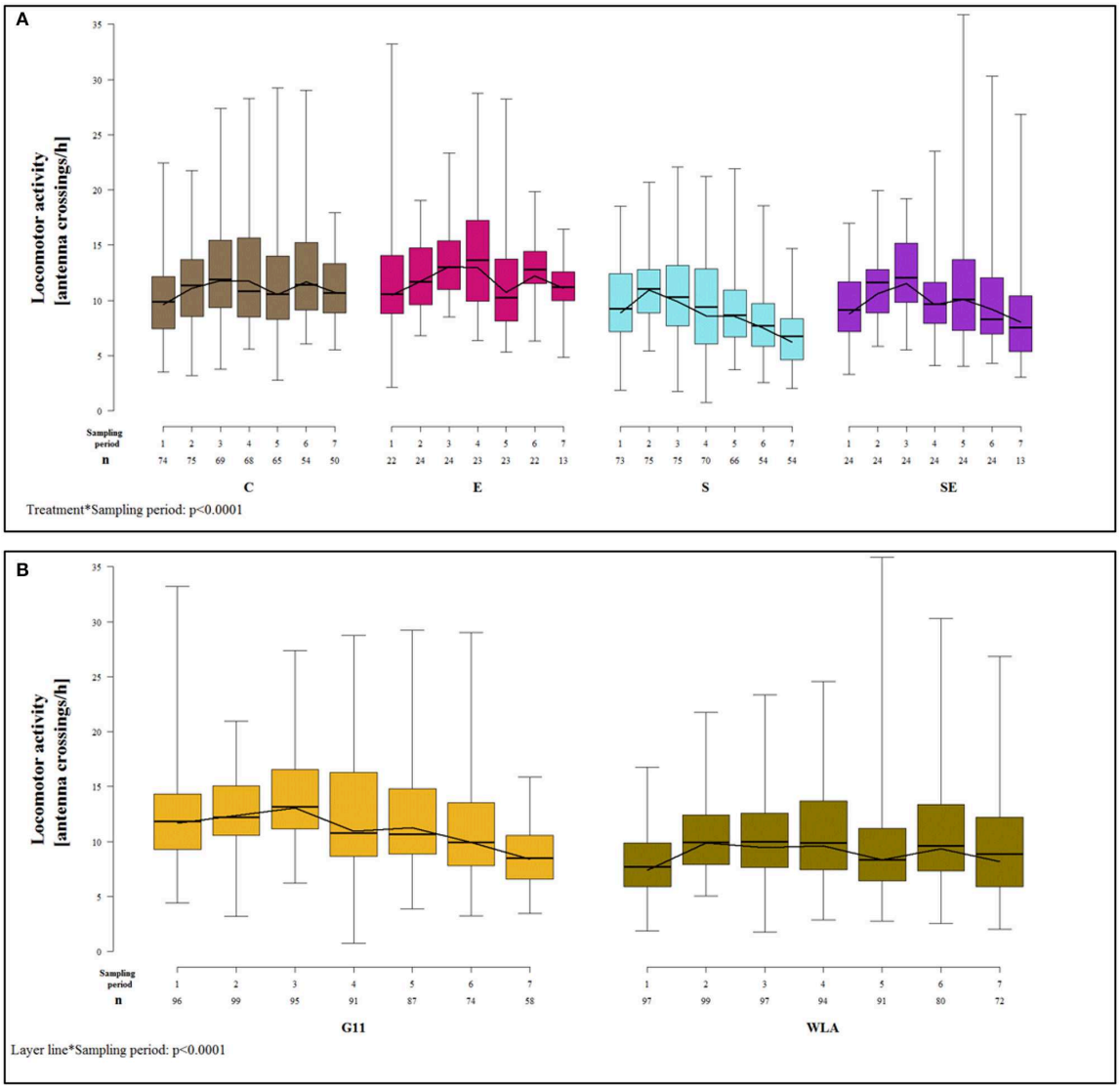

FIGURE 9 | Locomotor activity. Each boxplot represents the median, first and third quartile, and minimum and maximum of the locomotor activity of **(A)** a specific treatment group within a certain sampling period. The black lines represent the model estimates. Numbers of hens that were included in the analysis are given under each sampling period. **(B)** a specific layer line within a certain sampling period. The black lines represent the model estimates. Numbers of hens that were included in the analysis are given under each sampling period. C, control hens (egg laying); E, hens treated with estradiol-17ß (egg laying); S, hens treated with deslorelin acetate (non-egg laying); SE, hens treated with deslorelin acetate and estradiol-17ß (non-egg laying); G11, low performing layer line; WLA, high performing layer line; Sampling period 1 = 14th–15th week of age, 2 = 21st–22nd week of age, 3 = 28th–29th week of age, 4 = 34th–35th week of age, 5 = 41st–42nd week of age, 6 = 51st–52nd week of age, 7 = 61st–62nd week of age.

in section Layer Line and Keel Bone Damage. Radiographic density and BMD can be influenced by bone mass and the degree of mineralization. Thus, one of or both these characteristics may differ between the keel bone of egg laying and non-egg laying hens. It has been suggested that lack of bone mass is an underlying cause of keel bone fractures (13). It has also been suggested that this loss in bone mass takes place because during the laying period, only medullary bone and no structural bone is formed while osteoclastic resorption of structural bone continues and that structural bone formation only recommences when the hen goes out of lay (27). While this cycle of bone loss and regeneration seems to allow to maintain good bone

quality in female birds that lay eggs in clutches followed by incubation, commercial laying hens have been selected to remain in a continuously reproductive condition and their bones do not seem to have time to regenerate (27). While our data suggest that changes in bone mass may indeed contribute to the high prevalence of keel bone fractures, they do not allow to support the hypothesis that there is a continuous loss of bone mass during lay. Radiographic density only decreased between sampling periods 4 and 5 in egg laying hens but increased again thereafter. This may indicate that no loss of bone mass occurred after the 40th week of age and that the bones even recovered from the temporary loss of bone mass. It is important to note that the hypothesis about a continuous loss of bone mass was constructed based on studies that took place when the majority of hens was still housed in single cages (27) while the hens of the present study were kept in a floor housing system. It is possible that in caged hens in which movement is restricted, bone mass does indeed continuously decrease during lay while this is not the case if hens are kept in a housing system which allows for load-bearing movement. However, radiographic density as assessed in the present study includes structural and medullary bone. Thus, it is also possible that radiographic density was kept at a high level due to a high amount of medullary rather than structural bone in egg laying hens. While this type of bone shows a high degree of mineralization, it is weaker than structural bone, and thus, does not contribute to bone strength to the same extent as structural bone (27). This would explain why in egg laying hens radiographic density did not continuously decrease while fracture prevalence increased throughout the study. It is also important to mention that radiographic density as measured in the present study may also have been influenced by other factors, such as the breast muscles, and not by BMD alone. Thus, further examinations that allow for assessing BMD into more detail and for distinguishing between structural and medullary bone are required in order to further analyze bone structure of egg laying and non-egg laying hens. These could include radiographic density assessment of isolated keel bones, chemical analyses or histological analyses.

Another factor that differed between treatment groups is the body weight. Hens of group C were heavier compared to hens of group S from sampling period 4 onwards in layer line G11 and in sampling periods 4 to 6 in layer line WLA. This is consistent with a previous study in which control hens were also heavier compared to hens treated with deslorelin acetate (28). In that previous study, we assumed that the higher body weight in egg laying compared to non-egg laying hens could be the result of the increased weight of the ovary and oviduct alone. However, hens of group SE of the current study were as heavy or even heavier than hens of group C although they did not show ovarian follicles and, thus, the weight of their ovary and oviduct is assumed to have been as low as that of group S. This indicates that estradiol-17ß seems to have an influence on body weight, but the mechanisms remain unknown to us. Body weight is discussed to affect the occurrence of keel bone fractures and deviations. On the one hand, it is assumed that higher body weight may increase the risk of keel bone fracture due to greater collision energies when colliding with a perch (5, 15). On the other hand, it is possible that a higher breast muscle mass, which also increases body weight, may have a protective effect on the keel bone because low breast muscle mass leaves the keel vulnerable to fracture (13). Furthermore, increased body weight has been found to be associated with increased bone strength in laying hens, probably due to increased mechanical loading on the bone (61). However, in the current study, it seems unlikely that differences in body weight development have influenced fracture risk due to the similar body weight in hens of groups C and SE but the much higher risk of fracture in group C compared to group SE.

Contrary to our hypothesis and in contrast to fractures, neither the risk of keel bone deviation nor the severity of the present deviations, i.e., POD, were clearly influenced by egg production. Although hens of group S showed a lower POD compared to control hens within layer line WLA, this effect was reverse in layer line G11 and, within this layer line, group S also showed a higher risk of deviation compared to group C. This indicates that keel bone fractures and keel bone deviations are two independent phenomena caused by different factors. This assumption is in accordance with findings of a previous study in which deviations were more severe in cage housed hens while fractures were more frequent in their floor housed siblings, indicating different risk factors for these two types of damage (19). Results of that previous study suggest that deviations are mainly caused by the pressure of the perch on the keel bone. Likewise, in the present study, all pens were equipped with perches and, thus, deviations may have been caused by the pressure on the keel bone while perching. This is in accordance with findings that in a perching laying hen, the peak force is ∼5 times higher on the keel bone compared to a single foot pad (62), indicating that most of the hen's weight is supported by the keel bone. Interestingly, suppressed egg production did not protect the keel bone against this kind of keel bone damage. In contrast, hens of group S even showed a higher risk of keel bone deviations compared to group C within layer line G11. It is possible that this was caused by a higher perch use in hens of this group but this has not been assessed in this study. Results of the present study are not in accordance with findings of another previous study in which POD was significantly higher in egg laying control compared to hens that were treated with a deslorelin acetate implant after the onset of lay (28). This may be explained by different genetics in both studies. In the previous study, we used the hybrid Lohmann Selected Leghorn (LSL) while in the current study, the purebred layer lines WLA and G11 were used. Furthermore, the influence of the treatment on the severity of deviations was also analyzed differently in both studies. While in the previous study, all hens were included in the statistical analysis of POD (28), only hens that actually showed a keel bone deviation were included in the statistical analysis of POD in the present study. This may also explain the different findings of the two studies.

