Universitat Autònoma de Barcelona

EFFECT OF MANAGEMENT AND NUTRITIONAL STRATEGIES ON SKELETAL DEVELOPMENT AND BEHAVIOR OF BROILER BREEDERS

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Ana Cristina Barroeta Lajusticia, catedràtica del Departament de Ciència Animal i dels Aliments de la Universitat Autònoma de Barcelona,

Certifica:

Que la memòria titulada "**Effect of management and nutritional strategies on skeletal development and behavior of broiler breeders**", presentada per Xavier Asensio Dávila amb la finalitat d'optar al grau de Doctor en Veterinària, ha estat realitzada sota la seva direcció i, considerant-la acabada, autoritza la seva presentació perquè sigui jutjada per la comissió corresponent.

I perquè consti als efectes oportuns, signa la present a Bellaterra, 30 de maig de 2019.

Dra. Ana Cristina Barroeta Lajusticia

La present memòria de tesi ha estat realitzada gràcies al pla de formació que Aviagen SAU em va proposar com a millora dels meus coneixements dintre del camp de les aus reproductores pesades: el seu maneig, fisiologia, alimentació i comportament.

A mi padre

Agraïments

Durant la meva vida professional he obert etapes i les he tancat, tant dintre del camp de la formació com a les empreses on he treballat. He tingut clars els objectius a cada una d'aquestes etapes i m'he esforçat per aconseguir-los; però això no ha sigut obstacle per veure quan una fase de la meva vida professional estava esgotada i calia buscar nous reptes que m'ajudessin a millorar els meus coneixements i per tant a ser un millor veterinari. D'altre banda, al llarg del temps també he hagut de deixar passar projectes que no es podien compaginar amb la meva vida personal i professional. Aquests projectes van quedar enrere poc a poc i es van anar difuminant. Tots menys un, un que sempre va perdurar, que pensava que ja era irrealitzable però que no desapareixia de la meva memòria perquè tenia un component que li donava força: la il·lusió. Estic parlar de la realització d'una tesi doctoral, d'aprofundir dintre del pensament científic i de culminar així la meva formació.

Des de el primer moment vaig tenir clar que compaginar la meva feina amb la realització de la tesi no seria fàcil, i ara que escric les últimes línies crec que no era conscient de l'esforç que em demanaria: no me'n penedeixo. Hi ha dues persones especials que han estat amb mi al llarg dels quatre anys en els que he realitzat la tesi. La primera és la meva dona, Maria, que m'ha recolzat sempre, ha sabut estar al meu costat quan era necessari i també agafar distància quan el meu humor ho requeria. Està clar que la intel·ligència emocional és una de les seves moltes virtuts. Aquest treball hauria sigut irrealitzable sense la seva comprensió, per tant en part també és seu. La segona persona és Ana Barroeta. He tingut molts professors al llarg de la meva formació, però crec que en el cas d'Ana la docència circula per les seves venes. En la meva opinió Ana valora els seus alumnes de forma molt amplia i això li permet veure més enllà dintre de les seves aptituds. Sempre ha estat positiva i a cada problema m'ha ajudat a trobar una solució, això m'ha servit com a flotador en moltes ocasions.

De vegades m'he imaginat la ment humana com una habitació amb finestres; hi ha gent que té les finestres tancades, altres a mitges i uns pocs que les tenen obertes de bat a bat. A l'habitació mental de Jesús Piedrafita totes les finestres estan obertes i corre un aire fresc de primavera. Veia l'estadística com una paret davant meu des del principi, però el dia que vaig entrar al despatx de Jesús vaig tenir clar que la saltaria. Hi va haver moments que podia escoltar els engranatges de la meva ment i estic segur que Jesús també els escoltava, però em va ajudar a refrescar i entendre els conceptes necessaris per fer l'anàlisi estadística.

Menció especial voldria fer de Pauline Finlay, la meva professora d'anglès. Ha llegit i corregit varies vegades les diferents versions de tots els apartats de la tesi; i crec que actualment, amb el seu coneixement del vocabulari avícola, podria treballar com a traductora simultània a congressos d'avicultura.

Al llarg del temps que ha durat la realització de la tesi he tingut contacte amb gent que m´ha ajudat i orientat. Me'n recordo especialment de Nedra Abdelli, que va col·laborar amb mi durant la realització del primer i segon experiment; de Luís Guerrero, que va tenir paciència infinita quan vam testar diverses vegades la millor manera de calcular la resistència del tendó del gastrocnemi; de Jorge Martínez, que va realitzar l'estudi histològic; de Anna Bassols, que em va orientar sobre els marcadors serològics de formació òssia; de Roser Sala, que va formar part de forma desinteressada de l'operatiu de xoc " presa de mostres 22 setmanes"; i de Marc Navarro, que em va ensenyar com fer una dissecció correcta del tendó del gastrocnemi; per cert, després de veure'l treballar amb les tisores i el bisturí i de veure com separava músculs, tendons .. vaig tenir del tot clar que mai una màquina podrà substituir l'ésser humà.

En l'àmbit de l'empresa he d'agrair l'acceptació del projecte per part de Sergi Illan. Parlant de la formació dintre de l'empresa, em va aconsellar que pensés com em volia veure professionalment passats uns anys i que llavors triés una formació en conseqüència. És d'agrair per part seva la disposició a la millora dels professionals de l´equip d 'Aviagen SAU. Així mateix, i també dintre de l'àmbit de l'empresa, me'n recordo d 'Álvaro Puente, jubilat en aquests moments, però que va estar al meu costat i em va ajudar amb la part de nutrició. I no em puc oblidar de Víctor Ferrando, crec que podria donar una classe magistral de com disseccionar el tendó del múscul gastrocnemi, en va fer molts i molts amb una precisió increïble.

Per la prova de camp em feia falta una granja de recria i col·laboradors dintre de la mateixa que fossin de confiança. Li vaig proposar a Javier Polo, propietari d'Avícola Sichar. Durant les 30 setmanes que va durar la prova em va ajudar en tot moment, i em va facilitar el personal necessari per les feines que vam realitzar. La seva filla ,Claudia,

em va tenir informat en tot moment i va mostrar un actitud de col·laboració que també agraeixo.

El segon experiment el vaig realitzar a Granja Solé. Enric Solé i Elvira Cunillera van preparar la nau i van supervisar la feina diària. Van fer un treball magnífic, les feines setmanals programades i les feines puntuals de presa de mostres es van realitzar sense cap problema; això em va permetre compaginar la meva feina amb el seguiment de la prova.

Resum

El bon maneig de les reproductores pesades durant les fases de recria i posta és essencial per obtenir produccions correctes, que finalment s`han de traduir en un nombre de pollets per au allotjada que correspongui amb el potencial genètic de les aus. En els últims anys s'ha fet inclús més important el bon maneig durant la fase de creixement de les polletes reproductores, en especial per aconseguir un bon desenvolupament esquelètic i evitar problemes locomotors; i així mateix, per criar les aus sota criteris de benestar, lliures d'estrès, ja que tenir les aus confortables a les instal·lacions avícoles és la base sobre la que aconseguir bones produccions. Per tot això, l'objectiu global d'aquesta tesi va ser investigar l'efecte de diferents estratègies de maneig i nutricionals sobre el desenvolupament esquelètic i el comportament de les aus reproductores pesades.

Addicionalment, també s'ha estudiat la utilitat de marcadors serològics, fosfatasa alcalina i osteocalcina, per avaluar de forma directa el desenvolupament esquelètic, i s'ha proposat una puntuació de la integritat de la ploma de les ales i de la cua com a sistema per avaluar el confort de les aus durant la fase de recria.

En el primer experiment, prova de camp, es va estudiar el maneig de la uniformitat de les aus reproductores pesades i el seu impacte en el desenvolupament esquelètic. Es va observar que les polletes amb un pes inferior a l'estàndard a les 5 setmanes d'edat tenien, una vegada finalitzat el seu desenvolupament esquelètic, tíbies més curtes, menys resistents i més dúctils, així com a tendons més dèbils. Aquestes aus van recuperar pes corporal en producció però no van recuperar mida d'esquelet ni van obtenir tendons amb estructura capaç de suportar tensió. Per una altra banda, per obtenir lots uniformes i evitar mortalitat durant la recria , es va comprovar la necessitat de seleccionar i agrupar únicament les polletes més lleugeres, mentre la resta d'aus podrien romandre juntes.

En el segon experiment, prova experimental, es va estudiar l'efecte de la densitat de la dieta i de la inclusió de vitamina C sobre la uniformitat, la mortalitat, les característiques de la carcassa, la fortalesa esquelètica i el comportament de les aus reproductores pesades. Es va observar que dietes diluïdes amb matèries primes fibroses

no afectaven ni a la uniformitat ni a la mortalitat. D'altra banda, aquesta estratègia nutricional va permetre a les aus acumular més greix abdominal i menys pit, la qual cosa és un gran avantatge per obtenir bones produccions. No obstant això, la deposició mineral òssia i la fortalesa esquelètica van ser més pobres. Així mateix, les dietes diluïdes van reduir els comportaments estereotípics, com el picatge de les plomes, i per tant van millorar la integritat de les plomes de les ales i de la cua. La inclusió de vitamina C en la dieta no va afectar a la fortalesa esquelètica ni va reduir els comportaments estereotípics; però va millorar la integritat de l'emplomament de les ales i de la cua.

Finalment, es va demostrar que els marcadors serològics poden ser una via directa per valorar el desenvolupament esquelètic i la deposició mineral òssia; i la puntuació de la integritat de les plomes de la cua pot ser un test pràctic per valorar el maneig correcte i el confort de les polletes reproductores pesades a les granges de recria.

Resumen

El buen manejo de las reproductoras pesadas durante las fases de recría y prepuesta es esencial para obtener producciones correctas, que finalmente se tienen que traducir en un número de pollitos por ave alojada que se corresponda con el potencial genético de las aves. Los últimos años se ha hecho incluso más importante el buen manejo durante la fase de crecimiento de las pollitas reproductoras, en especial para conseguir un buen desarrollo esquelético y evitar problemas locomotores; y así mismo, para criar las aves bajo criterios de bienestar, libres de estrés, ya que tener las aves confortables en las instalaciones avícolas debería ser la base sobre la que obtener buenas producciones. Por todo ello, el objetivo global de esta tesis fue investigar el efecto de diferentes estrategias de manejo y nutricionales sobre el desarrollo esquelético y el comportamiento de las aves reproductoras pesadas.

Adicionalmente, también se ha estudiado la utilidad de marcadores serológicos, fosfatasa alcalina y osteocalcina, para evaluar de forma directa el desarrollo esquelético, y se ha propuesto una puntuación de la integridad de las plumas de las alas y de la cola como un sistema para evaluar el confort de las aves durante la fase de recría.

En el primer experimento, prueba de campo, se estudió el manejo de la uniformidad de las aves reproductoras pesadas y su impacto en el desarrollo esquelético. Se observó que las pollitas con un peso inferior al standard a las 5 semanas de edad tenían, una vez finalizado su desarrollo esquelético, tibias más cortas, menos resistentes y más dúctiles, así como tendones más débiles. Estas aves recuperaron peso corporal en producción pero no recuperaron tamaño de esqueleto ni obtuvieron tendones con una estructura capaz de soportar tensión. Por otro lado, para obtener lotes uniformes y evitar mortalidad durante la recría, se comprobó la necesidad de seleccionar y agrupar únicamente las pollitas más ligeras, mientras el resto de aves podrían permanecer juntas.

En el segundo experimento, prueba experimental, se estudió el efecto de la densidad de la dieta y de la inclusión de vitamina C sobre la uniformidad, la mortalidad, las características de la carcasa, la fortaleza esquelética y el comportamiento de las aves reproductoras pesadas. Se observó que dietas diluidas con materias primas fibrosas no afectaron ni la uniformidad ni a la mortalidad. Por otro lado, esta estrategia nutricional

permitió a las aves acumular más grasa abdominal y menos pechuga, lo cual es una gran ventaja para obtener buenas producciones. Sin embargo, la deposición mineral ósea y la fortaleza esquelética fueron más pobres. Así mismo, las dietas diluidas redujeron los comportamientos estereotípicos, como el picoteo de las plumas, y por lo tanto mejoraron la integridad de las plumas de las alas y de la cola. La inclusión de vitamina C en la dieta no afectó la fortaleza esquelética ni redujo los comportamientos estereotípicos; sin embargo, mejoró la integridad del emplumamiento de las alas y de la cola.

Finalmente, se demostró que los marcadores serológicos pueden ser una vía directa para valorar el desarrollo esquelético y la deposición mineral ósea; y la puntuación de la integridad de las plumas de la cola puede ser un test práctico para valorar el correcto manejo y el confort de las pollitas reproductoras pesadas en las granjas de recría.

Summary

Good management of broiler breeders during the rearing and pre-breeder phases is essential to obtain correct production, which results in a number of chicks per hen housed according to the genetic potential of the birds. In recent years it is even more important to have good management during the growth of the pullets, especially to achieve correct skeletal development in order to avoid leg health issues; likewise, to rear the birds following welfare criteria, free of stress, since keeping birds comfortable in the poultry facilities should be the basis to achieve good production. For all this, the objective of this thesis was to investigate the effect of different management and nutritional strategies on skeletal development and behavior of broiler breeder pullets.

Additionally, the usefulness of serological markers, alkaline phosphatase and osteocalcin, was also studied as a way to evaluate directly skeletal development; and a feather score was proposed to evaluate wing and tail feather integrity, as a system to assess the comfort of the pullets during rearing.

In the first experiment, field trial, weight uniformity management of broiler breeders and its impact on skeletal development was studied. It was observed that the pullets with body weight under the standard at 5 wk had, once their skeletal development was completed, shorter, less resistant and more ductile tibias, as well as weaker tendons. These birds recovered body weight in production but they neither recovered their skeletal frame nor obtained tendons with a structure able to endure strain. On the other hand, to obtain uniform flocks and avoid mortality during rearing, the need of grading and grouping only the lightest pullets was proved, the rest could be kept together.

In the second experiment, experimental trial, the effect of diet density and vitamin C inclusion on uniformity, mortality, carcass traits, skeletal strength and behavior of broiler breeder pullets was studied. It was observed that diluted diets with fibrous raw materials did not affect both uniformity and mortality. On the other hand, this nutritional strategy allowed the birds to accumulate more abdominal fat and less breast meat, which is a great advantage to obtain good production. However, bone mineral deposition and skeletal strength were poorer. Likewise, diluted diets reduced stereotypic

behavior, such as feather pecking, and therefore they improved wing and tail feather integrity. Vitamin C inclusion in the diet neither affected skeletal strength not reduced stereotypic behavior; however, it improved wing and tail feather integrity.

Finally, it was demonstrated that serological markers can be a way to directly evaluate skeletal development and bone mineral deposition; and the score to evaluate tail feather integrity can be a practical test to assess correct management and comfort of the broiler breeder pullets in the rearing farms.

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Abbreviations

ADF	acid detergent fiber
ALP	alkaline phosphatase
AME	apparent metabolizable energy
AMEn	apparent metabolizable energy corrected to zero nitrogen retained
BMU	bone metabolism unit
BS	breaking strength
BW	body weight
CF	crude fiber
CP	crude protein
CV	coefficient of variation
DM	dry matter
EM	elastic modulus
FI	feed intake
GFP	grasping feather pecking
GPS	grand parent stock
GTT	gastrocnemius tendon thickness
GTW	gastrocnemius tendon width
H/L	heterophil to lymphocyte ratio
NDF	neutral detergent fiber
NFOP	non-food object pecking
OC	osteocalcin
PCC	plasma corticosterone concentration
RGT	rupture of the gastrocnemius tendon
TL	tibia length
TW	tibia width
WBCs	white blood cell frequencies

CHAPTER 1

Literature review

1.1. Genetic selection

Poultry companies of chicken meat production and also specialized in breeding began in the late 1940s. Since that time the improvement in chicken biological efficiency has been constant, and the modern fast growing broiler is a successful result of years of high-level genetic selection. Comparisons between selected and unselected or heritage lines provide estimates of yearly improvements over the last 50 years of around 50 g of body weight (**BW**), feed conversion rate improvements of 15 to 25 g feed/kg of BW, and around 0.2% increase in breast meat yield (Havenstein et al., 2003a, b; Fleming et al., 2007a, b; Mussini, 2012; Zuidhof et al., 2014). **Table 1.1** shows a live performance comparison between modern lines (2005) and their corresponding 1972 control lines (maintained as discrete populations, randomly selected to uphold their characteristics of the 1972 lines). During the last decades there have been significant improvements in live performance without increasing mortality.

Table 1.1. Broiler production traits progress (adapted from Fleming et al., 2007a, b). Modern (2005 – Ross 308) vs Control (1972 – Ross 308 random bred).

Genotype	Body weight g 42d	2.0 kg FCR	Mortality % 0-42d	2.0 kg Carcass Yield % live	2.0 kg Breast Yield % live
Modern	2665	1.650	4.15	68.00	17.40
Control	1210	2.230	5.00	65.30	11.05
Yearly change	44.1	-0.018	-0.026	0.082	0.192

There have been concerns over the sustainability of genetic improvement due to the possible undesired consequences of genetic selection in terms of musculoskeletal health and reproductive fitness (Dawkins and Layton, 2012; Hocking, 2014). To obtain sustainable genetics it is necessary to implement broad breeding goals including biological efficiency, environmental adaptability, reproductive fitness, welfare and product quality (Neeteson et al., 2013). This balanced breeding strategy demands the assessment and handling of antagonistic genetic correlations between trait groups in the breeding target, for example between biological performance and leg health (Kapell et al., 2012). As a consequence of this balanced breeding strategy, both experimental and industry data have shown sustained improvements in broiler leg health, liveability and product quality (Fleming et al., 2007a, b; National Chicken Council, 2016).

However, in addition to genetic selection, good management is necessary in the rearing farms to avoid leg health and welfare issues. Nowadays, broiler breeder flocks not well managed may have pullets under the advised standard BW (birds underweight); in fact, larger or aggressive pullets likely out-compete smaller or timid pullets, resulting in unequal access to feed and increasing flock BW variation (Zuidhof et al., 2015). Unequal access to feed and thus low uniformity may result in broiler breeder pullets not having received the nutrients required, and thus lead to leg health issues if their skeleton is not developed properly.

1.2. Broiler breeder objectives

At the present time, to obtain good technical and economic broiler breeder results it is necessary to meet different objectives during rearing and production periods. **Table 1.2** shows an example of several data objectives that have to be obtained together throughout growing and laying. These data objectives can vary depending on the broiler breeder strain, but currently are quite similar.

Table 1.2. Broiler breeder performance objectives (0-64 wk of age) (Adapted from Ross 308 Parent Stock Performance Objectives. Aviagen, 2016).

Rearing mortality %	Production mortality %	Hatching eggs	Hatchability %	Chicks/hen housed	Feed/100 chicks (kg)
4-5	8.0	175	84.8	148	37.7

To attain these technical targets, breeder companies have to focus mainly on the rearing period (from 0 to 25 wk of age). An adequate body condition is key at the end of this period, avoiding under-fleshed (thin) or over-fleshed (fat) subjects, in order to take advantage of the full genetic potential of the birds.

1.2.1. Broiler breeder growth rate and body condition

Broiler breeder pullets have to be feed intake (**FI**) controlled in order to achieve a correct growth rate, since it is critical to avoid excessive BW in rearing. At the same time, nutrient supply must be adequate for skeletal development and fleshing (breast condition), and to meet thresholds of BW and fat necessary for the onset of sexual maturity and persistent egg production (Lewis et al., 2007; Pishnamazi et al., 2014). For these reasons, and according to the advice of the genetic companies, at 25 wk of age (onset of laying), the body condition of the broiler breeder females should be:

- BW close to 3.0 kg.
- Optimal breast meat yield of 22% (taking into account pectoralis major and minor).
- Abdominal fat pad of 2%.

Table 1.3 shows an example of a broiler breeder female BW profile during rearing and early production, as well as daily feed allocation (g) and Kcal consumed to attain correct weekly growth.

Table 1.3. Broiler breeder female body weight profile, daily feed allocation and energy intake per bird. (Adapted from Ross 308 Parent Stock Performance Objectives. Aviagen, 2016).

Week	Body weight profile (g)	Daily feed allocation (g/bird)	Daily energy intake (kcal/bird)
1	125	26	73
2	240	33	92
3	360	38	105
4	480	41	115
5	600	45	125
6	740	51	133
7	870	54	140
8	990	56	147
9	1100	59	154
10	1200	62	162
11	1300	66	172
12	1400	70	183
13	1505	75	194
14	1610	79	206
15	1715	83	217
16	1825	87	235
17	1945	93	250
18	2070	99	267
19	2200	106	285
20	2340	112	303
21	2495	119	320
22	2655	126	341
23	2810	130	364
24	2955	134	375
25	3093	138	386
26	3223	148	414

1.2.2. Broiler breeder development and skeletal growth

During the rearing period of the broiler breeder pullets, particular attention has to be taken to ensure that birds end up with a correct skeletal frame size. In fact, pullets have to complete different development phases throughout the 25 wk that growing lasts; phases which are displayed and segmented in **Figure 1.1**. The most significant to be highlighted are:

- Feather coverage (0-4 wk).
- Skeletal frame development (90% completed at 13 wk).
- Accelerated growth and weight gain to prepare the hens for laying (16-25 wk).
- Rapid reproductive organs growth (21-25 wk).

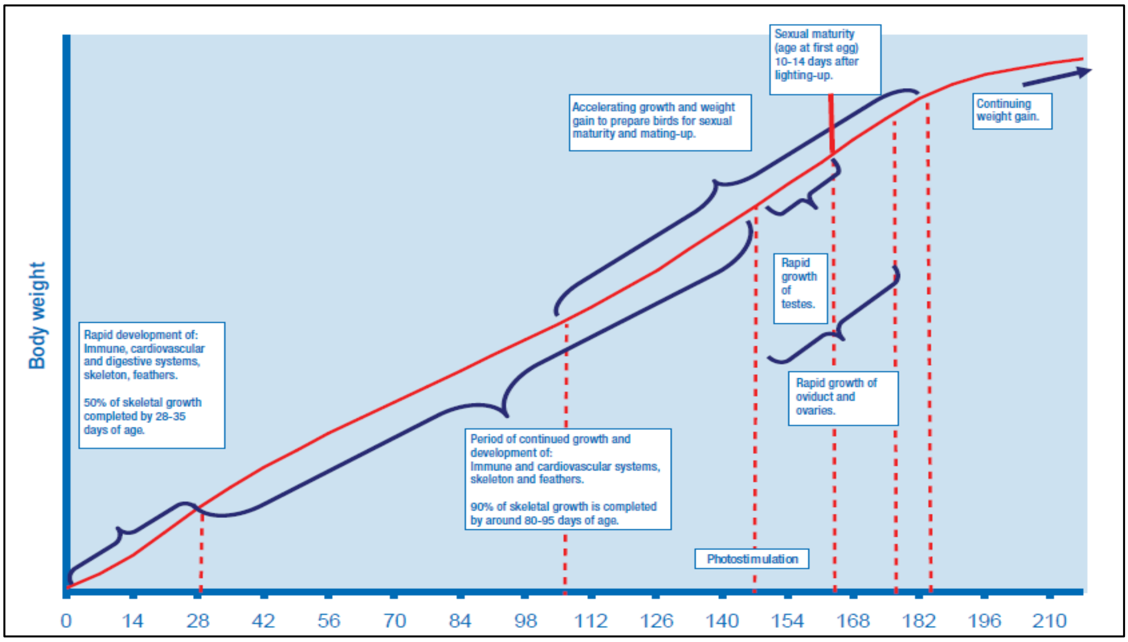

Figure 1.1. Phases of the female broiler breeder development.
(Adapted from Ross Parent Stock Management Handbook. Aviagen, 2013).

To ensure that birds have the correct skeletal frame size at the end of rearing, attention must be paid to the development of its components throughout this period. Tendons and bones are skeletal components, and together with the muscles, provide physical support for the body and determine its shape.

Tendon and bone specific functions, their structural features and composition, are explained below. The objective is to understand well the importance of their correct development.