Estradiol-17ß and Keel Bone Damage

Treatment with estradiol-17ß implants had a strong effect on estradiol-17ß plasma concentration but its impact on keel bone health differed between egg laying and non-egg laying hens.

Estradiol-17ß plasma concentration differed between treatment groups and confirmed the effectiveness of the administered implants. The concentration of this gonadal steroid was decreased by administration of the sustained release deslorelin acetate implant (i.e., in group S). This finding is consistent with findings about estradiol-17ß concentrations after administration of a sustained release deslorelin acetate implant in laying hens (28), Japanese quail (63), and ferrets (64). Administration of a subcutaneous implant containing estradiol-17ß in hens treated with deslorelin acetate (i.e., group SE) increased the plasma concentration of this hormone to a concentration which was comparable to that found in control hens (layer line WLA) or even higher (layer line G11). Similarly, administration of an estradiol-17ß implant in laying hens without deslorelin acetate (i.e., group E) increased the hormone concentration above the concentration which was found in control hens (group C). Thus, after administration of the implants, estradiol-17ß plasma concentration was highest in group E, followed by groups SE and C, and lowest in group S.

However, no clear effect of these different estradiol-17ß plasma concentrations on keel bone health was found. Concerning non-egg laying hens, hens of group SE, which showed a similar or even higher estradiol-17ß plasma concentration compared to control hens, were at a lower fracture risk compared to hens of group C. Interestingly and contrary to our hypothesis, hens of group SE also were at a lower risk of keel bone fracture compared to hens of group S which showed a much lower estradiol-17ß plasma concentration. Furthermore, radiographic density was higher in group SE from sampling period 4 onwards and, although only within layer line G11, risk of keel bone deviation and POD were higher in group S compared to group SE. Thus, treatment with an estradiol-17ß implant did not diminish the positive effect of deslorelin acetate on keel bone health by increasing estradiol-17ß plasma concentrations. In contrast, both implants seemed to have a synergistic, positive effect on keel bone health. This clearly shows that the lower risk of keel bone fracture in non-egg laying compared to egg laying hens was not caused by lower estradiol-17ß plasma concentrations in non-egg laying hens and that the positive influence of deslorelin acetate on the keel bone was not related to the decrease of estradiol-17ß caused by this implant. The results of groups S and SE also suggest that estradiol-17ß may have a protective effect on the keel bone if no egg production occurs. Within egg laying hens, the risk of keel bone fracture was moderately increased in hens treated with only estradiol-17ß (group E) compared to control hens (group C). In contrast, radiographic density of the keel bone did not differ between groups E and C and no clear relationship between treatment with estradiol-17ß and keel bone deviations was found either. Thus, our findings indicate that estradiol-17ß plasma concentrations above physiological (i.e., found in control hens) concentrations may lead to a higher keel bone fracture risk in egg laying hens but do not fully support findings by other authors about a large negative influence of estradiol on bone health in chickens. Urist and Deutsch (32) found that treating laying hens with exogenous estradiol led to a thinner cortex and lower breaking strength of the long bones and assumed that exogenous estradiol accentuated osteoporosis. Reduced bone strength and defects in the cortical bone were also found after treatment with exogenous estradiol in roosters (32) and capons (30). The fact that exogenous estradiol had a large influence on bone health in these studies but only a moderate influence on fracture risk in egg-laying hens in the present study may be explained by different amounts of exogenous estradiol. The hens in the study by Urist and Deutsch (32) received 100 mg exogenous estradiol per week for 4 weeks while the administered implant in the current study contained 75 mg estradiol-17ß and lasted for 12 weeks. Thus, it is possible that the concentration of the administered estradiol-17ß was too low to show a large effect on the skeleton. Furthermore, the other authors examined long bones while we assessed the keel bone, which may also explain different findings. Taken together, our results indicate that estradiol-17ß has a different effect on keel bone health in egg laying compared to non-egg laying hens and suggest that detailed analyses of the mechanisms behind these effects may help to better understand the causes of keel bone damage in laying hens.

Layer Line and Keel Bone Damage

Similar to egg production, layer line had an influence on keel bone fractures but not on keel bone deviations.

The high performing layer line WLA showed a higher risk of keel bone fracture compared to the low performing layer line G11. This is consistent with a previous study in which we also found more keel bone fractures in a high compared to a low performing layer line (19). In accordance with these findings, Habig et al. (65), who worked with the same layer lines, found a higher breaking strength and bone mineral density of long bones in the low compared to the high performing layer lines. Furthermore, Candelotto et al. (24) found a lower number of experimental keel bone fractures in an experimental line that descended from a dam line which had not been selected for any breeding goal for several years and a sire line which had been bred for dual egg and meat production compared to the lines that had been selected for high productivity. In a study by Hocking et al. (33), a higher radiographic density of the keel bone as well as a higher breaking strength of the humerus and tibiotarsus were found in traditional layer lines with a low laying performance compared to commercial layer lines with a high laying performance (33). The results of all studies together may indicate that selection for high laying performance has led to poor bone health and a higher risk of keel bone fractures. However, interestingly, WLA, although having a higher risk of keel bone fracture, had a higher radiographic density compared to G11 in the present study. This was true for the whole experimental period within groups S and SE and until sampling period 4 within groups C and E. There are different possible explanations for this phenomenon. On the one hand, it is possible that other differences between the layer lines that were not subject to the present study played a more important role in the etiology of keel bone fractures compared to BMD, i.e., bone mass and degree of mineralization. These could be a number of other differences in bone characteristics such as crystallinity, collagen characteristics, osteocyte viability,

or thickness and connectivity of the trabeculae (54, 55). There could also be behavioral differences, e.g., differences in perch use and in motor skills, i.e., flight and 3D-movement skills, between the layer lines that led to a higher fracture risk in WLA. However, on the other hand, it is also possible that radiographic density as assessed in the present study does not allow to readily draw conclusions about the degree of mineralization and amount of structural bone. As mentioned above, the higher radiographic density may also reflect a higher amount of medullary bone in WLA compared to G11 which is weaker than structural bone (27). Furthermore, also factors that are not directly related to the keel bone may have influenced the radiographic density in this study. WLA hens were heavier compared to G11 hens. Thus, it is possible that WLA had a higher breast muscle mass which may have led to the higher radiographic density. Again, further analyses of the BMD and the structure of the keel bone would be required to assess the relevance of the higher radiographic density in WLA compared to G11 in the present study. Differences in fracture risk between the two layer lines cannot directly be linked to the different laying performance as only one high and one low performing layer line have been examined that also differ in other characteristics. To name only one, age at onset of lay differed between layer lines of the present and at least some of the other mentioned studies that found differences in keel bone health between high and low performing layer lines (33, 65). High performing hens were younger when they started to lay eggs compared to low performing hens. Thus, the early onset of lay may additionally have influenced keel bone health. Gebhardt-Henrich and Fröhlich (66) found a negative correlation between the age of hens when laying their first egg and the probability of keel bone fracture presence at depopulation. Thus, it may be possible to decrease the prevalence of keel bone fractures by protracting the onset of lay in commercial laying hens, for example with the help of the lighting regime. However, more studies are required to assess the possible role of the early onset of lay in the etiology of keel bone fractures.

Contrary to our hypothesis, control hens of the high performing layer line WLA showed a lower risk of keel bone deviation compared to control hens of the low performing layer line G11. However, this difference was only very marginal (risk reduced by 2%) and, thus, the biological relevance of this finding is debatable. Concerning severity of deviations, within treatment groups C, E, and SE, POD was slightly higher in WLA compared to G11 while the opposite was the case within group S. This is partly in contrast to and partly consistent with findings of a previous study (19). In that study, prevalence of deviations was higher in a high performing compared to a low performing brown layer line and higher in WLA compared to G11 but not compared to another low performing white layer line (R11). Moreover, differences between the layer lines in terms of POD were only found for hens housed in cages, where it increased in the high performing lines and in R11 but not in the other two low performing lines. In hens housed in a floor system, no differences were found between layer lines in terms of POD, similarly to the current study. These differences may be explained by the overall higher POD in hens housed in cages which eases the detection of a possible difference between the layer lines. However, statistical analysis for the risk or presence of deviations was performed differently between the studies which may also explain different findings.

Taken together, our results confirm that KBD, mainly keel bone fractures, have a genetic component. Thus, it seems a promising approach to decrease the prevalence of keel bone fractures by selecting hens for a high bone stability as has been suggested by others (13, 67). In contrast, further selection on laying performance may amplify this animal welfare problem.

Age and Keel Bone Damage

As hypothesized, KBD increased with age. This is consistent with other studies (5, 14, 16–19). However, several authors found that the prevalence of keel bone fractures peaked at about 50 weeks of age. Petrik et al. (4) assessed the prevalence of keel bone fractures on various farms in Ontario, Canada, with palpation. Fracture prevalence increased between 20, 35, and 50 weeks of age, but showed similar values at 50 and 65 weeks of age. Similarly, in an experimental study by Stratmann et al. (15), fracture prevalence, as assessed by palpation, increased with age but not beyond 52 weeks of age. Furthermore, Toscano et al. (21) found that the likelihood of experimental keel bone fractures increased with age but then began leveling off and to reverse at ∼49.5 weeks of age. This was neither the case in the present study where prevalence increased until the end of the study nor in a previous study where we found more laying hens with keel bone fractures in the 72nd compared to the 51st week of age (19). However, in the present study, increase of prevalence of fractures and deviations was less pronounced at a higher compared to a younger age of the laying hens. The increase of prevalence was steepest between the 20th and 27th as well as between the 27th and 33rd week of age for deviations and between the 27th and 33rd as well as between the 33rd and 40th week of age for fractures, respectively. Thereafter, the increase was much less pronounced. Thus, it is possible that the keel bone is more susceptible to keel bone deviations and fractures until a certain age of about 40–50 weeks compared to higher ages. Further studies are required to get a better insight into the effect of age on KBD, especially into the possible mechanisms that may make the keel bone less susceptible to fractures and deviations after a certain age.