1.2.2.1. Tendons. Function and composition

Tendons are anatomic structures situated between the bones and the muscles. Their function is to transmit the force which has been created by the muscle to the bone and therefore, to allow movement. From a basic point of view, muscles have a proximal and a distal tendon, which join the muscle by a myotendinous junction and the bone by an osteotendinous junction (kannus, 2000). Healthy tendons should be a brilliant white color and fibro-elastic texture, and be able to cope with high mechanical loads. Related to their shape features, they can vary from wide to flat, cylindrical, fan-shaped and ribbon-shaped. Muscles that have to create powerful and resistive forces (e.g. quadriceps or triceps) have short and broad tendons, while those which are involved in subtle and delicate movement (e.g. finger flexors) have long and thin tendons (Kannus, 2000). Tendons consist of collagen (mostly type I collagen) and elastin embedded in a proteoglycan-water matrix; with collagen accounting for 65-80% and elastin approximately 1-2% of the dry mass of the tendon (Curwin, 1997; Hess et al., 1989; Jozsa et al., 1989; Kirkendall and Garrett, 1997; O'Brien, 1997; Tipton et al., 1975).

1.2.2.2. Bones. Function, composition and turnover

Bones are also anatomical structures, and among their functions are protection of the vital organs, body support and Ca storage (necessary for egg shell formation). There are two types of bones, intramembranous and endochondral. Intramembranous bones are flat and for example placed in the skull. Endochondral bones are short and long, they are named cartilaginous.

In both types, during their formation, mesenchymal cells migrate to the site of eventual bone formation. In the case of intramembranous bones, ossification involves the replacement of sheet-like connective tissue membranes with bony tissue. If they are endochondral bones, ossification involves mesenchymal cells becoming chondrocytes, which proliferate and stack into a very dense mass of cells (proliferating zone), devoid of blood vessels, and form cartilage in the shape of forming bone (Pines, 2007); later on chondrocytes grow in volume before degenerating and calcifying (hypertrophic zone) (**Figure 1.2**). Bone composition is approximately 70% mineral, 20% organic matrix and 10% water. The mineral part is composed of hydroxyapatite crystals and the organic matrix is composed mainly of collagenous proteins (type I collagen) and also of non-collagenous proteins (osteocalcin, 1% of bone organic matrix). As explained before, collagen is the major organic matrix component and confers tensile strength to the bone, whereas hydroxyapatite provides compressional strength (Rath et al., 2000).

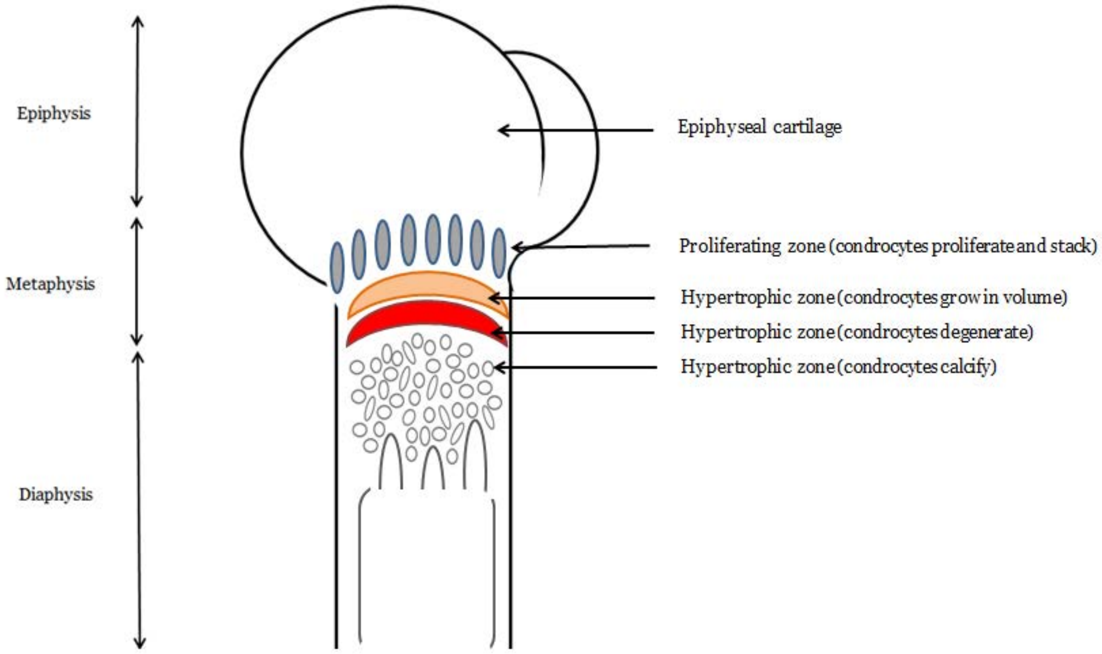

Figure 1.2. Growth plate of endochondral bones (between epiphysis and diaphysis) (Asensio, 2018).

After mature frame size is attained, bone development continues in order to repair fractures and for remodeling to meet different challenges (Pines, 2007). Bone is a metabolically active tissue which is continuously remodeling in two normally balanced processes, bone formation and bone resorption; this process is named bone turnover. In these two processes are involved osteoclast as responsible for resorption and osteoblast as responsible for formation. Under normal conditions, bone resorption and formation are tightly coupled to each other, so that the amount of bone removed is always equal to the amount of newly formed bone (Seibel, 2005).

As is displayed in **Figure 1.3** bone remodeling always begins in the quiescent phase (1). After activation is initiated, osteoclasts are attracted to a new bone metabolism unit (**BMU**) site (2); where they erode the bone matrix forming a lacunae; in this phase collagen breakdown products are released into the blood stream, such as hydroxyproline, collagen cross-links or telopeptides (Seibel, 2005). In a process requiring about 10 d, osteoclasts normally resorb bone until the lacunae is approximately 100 µm in diameter and 50µm deep (3); resorption is then halted and osteoblasts are recruited to the BMU site, reversal phase (4). Osteoblasts begin at the bottom of lacunae and lay down the organic matrix, composed mainly of type I collagen and other non-collagenous proteins such as osteocalcin. Likewise, alkaline phosphatase, as one product of osteoblast activity, is released (5). When the lacunae is filled with organic matrix, a process requiring about 80 d, this newly formed matrix is mineralized with hydroxyapatite, giving the BMU tensile strength (6). The remodeled area then passes into the quiescent phase to complete the 60- to 120- day bone cycle (Christenson, 1997).

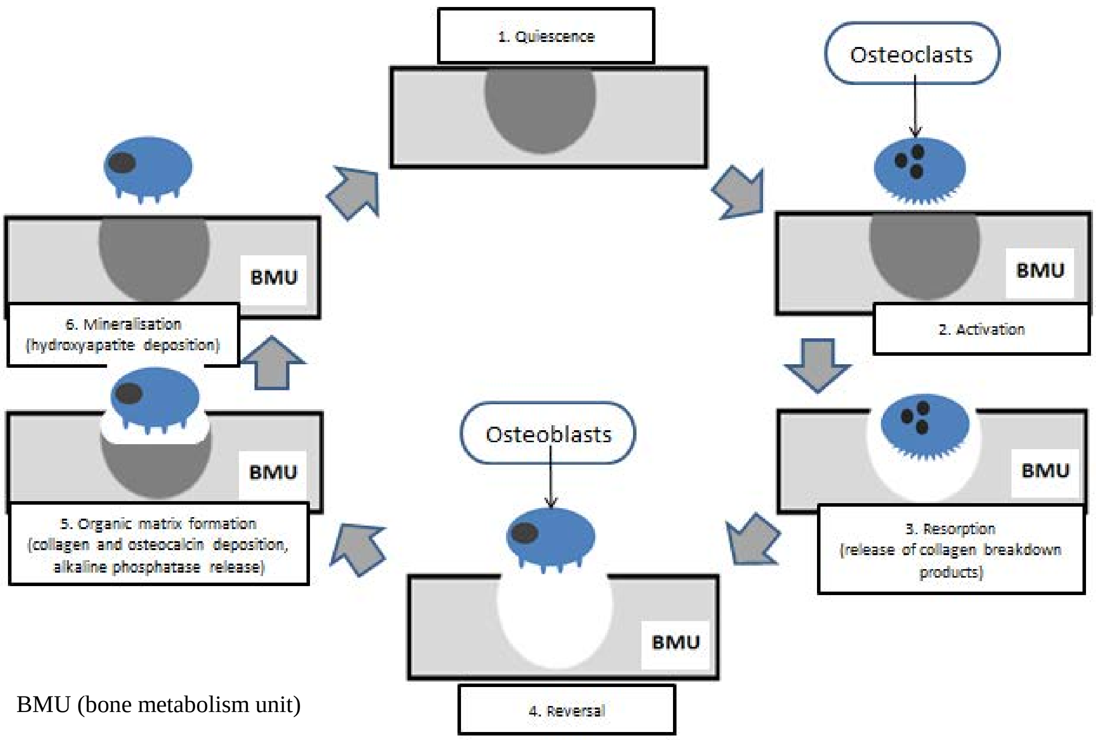

Figure 1.3. Bone remodeling cycle.
(Adapted from Christenson, 1997).

In contrast, somatic growth, ageing, metabolic bone diseases, states of increased or decreased mobility, are characterized by more pronounced imbalances in bone turnover. The results of such uncoupling in bone turnover are often changes in bone structure, strength and mass (Seibel, 2005). To detect the dynamics of the metabolic imbalance, molecular serological markers of bone metabolism are helpful (Lian and Stein, 1999; Rizzoli and Bonjour, 1999).

1.2.2.2.1. Serological markers of bone formation

Nowadays, the population of older people is increasing in many countries, and as a consequence of this fact there are more incidences of bone disorders and imbalance of bone mineral metabolism. At the same time, cellular and extracellular components of the skeletal matrix have been isolated, and thus, it has been possible to develop molecular markers of bone resorption and formation. Therefore, serological markers are now an important tool in the assessment and differential diagnosis of metabolic bone diseases in humans. However, these markers have never been used in broiler breeder pullets; where they can also be useful to evaluate bone resorption and formation and hence, metabolic bone disorders.

Available markers are normally classified as serological markers of bone resorption or bone formation, depending on the metabolic process.

Bone resorption serological markers are usually related to collagen breakdown products such as hydroxyproline or the various collagen cross-links and telopeptides. Hydroxyproline is formed intracellularly from the post-translational hydroxylation of proline that constitutes 12-14% of the total amino acid content of mature collagen. Collagen cross-links, which are formed during the extracellular maturation of fibrillary collagens, bridge several collagen peptides and stabilize mechanically the collagen molecule; and telopeptides are derived from specific regions of the collagen type I molecule (Seibel, 2005).

On the other hand, serological markers of bone formation are either by-products of collagen neosynthesis (e.g. propeptides of type I collagen), or osteoblast-related proteins such as osteocalcin (**OC**) and alkaline phosphatase (**ALP**) (Seibel, 2005). These two markers, OC and ALP, can be used as important tools for the assessment and monitoring of bone metabolism; their composition, origin and function as serological markers are explained below.

OC is a small hydroxyapatite-binding protein of 5.8 kDa, produced by osteoblasts during the organic matrix deposition (**Figure 1.3**, phase 5); in fact, it is the most abundant non-collagenous protein of the bone matrix (Power and Fottrell, 1991). Its synthesis is dependent on vitamin K, which posttranslationally modifies the gene product with gamma-carboxyglutamate (Gla) residues; due to this modification OC is also known as bone Gla protein (Power and Fottrell, 1991; Price, 1987).

OC exact function is unknown at present (Power and Fottrell, 1991; Price, 1987), although it is thought that during bone formation, the newly OC synthetized protein is either deposited in the bone organic matrix (mainly) or released into the blood stream and later excreted in urine due to its low molecular weight (Price et al., 1981; Delmas et al., 1983a, b). Likewise, as a component of bone organic matrix, during bone resorption, OC is either degraded or also released into the blood circulation (a majority, up to 70%). Therefore, since OC present in the blood stream can come from new synthesis during bone formation or released during resorption, there is some controversy about if OC should be considered a marker of osteoblast bone formation activity (Powel and Fottrell, 1991) or an indicator of bone matrix metabolism or turnover (Kleerekoper and Edelson, 1996). However, when formation and resorption are uncoupled, for instance during somatic growth or ageing (Seibel, 2005), OC is considered a marker of osteoblast activity (Brown et al., 1984; Charles et al., 1985; Bataille et al., 1987), and therefore of bone formation.

Several different tests have been developed to analyze OC, and among them, immunoassay test is frequently used in humans.

ALP is a ubiquitous, membrane-bound tetrameric enzyme attached to glycosyl-phosphatidylinositol moeities located on the outer cell surface (Stinson and Hamilton, 1994). Mechanistically, the enzyme may be clipped off the membrane and released into circulation. The precise function of the enzyme is yet unknown, but it obviously plays an important role in the organic matrix formation and mineralization (Harris, 1990); in fact, it is a product derived from osteoblast activity (**Figure 1.3**, phase 5).

Four ALP isoenzymes are commonly present in circulation (Christenson et al., 1996), and are relatively specific for the tissues with which they are associated: liver, bone, placental, and intestinal tissues. However, total ALP has been normally used for monitoring bone disease.

Because of the large molecular size of ALP, all analyses have been based on serum. A method of choice is a colorimetric test to assess ALP activity based on a catalytic transformation of p-nitrophenylphosphate as substrate. To be more precise, ALP activity is determined by measuring the rate of conversion of p-nitro-phenylphosphate (pNPP) in the presence of 2-amino-2-methyl-1-propanol (AMP) at pH 10.4 (**Figure 1.4**). The rate of change in absorbance due to the formation of pNP is measured bichromatically at 410/480 nm and is directly proportional to the ALP activity in the sample.

$$\text{pNPP} + \text{AMP} \xrightarrow[\text{Mg}^{2+}]{\text{ALP}} \text{pNP} + \text{AMP-PO}_4$$

Figure 1.4. Catalytic transformation of p-nitrophenylphosphate as substrate (IFCC method).

This method is used in the diagnosis of hepatobiliary disorders and bone disease associated with increased osteoblastic bone formation activity. Therefore, due to the high osteoblastic activity during growth, this method, in absence of liver pathology, can be also useful to assess bone formation during skeletal development.

1.3. Broiler breeder nutrition

To obtain correct broiler breeder skeletal growth and body condition in order to maximize reproductive potential and chick quality, is an essential objective of broiler breeder nutrition. To feed breeders correctly, means to follow BW profiles and to maintain good flock uniformity throughout rearing and production periods. To achieve these targets it is necessary to combine feed formulation and feeding management.

Nowadays, there are several nutritional specifications which can be consulted to formulate broiler breeder diets; as for example, those of FEDNA norms (2008) or those of Kleyn (2013b). In the case of FEDNA norms, values are based on the recommendations of NRC (1994), Mateos and Piquer (1994), Daghir (1995), Leeson and Summers (2005) and Rostagno et al. (2005); likewise, feed specifications handbooks from genetic companies were taken into account (Ross, 2005, 2007b; Cobb, 2006b and others).

Although the primary breeding companies give guideline feed specifications, in practice, what is recommended is to adjust specifications up or downwards accordingly depending on the farm manager's view of performance (Kleyn, 2013a). Likewise, environmental conditions, such as temperature or humidity, have also to be taken into account since they influence bird requirements.

1.3.1. Apparent metabolizable energy

In broiler breeders, energy is the driver of feed allocation. The prediction of energy requirements in birds is expressed as apparent metabolizable energy (**AME**), since faeces and urine are excreted together. AME values can also be expressed nitrogen corrected: apparent metabolizable energy corrected to zero nitrogen retention (**AMEn**); for this it is necessary to determine the quantity of nitrogen retained as protein tissue or excreted as uric acid. Feed energy for poultry requirements and energy content of raw materials, are expressed in most of the nutritional specification tables as AMEn (FEDNA norms, 2008); although usually the term ME is used to describe AMEn.

Energy contents of successive feeds should not vary widely. Hence, feed changes should be gradual and carefully controlled; especially when changing diets (e.g. transition from grower to breeder rations). Within a given diet, consistency in nutrient density and quality is critical.

As a result of modern broiler strains being selected for lean tissue deposition (Renema et al., 2007), they have reduced ability to store fat. However, if the abdominal fat pad is too small, there will be a drop in egg number. Therefore, it has to be ensured that pullets receive adequate energy allocation without supplying it with too much protein (Kleyn, 2013a).

The total energy required for a hen is the addition of the energy requirement for maintenance, growth and production of egg mass. The maintenance energy requirement is by far the largest component of the total energy needed. Maintenance energy requirement is based on the BW of the birds and is significantly affected by environmental conditions; and therefore, total energy requirement will vary with environmental temperatures, location and season.

1.3.2. Crude protein

Poultry species do not have specific requirements of crude protein (**CP**), but amino acids requirements. However, and as a security measure, a minimum and a maximum of CP are included in the nutritional specification tables. The minimum reduces the possibility that the essential amino acids, not taken into account in formulation, limits productivity; and the maximum helps to control environmental contamination and to reduce the incidence of wet litter and dirty eggs (FEDNA norms, 2008).

Variation of feed protein content should be minimized. Low protein levels during rearing were found to have a negative impact on ovary development and hence cause a drop in lifetime egg production. The opposite, too much protein during rearing, causes over fleshing (increase breast meat deposition) and excessive follicular development (Renema et al., 2007).

In poultry, amino acids that usually limit production are lysine, methionine and cysteine and followed closely by threonine. Lysine requirements have been determined based on scientific publications and practical experience. To predict and calculate the rest of the essential amino acids, the concept of ideal protein has been used, lysine always being the amino acid of reference (FEDNA norms, 2008). Likewise, the same ME: lysine ratio should be maintained and, as was said before, the other amino acids adjusted according to the ideal protein profile (Kleyn, 2013a).

The digestible amino acids are based on true faecal digestibility. Formulating diets on digestible amino acid provides a better balanced protein in feed, which better meets bird requirements.

1.3.3. Crude fiber

Crude fiber (**CF**) requirements and its effect on digestive physiology, intestinal health and poultry productivity are not well documented. It is widely thought that poultry feed has to include the minimum possible level of CF and it is accepted that its inclusion reduces palatability and digestibility of the poultry feed (FEDNA norms, 2008). On the other hand, there are recent studies which show that the acceptable level is higher than estimated until now; and that in any case, it depends on the type of fiber considered (González-Alvarado et al., 2007). In fact, different authors have indicated that when broilers are fed with diets including low levels of fiber, gastrointestinal tract development can be penalized and mainly the gizzard, provoking a decrease in nutrient digestibility and feed efficiency (Mateos et al., 2012). Likewise, diets including insoluble fiber sources can increase secretion of HCL, bile acids, and endogenous enzymes (Rogel et al., 1987; Svihus, 2011) which in turn would have an important role in the digestive processes.

Related to welfare, some studies have reported that the dilution of the ration with fiber can improve satiety by measuring subsequent motivation to feed in birds (Robert et al., 1997; Savory and Lariviere, 2000). In several studies, raw materials which are a source of fiber have been used to dilute the energy of the diets, such as oat hulls (Zuidhof et al., 1995; Sandilands et al., 2005, 2006; Hocking et al., 2004; Hocking, 2006; Enting et al., 2007a, b; Nielsen et al., 2011), sugar beet or potato pulp (Savory et al., 1996; Hocking et al., 2004; Enting et al., 2007a, b; Nielsen et al., 2011) and sawdust (Savory et al., 1996). However, these compounds were primarily designed to increase gastric satiety through delaying crop and gizzard emptying.

1.3.3.1. The role of fiber in the digestion

The role that fiber plays in the digestion process can be explained by following the different segments or parts of the gastrointestinal tract.

In the proximal part we find the crop, which is an esophageal diverticulum used by poultry to store feed. As explained before, if fibrous ingredients are consumed the particles are retained for a long time in this organ (Vergara et al., 1989), thus provoking a sensation of satiety which inhibits FI (Richardson, 1970).

These particles, after being released from the crop, reach the next organ, which is called proventriculus and is in fact a glandular stomach where pepsinogen and HCL are secreted. Next, the feed particles mixed with the proventriculus secretions arrive in the muscular stomach that is named the gizzard, the site of mechanical grinding in a low pH environment. There are several published studies that demonstrate the beneficial effects of fiber on gizzard function, mostly due to mechanical stimulation (Hetland et al., 2005; Hetland and Svihus, 2007).

Once feed particle size is reduced by the gizzard, the digesta passes to the small intestine where there are intensive gastroduodenal refluxes of the digesta to compensate for its small dimensions and fast rate transit (Sklan et al., 1978; Duke, 1986). Fiber inclusion in the diet is thought to improve the number and intensity of these refluxes (Hetland et al., 2003). Throughout the small intestine nutrients are digested and absorbed. However, poultry gastrointestinal tract does not produce the necessary enzymes to digest fiber and thus, some of the water soluble particles including different fiber fractions enter into the caecum by antiperistaltic movements. In this organ a large bacterial community breaks down indigestible plant material; in fact, these microorganisms ferment part of the fiber obtaining short chain fatty acids that reduce caecum pH and can be used as a preferential source of energy by the colonocytes and so by the bird.

1.3.3.2. Soluble and insoluble fiber

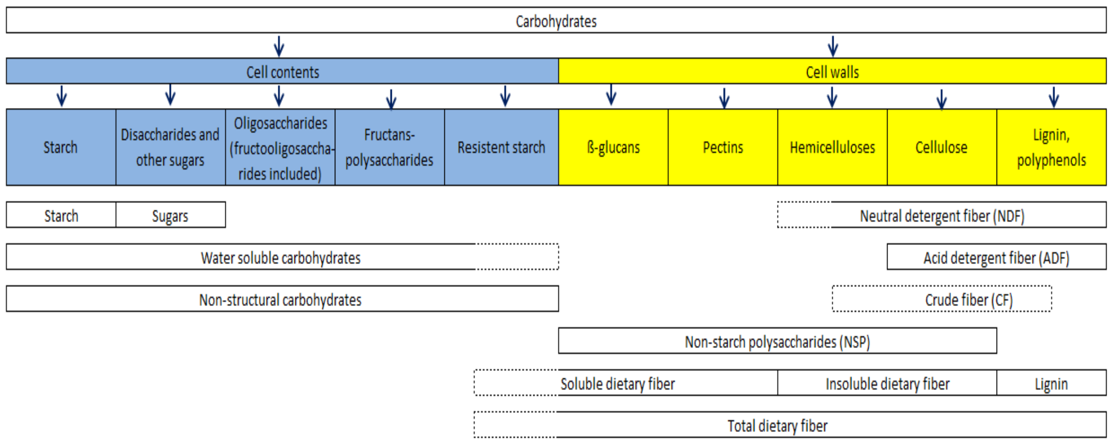

Figure 1.5. Classification of plant origin carbohydrates.
(Adapted from NRC, 2012).

The type of fiber is a major factor influencing the response of poultry to its inclusion in their diets. **Figure 1.5** shows the classification of plant origin carbohydrates, as well as soluble and insoluble dietary fiber, and lignin contents. This classification into soluble and insoluble provides important nutritional information because their effects on the digestion process and intestinal health are different (Bach Knudsen, 2001; Mertens, 2003).

Soluble fiber sources have a high content in pectins, and thus a high water holding capacity that might lead to an increase in the bulk and viscosity of the digesta (Mosenthin et al., 2001; Noblet and Le Goff, 2001), slowing transit time along the gastrointestinal tract due to the suppression of the intestinal contractions (Cherbut et al., 1990); and as a result, the mixing of the dietary components with digestive enzymes reduces, and likewise nutrient digestibility and utilization (McNab and Smithard, 1992; Johnston et al., 2003).

Insoluble fiber and lignin, coming for example from cereal straw and sunflower hulls, can provoke a shorter transit time of the digesta in the distal part of the gastrointestinal tract. As a result of this reduction, time available for enzymes to degrade nutrients of feed is lower, and so digestion process could be less effective (Morel et al., 2006). Otherwise, the high lignin content of most insoluble fiber sources leads to a longer retention of the feed in the gizzard, improving its muscular development and thus its function (Hetland and Svihus, 2001; Hetland et al., 2003; Jiménez-Moreno et al., 2010). Raw materials as a source of insoluble fiber and lignin are mainly used to dilute rations for broiler breeder pullets during the rearing period.

Apart from the type of fiber; in poultry diets, the particle size of the fiber also has to be taken into account. In fact, Sacranie et al. (2012) studied broiler chickens exposed to basal diets diluted with coarse hulls or the same hulls finely ground. The large particle size of the coarse hulls and their hardness as a result of the insoluble fiber content, explained why birds consuming the coarse hull diet developed the heaviest gizzards. The coarse hull particles are retained in the gizzard until they are ground to a certain critical size that allows them to pass through the pyloric sphincter (Clemens et al., 1975; Moore, 1999; Hetland et al., 2002, 2003). This leads to an increase in the volume of the organ’s contents and muscular adaptation to meet the greater demand for grinding. Birds fed the fine hull diet also exhibited heavier gizzards than those of the control group, although less so than those of the coarse hull group, suggesting that the fine particles were retained and partly induced the same response. They concluded that broiler chickens raised on diets diluted with hulls exhibit improved performance due to improved gizzard function and holding capacity.

1.3.4. Minerals

The macro minerals Ca and P are critical for proper skeletal development, reproductive performance, shell quality and other metabolic functions. They are primary inorganic nutrients because they form 95% of the mineral bone matrices, although there are several other inorganic elements present in the bone (Rath et al., 2000).

Ca deficiency it is not a common problem in poultry, although there can be problems of malabsorption that can impair intestinal Ca absorption (Perry et al., 1991). Other factors which can interfere with Ca absorption are high levels of cellulose fibers and phytate in the diet. Related to skeletal integrity, it has to be taken into account that Ca homeostasis is an important driving force in the maintenance of bone strength; likewise, an imbalance in P metabolism can affect skeletal structure, integrity and strength (Rath et al., 2000).

Nowadays, the use of phytases in poultry feed is widespread; it is a question of taking advantage of the phytic phosphorus of the raw materials as a source of P and to reduce the use of phosphates with the consequent benefit for the environment.

As a result of the complex interaction among Ca, P, vitamin D, and other calcitropic hormones, it is necessary to balance correctly the amount of Ca and P added in the poultry diet.

Levels of sodium, chloride, and potassium above required levels will increase water intake and reduce litter quality, having therefore a higher risk of foot pad dermatitis in rearing broiler breeders.

1.3.5. Vitamins

Vitamins, as organic catalysts present in small quantities in the majority of foods, are critical for the normal functioning of metabolic and physiological processes such as growth, reproduction and health. Poultry requirements can vary depending on genotypes, levels of yield and different production systems; likewise, under stressful conditions such as disease outbreaks or high environmental temperatures, birds can show a positive response to higher levels of certain vitamins.

Vitamin supplementation in poultry feed is nowadays essential, and their inclusion recommendations are suggested by various international scientific organizations such as

NRC, ARC and INRA. More recently, FEDNA norms (2008) and DSM OVN (2012) have also recommended inclusion of vitamin levels. Nowadays, requirements depending on type of production are not well known for most of the vitamins. In fact, vitamin premix composition is based on scientific studies carried out on levels of inclusion that prevent the appearance of classic deficiency symptoms (FEDNA norms, 2008). Likewise, premix includes safety margins to avoid also the appearance of subclinical problems in standard management conditions (Mateos et al., 2004). However, there is no agreement among authors about the optimum poultry premix composition (Ward, 1993; Villamide and Fraga, 1999; Allard, 2005).

1.3.5.1. Vitamin C

Regarding vitamin C (L-Ascorbic acid) in particular, there is even more controversy in the literature in relation to the beneficial effects of its supplementation in poultry feeds. In fact, nutritional tables in general do not recommend levels, as for example ARC (1975), INRA (1984), NRC (1994), Rostagno (2005), Leeson and Summers (2005) and FEDNA norms (2008). Only Whitehead et al. (1993) and DSM OVN (2012), recommend 200 mg and 100-150 mg respectively (DSM OVN only under heat stress conditions).

Anyway, if adding vitamin C in the premix, it has to be taken into account that its retention after pelleting at 71-75^{o}C is 60% and at 86-90^{o}C is 45%. If Ascorbyl phosphate is used, as another source of vitamin C, then its retention at 71-75^{o}C is 96% and at 86-90^{o}C is 93% (Coelho, 2002). This fact has to be considered when feeding broiler breeder pullets, since pellet presentation is the most common.

There are three main vitamin C functions related to skeletal development (collagen and bone formation) that have to be highlighted:

- Vitamin C is essential for the normal proliferation and differentiation of chondrocytes and also for the initial formation of bone and later remodelling (Whitehead and Keller, 2003). In fact, the role of vitamin C in chondrocytes development has been investigated in *in vitro* studies; which have shown that vitamin C is an essential component in the medium for cells to differentiate, as indicated by induction of alkaline phosphatase activity and production of type X collagen, in either monolayer (Leboy et al., 1989) or high density culture (Farquharson and Whitehead, 1995). Therefore, if there is a lack of vitamin C, cells are not able to secrete a normal connective tissue or produce other growth or regulatory factors, and this inhibits further cell development.

- Vitamin C is a necessary cofactor in the hydroxylation reactions that modify amino acids being incorporated into the collagen chains (e.g. conversion of proline and lysine into hydroxyproline and hydroxylysine respectively). These modifications allow the chains to form into the triple helical structure of procollagen. Therefore, a lack of vitamin C results in widespread connective tissue abnormalities, characteristic of scurvy, and with its origin coming from a disruption in collagen synthesis (Whitehead and Keller, 2003). In fact, since vitamin C is especially important in the formation of collagen, its presence increases the capacity for the healing of wounds (Rajkhowa et al., 1996). Therefore, vitamin C has an important role in the biosynthesis of collagen, which is an important component of the connective tissues; and hence of the cartilage and bone.
- Vitamin C dietary supplementation in poultry species stimulates cytochrome P-450 complex, of which 25-hydroxyvitamin D-1-hydroxylase is part (Takahashi et al., 1991). This can explain the enhancement of 1,25-dihydroxyvitamin D (1,25-D) production in vitamin C-supplemented chicks (Weiser et al., 1988). The importance of vitamin D and its metabolites in calcium metabolism provides a mechanism by which vitamin C can influence bone development.

Regarding dietary vitamin C supplementation in poultry, some studies have been carried out to investigate its influence in bone development. Below are summarized the results of these studies:

- In Franchini et al. (1994) study, broilers were fed up to 7 wk on a diet supplemented with 0, 250, 500 or 1000 mg vitamin C/kg. There were no statistically significant effects of vitamin C on bone dimensions, weights, ash content or bending stress at either 21 or 49 d, though bone Ca/P ratios were increased at 21 d and decreased at 49 d as dietary vitamin C increased.

- In Fleming et al. (1998a) study, vitamin C was tested in relation to bone growth during the rearing period of layer pullets, and dietary supplementation with 250 mg vitamin C/kg has not shown any effect in bone formation or structure up to 15 wk of age.
- Related to the importance of vitamin C to avoid skeletal abnormalities, Doan (2000) broiler study showed that later fed diets containing 8.5 or 11 g Ca in combination with 0 or 150 mg vitamin C/kg, had the lowest mortality and incidence of crooked legs and highest bone ash content with the diet containing 11 gr Ca and 150 gr vitamin C/kg and the highest mortality and incidence of crooked legs on the diet with lower Ca content and not supplemented with vitamin C. These results can suggest a complementary effect between dietary vitamin C and Ca concentrations.
- In McCormack et al. (2001) study, broilers were fed on a diet supplemented with 500 or 1000 mg vitamin C/kg and it was not found to have any effect on bone dimensions or composition at 42 d and no consistent effects on structure, as assessed by cortical porosity.

The previous studies show controversial results on bone development when feed is vitamin C supplemented. This could be because vitamin C is not essential in poultry since they have enzyme gulonolactone oxidase that is part of its biosynthetic pathway; an enzyme which is lacking for example in humans. On the other hand, it has to be pointed out that endogenous synthesis may not be adequate to meet always the full poultry necessities, since its requirements could increase under certain circumstances such as stressful conditions. In fact, vitamin C might be an essential nutrient in poultry when birds are subject to stress (Pardue and Thaxton, 1986).

Related to the importance of vitamin C in poultry in stressful situations, it has to be noted that some major physiological systems are involved in stress responses, which have as a result some biochemical changes, which affect corticosterone blood levels as well. Vitamin C is closely associated with corticosterone production; in fact, vitamin C is found in high concentration in the adrenal, where stress induces its depletion and this is associated with corticosterone release. Maintenance of high adrenal concentration of vitamin C by dietary supplementation has been found to limit the rise in circulating corticosterone concentrations in chickens under stress (Pardue et al., 1985). In the absence of stress, supplemental vitamin C has little effect on circulating corticosterone concentrations (Pardue et al., 1985).

Psychological stressors like fear can activate physiological responses to stress. Thus, plasma corticosterone concentration rose 5-fold in broilers stressed by catching, blood sampling and cooping for 10 minutes, but this rise was unaffected by prior provision of water containing 1 g vitamin C/kg for 24h. However, vitamin C treatment decreased non-specific, underlying fearfulness of birds in open field and tonic immobility tests (Satterlee et al., 1994).

Similar responses have been observed in quails. Thus, fear induced in quail by catching and cooping is able to elevate plasma corticosterone concentrations (Satterlee et al., 1993). Dietary treatment with vitamin C failed to affect corticosterone concentrations, but attenuated tonic immobility fear reactions of stressed and unstressed quail. Pretreatment with drinking water supplemented with vitamin C also reduced the fearfulness of quail, though it was not necessary to give treatment for more than 24h prior to the frightening event (Jones et al., 1996). Lines of quail have been selected for low and high adrenocortical response to stress. Both lines showed reduced fearfulness after vitamin C supplementation in water (Jones et al., 1999).

As a summary, there are three key points related to the role of vitamin C in poultry production that have to be noted:

- Several author studies show that vitamin C has important functions in skeletal development.
- Birds are able to synthesize vitamin C, but some studies show that under stressful conditions their endogenous synthesis can be insufficient.
- Studies show that vitamin C might help birds to cope with stressful situations (welfare involvement).

In relation with the points presented before, it has to be highlighted that modern broiler breeder pullets are FI controlled during the rearing period, when their skeletal development takes place. Therefore, if management is not correct and the pullets have not an equal access to feed and water, they could suffer from stress in a period when they are developing their skeletal frame. Therefore, it might be that because of this stressful situation, pullets are not able to produce the necessary vitamin C at a moment when their requirements are important to support skeletal development. Thus, it seems necessary to further study the possible benefits of vitamin C feed supplementation in broiler breeder pullets to support skeletal growth and to reduce stress issues.

1.4. Broiler breeder feeding program

The most widely used feed program in rearing and pre-breeder periods, consists of a Starter 1 (up to 21 d), a Starter 2 (22 to 35 d), a Grower (36 to 105 d) and a pre-breeder (106 d to 5% production), which would be followed by breeder layer feeds.

Starter 1 feed contains the highest protein and amino acid specifications, since in this period it has to support the rapid development of the immune, cardiovascular and digestive systems; likewise, skeleton and feathers. In fact, to achieve successful breeder performance it is essential to attain proper early growth and physiological development; for this reason, during the first and sometimes the second wk of age broiler breeder pullets are not FI controlled.

Starter 2 function is to smooth the transition from starter 1 to grower feed; therefore its protein and amino acid specifications are between both of them. This feed is intended to carry on supporting initial rapid development but at the same time to avoid early overfleshing.

A grower feed will follow immediately after the starters. During changes from Starter to Grower feed, BW should be carefully monitored to be sure that the correct growth rate is followed and bird requirements are met. In fact, in the growing period, daily growth rates are low and nutrient requirements, when expressed as daily intakes, are small. Therefore, a grower feed will generally contain lower crude protein and amino acid specifications than the starter diets so as to control body-gain.

A pre-breeder diet is necessary for the transition from growing period to sexual maturity. Its main features should be a higher amino acid and protein content compared to the grower diet, to enable their higher intake in order to achieve the correct development of the reproductive tissue. Related to minerals, in the pre-breeder period, a higher level of Ca has to be provided, so as to increase its deposition in the bone marrow and thus to support shell quality late in production.

1.4.1. Feed form presentation and feed quality controls

As a rule, fine crumbles or micro pellets should always be used to produce Stater 1; these are two feed presentations that, because of their small size, chicks are physically able to consume the first days. Likewise, during this phase mash presentation has to be avoided; since during this period pullets are able to select particles, which would mean that they do not consume a balanced diet.

From this moment, to feed Starter 2, Grower and Pre-breeder, there are three types of feed presentation that can be used: pellets, crumbles or mash.

These three feed presentations can be utilized if pans or tracks are the feeder material set up in the farm. But, if the feeder material is spin feeders (which in fact is the most common) and thus feed spread on the floor, pellet must be the feed form of choice. In this case, to have good pellet presentation is compulsory; and hence, to have durability close to 96% should be the objective. This is important to prevent losing fine feed particles which the pullets are unable to find after mixing with the litter.

Feed quality control is essential; thus, a program to monitor the quality of the finished feed presentation is necessary. One of the main objectives of this quality control is to avoid fine particles in the feed. This is essential to prevent the birds from selecting particles, which, as was explained before, is contrary to a balanced diet and to obtain good flock uniformity in rearing. Therefore, samples from each delivery should be taken and feed particle distribution be assessed. In **Table 1.4** are shown the minimum and maximum percentage targets of the particle sizes depending on the feed presentation.

Table 1.4. Particle distribution depending on the feed phase and form presentation (Asensio, 2018).

	Starter 1	Starter 2	Grower and pre-breeder
Form	Fine crumb	Pellet (3 mm)	Pellet (3 mm)
> 3 mm	< 15%	> 70%	> 70%
2.0 - 3.0 mm	40 - 45%		
1.0 - 2.0 mm	35 - 40%		
< 1.0 mm	< 10%	< 10%	< 10%

Another point to be taken into account in broiler breeder nutrition is the usefulness of heat treating the feed in order to reduce or eliminate its bacteriological contamination. Clostridium, Escherichia Coli and Salmonella are the most dangerous sources of this contamination. To produce pellets, raw materials are heat treated at a temperature that reaches around 75^{o}C for 30 seconds. Therefore, pelleting reduces feed contamination and this is another reason to use this type of feed presentation in rearing and production periods. In fact, depending on the level of heat treatment and time applied we can even manage to almost totally kill organisms; if temperature reaches 86^{o}C during 6 minutes, the total viable bacterial counts will generally reduce to less than 10 organisms per gram.

As a consequence of the feed being heat treated, it has to be considered that vitamins and amino acids can be damaged. Anyway, the levels of these components, which are recommended in the nutrition specifications, should cover losses from conventional conditioning and pelleting. However, more severe treatment may increase vitamin and/or amino acid destruction and thus supplementation may be necessary. However, tests to check on vitamin recovery and aminograms should be carried out periodically in order to be aware of the real level of vitamins and amino acids after heat treatment, and to decide if the some of these components should be supplemented.

1.5. Feed intake control of broiler breeders

As was explained before, when pullets are FI controlled during the rearing period, good farm management is necessary to allow all of the birds to consume the ration which corresponds to their requirements. If management is not correct larger or aggressive pullets likely out-compete smaller or timid pullets, resulting in unequal access to feed and increasing flock BW variation (Zuidhof et al., 2015).

As a consequence of this competition for feed and thus of the resulting increase of flock BW variation (lack of uniformity), companies have to grade pullets in rearing with the objective of reducing flock coefficient of variation (**CV**) and thus to obtain uniform flocks. In fact, birds in a similar physiological state respond uniformly to management factors and arrive synchronized at sexual maturity; which supports high peak production and enables producers to adjust feed allocations to match the nutrient requirements of a higher proportion of individual birds within flocks (Pishnamazi et al., 2008).

By grading, pullets are sorted into 2 or 3 sub-populations of different average weight so that each group can be fed to match their maintenance and growth requirements. Likewise, the aim is to achieve the target BW uniformly for each graded population within the period during which skeletal development is taking place (up to 13 wk of age).

From a practical point of view, grading involves segregation of the graded populations into pens or houses left empty at placement for this purpose; this depends on the farm/house design. First grading is best carried out when the flock is 28 d old and flock uniformity is usually within the range of CV from 10 to 14%. If completed later than this, the time available to restore flock uniformity is reduced, and the procedure is less effective. If CV is under 12% the flocks need to be split into 2 populations, light and normal birds (medium BW). The approximate percentage of birds required in each of the 2 populations is 20% light birds and 80% normal birds. When CV is above 12% the flocks need to be split into 3 populations; light, normal and heavy weight birds. The percentage of birds required in each of the 3 populations is approximately 20% light birds, 70% normal and 10% heavy birds.

Grading effectiveness to improve flock uniformity was studied by Zuidhof et al. (2015). They tested the effect of broiler breeder feeding management practices on pullet BW uniformity. Five different feeding management treatments were implemented in this study: 1) control: standard diet in mash form, fed daily; 2) high fiber: mash diet containing 25% lower nutrient density, fed daily; 3)scatter: standard diet in pellet form scattered on the litter, fed daily; 4) skip-a-day: standard mash diet, fed on alternate days; or 5) grading: standard mash diet, fed daily (birds sorted into low, average, and high BW groups every 4 wk). They concluded that grading yielded the highest BW uniformity at 22 wk of age, while control and high fiber treatment groups were the least uniform.

To control FI in broiler breeder pullets there are three strategies that could be used. There are some differences among them, such as their practical application. These different FI strategies are explained below.

1.5.1. Quantitative feed intake control

When pullets are FI controlled by using a quantitative strategy, daily feed allocation meets pullet requirements to achieve the correct growth rate in order to rear birds which are able to support production.

It is normal management to feed broiler breeder pullets in rearing by using spin feeders. With this system the ration is scattered on the floor, which has the advantage of decreasing the negative effects of competition for available food. However, even using spin feeders, when FI control is quantitative, good farm management, including low stock density and uniform floor feed distribution is necessary to avoid larger or aggressive pullets overcoming smaller or timid pullets, resulting in unequal access to feed and thus poor flock uniformity.

Therefore, it has been studied if broiler breeder pullets fed on qualitatively rather than quantitatively FI controlled diets during rearing, could achieve the desired growth rates, obtain good uniformity and improve welfare.

1.5.2. Qualitative feed intake control. *Ad libitum* access to feed

Qualitative FI control is a strategy that can be used to give broiler breeder pullets *ad libitum* access to feed and at the same time to try to match the standard BW profile (limit growth rate). There are different strategies to be applied, and they differ depending on if appetite suppressants, dietary diluents (fiber sources) or both of them are used.

From a practical point of view to feed pullets *ad libitum* in our current rearing facilities would imply replacing spin feeders with tracks or pans feeders; since by distributing pellets on the floor it is not possible to control the feed remaining and mixed with the litter. Hence, it is a difficult system to be applied; it would force the companies to face investments in their rearing farms.

Sandilands et al. (2006) investigated if broiler breeders could be reared to recommended BWs up to 20 wk of age with *ad libitum* access to qualitative controlled diets. They gradually increased, according to bird age, the inclusion level of an appetite suppressant (calcium propionate, CaP) or a dietary diluent (ground oat hulls, OH).

Results showed that both qualitative FI control strategies can limit growth rate close to target.

With regard to flock uniformity and thus the target of achieving low CV at the end of rearing period, Sandilands et al. (2006) study concluded that the BW CV tended to be higher for qualitative feed controlled treatments than for the quantitative control. The same results were noted in previous studies (Pinchasov et al., 1993; Savory et al., 1996). This may be because of individual variation in food intake, or efficiency of nutrient utilization, or variation in adaptation to the appetite suppressant (Pinchasov et al., 1993).

Nowadays, the utilization of the qualitative FI control strategy by the addition of bulking agents such as soybean hulls or appetite suppressants such as calcium propionate, have however failed to gain widespread acceptance within the broiler breeder industry (Mench, 2002; Sandilands et al., 2006), although some recent studies have shown promising results (Nielsen et al., 2011; Morrissey et al., 2014).

1.5.3. Quantitative and qualitative feed intake control. Partially diluted diets

Another feeding management is to mix quantitative and qualitative FI control strategies. It consists of a diluted ration daily distributed by adding different concentrations and sources of fiber. This system would not allow feeding pullets *ad libitum* but depending on the percentage of dilution would allow increasing feed allocation accordingly.

Related to flock uniformity, it has to be taken into account de Los Mozos et al. (2017) study. They assessed the effect of diet density (dilution) on BW uniformity. Diet was diluted by using different sources of fiber raw materials such as wheat bran, soybean hulls or cereal straw. They found that at 19 wk of age broiler breeder pullets fed diluted diets had lower BW CV and higher uniformity compared to the animals eating the control diet (13.1 and 14.2%, respectively); however, differences were not significant.

One advantage of this system is that to feed pullets with spin feeders, by distributing rations diluted with fiber sources, is possible; and no new material such as tracks or pan feeders would be necessary. Therefore, this dietary manipulation (quantitative and qualitative FI control) should result in minimal changes in the existing feed management practices. However, in practice the inclusion of sources of high fiber ingredients has limits, due to the difficulty of formulating rations to the desired specifications with high rates of fiber inclusion and also due to the friction induced during the pelleting process.

1.6. Behavior in broiler breeders

Poultry companies are most interested in following welfare rules in order to have birds in optimum conditions of comfort. However, nowadays, to obtain the targeted broiler breeder production objectives it is essential to control the growth rate during the rearing period, and thus FI control strategies are used in the rearing farms. Accordingly, it is necessary to correctly manage the pullets in order to provide them with equal access to feed and water, good environment and uniform feed distribution. The objective is to avoid having within the flock pullets with a lower growth rate and which could have gone through under consumption and stressful periods, which results in stereotypic behavior (e.g. feather pecking) and potential welfare issues.

1.6.1. Stress measurements in broiler breeders

To assess stress and thus welfare issues by monitoring farm data, for example temperature, wind speed or feed consumption, is essential in modern farms; but it is also important to take into account signals from the birds and their environment. The farm manager has to use his senses in order to build-up an awareness of the environment and check on the behavior of the flock. The first visual indicator of stress that can alert us to stereotypic behavior of the pullets is the sucking movement. The birds move the head forward and backward to the tail of other subjects as if they wanted to lick it but without touching the feathers. The next step is pecking; the movement is the same as before but now the birds peck the feathers, mainly of the tails, and thus, feather cover is quickly damaged.

Therefore, there are signs which can show us if the birds are suffering from stress in the farms. However, from the welfare point of view it is important to evaluate and measure this stress and the next systems, physiological indicators of stress and tail feather score, can be used for this purpose.

1.6.1.1. Physiological indicators of stress

Among the physiological indicators of stress, plasma corticosterone concentration (**PCC**), heterophil to lymphocyte ratio (**H/L**) and white blood cell frequencies (**WBCs**) are the most utilized.

Several authors have used PCC to assess the stress level experienced by birds due to quantitative FI control, since corticosterone is the principal glucocorticoid released by the avian adrenal gland (Whittow, 2000). In some studies, FI control was accompanied by increased plasma corticosterone levels (Hocking et al., 1998; de Jong et al., 2002). However, Savory et al. (1996) study, show that birds fed a quantitatively feed controlled diet to achieve target weight gains, had PCC not significantly different from birds that were fed twice as much with the same diet.

Likewise, several studies reported that a prolonged secretion of corticosterone leads to a suppression of the immune system as corticosterone is involved in lymphocyte apoptosis, which may allow the birds to save energy and/or to provide substrate for tissue repair or glucose production (Sapolsky et al., 2000).

The suppression of the immune system during chronic stress provokes that the number of granular leukocytes, such as heterophils, increases and the number of lymphocytes decreases, which affects the inflammatory response (Sapolsky et al., 2000; Gross and Siegel, 1983).

For this reason several authors refer to the measurement of heterophil to lymphocyte (H/L) ratio in order to determine the level of stress and obtain an indication of the chronic increase of corticosterone in blood (Gross and Siegel, 1983). A low number of lymphocytes results in a high H/L ratio which may be interpreted as a high stress response.

While some studies reported elevated H/L ratios, indicative of chronic stress (Maxwell, 1993) in FI controlled birds (Hocking et al., 1993; Hocking et al., 1996), others could not find this H/L increase in the same situation (Savory et al., 1996; Hocking et al., 1999; de Jong et al., 2002). In fact, de Jong et al. (2002) found that H/L ratios were not different even between FI controlled broiler breeders and the control group fed *ad libitum* with a standard diet at 6 wk of age.