Locomotor Activity

As hypothesized, locomotor activity differed between treatment groups over time. Locomotor activity decreased in non-egg laying hens (groups S and SE) toward the end of the study but not in egg laying hens (groups C and E) and, thus, reached higher levels in egg laying compared to non-egg laying hens. The increased general locomotor activity in egg laying compared to non-egg laying hens may be a result of nesting behavior in hens of groups C and E but not in hens of groups S and SE. Increased locomotor activity has been found prior to oviposition (44) and restlessness has been described as part of nesting behavior in laying hens (43). Interestingly, treatment with estradiol-17ß did not seem to have any influence on locomotor activity. Locomotor activity decreased in hens of group SE as it decreased in hens of group S and did not differ between these groups. Similarly, locomotor activity did not differ between groups C and E. Wood-Gush and

Gilbert (42) found that nesting behavior could be induced by administration of estrogen and progesterone in ovariectomized hens while administration of estradiol alone only led to nesting behavior in a small part of the hens. Concerning intact laying hens, it has been found that exogenous estrogen did not have any influence on nesting behavior (68). Thus, the fact that no differences in locomotor activity were found between groups S and SE as well as between C and E could strengthen the assumption that the increased general locomotor activity in egg laying compared to non-egg laying hens was caused by the increase in activity related to nesting behavior. However, nesting behavior was not explicitly assessed in the present study and, thus, it cannot be assured whether the increased locomotor activity in egg laying compared to non-egg laying hens was related to nesting behavior.

Findings on locomotor activity of the two layer lines differed from findings concerning treatment groups. The low performing layer line G11 was more active at the beginning of the study while there were no differences between the layer lines once hens had started to lay eggs. As G11 showed a lower keel bone fracture prevalence compared to WLA it is possible that the increased activity in chicks and pullets in this layer line led to a higher breaking strength.

The method to compare locomotor activity between groups presented in this work is only a very first approach to get an idea about the general locomotor activity of the hens which may also be influenced by other factors. For example, the preferred location of each hen may influence the measurement as the antennae could not cover the whole pen so that hens that spent more time in the center of the pen are likely to have been registered more often compared to hens that spent more time at the boundary. Furthermore, no information about the use of different structures such as nest boxes and perches was acquired with this method.

It is possible that the differences in locomotor activity had an influence on keel bone health. Increased mobility positively influences bone strength (35, 36) but can also lead to an increased fracture prevalence due to a higher risk of collisions (7, 38). Furthermore, if nesting behavior was the cause for the increased locomotor activity in egg laying compared to non-egg laying hens, this may also have had a direct effect on keel bone damage as the possible competition for nest box access may lead to a higher fracture risk. Thus, the difference in locomotor activity may be a confounding factor when comparing keel bone fracture prevalence between egg laying and non-egg laying hens. However, as differences in fracture prevalence were extremely large between treatment groups while differences in locomotor activity were relatively small, we assume that a higher susceptibility to fractures of the keel bone due to egg production played a more important role in the etiology of keel bone fractures than higher risk of collisions due to increased activity in egg laying hens. Nevertheless, a more detailed assessment of the hens' activity and behavior is required in order to determine the relevance of the findings on locomotor activity in relation to keel bone damage. This could be done by video monitoring and should include information about the behavior performed by hens while moving, about the use of structures such as perches and nest boxes, and about the kind of movement they are performing. It should also include wing movements such as wing-flapping and balancing movements when perching or ascending and descending perches as these activities may have a larger effect on the keel bone than general locomotor activity which is likely to have most impact on leg bones.

Limitations of This Study

Being the very first study to compare keel bone damage between hens in which egg production was suppressed and intact laying hens and, thus, including a number of methods that have not been applied in many studies before, the present study has some limitations that need to be kept in mind when drawing conclusions.

Sample sizes differed between groups. This was due to the very high costs of the estradiol-17ß implants which made it impossible to treat a higher number of hens with this implant. However, we decided not to orientate sample sizes of the other groups to the estradiol groups in order to have more hens to compare in groups S and C as our main focus was on differences between hens treated with deslorelin acetate and control hens. The differences in sample size also increased throughout the study due to higher mortality in groups E and SE compared to the other groups between sampling periods 6 and 7. Pathological investigations could not clarify whether mortality was associated with estradiol-17ß supplementation. Although an unbalanced sample size is not ideal, the methods used for statistical analyses do account for unbalanced sample sizes and allow to compare these groups and, thus, the influence of this limitation was kept as low as possible.

Sample size was also quite low in some groups, especially for analysis of keel bone fractures where only hens in which all radiographs could be assessed for fractures were included. Some radiographs could not be evaluated for fractures due to the legs overlying the keel bone when taking the radiograph. This occurred more often in non-egg laying compared to egg laying hens. Thus, sample size was lower in group S compared to group C for analysis of keel bone fractures. In future studies, radiographs could be taken with hens hanging upside down as shown by Sirovnik and Toscano (69) as well as Rufener et al. (70), which is likely to reduce the number of non-evaluable radiographs.

Furthermore, taking only lateral radiographs may have led to specific deviations, although being visible, being underestimated. However, postero-anterior radiographs of the keel bone, which would allow for a more detailed analysis of some kinds of deviations, are not useful as too many other parts of the body such as the vertebral column, being situated between the keel bone and the detector, overlie the keel bone and make the assessment of the keel bone impossible. Taken together, the assessment of the severity of deviations, i.e., POD, can only be an estimation of the actual amount of deviated keel bone area and must be interpreted with care, also because POD itself and differences in POD between the groups were small in the present study.

Control hens did not receive any sham operation or any placebo implant. Thus, it cannot fully be excluded that differences between treated and control hens were based on the treatment process itself rather than on egg production or estradiol-17ß plasma concentrations. However, although not being objectively quantified, treated hens did not show any evident behavioral changes after the implantation procedure. They fed and used the perches directly after having been brought back to the pen. Furthermore, all sampling procedures occurred throughout a broad time period while any possible effects of the small surgical procedure would only be expected to be relevant for a certain time after the procedure. In addition, hens of groups E, S, and SE were all subject to the same surgical procedure but hens of group E showed a higher prevalence of keel bone fractures compared to hens of groups S and SE. Thus, it is clear that differences between these groups are not caused by any effect of the surgical procedure and indicate that the procedure did not have a major influence on keel bone damage.

CONCLUSIONS

The present study clearly shows a strong association between egg production and keel bone fractures. In order to find solutions to decrease fracture prevalence, possible changes in the bone caused by egg production should be analyzed. One characteristic that differed between egg laying and non-egg laying hens was the radiographic density. This may reflect a lack of bone mass in egg laying hens. However, further studies are required to strengthen this assumption. Furthermore, genetic background and, possibly, selection for high laying performance have been found to influence keel bone fractures, indicating that selection for high bone quality may be a promising way to decrease their prevalence. In addition, the early onset of lay may negatively influence keel bone health and it seems worth to have a closer look at this possible relationship as this trait could be easily manipulated in commercial farms.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by Lower Saxony State Office for Consumer Protection and Food Safety, Postfach 39 49, 26029 Oldenburg.

AUTHOR CONTRIBUTIONS

BE, LS, and SP conceived and designed the experiments. SW bred the chicks of the layer line G11. BE and SP performed the experiments. AP and BE analyzed the data. BE wrote the original draft of the manuscript and visualized the data. AP, SP, LS, CT-R, and SW reviewed and edited the original draft of the manuscript. All authors read and approved of the final manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4 Concluding Discussion

The aim of this work was to get a better insight into the etiology of keel bone fractures and deviations, focusing on the possible role of different housing systems, age, genetic background, egg production, and estradiol-17β. A protocol for repeated examination of the keel bone using radiography and an animal model with non-egg laying hens were successfully established. Both were further used in several studies that revealed differences in KBD between layer lines differing in phylogenetic background and laying performance, between two housing systems, between time points as well as between egg laying and non-egg laying hens. In contrast, no large effect of exogenous estradiol on keel bone fractures or deviations was found.

4.1 Radiography as a Tool to Evaluate the Keel Bone in Laying Hens

To examine the development of the keel bone as well as prevalence of fractures and deviations over time, it is crucial to repeatedly assess the keel bone of the same hen throughout the laying period. This requires a method which can be applied in live birds. Thus, the first aim of the present work was to establish a protocol for taking and evaluating radiographs of keel bones in laying hens which included the assessment of both fractures and deviations and which allowed for repeatedly examining the keel bone throughout the entire life of laying hens. Lateral radiographs of the keel bone were taken with a portable radiographic system (Eusemann et al. 2018a). In contrast to previous studies (Clark et al. 2008; Richards et al. 2011), hens of the present work were neither dead nor anesthetized when being radiographed (Eusemann et al. 2018a). The fact that the laying hens were live when being radiographed made a longitudinal study on the same hens possible while the fact that they were awake made the procedure faster compared to radiographic examination on anesthetized hens. However, this procedure required two persons that restrained the birds and that were, consequently, close to the X-ray generator. In order to best protect these two persons from the X-ray radiation, they were equipped with lead aprons, lead gloves, and protective glasses. Furthermore, the radiation dosage they received was controlled with dosimeters at the fingers and the torso. The dosage was far below the maximal permitted dosage (data not shown). However, the safest way to avoid exposure to X-ray radiation is, of course, to keep back from the generator. This is not possible with the presented method. An alternative way to take radiographs of laying hens without anesthesia is to hang the hens upside down on a shackle inducing them to remain still (Širovnik and Toscano 2017). Thus, in order to meet the health and safety criteria best possible, this method is preferable to the method presented in the present work.
Similar to other studies (Richards et al. 2011; Rufener et al. 2018), the protocol that was used to evaluate the radiographs in the present work included the presence of fractures (Eusemann et al. 2018a). It was shown that radiography is a suitable tool to assess keel bone fractures as not only those with callus formation, but also small fractures and new ones that did not show callus formation were detected. Unlike most of the previous studies, the present protocol also included the detection and measurement of keel bone deviations (Eusemann et al. 2018a). Clark et al. (2008) were the first to present a method to estimate the extent of keel bone deviations. They measured the depth of the largest indentation of the keel bone and, thus, received one value for the deviation in each radiograph which is a good estimate for the severity of a deviation. However, as hens were euthanized before radiographs were taken, this method was not developed for longitudinal assessment of keel bone deviations throughout the life of laying hens. Thus, it does not allow for the comparison of the severity of keel bone deviations between different ages. Keel bones of young hens are smaller compared to those of mature hens. Therefore, a certain depth of an indentation may reflect a more severe deviation in the keel bone of a young compared to the keel bone of an adult hen. In contrast, the method presented in the present work (Eusemann et al. 2018a) allows for the comparison

of the severity of keel bone deviations between different ages. As the dimension of the deviation is given as a proportion of the total keel bone area (Eusemann et al. 2018a), it is possible to compare the relative dimension of keel bone deviation between small and large keel bones. This does not only enable the comparison of keel bone deviations between hens of different ages and the longitudinal assessment of keel bone deviations, but also the comparison of keel bone deviations between different layer lines which may also differ in keel bone size.