WBCs is also considered a stress marker by several authors (Beuving and Vonder, 1978; Gross and Siegel, 1983). On the other hand, Sandilands et al. (2006) used this indicator to assess stress in their study. They expected quantitative FI control to cause higher levels of this physiological indicator of stress, because it was associated with high levels of object pecking. However, this was not the case, and in the quantitatively FI controlled treatment there were not higher levels of WBCs per 100 cells.

Taking into account the results showed in previous studies, it is clear that there is controversy about the usefulness of PCC, H/L and WBCs as physiological indicators of stress; and it may be that they are not as sensitive as indicators of stress associated with FI control as was once assumed. Therefore, it seems evident that new stress tests should be developed to better assess stress issues in broiler breeder pullets.

1.6.1.2. Tail feather score

On a practical level, a common way to assess the stress that a broiler breeder flock has suffered in rearing is the integrity of the tail feathers. In fact, it is normally stated that if the birds have tails damaged they have been subjected to some type of stress, which could cause stereotypic behavior (tail feather pecking) and the cause has to be investigated.

Therefore, a tail feather score to assess stress associated with poor management could be a new indicator to complement physiological indicators of stress. This score would have five levels: 0) tail fully feathered , 1) rough tail, 2) some broken feathers, 3) heavily broken feathers, 4) tail almost bald and 5) tail bald. Score 0, 1 and 2 are the most common found in the rearing farms (**Figure 1.6**).

Figure 1.6. Tail feather score.
(Adapted from Protocol for Scoring Feather Cover of Broiler Breeders during the Laying Period. Aviagen, 2010).

1.6.2. *Ad libitum* feed available *vs* feed intake control. Behavior implications

Currently, FI control during rearing of broiler breeder pullets is an important management tool to avoid metabolic issues and mortality early in production as well as to reach the objectives of chicks per hen housed. In fact, FI control improves skeletal and cardiovascular health, fertility, and overall survivability, and is generally considered to improve the long-term welfare of these birds compared to ad libitum feed access (Renema and Robinson, 2004).

However, short-term, quantitative FI control combined with poor management may entail stereotypic behavior, such as pecking at non-food objects and increased pacing. In Sandilands et al. (2006) study broiler breeder pullets were reared up to 20 wk of age by using two different FI control strategies: quantitative and qualitative (diet fed *ad libitum*). Related to welfare, the high frequency of object pecking observed in the pullets submitted to quantitative FI control (47 – 54% in the grower periods) was virtually absent in the pullets qualitatively feed controlled. Therefore, the absence of pecking shows a clear benefit of the treatments where controlled intake was qualitative.

Regarding the combination of quantitative and qualitative FI control, Hocking et al. (2004), carried out an experiment in order to examine the effect of increasing concentrations (0, 50, 100 and 200 gr/kg) of three sources of fiber, on BW and stress measures in broiler breeders. The sources used were oat hulls, sugar beet pulp and sunflower meal. The pullets were fed controlled quantities of each ration to achieve the target BW gains recommended by the breeder company. Results show that the prevalence of spot pecking and skin damage was low with the lowest inclusion of sugar beet (50 g/kg). This ration was also associated with BWs that were closest to the control and could easily be adopted in commercial practice. The nearest alternative to 50 g/kg of sugar beet was the ration containing 200 g/kg oat hulls but a diet containing this concentration of oat hulls may be difficult to pellet commercially. Therefore, they concluded that there may be opportunities to reduce stress of FI controlled broiler breeders by modifying the fiber content of the ration.

Therefore, qualitative feed management strategies (addition of feed diluents) could be useful to increase portion size, minimize competition among the broiler breeder pullets and thus reduce stress.

1.7. Leg health issues in broiler breeders

During rearing pullets can present skeletal disorders which can lead to leg issues. These disorders can already appear during the growing or pre-breeder phases. Leg health is a key point to achieve optimum flock production and welfare. In fact, any deviation from normal bone growth will result in bone disorders, which will present a major economic problem to the poultry industry (Sullivan, 1994).

Leg health issues can be divided into infectious and non-infectious causes depending on their origin.

Within infectious causes there are diseases which impact on intestinal health, such as coccidiosis and viral or bacterial enteritis. These diseases can lead to dysbacteriosis, which is not a specific disease, but a secondary syndrome characterized by an imbalance in the gut microbiota as a consequence of an intestinal disruption. Dysbacteriosis presentation varies depending on severity, but generally provokes poor nutrient absorption, thinning of the gut wall along with gassy and watery gut contents (Bailey, 2013).

Non-infectious causes are related to nutritional deficiencies, whose origin could be poor management during the rearing period. The consequence will be poor flock uniformity and pullets under the advised standard BW (birds underweight), which would have not received the nutrients required. As was explained before, related to nutritional deficiencies it has to be taken into account that Ca and P are primary inorganic nutrients because they form 95% of the mineral matrices; therefore, together with other inorganic elements, are important for bone health and strength (Rath et al., 2000). Likewise, vitamin D, which is a calcitropic hormone involved in Ca absorption in the intestine, has a major regulatory role in bone metabolism and bone strength (National Research Council, 1994).

Nowadays, broiler breeders may be affected by different non-infectious leg health issues; among the most common are the rupture of the gastrocnemius tendon (**RGT**), varus&valgus or rickets. Studies have been carried out to find out the main causes leading to these problems and it seems that a holistic approach is necessary to properly analyze their multiple possible sources.

1.7.1. Rupture of the gastrocnemius tendon

The gastrocnemius tendon originates from the fusion of the aponeurotic insertions of the head of the gastrocnemius muscle and its laminar shape is the result of its insertion along the tarsometatarsal bone (Ghetie et al., 1981; Berge, 1981); **Figure 1.7** shows this.

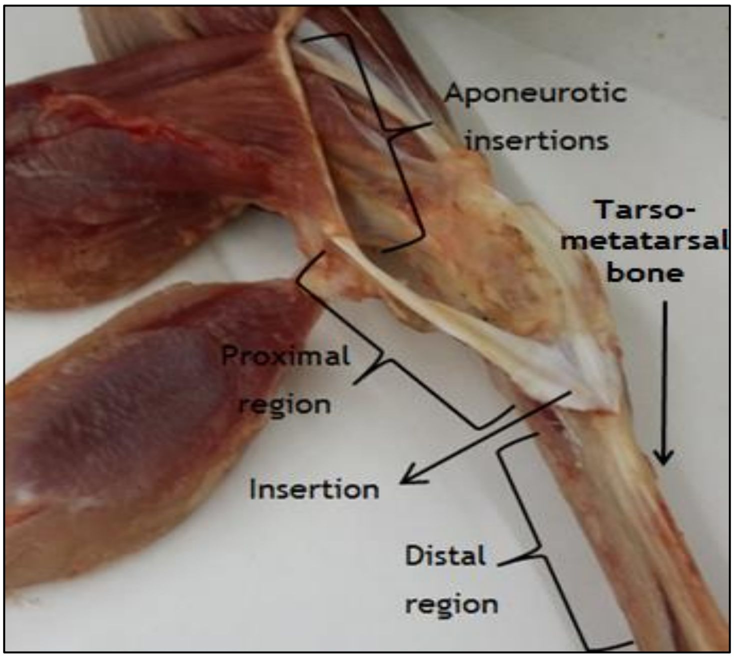

Figure 1.7. Gastrocnemius tendon from a broiler breeder pullet. Muscle aponeurotic insertion, proximal and distal regions, and insertion in the tarsometatarsal bone. (Asensio , 2018).

It has been reported in chickens that this tendon has regions to withstand only tensile loading (tensile region) and regions to withstand both compressive and tensile loading (compression region) (Benevides, 2004). The proximal region of the tendon, which is also shown in **Figure 1.7**, is subjected to compressive forces in addition to the tensile loading found throughout the extent of the tendon, and the distal region, mainly subjected to tensile forces (Nakagaki et al., 2007). In poultry, contraction of the gastrocnemius muscle permits the gastrocnemius tendon to extend the ankle joint and, secondarily, to influence plantar flexion of the toes (Nakagaki et al., 2007).

RGT has been in recent years a recognized cause of lameness in broiler breeders (Dinev, 2008). As is represented in **Figure 1.8**, RGT in broiler breeder hens happens in the distal part of the proximal region, where the tendon starts its insertion along the tarsometatarsal bone.

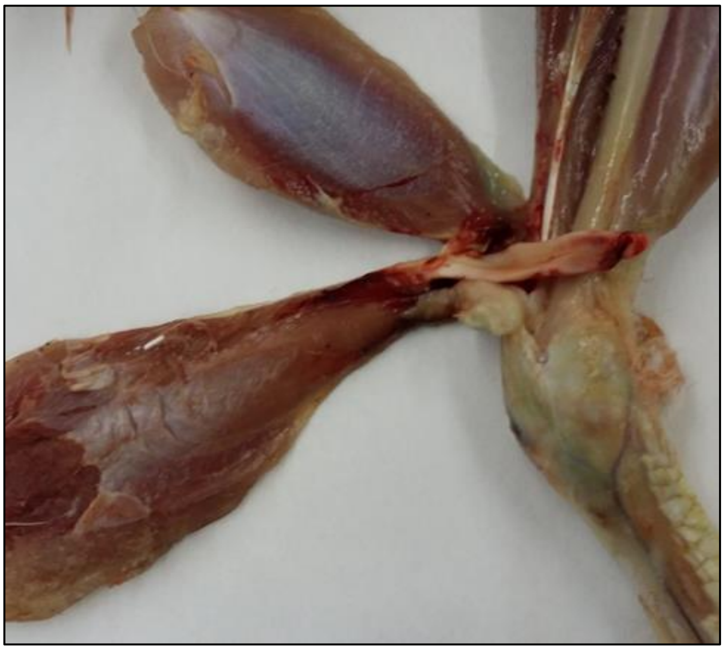

Figure 1.8. Ruptured gastrocnemius tendon (Asensio, 2018).

RGT affects mainly broiler breeder females at the onset of production, within a period which lasts from 25 to 40 wk of age. The incidence varies depending on the areas and companies and it has been impossible to establish clear relationships between management, nutrition, facilities, types of equipment, and its incidence. The only pattern which seems evident to producers and veterinarians is that RGT severity is higher in spring and summer.

Several origins of RGT have been reported; a reoviral tenosynovitis (Jones et al., 1981, Kibenge et al., 1982a; Jones and Kibenge, 1984), reoviruses associated with other predisposing factors (Duff and Randall, 1986) and a viral tenosynovitis associated with a secondary colonization of the tendon by mycoplasmas and staphylococci (Kibenge et al., 1982b; Dinev et al., 1995; Kleven, 2003). Likewise, RGT has also been related to non-infectious factors such as deficiency states, obesity, variation in tensile strength, glucosaminoglycan content and cellular structure of the tendon in the various species (van Walsum et al., 1981; Cook et al., 1983a, b; Bradshaw et al., 2002). Dinev (2008) study provides evidence for the spontaneous RGT in broiler breeder females. The findings were further confirmed by the lack of simultaneous tenosynovitis or arthritis. Furthermore, the absence of RGT in males reared together with females during both growth and productive periods, as well as the bacteriological and serological negative results, supported the thesis for a non-infectious etiology. Likewise, the histological results of this study revealed that lesions varied between birds. In about 50% of them, haemorrhages, dystrophic and necrobiotic changes in tendons were present. In the other 50% of cases, the resolving haematoma and ruptured tendon were surrounded by fibrous tissue growth, and no inflammatory changes were detected.

This is a major problem for producers; it represents huge economic losses as a result of the high mortality that it causes early in production, when the cost of the females is higher and they still have not laid eggs.

1.7.2. Varus and Valgus deformities

The term twisted legs was initially used to describe this leg deformity, but Randall and Mills (1981) and Julian (1984) suggested that this imprecise term should be replaced by varus-valgus angulation.

Varus and valgus angulations affect differently the bone morphometry. In the limb affected by varus angulation, the femur is internally rotated and the tibiotarsus has a shallow condyle groove. Likewise, a medial curvature of the metatarsus and external axial rotation of tibiotarsus can be observed (Leterrier et al., 1992). As a result, in varus deformity the parts forming the pelvic limb of the birds, with the tibiotarsal articulation in the middle, form an arc outward; and this is a consequence of a deviation of the osseous radius inward. In contrast, valgus angulation is characterized by the uprightness of the femur, increased distal bending and groove depth of the tibiotarsus and lateral curvature of the metatarsus (Leterrier et al., 1992). As a result, in valgus deformity (**Figure 1.9**) the deviation of the osseous radius is outward; hence, the parts of the pelvic limb form an arc inward. In both cases, varus and valgus, the angulation can be unilateral, bilateral or asymmetrical.

Figure 1.9. Broiler breeder male affected by a valgus deformity (Asensio, 2018).

In a study carried out with broilers by Leterrier et al (1992), varus angulation was always associated with tendinous displacement; it occurred suddenly at an early age and led immediately to severe locomotor handicap. On the other hand, in valgus angulation, displacement of the gastrocnemius tendon was less frequent and seemed to be a result of the valgus deformity. Its appearance came later and was progressive; consequently, locomotor problems were present in older birds.

Broiler breeders are more affected by valgus deformity. This bone abnormality appears during the rearing period and is usually evident from 15 – 16 wk of age onward. Males and females can be affected but it seems that males are more sensitive.

There are numerous causes of leg-bone abnormalities provoking gait disorders. In fact, severe defects greatly impair the walking ability of birds, and even mild deformities have been shown to cause discomfort. There is evidence that bone deformities, such as Varus and Valgus, leading to eventual lameness can be induced at an early age in chicks, and recent physiological studies have shown the importance of early nutrition in chick development (Fleming, 2008). The origins of Varus and Valgus issues are not clear, but producers and veterinarians agree that poor development during brooding period, as a consequence of bird under consumption and thus low bone mineralization, can be a predisposing factor. Therefore, to achieve BW targets during the starter phase and to avoid flock heterogeneity seems important in order to prevent this bone abnormality.

This is also a major problem for companies, since it is visually evident when the males are close to being transferred to the production farms. Therefore, it is an issue which affects hatchability and thus chicks produced per hen housed, and finally profitability.

1.7.3. Rickets

Rickets is a bone pathology which normally affects birds in growth and can cause a major disaster due to poor mineralization and resulting weak bones.

The proximal epiphysis of the tibia is used to assess rickets; and within it the growth plate, which is a transversal band to the bone, situated between the epiphysis and diaphysis. The growth plate calcifies, produces osseous tissue and thus makes it possible for the bone to grow in length. As was explained before, through this process, endochondral bones are formed from cartilaginous tissue. **Figure 1.10** (left picture) shows a normal growth plate with the two areas used to assess rickets. The first is the proliferating zone, which is the zone where the chondrocytes proliferate and stack. The second is the hypertrophic zone, where the chondrocytes, after taking distance from the epiphysis, grow in volume before degenerating and calcifying.

If the origin of rickets is a deficiency of Ca or vitamin D_3, the growth plate will show a wider proliferating zone (**Figure 1.10**, picture in the middle). When the origin is a P deficiency or a Ca excess, then the hypertrophic zone will be bigger and whitish in appearance; and the bone will be incurved (**Figure 1.10**, right picture).

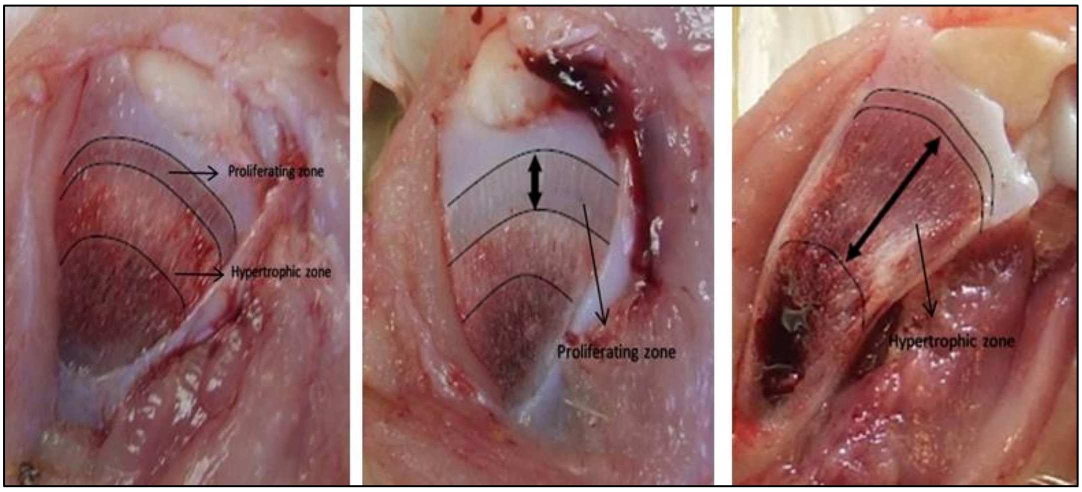

Figure 1.10. Growth plates of the proximal epiphysis of the tibia. Normal (left), calcium or vitamin D3 deficiency (middle) and phosphorus deficiency or calcium excess (right).
(Adapted from Dr. Fricke).

As a consequence of diseases, unbalanced diets or poor management broiler breeder pullets can suffer from rickets during the rearing period. An unequal access to feed might cause underconsumption and underweight of the timid pullets, which will not receive the necessary nutrients to meet their requirements (nutritional deficiencies). Pullets affected by rickets suffer from leg weakness and thus they have difficulty to move. Their necropsy can show soft bones which tend to deform and that can result in fractures, as well as thickening of epiphysis and rickety rosary.

Regarding management, it has to be taken into account that uniform feed distribution and correct stock density are key points to obtain flocks with good uniformity; and thus to avoid unequal access to feed and pullets which do not consume enough to meet their requirements.

If the origin is a disease such as coccidiosis and viral or bacterial enteritis; as was explained previously, the result can be dysbacteriosis and intestinal malabsorption, which in turn can impair Ca absorption (Perry et al., 1991).

Related to unbalanced diets as the origin of rickets, it has to be taken into account that an inadequate Ca level in blood (hypocalcemia) is likely to decrease bone strength. Similarly, an imbalance in P metabolism can affect skeletal integrity and strength. Because of the complex interaction among Ca, P, vitamin D, and other calcitropic hormones, it is necessary to judiciously balance the amount of Ca and P added in the diet (Rath et al., 2000). Likewise, besides vitamin D, vitamins B_6, C and K are integral to bone health because of their involvement in the synthesis of matrix constituents, such as collagen and osteocalcin, and formation of collagen crosslinks (Weber, 1999).

The consequence of rickets is that birds are not able to move normally, and thus producers have an important loss of profitability since they have to be removed.

CHAPTER 2

Background, hypotheses, and objectives

After reviewing literature, it is clear that good management is necessary in the rearing farms to avoid leg health and welfare problems. Nowadays, broiler breeder flocks which are not well managed may have pullets under the advised body weight (**BW**); in fact, large or aggressive pullets may overcome smaller or timid pullets, resulting in unequal access to feed and increasing BW variation. Unequal access to feed and thus low uniformity may result in broiler breeder pullets not having received the nutrients required and thus lead to leg health and welfare issues.

The idea for this thesis comes from the fact that leg health issues and welfare concerns have arisen in recent years in modern broiler breeder production. Therefore, the purpose of this thesis is to find realistic solutions to these problems which the poultry industry is facing at the present time. Likewise, serological markers, which have never been used in broiler breeder pullets, have also been studied to give producers direct ways to assess skeletal development; and, since literature shows controversial results when the current welfare tests are used, a new test will be proposed to assess the comfort of the pullets in the rearing period.

We hypothesize that poor management could lead broiler breeder flocks to low uniformity, and thus pullets underweight in an early phase of the rearing period, which might compromise their skeletal development and strength permanently. Likewise, that pullets fed fiber diluted diets, being able to consume more feed, might result in more uniform flocks, their carcass traits would be correct to sustain production, and their skeletal strength and welfare would be enhanced. Furthermore, it is also hypothesized that feed supplementation with vitamin C might increase skeletal strength and welfare.

Therefore, the global aim of this thesis is to investigate how management and nutritional strategies can affect skeletal development and strength, and welfare of broiler breeders. In addition, new tools to assess correct pullet development and possible welfare issues have also been investigated.

The specific objectives are:

- To study whether pullets with BW below standard at 5 wk of age, and never graded in rearing, could have permanently affected skeletal development and strength.

- To study whether using diets diluted with insoluble fiber, carcass traits are adequate to sustain production.
- To study whether using diets diluted with insoluble fiber could be useful to enhance flock uniformity, carcass traits, skeletal strength, and to reduce welfare issues.
- To study whether vitamin C supplemented in feed might increase skeletal strength and reduce welfare issues.
- To propose serological markers as a direct way to assess skeletal development.
- To propose a tail feather score to assess welfare of the broiler breeder pullets.

To attain these objectives, two different trials were carried out with pullet broiler breeders.

The first was a field trial (**chapter 3**), under commercial conditions, where non-graded light and heavy pullets were evaluated to determine their skeletal development and strength. The usefulness of serological markers was studied to assess skeletal development.

In the second trial (**chapter 4**), pullets were reared under experimental conditions. The objective was to compare, at the onset of production, the result of different nutritional strategies. The trial had two diet densities (control and diluted) and two vitamin C inclusions (0 and 200 mg/kg). Uniformity, mortality, carcass traits, skeletal development and strength, and behavior were assessed. Likewise, alkaline phosphatase (serological marker) was used to evaluate bone development and mineralization, and a tail feather score to evaluate stress and thus welfare.

CHAPTER 3

Weight uniformity management of broiler breeders and impact on their skeletal development

Weight uniformity management of broiler breeders and impact on their skeletal development

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Key words:

Broiler breeders, body weight, grading, skeletal development, gastrocnemius muscle tendon, alkaline phosphatase and osteocalcin.

Statement of primary audience:

Nutritionists, veterinarians and flock supervisors of poultry companies specialized in breeding.

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3.1. Summary

Uniformity of broiler breeders is an important indirect characteristic of good health and welfare, and part of carefully raising a flock. In uneven flocks larger or aggressive pullets may overcome smaller or timid pullets, which can result in unequal access to feed and decreasing flock body weight (**BW)** uniformity. A flock not managed well may contain pullets under the advised standard BW (birds underweight), not having received the nutrients required, and even lead to leg health issues if the skeleton is not developed properly.

This field report investigates the effect of grading on flock uniformity of broiler breeder pullets and their influence on skeletal development and strength. The results of this study show that without grading in rearing, pullets with BW below standard at 5 wk of age will have permanently shorter and less resistant tibias, along with weak tendons. Once a flock is graded, only the light pullets need to be managed separately, as medium and heavy birds can be managed in the same flock. This will simplify management, which can focus on the lighter birds more easily. Serological markers have been tested to evaluate their usefulness as new tools to assess, at an early age, whether bone formation of the pullets is correct so that management can be fine-tuned in time to ensure strong development of all the birds in the flock. Information related to serological markers used in humans to evaluate bone metabolism is also provided in order to understand their potential usefulness in the rearing of modern broiler breeders.

3.2. Description of the problem

Flock uniformity is important to ensure the weight and development of the pullets are even, and to avoid that stronger more developed pullets are aggressive towards lesser developed lighter pullets. Apart from aggression, uneven weight development can lead to underweight pullets not receiving the necessary nutrients to meet their requirements, which could lead to suboptimal bone structure and leg health issues. Genetic selection has concentrated on leg strength and heart and lung fitness during the last decades (Kapell et al., 2012; Hiemstra et al., 2013; Fancher, 2015). However, additional to genetic selection, good management is necessary in the rearing farms to obtain uniform flocks and thus pullets with strong skeletons. Grading is a management tool with the aim to sort the flock into 2 or 3 sub-populations of different average weights (physiological state) so that each group can be managed in a way that will result in bringing the whole population back to the target BW and in good whole flock uniformity at the point of lay.

Nowadays, to evaluate whether the skeletal and bone growth of the broiler breeders during the rearing phase is correct, pullet BW has to be weekly compared to the BW reference profiles provided by the genetic companies. While monitoring of BW is an effective way to assess correct development, markers can provide direct insight on skeletal development, particularly bone growth. Alkaline phosphatase (**ALP**) and osteocalcin (**OC**), which are serological markers of bone formation (Seibel, 2005), are currently used in humans to evaluate bone metabolism, and provide a real-time assessment of bone formation, resorption and turnover. However, they have not been used to date in broiler breeder pullets to assess their development.