Furthermore, a method to assess radiographic density of the keel bone in live hens has been presented (Eusemann et al. 2020). This method has been adapted from a method presented by Fleming et al. (2000) and uses an aluminum step-wedge that is radiographed together with each hen and serves for calibration purposes (Eusemann et al. 2020). In contrast to previous studies that applied this method to assess excised keel bones postmortem (Fleming et al. 2004) or to assess long bones in live hens (Fleming et al. 2000), this work is the first to describe the assessment of radiographic density of the keel bone in vivo. Thus, it provides a possibility to observe this characteristic throughout the life of laying hens. Radiographic density can give a first insight into the constitution of a bone and has, in long bones, been shown to predict breaking strength (Fleming et al. 2000).

Taken together, radiography has been shown to be a suitable tool to detect keel bone fractures and deviations, measure the dimension of the latter, i.e., estimating its severity, as well as to compare keel bone radiographic density between hens and time points. It can be used for longitudinal studies and, as the radiographic device is portable, this method can also be applied on-farm.

4.2 Inhibition of Egg Production in Laying Hens in Order to Obtain a Model with Non-Egg Laying Hens

In order to estimate the role of egg production in the etiology of KBD, a model that allowed for comparison of KBD between egg laying and non-egg laying hens was required. To this aim, a sustained release implant containing the GnRH agonist deslorelin acetate was tested on laying hens before and after the onset of lay (Eusemann et al. 2018b). This implant has been developed for chemical castration in male dogs (Ponglowhapan 2011) and has been shown to inhibit reproductive activity in several species, including birds such as Japanese quail (Petritz et al. 2013; Schmidt et al. 2013; Petritz et al. 2015) and pigeons (Cowan et al. 2014). It is available in two strengths: 4.7 mg deslorelin acetate per implant and 9.4 mg deslorelin acetate per implant. In the present work, the inhibitory effect of the sustained release deslorelin acetate implant on egg laying activity in chickens was shown (Eusemann et al. 2018b). One implant containing 4.7 mg deslorelin acetate totally suppressed egg production in all hens that were treated after the onset of lay and protracted the onset of lay in all hens that were treated before (Eusemann et al. 2018b). However, the duration of effectiveness of the implant was comparatively short. In dogs, one deslorelin acetate implant 4.7 mg suppresses reproductive function for six months, i.e., 24 weeks (Ponglowhapan 2011). In contrast, only 14 weeks after implantation, the group of hens that were treated with deslorelin acetate after the onset of lay did not differ significantly from the control group in proportion of hens with follicles anymore (Eusemann et al. 2018b). In two of the treated hens, the implant suppressed egg laying activity for no more than eight weeks (Eusemann et al. 2018b). This is in contrast to a study by Noonan et al. (2012) in which one sustained release implant containing 4.7 mg deslorelin acetate suppressed egg laying activity in chickens for almost 26 weeks. Differences between the studies may be explained by the higher age of the hens when being implanted in the study by Noonan et al. (2012). Also the use of different layer lines may explain the discrepancy. However, information about the layer line used in the study by Noonan et al. (2012) is lacking since it was not published in a peer-reviewed journal but as a scientific abstract. The results

about the relatively short duration of the tested implant (Eusemann et al. 2018b) show that in order to obtain a model with non-egg laying hens, administration of the implant has to be repeated regularly. Based on these results, it was decided to administer a new implant every 90 days, i.e., around every twelve weeks, in the third study of the present work (Eusemann et al. 2020). Also in this study, the deslorelin acetate implant 4.7 mg was very effective in the hens (Eusemann et al. 2020). Only one of the treated hens continued to lay eggs after implantation and was, thus, excluded from the analysis of results. All other hens treated with the implant did not show any ovarian follicles in the ultrasonographic examinations after implantation (Eusemann et al. 2020). This shows that the chosen interval of 90 days after which a new implant was administered was short enough and that the implant suppressed egg production for at least 90 days. Furthermore, as layer lines differed between the studies, i.e., the laying hybrid LSL was used in the second (Eusemann et al. 2018b), whereas the purebred layer lines G11 and WLA were used in the third study (Eusemann et al. 2020), it could be shown that the implant is effective in different layer lines.
Another important but not surprising finding concerning the effect of the slow release deslorelin acetate implant is that estradiol-17β plasma concentration was decreased in treated compared to control hens (Eusemann et al. 2018b; Eusemann et al. 2020). This is consistent with findings in other species such as ferrets (Wagner et al. 2005) and Japanese quail (Petritz et al. 2013). Prolonged administration of the GnRH agonist deslorelin acetate, e.g., by a sustained release formulation as in the present work, leads to a desensitization of the GnRH receptors in the pituitary gland (Belchetz et al. 1978; Rabin and McNeil 1980; Ottinger et al. 2002; Gobello 2007). This leads to a decreased release of the two gonadotropins FSH and LH from the pituitary gland which stimulate, under physiological conditions, the synthesis of estrogens, amongst others (Mans and Taylor 2008). Consequently, decreased estradiol-17β plasma concentrations are found in animals treated with sustained release deslorelin acetate implants. Thus, depending on the hypothesis, researchers working with the presented animal model should consider substituting this hormone as it may have a large influence on the examined trait. In the present work, estradiol-17β was substituted due to the possible influence of this gonadal steroid on the bone (Eusemann et al. 2020). Part of the hens in the study comparing KBD between egg laying and non-egg laying hens received a subcutaneous slow-release implant containing 75 mg estradiol-17β together with the deslorelin implant (Eusemann et al. 2020). Administration of this implant was repeated every 90 days as suggested by the manufacturer. It increased the plasma concentration of estradiol-17β to a concentration which was within the range of the plasma concentration in control hens (Eusemann et al. 2020). Thus, concentration of this hormone was comparable between these two groups. However, plasma concentration varied a lot throughout the experiment in hens that received both implants which was not the case in control hens (Eusemann et al. 2020). This variation was consistent with the repeated administration of the implant: estradiol-17β plasma concentration was highest shortly after administration of a subcutaneous estradiol-17β implant and then steadily decreased until the subsequent administration (Eusemann et al. 2020). Thus, it may be worth to seek for an alternative method to substitute estradiol-17β in hens treated with a sustained release deslorelin acetate implant in order to keep the plasma concentration as constant as possible.

Taken together, a model with non-egg laying hens can be achieved by regular administration (every twelve weeks) of a sustained release deslorelin acetate implant 4.7 mg. This procedure can successfully be applied in different layer lines. Furthermore, the implant can be used to experimentally protract the onset of lay when implanted before. In both cases, researchers should be aware of the decreasing effect on estradiol-17β plasma concentrations caused by the implant, and, based on the hypothesis, decide whether to substitute this hormone in (part of the) treated hens. The model can be used to investigate the relationship between egg production or the early onset of lay and different physiological processes or pathological circumstances. In the present work, the model with non-egg laying hens was used to investigate the role of egg production in the etiology of keel bone fractures and deviations (Eusemann et al. 2020). The model with the protracted onset of lay was not further used in the

present work but seems promising to investigate whether the early onset of lay in commercial laying hens negatively influences keel bone health.

4.3 The Influence of Housing System on Keel Bone Damage

One of the possible factors in the etiology of KBD that were investigated in the current work is the housing system of laying hens (Eusemann et al. 2018a). The prevalence of keel bone fractures and deviations as well as the severity of keel bone deviations were compared in two different housing systems: single cages and a floor housing system, representing an alternative housing system (Eusemann et al. 2018a). It was shown that the housing system has an influence on both prevalence of keel bone fractures and severity of keel bone deviations, although in opposite ways (Eusemann et al. 2018a).
In accordance with findings by other authors (Sherwin et al. 2010; Wilkins et al. 2011; Petrik et al. 2015), a higher prevalence of keel bone fractures was found in floor-housed compared to caged laying hens (Eusemann et al. 2018a). The higher fracture prevalence in non-cage compared to cage systems is usually explained by the higher risk of collisions with housing equipment such as perches and nest boxes as well as with conspecifics in non-cage systems (Sandilands et al. 2009; Wilkins et al. 2011). These findings emphasize the need of means that decrease the risk of collisions in alternative housing systems, especially when keeping in mind that all hens for table egg production are kept in furnished cages or alternative systems since the ban on conventional cages in the EU in 2012 (Appleby 2003). Several possibilities to decrease keel bone fracture prevalence in non-cage systems have been proposed. Ramps between the different tiers and elements of the housing equipment may facilitate the save vertical movement, especially in aviaries, and have been shown to decrease keel bone fracture prevalence (Heerkens et al. 2016a). Furthermore, hens housed in pens that were equipped with perches with a soft cushion showed fewer keel bone fractures compared to hens housed in pens with uncovered metal perches (Stratmann et al. 2015a). Also the arrangement of perches seems to influence the risk of collisions since aerial perches seem to be associated with a higher keel bone fracture prevalence (Wilkins et al. 2011). All these findings should be considered when choosing housing equipment for laying hens. However, as also hens housed in cages showed a remarkably high fracture prevalence of 50 % in the 72nd week of age (Eusemann et al. 2018a), it seems likely that not all keel bone fractures are of traumatic origin. Caged hens were not at a high risk of collisions with housing equipment and, as they were housed in single cages, could not collide with conspecifics at all (Eusemann et al. 2018a). Thus, keel bone fractures in caged hens, and maybe also part of the keel bone fractures in general, are likely to be pathologic fractures caused by bone weakness rather than by traumatic impact. This weakness in caged hens may be a form of disuse osteoporosis (compare chapter 2.2.3). However, these assumptions still have to be investigated into detail, e.g., with histological and chemical analyses of the keel bone.