The aim of this study is to evaluate whether 5 wk old pullets with a lower BW than standard and never graded in rearing, are affected in their development and skeletal strength, in particular in tibia and gastrocnemius muscle tendon status. Likewise, the usefulness of two different serological markers is proposed to assess the skeletal development throughout the initial phase of the broiler breeders in rearing period.

3.3. Materials and methods

A field trial was designed to evaluate skeletal development and bone strength of the lightest pullets within a flock. The objective was to reproduce commercial conditions where pullets with a lower BW than standard were present, to assess whether their skeletal development and strength could be permanently affected negatively. Also, the lightest and the heaviest pullets were blood sampled to investigate the usefulness of serological markers of bone formation for the assessment of skeletal development and strength.

3.3.1. Birds and facility

The experimental procedures were in compliance with the European Union guidelines for the care and use of animals in research (European Parliament, 2010).

A total of 1988 broiler breeders, Ross 308 (**FLOCK**) female chicks from a commercial flock in Spain were placed in an all in all out farm during a period from 0 to 30 wk of age. Pullets were infrared beak treated with a Nova-Tech machine and vaccinated at the hatchery against Marek and Infectious Bronchitis. A vaccination program adapted to the epidemiology of the farm area was followed during the rearing period.

Until 21 wk of age, pen dimensions were 48.50 m long by 6.5 m wide, 315.25 m^2 in total with 6.30 birds/m^2. Feed space was 0.87 birds per pan hole and drinking space was 5.58 birds per nipple. At 21 wk old these could use the whole house and mixed with the males (ratio 10/1). From this moment, stock density was 5.30 birds/m^2, feed space was 0.74 birds per pan hole and drinking space was 4.74 birds per nipple. Throughout the whole trial feed and drinker space was sufficient for all hens to reach feed and water at the same time. Wood shavings were utilized as litter material and litter was topped up weekly to keep it dry.

The house was environmentally controlled and the temperature set point was modified depending on the bird age, starting with 32^{o}C at one day old and decreasing on a regular basis until 20^{o}C at 27 d old; this set point was kept until the end of the trial. The lighting program was 23 h during the first 4 d and then, gradually reduced to 8 h until 14 d. From 2 to 20 wk the lighting program was 8 h of light; later on light was increased 1 h per wk until 14 h (26 wk). Light intensity was 100-80 lux the first wk, 20 lux from 2 to 20 wk and 60 lux later on. Management data such as light program, light intensity and environmental temperature were weekly recorded.

3.3.2. Experimental design

At 5 wk of age 160 pullets were randomly selected within the FLOCK to create a control (**CTR**) group. Pullets from the CTR were individually identified and two experimental groups of weight were created: the first quartile of the 40 lightest birds which was named **L group** and the forth quartile of the 40 heaviest birds which was named **H group**.

After 5 wk of age and until 21 wk, the FLOCK was graded three times whereas the CTR was never graded or grouped according to BW. All the pullets from the FLOCK were weighed individually at 6 wk of age and BW coefficient of variation (**CV**) calculated. Since BW CV result was higher than 12%, it was decided to divide the initial pen in three sub-populations: 20% of the surface for the lightest pullets, 10% for the heaviest and 70% for the rest (medium BW). After grading, each sub-population was managed separately according to its weight with the objective of bringing all the population back to the target BW. The FLOCK was graded two more times, at 12 and 18 wk of age. In both cases BW CV of the FLOCK was lower than 12%, thus the pen was only divided into two sub-populations: 20% of the surface for the lightest pullets and 80% for the rest (medium BW). The aim was to obtain a correct uniformity of the FLOCK; mainly during the period where skeletal development was taking place (before 13 wk of age). The pullets of the CTR were always in the pen of the medium BW sub-population, and as was explained before, they were never graded or grouped according to BW. The aim was to study these pullets throughout the trial without any grading.

3.3.3. Diets

Feed was produced in a feed mill of CAG (Tarragona, Spain). The different diets were formulated according to the Ross 308 Parent Stock Nutrition Specifications (Aviagen, 2014). A five diet phase feeding program was followed: starter I (from 0 to 3 wk of age), starter II (from 4 to 6 wk), grower (from 7 to 15 wk), pre-breeder (from 16 to 24 wk) and breeder (from 25 to 30 wk). The ingredients and nutrient composition are shown in **Table 3.1**.

Feed was provided ad libitum during the first 2 wk in order to ensure a correct initial development. From this point onwards, in order to follow the standard BW profile, the total daily feed distributed was calculated by multiplying all the pullets of the FLOCK (present at the moment) by the daily AMEn allowance per bird and divided by the AMEn of the diet. Both, BW profile and daily AMEn allowance per pullet were based on Ross 308 Parent Stock Performance Objectives (Aviagen, 2012). **Figures 3.1** and **3.2** show respectively feed intake (standard and real) and BW (standard profile, FLOCK and CTR) throughout the trial. In addition, a ratio water/feed of 2 was used during the rearing and production phases.

Table 3.1. Ingredients (%) and nutritional composition of the experimental diets.

	Starter I 0 to 3wk	Starter II 4 to 6 wk	Grower 7 to 15 wk	Pre-breeder 16 to 24 wk	Breeder I 25 to 30 wk
Wheat	35.2	36.2	27.7	33.1	30.8
Soybean meal	25.6	17.8	-	6.2	15.8
Corn	24.5	31.6	30.0	35.0	38.0
Sunflower 28-30%	5.00	6.00	15.76	10.50	4.10
Wheat bran	3.00	1.23	19.70	7.00	-
Dicalcium phosphate	2.33	2.23	1.87	1.53	1.70
Rapeseed meal	-	2.00	1.57	3.00	-
Soybean oil	2.00	1.00	1.20	0.77	2.00
Limestone	0.97	0.90	0.90	1.87	6.57
Premix[1]	0.30	0.30	0.30	0.30	0.30
Sodium bicarbonate	0.27	0.24	0.22	0.20	0.18
Salt	0.23	0.23	0.21	0.23	0.24
L-lysine sulfate 54.6%	0.23	-	0.26	0.12	0.03
Methionine	0.20	0.10	0.11	0.10	0.14
Choline Cl-60%	0.10	0.10	0.10	0.07	0.07
Calcium pidolate	0.05	0.03	0.03	-	-
Threonine	0.05	0.03	0.04	0.01	0.04
AMEn, Kcal/Kg	2810	2801	2550	2701	2800
Dry matter %[2]	89.0	88.6	88.6	88.7	89.4
Crude Protein %[2]	20.7	16.9	13.3	14.4	14.8
dig Lys %	0.95	0.67	0.50	0.52	0.58
dig Met %	0.47	0.35	0.32	0.32	0.35
dig M+C %	0.74	0.60	0.53	0.54	0.56
dig Thre %	0.64	0.54	0.39	0.42	0.47
dig Trp %	0.20	0.17	0.13	0.14	0.14
Crude Fiber %[2]	4.17	4.48	7.99	5.99	3.46
Crude Fat %[2]	4.06	3.05	3.98	3.02	4.13
Ash %[2]	5.87	5.68	5.30	5.92	10.21
Ca %	1.06	1.00	0.91	1.19	2.99
dig P %	0.48	0.46	0.42	0.36	0.36

[1]Premix provided per kilogram of diet: vitamin A (retinyl acetate), 10000 UI; vitamin D_3 (cholecalciferol), 1500 UI; 25-hydroxycholecalciferol, 0.0375 mg; vitamin E (DL-α-tocopheryl acetate), 150 mg; vitamin K_3, 10.00 mg; Vitamin B_1(thiamin), 7.00 mg; vitamin B_2 (riboflavin), 20.00 mg; vitamin B_5 (pantothenic acid), 27.22 mg; vitamin B_6 (pyridoxine hydrochloride), 10.00 mg; vitamin B_{12} (cyanocobalamin), 0.07 mg; vitamin B_3 (nicotinic acid), 87.09 mg; vitamin B_9 (folic acid), 5.00 mg; vitamin B_7 (biotin), 0.60 mg; vitamin C (ascorbyl phosphate), 150 mg; iodine, 2.00 mg; Mn (manganous oxide), 80.00 mg; Mn (manganese chelate), 40.00 mg; Cu (cupric sulfate), 6.00 mg; Cu (copper chelate), 10.00 mg; Zn (zinc oxide), 60 mg; Zn (zinc chelate), 40.00 mg; Se (sodium selenite), 0.20 mg; Se (selenium organic form), 0.20 mg; Fe (ferrous carbonate), 65.00 mg.
[2]Analyzed values.

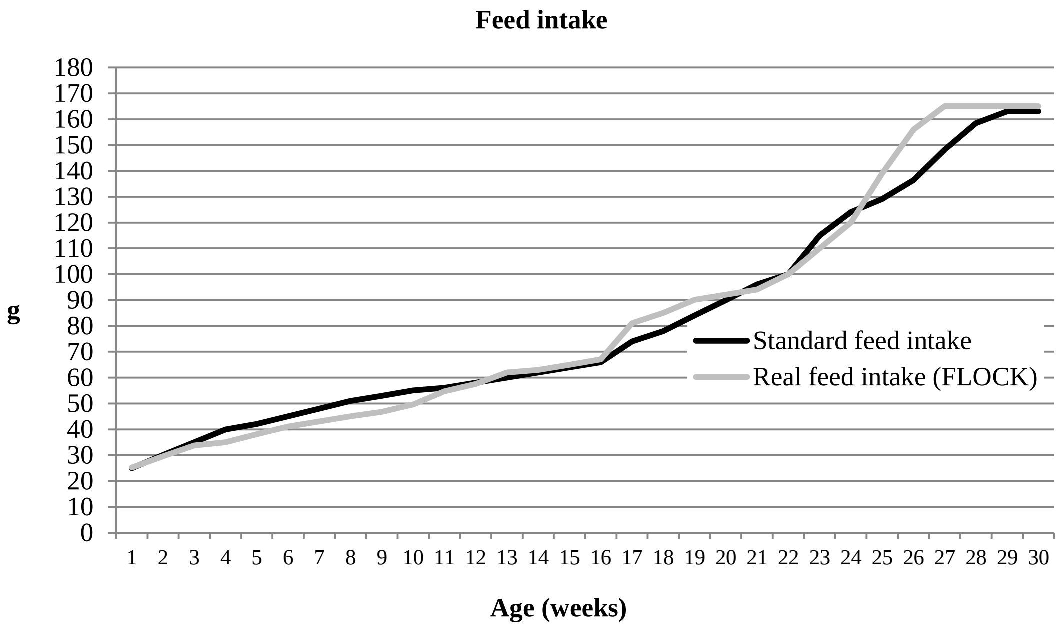

Figure 3.1. Standard feed intake (g/d) based on Ross 308 Parent Stock Performance Objectives (Aviagen, 2012) to follow the target body weight profile; and real feed intake (g/d) provided to all the pullets of the FLOCK (1988 Ross broiler breeder hens kept in a standard breeder farm) throughout the trial (from 5 to 30 weeks).

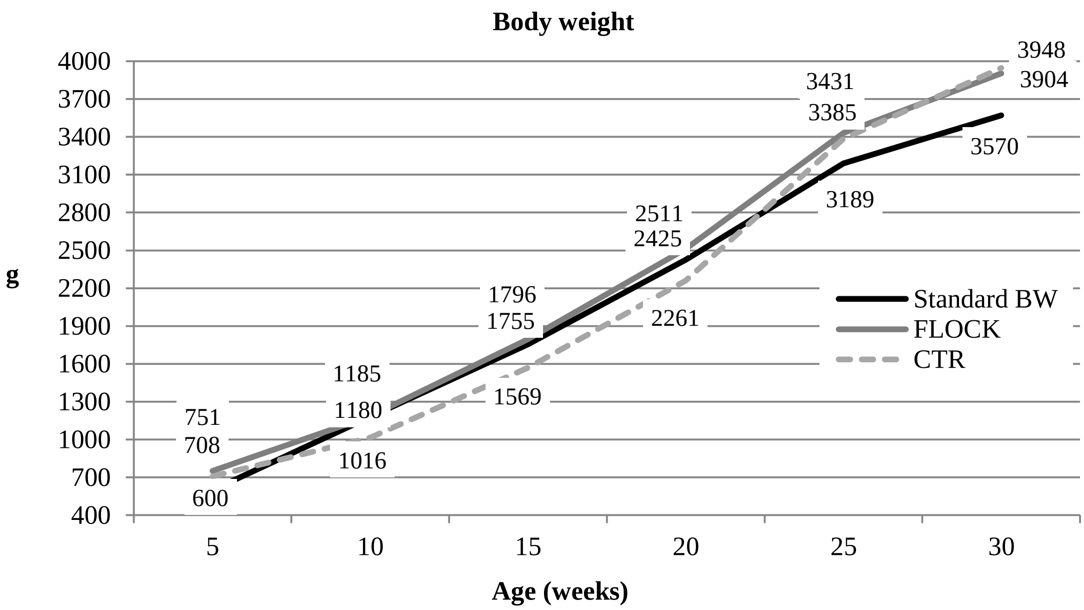

Figure 3.2. Standard body weight profile based on Ross 308 Parent Stock Performance Objectives (Aviagen, 2012); and body weight of the FLOCK (1988 Ross broiler breeder hens kept in a standard breeder farm) and CTR (160 Ross broiler breeder hens randomly selected at 5 wk of age within the FLOCK) throughout the trial (from 5 to 30 weeks).

3.3.4. Collected data, sampling and analytical determinations

Experimental feed samples were collected from the different diets supplied during the rearing and production periods of the field trial. These samples were ground and stored at 5^{o}C until their analysis. Diet proximate analyses were performed according to the method of AOAC International (2005): dry matter (Method 934.01), crude protein (Method 968.06), crude fiber (Method 962.09), crude fat (Method 2003.05), and ash content (Method 942.05). All the analyses were carried out in the SNiBA lab of the UAB (Barcelona, Spain).

The FLOCK was monitored weekly by sampling BW and calculating the CV. The pullets were randomly caught (minimum 2% of the FLOCK) by using catching frames, and all the birds rounded up were weighed in order to eliminate any selective bias. All the pullets of the CTR were individually weighed every 5 wk, starting when they were 5 wk and finishing at 30 wk old. BW and CV were also recorded.

Mortality of the FLOCK, CTR and L and H groups was recorded every day and accumulated mortality calculated in order to make comparisons among the different groups.

To determine ALP, the 14 lightest pullets of the L group and the 14 heaviest pullets of the H group were blood sampled at 5 and 10 wk of age. Blood was spun at 3000 rpm during 20 min to obtain the serum, which was stored at -20^{o}C until the moment of testing. To determine OC, serums coming from the same pullets were used, but only those of the 10 lightest birds of the L group and those of the 10 heaviest birds of the H group.

To test ALP a colorimetric method based on catalytic transformation of p-nitrophenylphosphate as substrate (IFCC method) on the Beckman Coulter AU analyzer was used. In the case of OC, the Rat-MIDTM Osteocalcin EIA (ELISA) was the test performed. ALP and OC analyses were carried out by the Departament de Bioquímica i Biologia Molecular of the UAB (Barcelona, Spain).

To perform post-mortem analyses, 3 pullets from the L group and 3 pullets from the H group were slaughtered every 5 wk (starting at 5 wk and finishing at 30 wk of age).

Right legs were used to dissect tibias, which were utilized to determine tibia length (**TL**), tibia breaking strength (**BS**) (maximum load endurance) and elastic modulus

(**EM**). All the right tibias were used to determine TL; but only the right tibias of the birds slaughtered at 15, 25 and 30 wk of age were used to determine BS and EM.

Left legs of the birds slaughtered at 20 and 25 wk of age were used to dissect the gastrocnemius muscle tendon in order to carry out histological analyses and to assess the presence of inflammation and fibrosis.

TL was measured by using a calliper (0.03 mm precision and 0.01 mm resolution); and tibia BS and EM were measured by using a MTS Alliance RT/5 material testing system (force resolution 0.001 N and distance resolution 0.001 mm). These studies were carried out by IRTA (Institut de Recerca i Tecnologia Agroalimentaria, Girona, Spain).

To carry out the histological analysis of the gastrocnemius muscle tendon, samples were fixed in 10% buffered formalin and routinely processed for histopathology. Slides were cut at 3 μm, stained with hematoxylin-eosin and examined in a “blind-fashion” manner. The presence of inflammation and fibrosis was assessed by using a semi-quantitative approach: (0) absence, (1) low amount, (2) moderate amount, (3) large amount. Inflammation, when present, was identified by the presence of mononuclear cells (lymphocytes, plasma cells and few macrophages). The samples were evaluated by a specialist in veterinary pathology (Dipl. ECVP) from the Servei de Diagnòstic de Patologia Veterinaria of the UAB (Barcelona, Spain).

3.3.5. Statistical analysis

The statistical analysis was performed with the R® program (version 3.3.3, 2017). A one way ANOVA was carried out to analyze BW data. A mixed model was used to analyze ALP and OC; BW (L and H groups) and age (wk) were fixed effects and the bird was a random effect. Factorial ANOVA was carried out to analyze TL and tibia BS and EM; the factors were BW (L and H groups) and age (wk). Means were compared through a Tukey test. In all cases, *P*-values lower than 0.05 were considered to be significant.

3.4. Results and discussion

3.4.1. Body weight and coefficient of variation evolution

Evolution of BW in L and H groups from 5 to 30 wk of age is presented in **Table 3.2.** BW of the lightest pullets (L group) was lower and significantly different from that of the heaviest (H group) until 25 wk of age. However, at 30 wk of age, BW of the L and H groups were similar (3944 and 3995 g respectively), and no significant differences were observed between them.

Table 3.2. Weekly broiler breeder body weight evolution (L and H groups).

	Body weight (g)			
AGE (wk)	L group[1]	H group[2]	SEM (n=40)	*P*-value
5	587	831	6.2	<0.001
10	829	1184	16.9	< 0.001
15	1346	1762	24.6	< 0.001
20	2035	2438	42.3	< 0.001
25	3292	3543	48.0	< 0.01
30	3944	3995	59.8	0.571

[1]The 40 lightest hens of the CTR (160 Ross broiler breeder hens randomly selected at 5 wk of age within the FLOCK).

[2]The 40 heaviest hens of the CTR.

Evolution of CV of the FLOCK, CTR and L and H groups from 5 to 30 wk of age is shown in **Figure 3.3**. From 10 to 25 wk of age, the FLOCK and H group had a CV lower than 10%, whereas the CTR and L group had higher CV, with values over 10% (except for L group at 25 wk) . The objective of good management should be to obtain a CV of maximum 10% (Uniformity of Female Broiler Breeders. Aviagen, 2016). At 30 wk of age, the CV of the FLOCK, CTR and L and H groups were 6.9, 5.8, 7.3 and 7.3% respectively; therefore, all groups came closer and were under the 10% target.

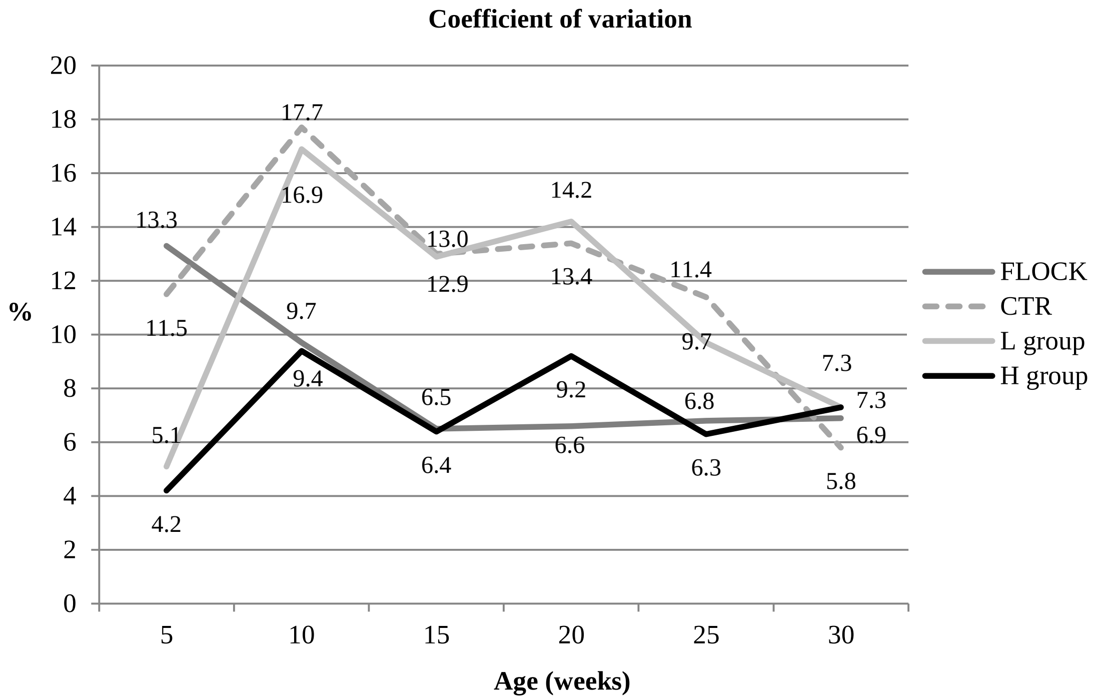

Figure 3.3. Evolution of the coefficient of variation (%) of the hens from the FLOCK (1988 Ross broiler breeder hens kept in a standard breeder farm), CTR (160 Ross broiler breeder hens randomly selected at 5 wk of age within the FLOCK), L group (the 40 lightest hens of the CTR) and H group (the 40 heaviest hens of the CTR).

Similar results were found in a study (Renema et al., 1999) carried out with broiler breeders. It was reported that in feed intake (**FI**) controlled broiler breeders, the 20% difference between the standard and low or high bird BW at 21 wk declined at sexual maturity to 5% and 4% for the low and high bird BW respectively. Likewise, by reducing the BW difference between treatments, uniformity also improved. In the current study feed allocation was the same for all the birds of the FLOCK, whereas the birds of mentioned study (Renema et al., 1999) were fed at the level that maintained constant rates of gain in each body size group.

Related to CV evolution, research carried out to study the effect of broiler breeder feeding management practices, showed that the grading treatment resulted in the highest flock BW uniformity at 22 wk of age, with a BW CV of 6.2% (Zuidhof et al., 2015). In the present study a similar BW CV (6.6%) at 20 wk of age was obtained with the FLOCK, which was the only one graded (unlike the CTR and thus L and H groups).

The results of this study show that after 25 wk of age, BW of the lightest hens (L group) almost matched that of the heaviest (H group) and CV of the L group decreased to a correct level (the same percentage as the H group). This could be explained by more feed availability, more feed space and a longer clean-up time in the production period; hence, the lightest hens would be able to obtain more feed. An outcome which is related to one of the objectives of grading: providing fair competition and reducing aggression between the lighter and the heavier pullets.

In the absence of grading in rearing, uniformity will be lower as probably the lightest pullets would consume below their requirements. This study shows that indeed the lack of uniformity of the CTR comes from the lightest pullets (L group); in fact, the heaviest (H group), which were also not graded, kept a correct CV (lower than 10%) throughout the trial. Therefore, to obtain a correct uniformity in field conditions it is only necessary to separate the lightest pullets, while the sub-populations with the heaviest and medium BW pullets can stay together.

3.4.2. Mortality

Accumulated mortality (%) displayed in **Figure 3.4**, shows that at 30 wk of age the pullets of the FLOCK, which had been graded, had lower mortality (11.5%) than that of the CTR (14.4%), which were not graded. Within the CTR, the lightest pullets (L group) had higher mortality (22.5%) than that of the heaviest (H group) (7.5%).

It is relevant that during the period from 5 to 15 wk of age (when the weekly feed increase is lower), the lightest pullets (L group) had their highest mortality (1.25%/wk), while between 15 and 20 wk (feed stimulation period) their mortality was the lowest (0.50%/wk). Likewise, it has to be highlighted that from 5 to 20 wk of age the heaviest pullets (H group) had no mortality. Between 20 and 30 wk of age mortality increased in the FLOCK, CTR, L and H groups (0.64, 0.75, 0.75 and 0.75%/wk respectively). This is a critical period in which the hens are mixed with the males and they start laying, which could be a likely origin of a higher mortality.