In contrast, prevalence of keel bone deviations was not affected by housing system and, within some of the tested layer lines, the severity of keel bone deviations was higher in caged compared to floor-housed hens and increased with age in cages but not in the floor housing system (Eusemann et al. 2018a). This is in contrast with Keutgen et al. (1999) who found a higher prevalence of deviations in free-range and deep-litter stocks than in conventional cages. These different findings may be explained by different definitions of keel bone deviations or by the presence of perches in cages in the present work (Eusemann et al. 2018a) but not in the cages tested by Keutgen et al. (1999). It is assumed that the constant pressure on the keel bone caused by the perch, in combination with lack of movement, led to the high and increasing severity of keel bone deviations (Eusemann et al. 2018a). This assumption is supported by findings that in perching laying hens, the peak force is approximately 5 times higher on the keel bone compared to the peak force on a single foot pad (Pickel et al. 2011), indicating that most of the animals' weight is supported by the keel bone. Future studies could investigate

whether providing hens with grids instead of perches decreases the prevalence of deviations by reducing the pressure on the keel bone. Different perch designs and materials may also help to diminish keel bone deviations.

Taken together, the opposite ways of the effect of housing system on keel bone fractures on the one hand and keel bone deviations on the other hand show the importance of distinguishing between these two phenomena which seem to be influenced by different risk factors.

4.4 The Influence of Genetic Background on Keel Bone Damage

As findings about the influence of genetic background on KBD were not consistent throughout studies by different research groups (compare chapter 2.1.4), another aim of the present work was to assess the role of phylogenetic background and selection for high laying performance on the keel bone. To that aim, a model with three white and two brown purebred layer lines was used (Eusemann et al. 2018a). One of the white and one of the brown layer lines have been selected for high productivity (around 320 eggs per year) while the other lines show a moderate laying performance (around 200 eggs per year) (Lieboldt et al. 2015). This model has already been used and described into detail in several studies of the Friedrich-Loeffler-Institut (e.g., Lieboldt et al. 2015; Habig et al. 2017; Dudde et al. 2018). It allows analyzing the effect of phylogenetic background and selection for high laying performance on different traits within one study. Two white layer lines of this model, a low performing and a high performing one, were also used in the study comparing KBD between egg laying and non-egg laying hens (Eusemann et al. 2020).
In accordance with other studies (Wahlström et al. 2001; Hocking et al. 2003; Vits et al. 2005; Habig and Distl 2013; Heerkens et al. 2016a), the present results revealed a relationship between phylogenetic background and keel bone fractures and deviations (Eusemann et al. 2018a). Prevalence of keel bone fractures was higher in brown compared to white layer lines (Eusemann et al. 2018a). This is consistent with findings by Heerkens et al. (2016a). In contrast, prevalence of deviations did not differ significantly between white and brown layer lines while severity of deviations tended to be higher in white layer lines (Eusemann et al. 2018a). This contrasts with findings by Habig and Distl (2013) who found more severe deviations in brown compared to white laying hens. This discrepancy may be explained by different white and brown layer lines that were used in the two studies. The mechanism behind different susceptibility to keel bone fractures and deviations between layer lines with different phylogenetic background is not yet clear. On the one hand, it is possible that layer lines differ in bone structure, composition, and strength. On the other hand, they may differ in behavior such as flight abilities or flightiness (Heerkens et al. 2016a). In order to disentangle these possible influencing factors, Candelotto et al. (2017) assessed the likelihood of getting an experimental keel bone fracture with an impact test apparatus in recently euthanized birds. Hereby, behavioral confounds were minimized. The authors found a reduced likelihood of experimental fractures in the crossbred line Experimental Brown compared to all other lines and a reduced likelihood of experimental fractures in the commercial line Dual Brown compared to the commercial line Dekalb White. This indicates that brown layer lines have stronger keel bones than white layer lines. Thus, it is likely that the higher keel bone fracture prevalence in brown layer lines in the present work (Eusemann et al. 2018a) as well as in the study by Heerkens et al. (2016a) was caused by behavioral differences between the layer lines rather than by differences in bone structure. For example, white layer lines have been found to show higher flight and 3D movement skills compared to brown layer lines (Heerkens et al. 2016a) and to have a higher proportion of safe landings on different perches (Scholz et al. 2014). This may indicate that brown layer lines are more likely to collide with housing equipment (Heerkens et al. 2016a) or to break the keel bone while unsafely landing on a perch.

The layer lines in the study by Candelotto et al. (2017) also differed in laying performance and breeding goals. Number of experimental fractures was much lower in one experimental line

which descended from a dam line that had not been selected for any breeding goal for several years and a sire line that had been bred for dual egg and meat production compared to the lines that had been selected for high productivity (Candelotto et al. 2017). This is consistent with results of the present work (Eusemann et al. 2018a; Eusemann et al. 2020) and with findings by Hocking et al. (2003). In the present work, a higher prevalence of keel bone fractures was found in the high performing compared to the low performing brown layer line (Eusemann et al. 2018a). Although this effect of laying performance was not seen among the white layer lines in the same study (Eusemann et al. 2018a), probability of keel bone fracture was higher in the high performing compared to the low performing white layer line in the third study (Eusemann et al. 2020). The influence of selection for high productivity on prevalence and severity of keel bone deviations was less clear (Eusemann et al. 2018a; Eusemann et al. 2020). One of both low performing white and the low performing brown layer line showed fewer deviations compared to their high performing counterparts (Eusemann et al. 2018a). Similarly, the severity of deviations did not increase in these two layer lines while it increased in both high performing layer lines when kept in cages (Eusemann et al. 2018a). However, the other low performing white layer line was very susceptible to keel bone deviations and also showed an increasing severity of deviations when kept in cages (Eusemann et al. 2018a). In the study by Hocking et al. (2003), radiographic density of the keel bone and tibiotarsus as well as breaking strength of the humerus and tibiotarsus were higher in traditional layer lines with a low laying performance compared to commercial layer lines with a high laying performance. This is consistent with findings by Habig et al. (2017) that bone mineral density and breaking strength of long bones are higher in the low compared to the high performing layer lines when working with the same purebred layer lines model as in the present work. The results of all studies together indicate that selection for high laying performance has led to poor bone health which leads to higher susceptibility of keel bone fractures and deviations. However, this selection has not only led to a higher amount of eggs in general, but also to differences in other traits. For example, high performing layer lines come into lay earlier compared to low performing layer lines (Hocking et al. 2003; Eusemann et al. 2018a; Eusemann et al. 2020). This early onset of lay may additionally influence KBD. The keel bone ossifies comparatively late in life, i.e., at about 35 weeks of age (FAWC 2010), when laying hens have already been laying eggs for several weeks. Thus, ossification may be disturbed by the competing demand for calcium to produce the eggshell, leading to a weak keel bone and this effect may be more pronounced the earlier a hen comes into lay. To examine the possible influence of an early onset of lay on keel bone fractures and deviations, deslorelin acetate implants could be administered to hens before the onset of lay in order to protract the same, as has been presented and discussed (Eusemann et al. 2018b; see also chapter 4.2). This would allow to investigate this possible influencing factor isolated from other factors as hens of the same layer line and age could be used and kept together under identical housing conditions. Furthermore, layer lines with a low and a high laying performance may also differ in behavior. For example, the low performing white layer line was found to show a higher locomotor activity compared to its high performing counterpart before the onset of lay (Eusemann et al. 2020). This increased activity of the pullets may additionally have led to stronger bones in adult laying hens of the low performing layer line.

The differences in KBD between different layer lines indicate that this large animal welfare problem may be alleviated by including bone strength into breeding programs. In accordance with that, several authors have shown that it is possible to decrease fracture prevalence in the keel bone and in long bones by selecting hens for increased bone stability (Bishop et al. 2000; Stratmann et al. 2016).

4.5 The Influence of Age on Keel Bone Damage

In accordance with findings by Heerkens et al. (2016a), prevalence of keel bone fractures increased with age (Eusemann et al. 2018a). This can be explained by deterioration of bone strength with age (Fleming et al. 1998c; EFSA 2015). However, several authors observed that fracture prevalence did not increase until the end of the laying period but peaked at about 50 weeks of age and then leveled off or even decreased (Petrik et al. 2015; Stratmann et al. 2015a; Toscano et al. 2018). This was only partly confirmed in the present work. In the first study, fracture prevalence was higher in the 72nd compared to the 51st week of age (Eusemann et al. 2018a), which does not support findings about a peak of fracture prevalence around the 50th week of age. However, in the third study, most fractures occurred between the 27th and 40th week of age while only comparatively few fractures occurred thereafter (Eusemann et al. 2020). Taken together, it is possible that laying hens are more susceptible to keel bone fractures until the 50th week of age than at higher ages or that those hens that are at a high risk of keel bone fractures usually get a fracture before the 50th week of age while the hens that survive without any fracture until that age are unlikely to break their keel bone thereafter. It seems worth to further investigate the possible mechanisms into detail as well as to find out why different studies come to different results about the exact influence of age on keel bone fractures. Different methods to assess the keel bone may explain part of the discrepancy.
In contrast to other studies (Wahlström et al. 2001; Habig and Distl 2013), prevalence of keel bone deviations did not increase with age (Eusemann et al. 2018a). The severity of deviations increased from the 27th to the 35th week of age in the second study (Eusemann et al. 2018b) but was not influenced by age in the third study (Eusemann et al. 2020). In the first study, severity of deviations only increased in hens of specific layer lines housed in cages (Eusemann et al. 2018a). Thus, the influence of age on keel bone deviations seems to depend on genetic background and on housing system. Possibly, prevalence and severity of deviations only increase in very susceptible layer lines or in hens that are kept in cages and, thus, experience a constant pressure on the keel bone caused by the perch.