Therefore, in field conditions grading increases livability of the lightest birds of a flock, mainly during the period between 5 and 15 wk of age.

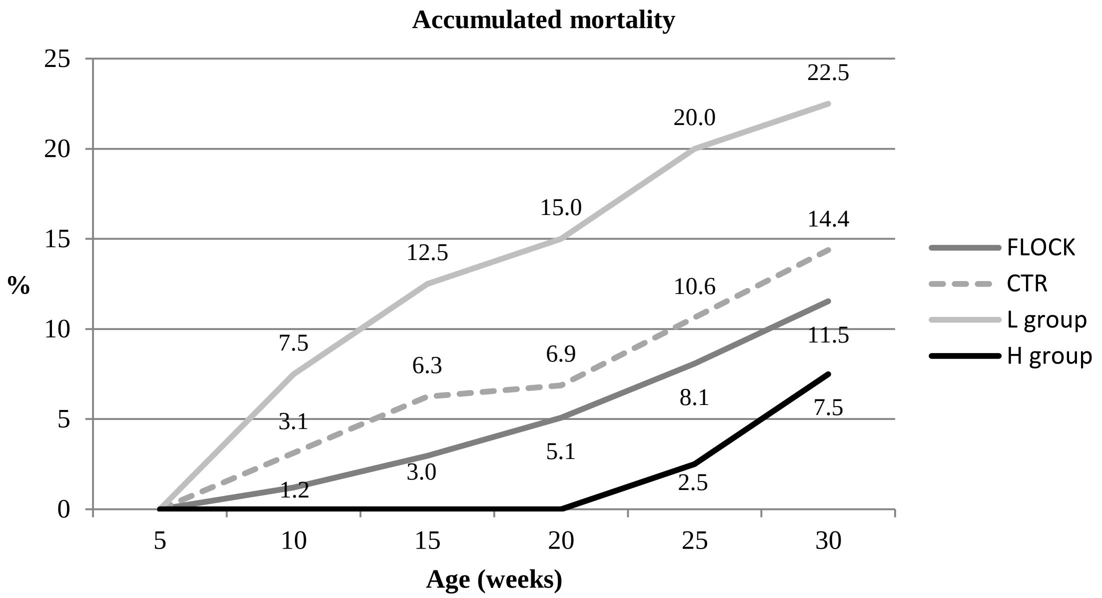

Figure 3.4. Accumulated mortality (%) of the hens from the FLOCK (1988 Ross broiler breeder kept in a breeder farm), the CTR (160 Ross broiler breeders randomly selected at 5 wk of age within the FLOCK), L group (the 40 lightest hens of the CTR) and H group (the 40 heaviest hens of the CTR).

3.4.3. Alkaline phosphatase and osteocalcin

ALP is a ubiquitous enzyme attached on the outer cell surface (Stinson and Hamilton, 1994). The precise function of the enzyme is yet unknown, but it obviously plays an important role in osteoid formation and mineralization (Harris, 1990). In a recent study (Ekmay et al., 2012) it has been used to assess broiler breeder growth and bone deposition during transition into sexual maturity (24 to 16 wk of age); in fact, it is considered a serological marker of bone formation.

Evolution of ALP serological levels is presented in **Table 3.3**. An interaction BW/age (wk) shows that at 5 wk of age the heaviest pullets (H group) had a significant higher ALP level than the lightest pullets (L group) (3839 *vs.* 2781 UI; P = 0.023). Likewise, ALP serological level of the lightest pullets (L group) differs significantly between 5 and 10 wk of age (5 wk: 2781 *vs.* 10 wk: 1798 UI; P = 0.023).

OC is considered a specific marker of osteoblast function (Brown et al., 1984), and thus also a serological marker of bone formation. It is estimated that, directly after its release from the osteoblast, the largest part of the newly synthesized protein is incorporated into the extracellular bone matrix where it constitutes approximately 15%

Table 3.3. Alkaline phosphatase and osteocalcin levels according to broiler breeder body weight and week of age.

	ALP[3] (UI)	OC[4] (ng/ml)
Body Weight		
L group[1]	2290	976
H group[2]	2949	1238
n	28	20
SEM	108.6	56.6
Age (wk)		
5	3374	1189
10	1944	1025
n	28	20
SEM	108.6	56.6
BW/Age (wk)		
L group/5	2781[b]	1032
L group/10	1798[c]	920
H group/5	3839[a]	1346
H group/10	2059[bc]	1130
n	14	10
SEM	153.6	79.6
P - value		
BW	0.032	0.029
Age (wk)	< 0.001	0.050
Interaction	0.023	0.527

[a-c]Means within a column and within a source with no common superscript differ significantly.

[1]The 40 lightest hens of the CTR (160 broiler breeder hens selected within the FLOCK at 5 wk of age).

[2]The 40 heaviest hens of the CTR.

[3]Alkaline Phosphatase.

[4]Osteocalcin.

of non-collagenous protein fraction. A smaller fraction is released into the circulation where it can be detected by immunoassays (Delmas et al., 1983a, b; Gundberg et al., 1985; Taylor et al., 1988; Bouillon et al., 1992; Monaghan et al., 1993; Chen et al., 2009; Parviainen et al., 2009).

Evolution of OC serum levels, according to BW (L and H groups) and age (wk), is also presented in **Table 3.3**. Related to BW, the heaviest pullets (H group) had a higher OC level than the lightest pullets (L group) (1238 *vs.* 976 ng/ml; $P = 0.029$). If age (wk) is taken into account, results show that at 5 wk OC level was higher than at 10 wk of age (1189 *vs.*1025 ng/ml; $P = 0.05$).

In the current study, ALP and OC serum levels decrease from 5 to 10 wk of age, which could confirm that when the pullets reach 10 wk of age the rate of skeletal growth has already reduced.

There are no studies which have used ALP or OC to assess skeletal growth in broiler breeders in rearing. However, in chickens, elevated ALP activity has been predominantly related to increased osteoblastic activity and used as a marker for evaluating skeletal health and bone disease, such as skeletal growth, rickets, fracture repair and osteomyelitis (Lumeij and Westerhof, 1987). In the present study, and related to skeletal or bone growth, it may be concluded that during the first 10 wk of rearing the heaviest pullets (H group) had higher serological levels of ALP and OC, which suggests that there is a relation between BW and bone development. Likewise, these two markers may be useful as additional information of BW and uniformity, in order to assess if bone development during the first 10 wk of the rearing period of broiler breeders is correct. Serological levels of ALP and OC are directly related to skeletal or bone development, whereas BW is only an indicator and does not relate directly to skeletal development.

From a management point of view, lower ALP and OC serological levels at 10 wk of age, suggests that at this age their skeletal development has already reduced and, therefore, it is worth grading the pullets early in rearing in order to recover the skeletal frame size of the lightest birds before their development starts to reduce.

3.4.4. Tibia length, tibia breaking strength and elastic modulus

Evolution of TL, BS and EM, according to BW (L and H groups) and age (wk), is presented in **Table 3.4**; and TL differences from 5 to 30 wk of age, depending on BW (L and H groups), are shown in **Figure 3.5**.

According to BW, the lightest pullets (L group) had shorter tibias compared to the heaviest (H group) (108 *vs.* 120 mm; $P < 0.001$). To be more specific, **Figure 3.5** shows that TL of the lightest pullets (L group), compared to that of the heaviest (H group), is always shorter throughout the trial (5 to 30 wk of age). According to age (wk) TL increased and differed significantly until 15 wk of age. Within the period from 15 to 30 wk of age, TL was not significantly different .These results are in line with the statement that 90 % of the skeleton of the broiler breeders is already developed at 11-13 wk (Ross Parent Stock Management Handbook. Aviagen, 2013).

In relation to BS and EM, it is worth pointing out that under constant increments of loads, the bone undergoes elastic deformation until the point is reached where it is no longer resilient. The load required to reach this point is named BS (maximum load endurance). BS reflects the rigidity of bone as a whole, whereas the slope of the linear region of the stress *vs.* strain-curve is called EM and reflects the intrinsic stiffness or rigidity and material properties of bone (Turner and Burr, 1993).

Results of tibia BS, according to BW (L and H groups), show that tibias of the lightest hens (L group) resisted less weight before breaking compared to the tibias of the heaviest hens (H group) (64284 and 71373 gf/mm^2; $P = 0.085$). According to age (wk), tibia BS at 15 wk was lower than at 25 and 30 wk of age (38899 *vs*. 80255 and 80102 gf/mm^2; $P < 0.001$); during the period between 15 and 25 wk of age tibias reached their maximum strength.

Results of tibia EM, according to BW (L and H groups), show that tibias of the lightest hens (L group) were intrinsically less rigid or more elastic compared to the tibias of heaviest hens (H group) (61.5 and 68.9 Kgf/mm^2; $P = 0.072$). According to age (wk), EM at 15 wk was lower than at 25 and 30 wk of age (37.9 *vs.* 75.6 and 78.2 Kgf/mm^2; $P < 0.001$); during the period between 15 and 25 wk tibias reached their maximum rigidity.

Table 3.4. Tibia length, breaking strength and elastic modulus data according to broiler breeder body weight and week of age.

	TL[3] (mm)	BS[4] (gf/mm^2)	EM[5] (Kgf/mm^2)
BW			
L group[1]	108	64284	61.5
H group[2]	120	71373	68.9
n	18	9	9
SEM	1.0	2606.1	2.58
AGE (wk)			
5	86[c]		
10	102[b]		
15	120[a]	38899[b]	37.9[b]
20	126[a]		
25	125[a]	80255[a]	75.6[a]
30	126[a]	80102[a]	78.2[a]
n	6	6	6
SEM	1.8	3191.8	3.16
P - value			
BW	< 0.001	0.085	0.072
AGE (wk)	< 0.001	< 0.001	< 0.001
Interaction	0.281	0.905	0.438

[a-c]Means within a column and within a source with no common superscript differ significantly.

[1]The 40 lightest hens of the CTR (160 broiler breeder hens selected within the FLOCK at 5 wk of age).

[2] The 40 heaviest hens of the CTR.

[3]Tibia length.

[4]Breaking strength (maximum load endurance).

[5]Elastic modulus (rigidity of the bone).

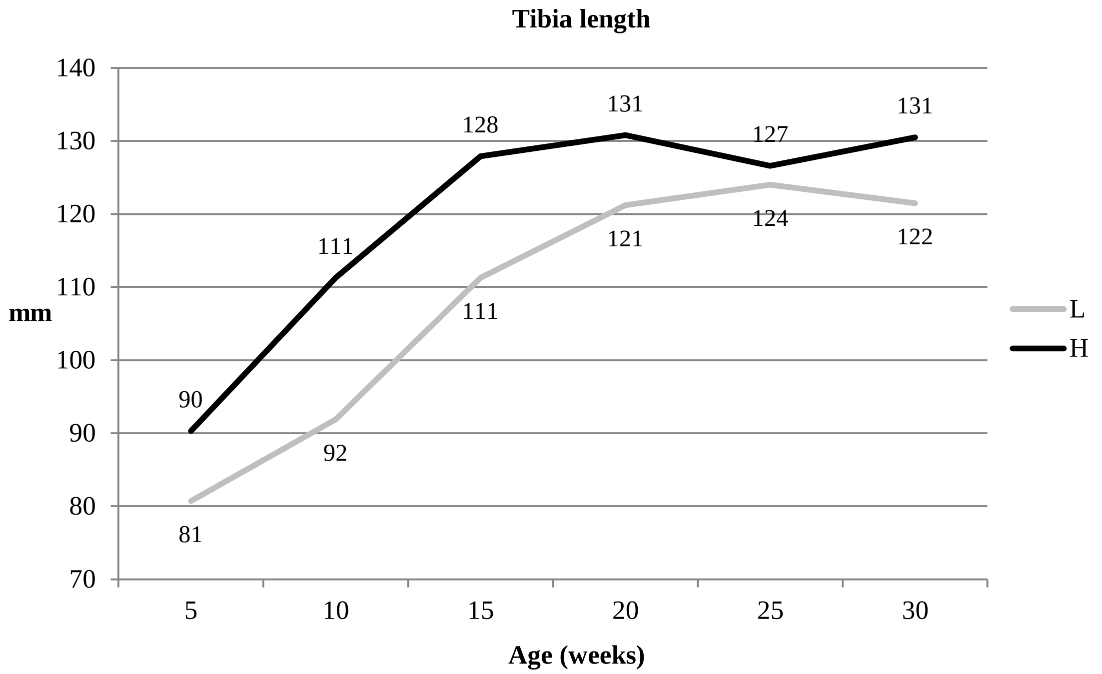

Figure 3.5. Tibia length (TL) of the hens from the L group (the 40 lightest of the CTR) and the H group (the 40 heaviest of the CTR). CTR (160 Ross broiler breeder hens randomly selected within the FLOCK). FLOCK (1988 Ross broiler breeder hens kept in a standard breeder farm).

These results show that the heaviest pullets (H group) had the longest tibias, which in turn also confirms that in the rearing period BW and skeletal growth are related as had been observed by other authors (Robinson et al., 2007).

Related to BS, it might be concluded that during the period between 15 and 25 wk of age tibias reached their maximum strength. This result differs from the conclusions of a study carried out with male broiler breeders, where they found that BS reached its maximum at 35 wk of age (Rath et al., 2000).

After TL results it might be concluded that the lightest pullets (L group) will always have shorter tibias than the heaviest pullets (H group) and that skeletal growth takes place until 15 wk of age. Likewise, BS and EM results show that tibias of the lightest pullets (L group), compared to the heaviest pullets (H group), have lower load endurance and are more elastic, thus more vulnerable. This could be related to a bone with lower EM being less mineralized, as may also be expected of a rachitic bone (Turner and Burr, 1993). The lightest pullets in breeder rearing farms may have poor bone mineralization, and to recover their bone development (to obtain compensatory growth) it might be necessary to grade them early in rearing, since TL reaches its maximum at 15 wk of age and tibia BS and EM between 15 and 25 wk of age.

3.4.5. Inflammation and fibrosis of the gastrocnemius tendon

In the present study, histological analyses were carried out to assess if there was any structural difference in the gastrocnemius muscle tendon of the lightest hens (L group) in comparison to the heaviest hens (H group). The percentage of hens (L and H groups at 20 and 25 wk of age) in each of the inflammation and fibrosis scores is shown in **Figures 3.6** and **3.7**. Results showed that at 20 and 25 wk of age, the lightest hens (L group) had more incidences of inflammation and fibrosis than the heaviest hens (H group).

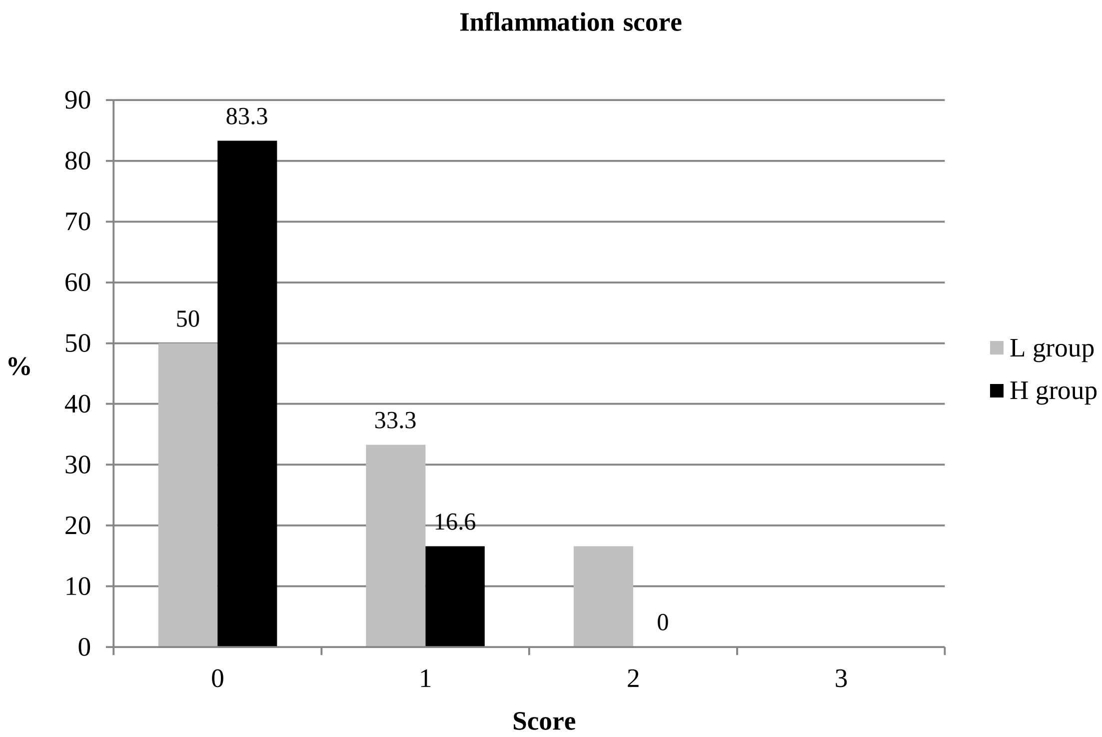

Figure 3.6. Percentage of hens from the L group (the 40 lightest of the CTR) and from the H group (the 40 heaviest of the CRT) in each inflammation score (at 20 and 25 wk of age). Inflammation score: (0) absence, (1) low amount, (2) moderate amount and (3) large amount.
CTR (160 Ross broiler breeder hens randomly selected within the FLOCK). FLOCK (1988 Ross broiler breeder hens kept in a standard breeder farm).

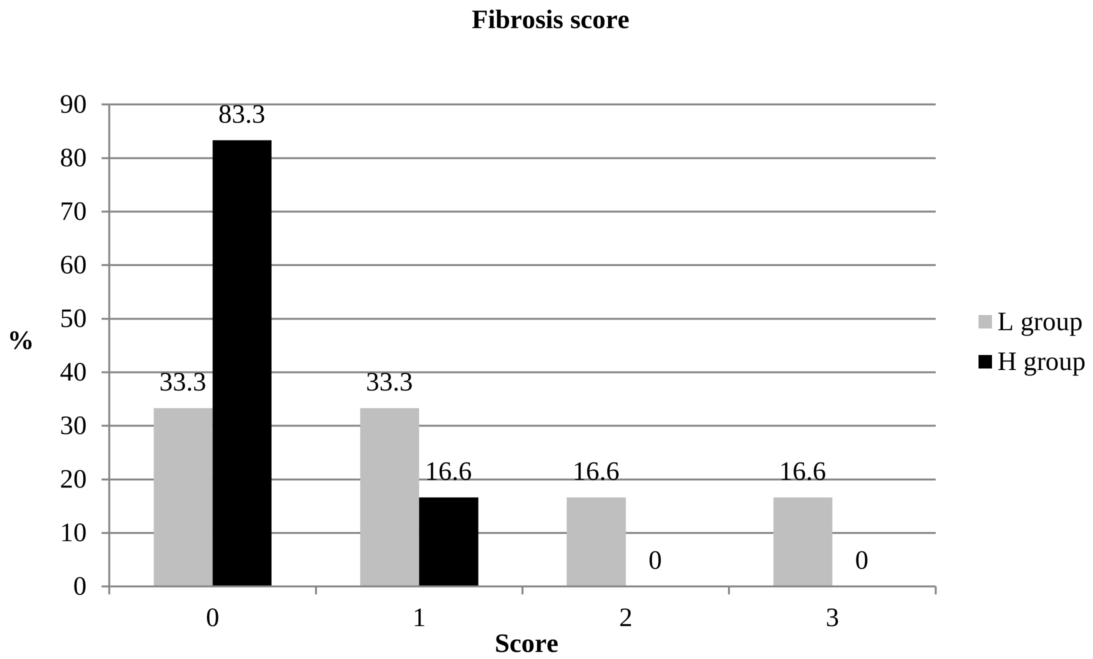

Figure 3.7. Percentage of hens from the L group (the 40 lightest of the CTR) and from the H group (the 40 heaviest of the CRT) in each fibrosis score (at 20 and 25 wk of age). Fibrosis score: (0) absence, (1) low amount, (2) moderate amount and (3) large amount.
CTR (160 Ross broiler breeder hens randomly selected within the FLOCK). FLOCK (1988 Ross broiler breeder hens kept in a standard breeder farm).

Related to these results, it has to be taken into account that damaged tissues may result from mechanical injuries, which can lead to fibrosis as a result of chronic inflammation (Wynn, 2007). On the other hand, a study reported that in 50% of the rupture of the gastrocnemius tendon (**RGT**) cases, the resolving heamatoma and ruptured tendon were also surrounded by fibrous tissue growth, but no inflammatory changes were detected (Dinev, 2008).

Although leg issues have greatly reduced over the last decades due to improved genetics and balanced breeding, development of strong legs is and will always be important when raising broiler breeders. One of the possible issues is RGT, which can appear when management is suboptimal. The rupture happens in the distal part of the proximal region of the tendon, where its insertion along the tarsometatarsal bone starts. The problem was initially associated with viral (reoviral) tenosynovitis (Jones et al., 1981; Kibenge et al., 1982a; Jones and Kibenge, 1984). Later it was suggested that reoviruses could not provoke ruptures by themselves, but required the involvement of other predisposing factors (Duff and Randall, 1986). Likewise, the absence of RGT in male birds, reared together with females, and the negative bacteriological and serological results, support the thesis for a non-infectious etiology (Dinev, 2008). In fact, cases of RGT in the absence of tenosynovitis were observed by other investigators as well (Randall, 1991). Likewise, in some investigations, the tendon rupture was associated with several non-infections factors such as deficiency states, variations in tensile strength, glucosaminoglycan content, etc. (van Walsum et al., 1981; Cook et al., 1983a, b; Bradshaw et al., 2002).

The origin of damaged tendons of the lightest broiler breeder hens might come from feed under consumption, and thus from a lack of the necessary nutrients (deficiency states) to build a tendon able to endure strain and BW at the onset of production. Therefore, to grade the lightest pullets in order to allow them to consume more feed and to recover BW and thus their skeletal development; seems to be important to avoid having RGT issues.

3.5. Conclusions and applications

The results of this study show that the lightest broiler breeder hens (L group), with smaller skeletons and lower BW at 5 wk of age than the heaviest broiler breeder hens (H group), compensate intake in production (25 to 30 wk) until the point of nearly matching the BW of the heaviest hens at 30 wk of age. At this moment bone development has settled. Therefore, feeding the pullets evenly during the rearing period and to provide them with the necessary nutrients to accurately satisfy their requirements is essential to achieve uniform broiler breeder flocks, with a similar skeletal development. This will allow a similar body frame at the beginning of production, which is key to the future reproductive success of the flock.

With regards to mortality, the lightest hens (L group) had the highest accumulated mortality, which occurred mainly in the period from 5 to 15 wk of age, when the weekly feed increase is lower. These pullets may have been the most vulnerable birds, more sensitive to e.g. disease, and thus lighter. During these periods of decreased fitness they will likely consume less feed, grow less and will be lighter pullets, and in a non graded flock may have trouble competing for feed with the heaviest pullets. After grading, the sensitive pullets are together in the same pen, which results in a more equal competition for feed, thus they are able to catch up and build strong bones in time.

As another practical application, ALP and OC have proved to be efficient ways to measure and assess correct bone development of breeder pullets during the first 10 wk of rearing, the phase of major skeletal development. Their serological levels related to correct bone formation are 3839 UI at 5 wk and 2059 UI at 10 wk for ALP and 1346 ng/ml at 5 wk and 1130 ng/ml at 10 wk for OC. Therefore, these two serological markers can be useful as additional information of BW and CV so as to evaluate if the skeletal development in the rearing period of the broiler breeders is correct.

It is also relevant that pullets with BW below standard at 5 wk of age will have shorter, less resistant and more ductile tibias , along with weak tendons, maybe resulting in lower strain resistance and as a result a higher risk of inflammation, fibrosis and breaking. An insufficient intake of nutrients during the initial phases of development of broiler breeders could be related to this inferior bone development and poor tendon structure; and even the lightest hens recovering BW, are not able to recover their skeletal frame and, as the histological analyses show, to obtain a tendon structure to endure strain.

Therefore, one of the main practical applications of this study is to show the importance of grading and grouping the pullets according to BW in order to have uniform broiler breeder flocks during rearing and early in production; and therefore, to prevent having leg health issues.