4.6 The Influence of Egg Production and Estradiol on Keel Bone Damage

The results of the current work clearly demonstrate an existing relationship between egg production and keel bone fractures while the effect of egg production on keel bone deviations is much less pronounced.
A higher prevalence of keel bone fractures in egg laying compared to non-egg laying hens was found in the second study (Eusemann et al. 2018b). In fact, none of the non-egg laying hens was affected by a fracture while fracture prevalence was up to 40 % in egg laying hens (Eusemann et al. 2018b). In the third study, it was confirmed that egg laying hens were at a much higher risk of keel bone fracture compared to non-egg laying hens (Eusemann et al. 2020). These huge differences and the almost nonexistence of keel bone fractures in non-egg laying hens clearly indicate that there is a fundamental weakness of the keel bone in laying hens caused by egg production which makes it very susceptible to fractures. Davison et al. (2006) reviewed that bone strength is determined by material properties such as degree of mineralization, crystallinity, collagen characteristics, and osteocyte viability and by structural properties such as thickness and porosity of the cortex as well as thickness and connectivity of the trabeculae. It is crucial to find out in which of these characteristics the keel bone of egg-laying hens differed from that of non-egg laying hens in order to find solutions against keel bone fractures. A first hint is the radiographic density of the keel bone which increased more markedly and more steadily and, thus, reached higher values in non-egg laying compared to egg laying hens (Eusemann et al. 2020). This indicates that either the bone mass or the degree of mineralization was lower in aged egg laying compared to aged non-egg laying hens which

may be an underlying cause of keel bone fractures. This is in accordance with Fleming et al. (2004) who suggested that lack of bone mass was the cause of keel bone fractures and deviations.

Results about the relationship between egg production and keel bone deviations were less clear. In the second study, non-egg laying hens of one subgroup showed less severe deviations compared to egg laying hens of the same subgroup (Eusemann et al. 2018b). However, no difference was found in the risk of keel bone deviation or in the severity of deviations between egg laying and non-egg laying hens in the third study (Eusemann et al. 2020). This discrepancy may be explained by the different layer lines that were used in the studies. Differences in the severity of deviations were found when comparing egg laying with non-egg laying LSL hens (Eusemann et al. 2018b) while no differences were found when working with the layer lines WLA and G11 (Eusemann et al. 2020). The values representing the severity of deviations were also higher in LSL compared to WLA and G11 although this was not tested statistically as the layer lines were part of two independent studies. Thus, it is possible that LSL hens are more susceptible to severe keel bone deviations than WLA and G11 and that this makes it easier to detect differences between treatment groups in this laying hybrid. However, differences in the severity of deviations were also analyzed differently in both studies. While all hens were included in the statistical analysis of proportion of deviated keel bone area when comparing egg laying and non-egg laying hens in LSL (Eusemann et al. 2018b), only hens that actually showed a keel bone deviation were included when working with WLA and G11 (Eusemann et al. 2020). This may also explain the different outcome of the studies.

Different theories about the role of estrogens in the etiology of bone diseases in laying hens have been established in the past. On the one hand, it is suggested that the rise in estrogen plasma concentration at the onset of lay stimulates the osteoblasts to form medullary rather than structural bone, leading to depression in structural bone formation and osteoporosis (Whitehead and Fleming 2000). On the other hand, it is argued that estradiol has a positive effect on bone formation and calcium homeostasis and that it is unlikely that an increase in estradiol concentration should lead to osteoporosis in laying hens while a decrease of the same hormone leads to osteoporosis in women (Beck and Hansen 2004). In the present work, non-egg laying hens that were substituted with estradiol-17β showed a plasma concentration of this gonadal steroid which was comparable to that of control hens. However, the risk of keel bone fracture was comparable to or even lower than that of non-egg laying hens without substitution which showed a much lower estradiol-17β plasma concentration (Eusemann et al. 2020). Thus, the higher fracture risk in egg laying compared to non-egg laying hens did not seem to be mediated by estrogens. Within egg laying hens, administration of an estradiol-17ß implant slightly increased the risk of keel bone fracture but did not influence radiographic density of the keel bone (Eusemann et al. 2020). A more marked influence of estradiol on bone health was found by Chen et al. (2014) as well as Urist and Deutsch (1960). The latter found a lower breaking strength, thinner cortex, and large defects within the cortical bone in hens and roosters treated with exogenous estradiol compared to control hens and roosters, respectively (Urist and Deutsch, 1960). Chen et al. (2014) found the same effects in capons treated with exogenous estradiol. The larger effect of exogenous estradiol on the bone found in these studies compared to the present work may be explained by different estradiol concentrations that were administered or by the fact that long bones and not the keel bone were assessed by Urist and Deutsch (1960) as well as Chen et al. (2014) in contrast to the present work.

4.7 Conclusions

The present work has presented new methods to assess the keel bone and the influence of egg production on different traits in laying hens and has contributed to the clarification of the etiology of keel bone fractures and deviations.
The radiographic method presented in chapter 3.1 allows for detecting keel bone fractures and deviations, for measuring the latter, and for clearly differentiating between both forms of KBD. It is suitable for repeated examinations of the same hens in longitudinal studies and can also be applied on-farm. Furthermore, a method to repeatedly assess the radiographic density of the keel bone has been added as described in chapter 3.3. This allows to draw first conclusions about bone composition in live hens as well as to detect changes in radiographic density throughout the life of laying hens.

Furthermore, an animal model with non-egg laying hens as well as a method to experimentally protract the onset of lay have been presented. Both can be obtained by repeatedly administering a sustained release deslorelin acetate implant. These models can be used by other researchers to assess the influence of egg production or the early onset of lay on different traits and disorders in laying hens.

Lastly, this work has helped to further elucidate the etiology of keel bone fractures and deviations. It has been shown that keel bone fractures and deviations should be considered as two separate disorders with a different etiology. They are multifactorial disorders and both external (here: the housing system) as well as internal factors (here: genetic background, age, and egg production) play a role in their etiology. However, concerning keel bone fractures, the largest differences were found when comparing egg laying with non-egg laying hens. This indicates that a fundamental weakness of the keel bone, caused by egg production, underlies the high prevalence of keel bone fractures in commercial laying hens. It seems very important and necessary to find out more about the involved mechanisms, i.e., whether egg production leads to a metabolic imbalance or a decrease in the amount of structural bone or others. Detailed knowledge about these mechanisms is likely to allow for developing strategies to improve keel bone quality and, consequently, reduce fracture prevalence. Including bone strength into breeding programs seems to be a promising way as differences in fracture prevalence have been found between different layer lines and because successful selection for bone strength has been presented by other authors. According to the present findings, improving external factors such as the housing system is important to further decrease keel bone fracture prevalence but unlikely to solve this problem if not combined with other means. In contrast to keel bone fractures, the influence of egg production on keel bone deviations was less pronounced and not consistent throughout the different studies. In this case, the housing system seems to play a more important role. Especially the pressure on the keel bone while perching seems to have a huge impact on the etiology of deviations. Thus, keel bone deviations may be diminished by providing grids instead of perches or by changing the perch design. However, more research is required to give practicable advice.

4.8 Outlook

It was shown that several factors such as housing system, egg production, and genetic background play a major role in the etiology of keel bone fractures and deviations. However, the mechanism behind these relationships has not been investigated in this work. Thus, further studies should aim at analyzing in which characteristics the keel bone differs between treatment groups and layer lines, making it more susceptible to fractures and deviations in some groups compared to others. Adequate methods to assess the structure and composition of the keel bone are histological techniques as well as chemical analyses. Histology of the keel bone would allow to compare the amount of structural, i.e., cortical and trabecular bone as well

as medullary bone between treatment groups or layer lines. Furthermore, the number of active osteoclasts could be assessed. Chemical analyses would give an insight into the composition of the keel bone, i.e., amount of water, organic, and inorganic matter as well as degree of mineralization and bone maturity. Both methods together could reveal whether any metabolic bone disease such as osteoporosis, osteomalacia or osteodystrophia fibrosa underlies KBD. All this information about the constitution of the keel bone in different treatment groups (e.g., egg laying and non-egg laying) as well as layer lines could then help to find solutions to make the keel bone stronger and less susceptible to fractures and deviations.
In addition, the established animal model with non-egg laying hens could be applied to an aviary system. In the current work, the influence of egg production on KBD was only assessed in hens that were kept in a floor housing system where the equipment was placed at a maximum height of 146 cm. Thus, the risk of traumatic fractures was relatively low compared to the risk in aviaries which can reach a height of several meters and where the distance between different furniture elements is larger. It seems worth to investigate whether suppressed egg production also protects the keel bone from fractures in these housing systems where collisions are assumed to have a higher impact than in floor housing systems.

Another aspect of high interest is the possible relationship between the early onset of lay and KBD. Future studies could focus on this aspect using the presented animal model. In case of an existing role of the early onset of lay in the etiology of KBD, this trait could easily be manipulated by means of lighting regime, amongst others, in order to reduce the prevalence of keel bone fractures and deviations in commercial laying hen farms.