CHAPTER 4

Effect of diet density and vitamin C inclusion on uniformity, carcass traits, skeletal strength and behavior of broiler breeder pullets

Effect of diet density and vitamin C inclusion on uniformity, carcass traits, skeletal strength and behavior of broiler breeder pullets

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Key words:
Broiler breeders, fiber diluted ration, vitamin C, skeletal strength, alkaline phosphatase and behavior.

Scientific section: NUTRITION AND BEHAVIOR

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4.1. Abstract

To prevent leg health and behavior issues, management and nutritional strategies have been used in the broiler breeder industry. This experiment studied the effect of broiler breeder nutritional strategies on uniformity, carcass traits, skeletal strength and behavior during rearing and pre-breeder periods (to 22 wk of age). The experiment included 4 treatments arranged as a 2 x 2 factorial with two diet densities (control *vs*. fiber diluted diet) and two vitamin C feed inclusions (0 *vs*. 200 mg/kg). Pullets (n = 384) were fed to obtain a similar body weight (**BW**) profile in the four treatments. At 6, 15 and 22 wk blood sampling was carried out to determine alkaline phosphatase serological levels and behavior was observed by visual scan. At 22 wk, samples from two birds per pen were taken to assess carcass traits, tibia and gastrocnemius tendon parameters, to perform histology of gastrocnemius tendon and intestinal mucosa, and to score tail feather integrity. Diluted diet, compared to control diet, did not modify BW uniformity, mortality or tibia and gastrocnemius tendon growth. Pullets fed the fiber diluted diet had lower tibia breaking strength, elastic modulus and ash content values ($P < 0.05$); their breast meat yield was lower (18.5 *vs*. 19.8%, $P = 0.001$) and their fat pad deposition higher (1.14 *vs*. 0.87%, $P = 0.031$). Likewise, at 15 and 22 wk they performed on average 95% less grasping feather pecking and 45% less non-food object pecking behaviors, and their tail feather score was lower at 22 wk ($P < 0.05$). Vitamin C inclusion reduces tail feather score of the control treatment at the same level as did the diluted treatments ($P < 0.05$). Broiler breeders fed fiber diluted diets improve carcass traits, have poorer bone mineral deposition and thus skeletal strength, and show a reduction in stereotypic behavior and an improvement in tail feather integrity. Vitamin C inclusion improves tail feather integrity. Alkaline phosphatase and tail feather score are useful to evaluate bone mineral deposition and stress respectively.

Key words: broiler breeder, diet density, vitamin C, skeletal strength, behavior

4.2. Introduction

Correct management of broiler breeder flocks is essential to prevent larger or aggressive pullets likely out-competing smaller or timid pullets, resulting in unequal access to feed and increasing flock BW variation (Zuidhof et al., 2015). Low uniformity may result in broiler breeder pullets under the advised standard BW, not having received the nutrients required, and thus with poor body development which may lead to leg health and behavior issues, and to difficulties to sustain production.

To dilute the ration daily distributed by adding different concentrations and sources of fibrous raw materials could be a useful management and nutritional strategy to help the pullets to consume uniformly. However the use of these bulky agents has failed to gain widespread acceptance within the broiler breeder industry (Mench, 2002; Sandilands et al., 2006), although some studies have shown promising results (Nielsen et al., 2011; Morrissey et al., 2014).

Likewise, the usefulness of vitamin C, which is not included in poultry premixes, has to be studied as another new nutritional strategy. Its dietary supplementation enhances 1,25-dihydroxyvitamin D (1,25-D) production (Weiser et al., 1988), which influences calcium metabolism and thus affects bone development; and its deficiency results in widespread connective tissue abnormalities, whose origin comes from a disruption in collagen synthesis (Whitehead and Keller, 2003). Vitamin C endogenous synthesis in poultry may not be adequate to always meet the full poultry necessities, specifically during stressful periods, when the requirements may exceed the synthesizing ability (Gous and Morris, 2005).

To evaluate whether the skeletal and bone growth of the broiler breeders during the rearing phase is correct, pullet BW has to be weekly compared to the BW profiles provided by the genetic companies; however, this is an indirect form to assess correct development. Alkaline phosphatase (**ALP**) is a serological marker directly related to skeletal development and in particular to bone formation and growth (Seibel, 2005). ALP is a ubiquitous enzyme attached on the outer cell surface (Stinson and Hamilton, 1994); the precise function of the enzyme is yet unknown, but it obviously plays an important role in osteoid formation and mineralization (Harris, 1990). This serological marker is now an important tool in the assessment and differential diagnosis of metabolic bone diseases in humans; however, it has never been used in broiler breeder pullets to assess development during the rearing period.

Physiological indicators of stress, such as plasma corticosterone concentrations (**PCC**), heterophil to lymphocyte ratio (**H/L**) and white blood cell frequencies (**WBCs**), have been used to evaluate welfare of broiler breeder pullets (Savory et al., 1996;

Hocking et al., 1998, 1999; de Jong et al., 2002; Sandilands et al., 2006). Apart from these analyses, it would be practical to have a test to quickly audit pullet stress in the broiler breeder rearing farms. Morrissey et al. (2014) used feather pecking to evaluate stress and welfare, their study showed that broiler breeders fed fiber diluted diets feather pecked significantly less often. Therefore, since feather pecking results in damaged feather cover, the assessment of tail feather integrity might be a practical way to evaluate stress, and, if necessary, to quickly apply corrective management actions.

The aim of this study is to investigate how nutritional strategies, diets diluted with insoluble fiber and vitamin C supplemented, can affect body development and carcass traits, skeletal strength and behavior. ALP has been proposed as a direct way to assess skeletal development; and tail feather score to assess stress in broiler breeder pullets.

4.3. Material and methods

4.3.1. Birds and facility

All the animal experimentation procedures used in the experiment were approved by the animal Ethics Committee of the Universitat Autònoma de Barcelona and were in compliance with the European Union guidelines for the care and use of animals in research (European Parliament, 2010).

A total of 384 one day old Ross 308 broiler breeder chicks were randomly distributed in 32 pens (12 chicks/pen) in the experimental facility Granja Solé (Vila-rodona, Tarragona, Spain) during a period from 0 to 22 wk of age. Each pen had an area of 1.5 m^2 and contained a bell drinker. Feed and drinker space was sufficient for all the hens to reach feed and water at the same time. Wood shavings were utilized as litter material and litter was topped up weekly to keep it dry.

A vaccination program adapted to the epidemiology of the farm area was followed during the rearing period. Management applied was according to the guidelines of the Ross Parent Stock Management Handbook (Aviagen, 2013).

4.3.2. Experimental design

The experiment included 4 treatments arranged as a 2 x 2 factorial with two diet densities, control diet (CTR) and fiber diluted diet (DIL); and two vitamin C inclusions, diet not vitamin C supplemented (C-) and diet vitamin C supplemented (200 mg/kg) (C+). Therefore, the treatments were CTR/C-, CTR/C+, DIL/C- and DIL/C+ and each of them was replicated 8 times.

Birds were randomly allotted to the different replicates, with an initial BW (g) of 43.2 ± 2.94 and a BW coefficient of variation (**CV**) (%) of 7.1 ± 0.51.

4.3.3. Feeding program, diets and feed intake

Feed was produced in the feed mill of IRTA Mas Bové (Constantí, Tarragona, Spain). A four phase diet feeding program was followed: starter I (the first wk), starter II (from 2 to 6 wk), grower (from 7 to 15 wk) and pre-breeder (from 16 to 22 wk). Starter I was the only identical feed, shared by the four treatments. Feed form presentation was crumble for Starter I, short pellet for starter II and regular pellet for grower and pre-breeder. Feed was always distributed on the floor.

The CTR diets were formulated following the requirements of the broiler breeders for each phase of the rearing and pre-breeding periods (Ross 308 Parent Stock Nutrition Specifications. Aviagen, 2016). Raw materials, source of insoluble fiber, were included in the DIL diets to dilute 15% their AMEn and nutritional values (crude protein and amino acids, digestible phosphorous and calcium, and vitamin and mineral premix). Among these a forage pellet, containing 67% ray grass and 33% barley straw, was used. AMEn estimated value of the forage pellet was 892 kcal, and its nutritional analyzed values were: dry matter 89.7%, CP 11.6%, crude fiber (**CF**) 24.8%, neutral detergent fiber (**NDF**) 48.1%, acid detergent fiber (**ADF**) 30.1%, lignin 8.1%, crude fat 1.3% and ashes 11.7%. In the diets supplemented with vitamin C, 200 mg/kg of a stabilized (phosphorylated) Na/Ca salt of L-ascorbic acid were included.

Ingredients and nutrient composition of the experimental diets are shown in **Table 4.1** and **4.2** respectively.

Table 4.1. Ingredients (%) of the experimental diets.

	Starter I	Starter II		Grower		Pre-breeder	
		CTR	DIL	CTR	DIL	CTR	DIL
Wheat	35.2	39.4	-	43.8	-	43.5	-
Corn	24.9	24.9	36.9	16.3	22.9	24.8	21.8
Soybean meal	25.2	25.5	10.5	-	-	6.3	-
Rye	-	-	12.0	-	28.7	-	33.1
Forage pellet[1]	-	-	5.4	-	16.3	-	11.5
Sunflower 28-30%	5.0	-	15.0	17.0	8.7	12.0	9.7
Wheat bran (high starch)	3.0	5.0	16.1	17.6	20.0	8.0	20.0
Dicalcium phosphate	2.33	2.17	1.70	1.83	1.40	1.53	0.97
Soybean oil	2.00	0.50	-	0.98	-	0.57	-
Limestone	0.97	0.97	0.80	0.90	0.63	1.97	1.63
Premix[2]	0.30	0.50	0.42	0.50	0.42	0.50	0.42
Sodium bicarbonate	0.27	0.31	0.41	0.36	0.40	0.29	0.37
L-lysine sulfate 54.6%	0.24	-	-	-	-	-	-
L-Lysine HCL 65%	-	0.19	0.32	0.22	0.16	0.12	0.15
Salt	0.23	0.20	0.05	0.17	0.05	0.22	0.06
DL-Methionine	0.20	0.22	0.16	0.112	0.11	0.11	0.10
Choline Cl-60%	0.10	0.10	0.10	0.10	0.10	0.07	0.08
L-Threonine	0.05	0.09	0.09	0.10	0.06	0.03	0.03
Xylanase + ß- glucanase	-	0.01	0.01	0.01	0.01	0.01	0.01
Vitamin C mg/kg	200	-	-	-	-	-	-
Vitamin C mg/kg C+[3]	-	200	200	200	200	200	200
Vitamin C mg/kg C-[4]	-	0	0	0	0	0	0

CTR: control diet and DIL: diluted diet.

[1]Forage pellet composition: Ray grass (67%) and barley straw (33%).

[2]Premix provided per kilogram of diet: vitamin A (retinyl acetate), 11000 UI; vitamin D_3 (cholecalciferol), 3500 UI; vitamin E (DL-α-tocopheryl acetate), 100 mg; vitamin K_3, 3.00 mg; Vitamin B_1(thiamin), 3.00 mg; vitamin B_2 (riboflavin), 6.00 mg; vitamin B_5 (pantothenic acid), 13.00 mg; vitamin B_6 (pyridoxine hydrochloride), 4.00 mg; vitamin B_{12} (cyanocobalamin), 0.02 mg; vitamin B_3 (nicotinic acid), 30.00 mg; vitamin B_9 (folic acid), 1.50 mg; vitamin B_7 (biotin), 0.20 mg; iodine (calcium iodate), 1,25 mg; Mn (manganous oxide), 120.00 mg; Cu (cupric sulfate), 16.00 mg; Zn (zinc oxide), 110 mg; Se (sodium selenite), 0.30 mg; Fe (iron sulphate), 40.00 mg.

[3]C+: Diets vitamin C supplemented.

[4]C-: Diets not vitamin C supplemented.

Table 4.2. Metabolizable energy and nutritional composition of the experimental diets.

	Starter I	Starter II		Grower		Pre-breeder	
		CTR	DIL	CTR	DIL	CTR	DIL
AMEn, Kcal/Kg	2824	2804	2399	2600	2240	2700	2295
Dry matter %[1]	87.7	88.0	87.8	88.5	87.4	88.4	88.1
Crude Protein %[1]	17.9	18.3	15.7	13.1	11.8	13.8	11.8
dig Lys %	0.95	0.96	0.81	0.54	0.43	0.54	0.43
dig Met %	0.47	0.48	0.40	0.34	0.28	0.33	0.27
dig M+C %	0.74	0.76	0.63	0.56	0.45	0.56	0.45
dig Thre %	0.64	0.67	0.56	0.46	0.37	0.43	0.34
dig Trp %	0..20	0.20	0.16	0.14	0.11	0.15	0.11
Crude Fiber %[1]	4.53	3.23	8.13	7.21	8.83	5.03	7.49
Neutral detergent fiber %[1]	11.6	10.3	17.6	17.5	19.9	12.2	18.4
Acid detergent fiber %[1]	5.02	3.69	8.25	7.92	9.23	5.17	7.98
Acid detergent lignin %[1]	0.93	0.76	2.42	2.34	2.56	1.60	2.64
Crude Fat %[1]	4.25	2.51	2.89	3.16	2.34	2.72	2.36
Ash %[1]	6.42	5.87	6.44	5.71	6.11	6.40	6.55
Ca %	1.06	1.00	0.88	0.90	0.78	1.22	1.02
dig P %	0.48	0.45	0.40	0.42	0.35	0.36	0.29
Vitamin C mg/kg	117	-	-	-	-	-	-
Vitamin C mg/kg C+[1,2]	-	134	164	152	132	150	117
Vitamin C mg/kg C-[1,3]	-	< 5	< 5	< 5	< 5	< 5	< 5

CTR: control diet and DIL: diluted diet.

[1]Analyzed values.

[2]C+: Diets vitamin C supplemented.

[3]C-: Diets not vitamin C supplemented.

Feed was provided ad libitum during the first wk in order to ensure a correct initial development. From this moment, to follow the standard BW profile, the total daily feed distributed per pen was calculated by multiplying all the pullets present at the moment in the pen by the daily AMEn allowance per bird and divided by the AMEn of the diet.

Both, BW profile and daily AMEn allowance per pullet were based on Ross 308 Parent Stock Performance Objectives 2016.

4.3.4. Collected data, sampling and analytical determinations

4.3.4.1. Body weight, uniformity and mortality

Pullets were individually weighed every week and average BW and BW CV calculated per pen. Mortality was recorded every day and accumulated mortality calculated in order to make comparisons among the different treatments.

4.3.4.2. Feed

Experimental feed samples were collected from the different diets supplied during the rearing and pre-breeder periods of the trial. These samples were ground and stored at 5^{o}C until their analysis. Diet proximate analyses were performed according to the method of AOAC International (2005): dry matter (Method 934.01), crude protein (Method 968.06), crude fiber (Method 962.09), crude fat (Method 2003.05) and ash content (Method 942.05). All the analyses were carried out in the SNiBA laboratory of the UAB (Barcelona, Spain).

4.3.4.3. Carcass traits

At 22 wk, after weighing all the pullets, the 2 closest birds to the average BW of each pen were euthanized to perform measurements and take samples in order to study carcass traits, tibia and gastrocnemius tendon parameters, and intestinal mucosa morphometry.

Breast meat (pectoralis major plus pectoralis minor), abdominal fat pad (surrounding the gizzard and peri-cloacal), empty gastrointestinal tract (from proventricle to cloaca, both included), and oviduct were extracted and weighed to calculate their BW percentage.

4.3.4.4. Intestinal mucosa morphometry

Tissue samples for histology were taken from the proximal portion of the jejunum. Samples were fixed in 10% buffered formalin solution, dehydrated in a graded ethanol series and embedded in paraffin. Sections of 4 µm were stained with haematoxylin and eosin, the method of Hampson (1986) was taken into account. Histomorphological measurements of the intestinal mucosa were carried out; the height of intact villi (distance from the villus tip to the villus-crypt junction) and the depth of the crypts of Lieberkühn (depth of the invagination between adjacent villi) were measured and the ratio between both was calculated. The measurements were taken from digital photographs of the preparations, which were captured in a Leica SCN400 scanner (40x) and the images obtained were analyzed by the Digital Image Hub image analysis software (Leica Microsystem). The samples were evaluated by a specialist in veterinary pathology from the Universidad CEU Cardenal Herrera, Valencia, Spain.

4.3.4.5. Tibia and gastrocnemius tendon parameters

Tibias of right legs were used to determine tibia length (**TL**) and width (**TW**), tibia breaking strength (**BS**) (maximum load endurance), apparent elastic modulus (**EM**) and ash content. TL was measured from the intercondylar eminence to the tip of the lateral malleolus and TW was measured in the narrowest part of the tibia diaphysis. To perform these two measurements a calliper (0.03 mm precision and 0.01 mm resolution) was used. To determine tibia BS and EM, the method of 3-point flexural bending test was carried out (Fleming et al., 1998b); and measurements were made by using a MTS Alliance RT/5 material testing system (force resolution 0.001 N and distance resolution 0.001 mm). These studies were carried out by the IRTA (Institut de Recerca i Tecnologia Agroalimentaria, Girona, Spain). To determine the percentage of ash content, right tibias were boiled and any adherent tissue cleaned off, weighed, dried (109ºC, 12h), chemically cleaned (in acetone for 48h), dried (109ºC, 12h), weighed again, and burned in a muffle-oven overnight (550ºC). This analysis was carried out in the SNiBA laboratory of the UAB (Barcelona, Spain).

Left legs were used to dissect the gastrocnemius muscle tendon in order to carry out measurements and histological analyses to assess the presence of inflammation and fibrosis. The gastrocnemius tendon width (**GTW**) and thickness (**GTT**) were measured between the proximal and distal regions of the tendon, where its insertion with the tarso-metatarsal bone starts. To perform this measurement a calliper (0.03 mm precision and 0.01 mm resolution) was used. Tendon samples were fixed in 10% buffered formalin and routinely processed for histopathology. Slides were cut at 3 μm, stained with hematoxylin-eosin and examined in a “blind-fashion” manner. Fibrosis and inflammation were assessed by using the semi-quantitative approach: (0) absence, (1) low amount, (2) moderate amount, (3) large amount. Inflammation, when present, was composed of mononuclear cells (lymphocytes, plasma cells and few macrophages). The samples were evaluated by a specialist in veterinary pathology (Dipl. ECVP) from the Servei de Diagnòstic de Patologia Veterinaria of the UAB (Barcelona, Spain).

4.3.4.6. Alkaline phosphatase

To determine ALP, the 4 closest pullets to the average BW of each pen were chosen at 6 wk and blood sampled at 6, 15 and 22 wk of age. Blood samples were spun at 3000 rpm during 20 min to obtain the serum, which was stored at -20^{o}C until the moment to be tested. To test ALP a colorimetric method based on catalytic transformation of p-nitrophenylphosphate as substrate (IFCC method) on the Beckman Coulter AU analyzer was used. ALP analyses were carried out by the Servei de Bioquímica Clínica Veterinària (SBCV) of the UAB (Barcelona, Spain).

4.3.4.7. Behavior

At 6, 15 and 22 wk of age, behavior of the pullets was observed by using an instantaneous visual scan sampling technique, the method performed by Hocking et al. (2004) was taken into account. Grasping feather pecking (**GFP**) (grasping tail and back feathers of other pullets) and non-food object pecking (**NFOP**) (pecking at drinkers and pen walls) behaviors were observed during two 20-min sessions a day, once during the morning (9h.30 to 12h.30) and once in the afternoon (13h.30 to 16h.30). Observations were made per block of four pens at one minute intervals, in a random order, after all feeding related activities had ceased and after allowing 5 min for the birds to adjust to the presence of the observer. The number of chicks engaged in each behavioral category was recorded and expressed in proportion to the total number of active animals. All observations were performed by the same observer.

The integrity of the tail feathers of all the pullets was scored at 22 wk of age. A feather score between 0 and 5 was used: (0) fully feathered, (1) rough, (2) some broken feathers and small bald areas, (3) heavily broken feathers and some bald areas, (4) almost bald/large bald areas and (5) bald (no feather cover). To perform this score the Protocol for Scoring the Feather Cover of Broiler Breeders during the Laying Period (Aviagen, 2010) was taken into account.

4.3.5. Statistical analysis

The statistical analysis was performed with the R® program (version 3.3.3, 2017). Factorial ANOVA was carried out to analyze BW, BW CV, mortality, ALP, carcass traits, tibia ash content, intestinal mucosa morphometry, GFP and NFOP. The factors were diet density (CTR and DIL) and VC inclusion (C- and C+). Means were compared through a Tukey test. ALP evolution was analyzed using a mixed model; diet density (CTR and DIL) and VC inclusion (C- and C+) were fixed effects in interaction with the linear and quadratic coefficients of the week, the bird being a random effect. A Kruskal-Wallis test was used to analyze gastrocnemius tendon fibrosis and inflammation, and tail feather cover; the factors were diet density (CTR and DIL) and VC inclusion (C- and C+). Means were compared through a Wilcoxon test. In all cases, *P*-values lower than 0.05 were considered to be significant.

4.4. Results

4.4.1. Body weight, uniformity and mortality

Table 4.3 shows that BW and BW CV at 6, 15 and 22 wk and accumulated mortality at 22 wk of age were not affected by diet density or vitamin C inclusion; there was no interaction either ($P > 0.05$). BW CV between the pullets fed the CTR and DIL diets were similar at 6 wk (11.36 and 11.59 %, $P = 0.818$), 15 wk (9.71 and 10.32 %, $P = 0.449$) and 22 wk (8.68 and 9.02 %, $P = 0.714$).

Table 4.3. Effects of diet density and vitamin C inclusion on body weight (BW) and body weight coefficient of variation (CV) at 6, 15 and 22 wk of age; and on % mortality at 22 wk of age.

	BW (g)[1]			BW CV (%)[2]			% mort (%)[2]
Effects	6 wk	15 wk	22 wk	6 wk	15 wk	22 wk	22 wk
Diet density							
CTR	700	1831	2693	11.36	9.71	8.68	3.65
DIL	687	1818	2684	11.59	10.32	9.02	2.08
Vitamin C							
C-	695	1831	2692	10.56	10.32	9.24	0.52
C+	691	1818	2685	12.39	9.72	8.46	5.21
SEM	5.2	12.0	16.0	0.213	0.572	0.644	1.691
P-values							
Diet density	0.085	0.460	0.692	0.818	0.449	0.714	0.518
Vitamin C	0.565	0.443	0.804	0.081	0.461	0.393	0.059
Interaction	0.309	0.892	0.730	0.281	0.180	0.258	0.829

CTR: control diet, and DIL: diluted diet.

C-: not vitamin C supplemented diet, and C+: vitamin C supplemented diet.

[1]n=192 for diet density and vitamin C effects and n=96 for the interaction.

[2]n=16 for diet density and vitamin C effects and n=8 for the interaction.

$P < 0.05$ was considered significant.

4.4.2. Feed, nutrient and energy intake

Feed intake between 0 and 22 wk of age is shown in **Figure 4.1**. After weekly adjustments to follow the standard BW profile in all treatments, accumulated feed intake (g) at 22 wk of the DIL treatments was 17.3% higher than that of the CTR (DIL/C-: 12431 ± 9.9 and DIL/C+: 12407 ± 8.8 *vs*. CTR/C-: 10268 ± 52.5 and CTR/C+: 10264 ± 36.0) (*P* < .0001).

The analyzed values of the experimental diets showed that on average the diluted rations had 12.9% less CP, 8.5% less crude fat, and 37.5% more CF, 29.1% more NDF, 34.7% more ADF and 38.9% lignin. Vitamin C supplemented diets of the treatments CTR/C+ and DIL/C+ presented an average vitamin C concentration (mg/kg) of 138 ± 18.2; whereas in the not supplemented diets of the treatments CTR/C- and DIL/C-, vitamin C concentration (mg/kg) was < 5 (mg/kg).

Table 4.4 shows AMEn and CP consumption per BW (g) at 22 wk of age. Pullets fed the DIL diet consumed more energy (10.8 *vs*. 10.4 kcal, *P* < .0001) and also more CP (0.*58 vs*. 0.55 g, *P* < .0001) per BW (g), compared to those fed the CTR diet.