5 Summary

The Influence of Egg Production, Genetic Background, Age, and Housing System on Keel Bone Damage in Laying Hens

The keel bone is the prominent ventral part of the sternum in birds where the flight muscles attach. It is fractured or deviated, i.e., deformed, in up to 97 % or 83 % of laying hens within one flock, respectively. Both symptoms are often summarized to the term “keel bone damage” (KBD). Keel bone fractures and possibly also deviations are likely to cause pain and impair the mobility of affected hens. For these reasons, KBD is considered to be one of the most severe animal welfare problems in the egg production industry. The etiology of KBD is not yet fully understood but it is widely defined as a multifactorial disorder. There are external factors such as housing system and nutrition as well as internal factors such as genetic background and age influencing the prevalence of KBD. However, there is no agreement about the direction of these effects and knowledge about the extent to which each of these factors contributes to the etiology of fractures and deviations is lacking. Another internal factor that may influence the keel bone is egg production. There is a high demand of calcium for the eggshell. To meet this demand, female birds possess a special kind of woven bone which is located in the medullary cavity of some bones. It is suggested that once the hen comes into lay, osteoblasts change their function from forming structural bone to forming medullary bone which leads to a decrease in the amount of structural bone and, thus, in bone strength. It is further suggested that these mechanisms are mediated by estrogens. However, the role of egg production and estrogens in KBD has never been investigated into detail. The aim of the present work was to get a better insight into the etiology of KBD. A special focus was put on comparing the external factor housing system with the internal factors genetic background, age, egg production, and estradiol-17β. To that aim, three studies were carried out.

In the first study, a method to assess keel bone fractures and deviations in living hens was established using radiography. Furthermore, hens of five layer strains differing in phylogenetic background (brown versus white layer lines) as well as laying performance (high versus low performing) were kept in two different housing systems (single cages versus floor housing) and repeatedly radiographed. Brown layer lines showed more keel bone fractures while the severity of keel bone deviations tended to be higher in white layer lines. Within the brown layers, the high performing layer line showed more keel bone fractures and deviations compared to the low performing layer line. More fractures were found in the floor housing system whereas keel bone deviations were more severe in cages within some of the layer lines. Fracture prevalence increased with age. The presented radiographic examination of the keel bone allowed to clearly differentiate between fractures and deviations and to assess the severity of the latter. It was further shown to be a suitable and quick method for longitudinal studies on KBD.

The aim of the second study was to establish an animal model with non-egg laying hens which could further be used to assess the influence of egg production on different traits in laying hens. 40 hens were kept in a floor housing system. Ten hens received a sustained release implant containing the gonadotropin releasing hormone (GnRH) agonist deslorelin acetate before and ten hens after the onset of lay. The remaining 20 hens were kept as control hens. The implant inhibited egg laying activity in all hens that were treated after the onset of lay and protracted the onset of lay in all hens that were treated before. However, duration of effectiveness was relatively short and showed that a new implant should be administered after approximately twelve weeks in order to constantly inhibit egg laying activity. Furthermore, estradiol-17β plasma concentration was decreased in treated hens. All hens of this study were also radiographed twice. Egg laying control hens showed significantly more keel bone fractures and more severe keel bone deviations compared to non-egg laying hens within the group that was treated after the onset of lay. Furthermore, severity of keel bone deviations increased with age in this group.

The results of the first two studies were used for the third study whose aim was to assess the influence of egg production and estradiol-17β on KBD. A total of 200 laying hens of two strains differing in laying performance were kept in a floor housing system. Half of each layer line was administered a deslorelin acetate implant every 90 days and, thus, did not lay eggs. Part of these hens as well as of the egg laying hens was further given an implant with estradiol-17β. All hens were repeatedly radiographed and fracture prevalence as well as prevalence and severity of deviations were compared between the four treatment groups and both layer lines. Furthermore, radiographic density of the keel bone was assessed. The risk of keel bone fracture was much lower in non-egg laying compared to egg laying hens while no effect of egg laying activity on keel bone deviations was found. Radiographic density of the keel bone was higher in aged non-egg laying hens compared to aged egg laying hens. Treatment with exogenous estradiol only showed a relatively small effect on keel bone fracture risk within egg laying hens and no effect on deviations or radiographic density. The high performing layer line showed a higher risk of keel bone fracture than the low performing layer line but layer lines did not differ in terms of keel bone deviations.

Taken together, a method to assess keel bone fractures, deviations and radiographic density in a longitudinal study as well as a model with non-egg laying hens have been established and can be used in further studies. Different risk factors have been found for keel bone fractures and deviations indicating that these are two different and independent phenomena and that it is very important to clearly differentiate between them. Both external and internal factors have been found to contribute to the etiology of keel bone fractures and deviations. Part of the keel bone fractures seem to be caused by collisions with housing equipment. However, the very large difference in risk of keel bone fracture between egg laying and non-egg laying hens clearly indicates that there is a fundamental weakness of the keel bone in laying hens caused by egg production which makes it very susceptible to fractures. Findings about the higher prevalence of keel bone fractures in high compared to low performing layer lines support this assumption. It is, thus, necessary to figure out which are the differences in bone structure and composition between egg laying and non-egg laying hens in order to find solutions against this huge animal welfare problem.

6 Zusammenfassung

Der Einfluss von Legetätigkeit, Genetik, Alter und Haltungssystem auf die Entstehung von Brustbeinschäden bei Legehennen

Der Begriff „Brustbeinschäden“ umfasst Frakturen und Deformationen der *Carina sterni*, die bei flugfähigen Vögeln sehr ausgeprägt ist und als Ansatzfläche für die Flugmuskulatur dient. Brustbeinschäden kommen bei Legehennen sehr häufig vor: Bis zu 97 % der Hennen einer Herde können von Frakturen und bis zu 83 % der Hennen von Deformationen betroffen sein. Da Brustbeinfrakturen und möglicherweise auch -deformationen mit hoher Wahrscheinlichkeit schmerzhaft sind und die Bewegungsfähigkeit der betroffenen Tiere beeinträchtigen, werden Brustbeinschäden als eines der größten Tierschutzprobleme in der Legehennenhaltung betrachtet. Die Ursachen von Brustbeinfrakturen und -deformationen sind noch nicht ausreichend geklärt. Es wird davon ausgegangen, dass es sich um ein multifaktorielles Krankheitsbild handelt, auf dessen Entstehung sowohl exogene Faktoren wie das Haltungssystem und die Fütterung als auch endogene Faktoren wie die Genetik und das Alter der Hennen Einfluss haben. Jedoch widersprechen sich die vorhandenen Studien teilweise bezüglich der Richtung der Effekte und auch das Ausmaß, in welchem die einzelnen Faktoren jeweils Einfluss auf die Entstehung von Brustbeinschäden nehmen, ist unbekannt. Ein weiterer endogener Faktor, der eine Rolle in der Entwicklung von Brustbeinschäden spielen könnte, ist die Legetätigkeit. Legehennen haben durch die Eischalenbildung einen sehr hohen Calciumbedarf. Dieser Bedarf wird teilweise durch Bereitstellung von Calcium aus dem Skelett, v.a. dem medullären Knochen, gedeckt. Dies ist ein spezielles geflechtartiges Knochengewebe, das bei weiblichen Vögeln in der Markhöhle einiger Knochen zu finden ist. Es besteht die Theorie, dass die Osteoblasten mit Legebeginn der Henne nur noch medullären und keinen kortikalen sowie trabekulären Knochen mehr bilden, was zu einer Abnahme der Knochenstabilität führt. Es wird weiterhin vermutet, dass diese Mechanismen durch Östrogene gesteuert werden. Jedoch gibt es bis heute keine Studien, die den Einfluss der Legetätigkeit und von Östrogenen auf die Brustbeingesundheit untersucht haben. Das Ziel der vorliegenden Arbeit war es, einen tieferen Einblick in die Ätiologie von Brustbeinschäden zu gewinnen. Hierbei wurden insbesondere der exogene Faktor Haltungssystem und die endogenen Faktoren Genetik, Alter, Legetätigkeit und 17β-Östradiol untersucht. Zu diesem Zweck wurden drei verschiedene Studien durchgeführt.

Das Ziel der ersten Studie war es, eine Röntgenmethode zu entwickeln, die eine Verlaufsuntersuchung von Brustbeinfrakturen und -deformationen sowie eine zuverlässige Unterscheidung dieser beiden Symptome und eine Vermessung von Deformationen erlaubt. Des Weiteren wurden fünf Legelinien und zwei Haltungssysteme miteinander verglichen. Die Legelinien unterschieden sich in ihrer phylogenetischen Herkunft (Braun- und Weißleger) sowie in ihrer Legeleistung (Hoch- und Minderleistung). Von jeder Legelinie wurde jeweils die Hälfte der Tiere in Bodenhaltung bzw. in Einzelkäfigen gehalten. Das Brustbein von allen Tieren wurde regelmäßig geröntgt. Die beiden braunlegenden Legelinien hatten mehr Brustbeinfrakturen als die drei weißlegenden Linien, während die Deformationen bei den Weißlegern tendenziell größer waren. Innerhalb der Braunleger zeigte die Hochleistungslinie mehr Frakturen und Deformationen als die Minderleistungslinie. Die Prävalenz von Frakturen war in der Bodenhaltung höher als in Einzelkäfigen, während innerhalb einiger Legelinien die Deformationen im Käfig größer waren als in der Bodenhaltung. Die Frakturprävalenz nahm mit dem Alter der Hennen zu. Die vorgestellte Röntgenmethode, die auch im Stall eingesetzt werden kann, erwies sich als eine zuverlässige und schnell durchzuführende Methode, um Brustbeinfrakturen und -deformationen wiederholt an denselben Tieren zu diagnostizieren und zu vermessen.

Das Ziel der zweiten Studie war es, ein Tiermodell mit nicht-legenden Hennen zu entwickeln, welches die detaillierte Untersuchung des Einflusses der Legetätigkeit auf unterschiedliche Erkrankungen bei Hennen, u.a. Brustbeinschäden, erlaubt. Hierfür wurde jeweils zehn Hennen

kurz nach sowie zehn Hennen vor Legebeginn ein subkutanes Implantat mit dem Gonadotropin-Releasing-Hormon (GnRH)-Agonisten Deslorelinazetat subkutan appliziert. Jeweils zehn weitere Hennen wurden als Kontrolltiere für beide Gruppen (nach / vor Legebeginn) gehalten. Das Implantat unterband die Legetätigkeit bei allen Hennen, die nach Legebeginn behandelt wurden, und zögerte den Legebeginn bei allen Hennen, die es davor erhalten hatten, hinaus. Die Wirkungsdauer war jedoch relativ kurz und es zeigte sich, dass die Gabe des Implantates im Abstand von ca. zwölf Wochen wiederholt werden sollte, um eine durchgängige Unterdrückung der Legetätigkeit zu erreichen. Des Weiteren zeigte sich ein niedrigerer 17β-Östradiol-Plasmaspiegel bei behandelten im Vergleich zu Kontrolltieren. Die Hennen dieses Versuches wurden ebenfalls zweimal während des Versuchszeitraums geröntgt. Innerhalb der Gruppe, die das Implantat nach Legebeginn erhielt, zeigten die legenden Hennen (Kontrolltiere) signifikant mehr Brustbeinfrakturen und größere Deformationen als die nicht-legenden Hennen. Die Größe der Deformationen nahm in dieser Gruppe insgesamt mit zunehmendem Alter der Hennen zu.

Die Ergebnisse aus diesen beiden Studien wurden für die dritte Studie genutzt, deren Ziel es war, den Einfluss der Legetätigkeit sowie von 17β-Östradiol auf die Entstehung von Brustbeinschäden zu untersuchen. Es wurden jeweils 100 Hennen einer Hochleistungs- und 100 Hennen einer Minderleistungslinie in Bodenhaltung gehalten. Bei jeweils der Hälfte der Tiere beider Legelinien wurde die Legetätigkeit durch wiederholte Gabe eines Deslorelinazetat-Implantates unterbunden. Ein Teil dieser sowie der legenden Hennen bekam zusätzlich ein Implantat mit dem Steroidhormon 17β-Östradiol. Das Auftreten und die Schwere von Brusbeinfrakturen und -deformationen wurden durch wiederholte Röntgenuntersuchungen beurteilt. Des Weiteren wurde die Röntgendichte des Brustbeins ermittelt. Die Wahrscheinlichkeit, innerhalb des Versuchszeitraumes eine Fraktur zu erleiden, war bei den nicht-legenden Hennen sehr viel niedriger als bei den legenden Hennen, während kein Effekt der Legetätigkeit auf Brustbeindeformationen festgestellt werden konnte. Die Röntgendichte des Brustbeins war am Ende des Versuchszeitraums bei den nicht-legenden Hennen höher als bei den legenden. Die Gabe von exogenem Östradiol führte nur innerhalb der legenden Hennen zu einem moderat erhöhten Frakturrisiko und hatte keinen Effekt auf das Auftreten von Brustbeindeformationen sowie auf die Röntgendichte. Die hochleistende Legelinie wies eine höhere Wahrscheinlichkeit für Brustbeinfrakturen auf als die minderleistende Legelinie, während sich die beiden Linien in Bezug auf Deformationen nicht unterschieden.

Zusammenfassend lässt sich sagen, dass in der vorliegenden Arbeit sowohl eine Methode zur Beurteilung der Brustbeingesundheit in Verlaufsstudien als auch ein Tiermodell zur Untersuchung des Zusammenhangs der Legetätigkeit sowie des frühen Legebeginns und verschiedenen Erkrankungen bei Legehennen erfolgreich etabliert und präsentiert wurden. Des Weiteren wurden unterschiedliche Risikofaktoren für das Auftreten von Brustbeinfrakturen einerseits und -deformationen andererseits gefunden. Dies zeigt, dass es sich hierbei um zwei voneinander unabhängige Symptome zu handeln scheint, weshalb eine klare Abgrenzung zwischen den beiden immens wichtig ist. Es wurde gezeigt, dass sowohl der untersuchte exogene Faktor als auch die untersuchten endogenen Faktoren einen Einfluss auf die Entstehung von Brustbeinschäden haben. Ein Teil der Frakturen scheint durch Kollisionen mit Einrichtungsgegenständen zu entstehen. Der enorme Unterschied zwischen legenden und nicht-legenden Hennen in Bezug auf die Wahrscheinlichkeit, eine Fraktur zu erleiden, zeigt jedoch sehr deutlich, dass eine durch die Legetätigkeit verursachte Schwäche des Brustbeins besteht, die diesen Knochen sehr anfällig für Frakturen macht. Der festgestellte Unterschied zwischen Hoch- und Minderleistungslinien untermauert diese Schlussfolgerung. Es erscheint daher notwendig, das Brustbein in weiteren Studien genauer zu untersuchen, um Unterschiede in der Knochenstruktur und -zusammensetzung zwischen legenden und nicht-legenden Hennen zu finden, welche die unterschiedliche Frakturanfälligkeit bedingen. Basierend auf diesen Ergebnissen könnten neue Ansatzpunkte zur Vermeidung dieses gravierenden Tierschutzproblems entwickelt werden.

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8 List of Own Publications

8.1 Research Papers in Peer-Reviewed Scientific Journals

Eusemann BK, Baulain U, Schrader L, Thöne-Reineke C, Patt A, Petow S (2018) Radiographic examination of keel bone damage in living laying hens of different strains kept in two housing systems. PLOS ONE, 13(5), e0194974.

Eusemann BK, Sharifi AR, Patt A, Reinhard A-K, Schrader L, Thöne-Reineke C, Petow S (2018) Influence of a sustained release deslorelin acetate implant on reproductive physiology and associated traits in laying hens. Frontiers in Physiology, 9:1846, doi: 10.3389/fphys.2018.01846.

Eusemann BK, Patt A, Schrader L, Weigend S, Thöne-Reineke C, Petow S (2020) The role of egg production in the etiology of keel bone damage in laying hens. Frontiers in Veterinary Science, 7:81, doi: 10.3389/fvets.2020.00081.

8.2 Oral Presentations

Eusemann B, Schrader L, Petow S: Brustbeinschäden bei Legehennen - Welchen Einfluss haben die Eiproduktion, Estradiol und UV-B-Bestrahlung? Graduiertenkurs der Gesellschaft für Tierzuchtwissenschaften „Methodik der Nutztierethologie“ an der Universität Hohenheim, September 7-12 2015, Eningen unter Achalm, Germany.

Eusemann BK, Schrader L, Petow S: Keel bone damage in layers – How do egg production, estrogens and UV irradiation influence the health of the skeleton? FLI Junior Scientist Symposium, September 21-23 2015, Greifswald – Isle of Riems, Germany.

Eusemann BK, Schrader L, Petow S: Brustbeinschäden bei Legehennen - Welchen Einfluss haben die Eiproduktion und Estradiol auf die Knochengesundheit? 47. Internationale Tagung Angewandte Ethologie, November 19-21 2015, Freiburg i. Br., Germany.

Eusemann BK, Baulain U, Schrader L, Petow S: Radiographic examination of deformities and fractures of keel bones in laying hens. 16th International Conference on Production Diseases in Farm Animals, June 20-23 2016, Wageningen, The Netherlands.

Eusemann BK, Baulain U, Schrader L, Petow S: Radiographic examination of deformities and fractures of keel bones in laying hens. FLI Junior Scientist Symposium, September 21-23 2016, Jena, Germany.

Eusemann BK, Weigend S, Schrader L, Petow S: Radiographic examination of keel bones in laying hens. COST KeelBoneDamage WG/CG meeting, March 21-22 2017, Ljubljana, Slovenia.

Eusemann BK, Reinhard A-K, Schrader L, Sharifi AR, Petow S: The influence of deslorelin acetate on physiology in laying hens. FLI Junior Scientist Symposium, September 20-22 2017, Brunswick, Germany.

Eusemann BK, Patt A, Rodríguez Navarro A, Schrader L, Petow S: Differences in (keel) bone characteristics and 17β estradiol between hens treated with deslorelin actetate and control hens. COST KeelBoneDamage MC and Research Coordination Meetings, January 25-26 2018, Bratislava, Slovakia.

8.3 Poster Presentations

Eusemann BK, Schrader L, Thöne-Reineke C, Petow S, Ursachen und Entwicklung von Brustbeinschäden bei Legehennen. Lange Nacht der Wissenschaften an der Freien Universität Berlin, July 24 2017, Berlin, Germany.

Eusemann BK, Schrader L, Thöne-Reineke C, Petow S, Ursachen und Entwicklung von Brustbeinschäden bei Legehennen. Tag der offenen Tür im Bundesministerium für Ernährung und Landwirtschaft, August 25-26 2017, Berlin, Germany.

Eusemann BK, Petow S: Cranial stumps of premature oviducts redeveloped into fully differentiated oviducts in laying hens. 51st Annual Conference of Physiology and Pathology of Reproduction and 43rd Mutual Conference of Veterinary and Human Reproductive Medicine, February 21-23 2018, Hannover, Germany.

Eusemann BK, Petow S, Sanchez-Rodriguez E, Benavides-Reyes C, Dominguez-Gasca N, Gonzalez-Lopez S, Rodriguez-Navarro AB: Influence of egg production on tibiae bone properties of laying hens. 45th European Calcified Tissue Society Congress, May 26-29 2018, Valencia, Spain.

Eusemann BK, Reinhard A-K, Schrader L, Petow S: Better foot health and fewer keel bone deviations in laying hens displaying a reduced laying rate after treatment with deslorelin acetate. UFAW Animal Welfare Conference, June 28 2018, Centre for Life, Newcastle, UK.

Eusemann BK, Rodriguez-Navarro AB, Sanchez-Rodriguez E, Patt A, Benavides-Reyes C, Dominguez-Gasca N, Schrader L, Petow S: The influence of egg production on keel and long bone quality in laying hens. 15th European Poultry Conference, September 17-21 2018, Dubrovnik, Croatia.

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Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

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