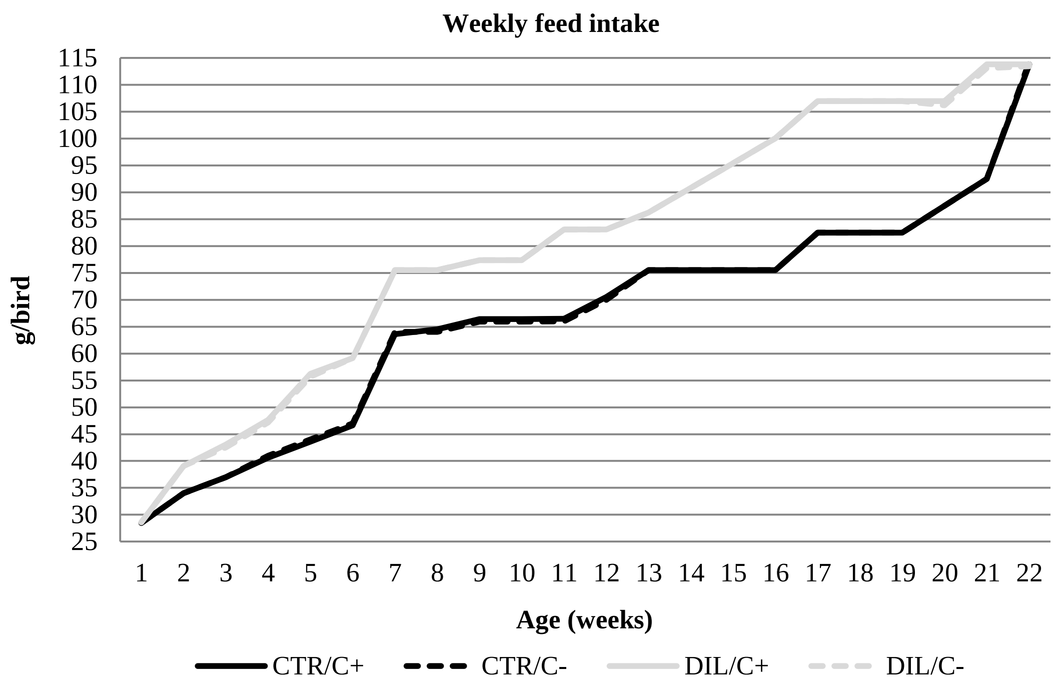


Figure 4.1. Weekly feed intake of the broiler breeder pullets from 0 to 22 wk of age depending on diet density and vitamin C inclusion.
Values are pooled means of 8 replicates per treatment.
CTR/C-: control diet not vitamin C supplemented, CTR/C+: control diet vitamin C supplemented, DIL/C-: diluted diet not vitamin C supplemented and DIL/C+: diluted diet vitamin C supplemented.

Table 4.4. Effects of diet density and vitamin C inclusion on AMEn (kcal) and CP (g) consumption per body weight (BW, g) at 22 wk of age.

Effect	AMEn:BW (kcal/g)	CP:BW (g/g)
Diet density		
CTR	10.4	0.55
DIL	10.8	0.58
Vitamin C		
C-	10.6	0.57
C+	10.6	0.57
SEM (n=16)	0.04	0.002
P-values		
Diet density	<.0001	<.0001
Vitamin C	0.589	0.896
Interaction	0.495	0.400

CTR: control diet, and DIL: diluted diet.

C-: not vitamin C supplemented diet, and C+: vitamin C supplemented diet.

P < 0.05 was considered significant.

4.4.3. Carcass traits

The effects of diet density and vitamin C inclusion on carcass traits (relative to BW) at 22 wk of age are shown in **Table 4.5**. There was no vitamin C inclusion effect, but pullets fed the DIL diet, compared to those fed the CTR diet, had lower breast meat yield (18.5 *vs*. 19.8 %, *P* = 0.001), and higher abdominal fat pad deposition (1.14 *vs*. 0.87 %, *P* = 0.031), total empty gastrointestinal tract (5.07 *vs.* 4.63 %, *P* = 0.001), proventricle plus gizzard (2.04 *vs*. 1.94 %, *P* = 0.035) and caecum plus rectum (0.90 *vs.* 0.64 %, *P* <.0001). However, there was not a significant difference among the pullets fed the DIL diet and those fed the CTR diet, neither for small intestine (2.13 and 2.05 %, *P* = 0.136) nor for oviduct (12.5 and 16.2 g/10 kg BW, *P* = 0.227).

Table 4.5. Effects of diet density and vitamin C inclusion on carcass traits (% BW, oviduct: g/10 Kg BW) at 22 wk of age.

	Carcass traits						
Effects	Breast meat	Abdominal fat pad	Total gastrointestinal tract	Proventricle plus gizzard	Small intestine	Caecum plus rectum	Oviduct
Diet density							
CTR	19.8	0.87	4.63	1.94	2.05	0.64	16.2
DIL	18.5	1.14	5.07	2.04	2.13	0.90	12.5
Vitamin C							
C-	19.0	1.03	4.88	1.97	2.12	0.79	13.4
C+	19.3	0.98	4.81	2.02	2.08	0.74	15.3
SEM (n=32)	0.23	0.082	0.081	0.033	0.042	0.041	2.12
P-values							
Diet density	0.001	0.031	0.001	0.035	0.136	<.0001	0.227
Vitamin C	0.345	0.601	0.526	0.368	0.265	0.343	0.536
Interaction	0.595	0.105	0.897	0.530	0.365	0.895	0.439

CTR: control diet, and DIL: diluted diet.

C-: not vitamin C supplemented diet, and C+: vitamin C supplemented diet.

$P < 0.05$ was considered significant.

4.4.4. Intestinal mucosa morphometry

Histomorphological parameters of the intestinal mucosa of proximal portion of the jejunum are shown in **Table 4.6**. There was neither diet density nor vitamin C inclusion effect on the intestinal morphology: villus height, Lieberkühn crypt depth and their ratio.

Table 4.6. Effects of diet density and vitamin C inclusion on the histomorphological parameters of the intestinal mucosa of proximal portion of the jejunum at 22 wk of age.

Effects	Villi height (µm)	Lieberkühn crypt depth (µm)	Ratio height/depth
Diet density			
CTR	974	160	6.12
DIL	935	166	5.67
Vitamin C			
C-	982	162	6.00
C+	925	165	5.73
SEM (n=21)	32.6	3.5	0.250
P-values			
Diet density	0.471	0.411	0.323
Vitamin C	0.318	0.618	0.570
Interaction	0.955	0.884	0.905

CTR: control diet, and DIL: diluted diet.

C-: not vitamin C supplemented diet, and C+: vitamin C supplemented diet.

$P < 0.05$ was considered significant.

4.4.5. Tibia and gastrocnemius tendon parameters

The effect of diet density and vitamin C inclusion on tibia BS, EM and ash content at 22 wk of age is shown in **Table 4.7**. There was no vitamin C inclusion effect, but tibias from the pullets fed the CTR diet, compared to those fed the DIL diet, were able to endure more weight before breaking, their BS values were higher (45138 *vs*. 41422 gf/mm^2, $P = 0.009$), were also more rigid or less ductile, their EM values were higher

(43.3 *vs*. 36.4 kgf/mm^2, P = 0.001), and had higher ash content (41.8 *vs*. 40.0 %, P = 0.001).

Table 4.7. Effects of diet density and vitamin C inclusion on tibia breaking strength (BS), elastic modulus (EM) and ash content at 22 wk of age.

Effects	BS (gf/mm^2)	EM (kgf/mm^2)	Ash content (%)
Diet density			
CTR	45138	43.3	41.8
DIL	41422	36.4	40.0
Vitamin C			
C-	43516	40.7	41.2
C+	43044	39.0	40.6
SEM (n=32)	972.3	1.26	0.37
P-values			
Diet density	0.009	0.001	0.001
Vitamin C	0.732	0.351	0.240
Interaction	0.988	0.632	0.760

CTR: control diet, and DIL: diluted diet.

C-: not vitamin C supplemented diet, and C+: vitamin C supplemented diet.

P < 0.05 was considered significant.

Regarding tibia and gastrocnemius tendon measurements, no effect of diet density and vitamin C inclusion was found. Pullets fed the CTR diet, compared to those fed the DIL diet, had neither significant differences in tibia values TL and TW (TL: 130 and 131 mm, P = 0.147; TW: 7.01 and 7.09 mm, P = 0.277) nor in tendon values GTW and GTT (GTW: 7.46 and 7.57 mm, P = 0.510; GTT: 1.12 and 1.11 mm, P = 0.708) at 22 wk of age.

There was no diet density or vitamin C inclusion effect, nor interaction (P > 0.05), on gastrocnemius tendon inflammation. Pullets fed the DIL diet had not a significantly different score from those fed the CTR diet (DIL: 0.09 and CTR: 0.03, P = 0.529), nor did pullets fed a vitamin C supplemented diet related to those not fed vitamin C (C+: 0.03 and C-: 0.09, P = 0.529). Inflammation was observed in just two pullets from the treatments: CTR/C+ (score 3) and DIL/C- (score 1). Likewise, there was no diet density or vitamin C inclusion effect, nor interaction ($P > 0.05$), on gastrocnemius tendon fibrosis. Pullets fed the DIL diet had not a significantly different score from those fed the CTR diet (DIL: 0.09 and CTR: 0.06, $P = 0.647$), nor did pullets fed a vitamin C supplemented diet related to those not fed vitamin C (C+: 0.12 and C-: 0.03, $P = 0.173$). Fibrosis was also observed in a low number of pullets from the treatments: CTR/C+ (1 bird, score 1), DIL/C- (2 birds, score 1 both) and DIL/C+ (2 birds, score 1 both). Neither inflammation nor fibrosis was detected in the CTR/C- treatment.

4.4.6. Alkaline phosphatase

The effect of diet density and vitamin C inclusion on ALP serological levels at 6, 15 and 22 wk is shown in **Table 4.8**.

Table 4.8. Effects of diet density and vitamin C inclusion on alkaline phosphatase (ALP) serological level at 6, 15 and 22 wk of age.

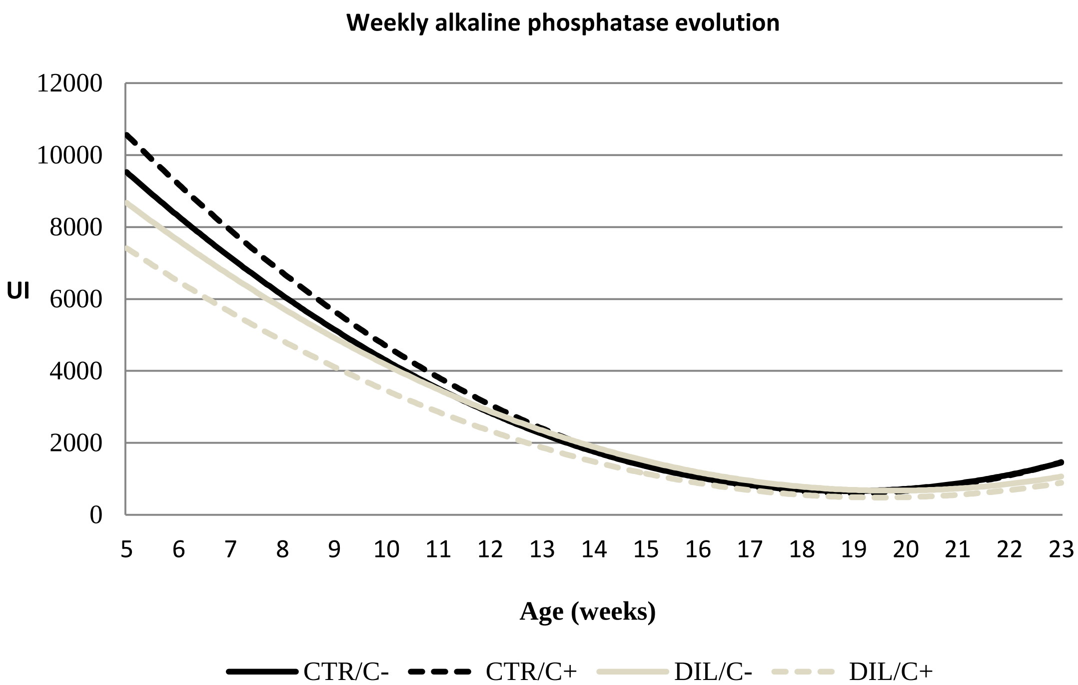
	ALP (UI)		
Effects	6 wk	15 wk	22 wk
Diet density			
CTR	10045	1371	1107
DIL	8042	1344	775
Vitamin C			
C-	8989	1408	987
C+	9098	1304	890
SEM (n=64)	424.5	61.4	41.7
P-values			
Diet density	0.001	0.765	<.0001
Vitamin C	0.856	0.247	0.114
Interaction	0.058	0.104	0.220

CTR: control diet, and DIL: diluted diet.

C-: not vitamin C supplemented diet, and C+: vitamin C supplemented diet.

$P < 0.05$ was considered significant.

At 6 wk and 22 wk, there was no vitamin C inclusion effect, but pullets fed the CTR diet, compared to those fed the DIL diet, had higher ALP serological levels (6 wk: 10045 *vs.* 8042 UI, $P = 0.001$; 22 wk: 1107 *vs.* 775 UI, $P < .0001$). However, at 15 wk there was neither diet density nor vitamin C inclusion effect on ALP serological level. **Figure 4.2** shows the quadratic regression curves of the ALP serological levels of the four treatments (CTR/C-, CTR/C+, DIL/C- and DIL/C+), which permits the prediction of their ALP values throughout rearing and pre-breeder periods. Curves of the treatments CTR/C-, CTR/C+ and DIL/C- do not differ significantly ($P > 0.05$), but curves of the treatments CTR/C- and DIL/C+ differ significantly ($P < 0.05$).



Treatments	Equations	*P*-values
CTR/C-	$y = 46.11x^2 – 1739.63x + 17070.86$	
CTR/C+	$y = 51.48x^2 – 1947.03x + 19013.24$	0.232
DIL/C-	$y = 36.81x^2 – 1453.32x + 15017.51$	0.207
DIL/C+	$y = 33.02x^2 – 1287.04x + 13022.98$	0.014

***Figure 4.2**. Age evolution (6 to 22 wk of age) of alkaline phosphatase serological levels (UI) depending on diet density and vitamin C inclusion.*
CTR/C-: control diet not vitamin C supplemented, CTR/C+: control diet vitamin C supplemented, DIL/C-: diluted diet not vitamin C supplemented and DIL/C+: diluted diet vitamin C supplemented.
P-values are related to CTR/C-. P < 0.05 was considered significant.
n = 32

4.4.7. Behavior and tail feather integrity

The effect of diet density and vitamin C inclusion on bird behavior (GFP and NFOP) at 6, 15 and 22 wk, and on tail feather score at 22 wk of age is shown in **Table 4.9**.

Table 4.9. Effects of diet density and vitamin C inclusion on the percentage of birds performing grasping feather pecking (GFP) and non-food object pecking (NFOP) at 6, 15 and 22 wk; and on tail feather score at 22 wk of age.

	GFP[1]			NFOP[1]			Tail feather score[2]
Effects	6 wk	15 wk	22 wk	6 wk	15 wk	22 wk	22 wk
Diet density							
CTR	0	7.74	5.17	12.4	24.0	35.1	0.73
DIL	0	0.29	0.05	12.9	14.1	18.5	0.20
Vitamin C							
C-	0	5.83	3.73	13.4	18.4	28.2	0.66
C+	0	2.20	1.49	11.9	19.7	25.4	0.25
Treatments							
CTR/C-	0	11.53	7.46	14.6	22.4	35.2	1.15[a]
CTR/C+	0	3.96	2.88	10.2	25.7	35.1	0.30[b]
DIL/C-	0	0.14	0	12.2	14.4	21.2	0.19[b]
DIL/C+	0	0.45	0.10	13.6	13.7	15.8	0.21[b]
SEM	0	3.070	1.927	3.32	3.41	4.23	0.050
P-values							
Diet density	0	0.018	0.010	0.888	0.005	0.001	<.0001
Vitamin C	0	0.242	0.250	0.658	0.707	0.516	<.0001
Interaction	0	0.202	0.227	0.381	0.566	0.529	<.0001

[a,b]Means within a column and within a source with no common superscript differ significantly.

Tail feather score: (0) fully feathered, (1) rough, (2) some broken feathers and small bald areas, (3) heavily broken feathers and some bald areas, (4) almost bald/large bald areas and (5) bald (no feather cover).

Treatments: CTR/C-: control diet not vitamin C supplemented, CTR/C+: control diet vitamin C supplemented, DIL/C-: diluted diet not vitamin C supplemented and DIL/C+: diluted diet vitamin C supplemented.

[1]n=32 for diet density and vitamin C effects and n= 16 for the interaction.

[2]n=192 for diet density and vitamin C effects and n=96 for the interaction.

$P < 0.05$ was considered significant.

At 6 wk pullets did not perform GFP at all, and there was no diet density or vitamin C inclusion effect on NFOP; at 15 wk pullets fed the DIL diet, compared to those fed the CTR diet, showed lower GFP (0.29 *vs.* 7.74 %, P = 0.018) and lower NFOP (14.1 *vs.* 24.0 %, P = 0.005); and likewise, at 22 wk pullets fed the DIL diet showed lower GFP (0.05 *vs*. 5.17 %, P = 0.010) and lower NFOP (18.5 *vs*. 35.1 %, P = 0.001). Related to tail feather score, an interaction (P < .0001) shows that there are no significant differences among the CTR/C+, DIL/C+ and DIL/C- treatments; their scores were lower and significantly different compared to that of the CTR/C- treatment.

4.5. Discussion

Pullets from the CTR and DIL treatments had similar BW profiles throughout the trial. However, to obtain this target, the energy and protein consumed / g BW by the pullets fed the diluted diet were respectively 3.91 and 5.74% higher than the control group. Contrary to the results of this study, Zuidhof et al. (2015) diluted 25% a broiler breeder pullet diet by using oat hulls with the result of better nutrient efficiency; energy and protein consumed / g BW were 4.8 and 6.0% lower than the control group.

Feed dilution did not influence BW CV, the uniformities of the DIL and CTR treatments being similar at 22 wk of age. One of the objectives of diluted diets is to increase feed allocation and thus to reduce feed competition and improve uniformity; however, the results of this study show that feed dilution does not enhance uniformity, and are congruent with those of other authors (Zuidhof et al., 2015; de los Mozos et al., 2017). Likewise, mortality is not significantly reduced either by feed dilution, which is also congruent with the results of de los Mozos et al. (2017) study. Therefore, more feed allocation per pullet, which is supposed to reduce pullet competition, has no influence on reducing mortality or increasing flock uniformity.

Diet dilution affected final carcass traits. Relative weights of abdominal fat pad, empty gastrointestinal tract, proventricle plus gizzard and caecum plus rectum at 22 wk of age were higher in the pullets fed the diluted diet; however, breast meat yield was lower. Nowadays, the target is to control excess of breast deposition and to stimulate abdominal fat pad deposition before the onset of production, since fat content in rearing period is related to egg persistence in the second half of the production phase (van

Emous et al., 2015). Therefore, to have the pullets fed the diluted diet, less breast meat yield and more abdominal fat pad, is an important advantage of this feed strategy. The higher development of proventricle plus gizzard, could be explained since the high lignin content of most insoluble fibre sources leads to a longer retention of the feed in the gizzard, improving its muscular development and thus its function (Hetland and Svihus, 2001; Hetland et al., 2003; Jiménez-Moreno et al., 2010). Likewise, Sacranie et al. (2012) studied broiler chickens exposed to basal diets diluted with coarse hulls or the same hulls finely ground. The large particle size of the coarse hulls and their hardness as a result of the insoluble fiber content, explained why birds consuming the coarse hull diet developed the heaviest gizzards. However, birds fed the fine hull diet also exhibited heavier gizzards than the control group, although less so than those of the coarse hull group.

Pullets fed the diluted diet had tibias with significantly lower BS, EM and ash content. These results might signal poor bone mineral deposition early in rearing, since it is stated that 90% of the skeleton of the broiler breeders is already developed at 11-13 wk of age (Ross Parent Stock Management Handbook. Aviagen, 2013). BS represents maximum load endurance, less mineralized bones have lower values; EM reflects the intrinsic stiffness or rigidity of the bone, less mineralized bones have also lower values (Turner and Burr, 1993); and since bone mineralization provides compressional strength to bone, the bone ash content has been used as an indicator of bone strength (Rath el al., 2000). The results of the present study suggest that skeletal strength of the pullets fed diluted diets is poorer and thus mineral absorption could have been impaired. This would not be related to changes in the intestinal morphology, villus height and Lieberkühn crypt depth, produced by the diet diluted with fiber raw materials, since the results of the present study show no effect of diet density on these parameters. However, Enting et al. (2007a) found that low-density diets resulted in shorter mean retention times in almost all sections of the gastrointestinal tract, and according to results of Leeson at al. (1991), the shorter mean retention times seem to be related to an increase in the insoluble fiber content of the low-density diets. Furthermore, the lower time that digesta spends in the jejunum and ileum is negatively related to the amount of Ca and P absorbed by paracellular absorption, passive and non-saturable way (Fujita et al., 2008; Adedokun and Adeola, 2013).

There are no studies which have used ALP to assess skeletal development (bone mineral deposition) in broiler breeder pullets in rearing. However, in chickens, elevated ALP activity has been predominantly related to increased osteoblastic activity and used as a marker for evaluating skeletal health and bone disease, such as skeletal growth, rickets, fracture repair and osteomyelitis (Lumeij and Westerhof, 1987). More recently, Ekmay et al. (2012) used ALP serological levels to evaluate broiler breeder growth and bone mineral deposition during transition into sexual maturity (24 to 26 wk of age). In the present study, pullets fed the diluted diet had lower ALP serological levels at 6 and 22 wk, which suggest that their bone mineral deposition during the starter and pre-breeder periods was poorer compared to the control. It has to be highlighted that ALP serological level is directly related to bone development and mineral deposition; and thus it is a better indicator of skeletal development than BW, which is an indirect indicator.

In the present study diet dilution does not affect bone growth; TL and TW were not affected. However, ALP serological levels at 6 wk, and BS, EM and ash content at 22 wk of age signal poorer bone mineral deposition of the birds fed the diluted diet (lower ALP serum values and lower BS and EM parameters, and ash content of the tibias). Therefore, results show that feed dilution does not affect skeletal growth, however mineral deposition is affected.

On the other hand, and related to the gastrocnemius tendon, the histological analyses showed no effect of diet dilution; there were no structural differences of the tendons among treatments and inflammation and fibrosis were almost absent. Therefore, pullets from all the four treatments had a tendon structure capable of enduring the increase of weight during the rearing and pre-breeder periods without suffering injuries. In fact, damaged tissues may result from mechanical injuries, which can lead to fibrosis as a result of chronic inflammation (Wynn, 2007).

Pullets fed the fiber diluted diet showed a reduction on GFP and NFOP behaviors at 15 and 22 wk of age. This is in agreement with other study results, which show a reduction in stereotypic behavior in broiler breeders fed diluted diets with fibrous raw materials (Zuidhof et al., 1995; de Jong et al., 2005). Hocking et al. (2004) observed decreased spot pecking in broiler breeders fed diets with 20% oat hulls and 5% sugar beet pulp. In the present study GFP reduction is related to tail feather integrity; in fact, birds fed the diluted diet showed lower GFP behavior and had also lower tail feather score. Likewise, in the case of tail feather score, an interaction shows that vitamin C inclusion in the control diet is able to enhance tail feather integrity at the same level as the diluted diet. Several studies show that vitamin C could help birds to cope with stressful situations (Satterlee et al., 1993, 1994; Jones et al., 1996, 1999).

4.6. Conclusions

Low-density diets, formulated with raw materials which are a source of insoluble fiber, do not modify broiler breeder pullet uniformity and mortality percentage. Pullets fed diluted diets have less breast meat yield and more abdominal fat pad, which is a great advantage for later production; their grasping feather pecking and non-food object pecking behaviors are lower and thus their tail feather integrity is better, which means they undergo less stress; however, even their skeletal growth not being modified, their bone mineral content and thus skeletal strength are poorer. Vitamin C inclusion does not affect skeletal strength, but is effective in improving tail feather integrity. It can be concluded, that diluted diets improve carcass traits and reduce stress, ALP could be a direct way to assess bone mineral deposition, and the tail feather score could be a practical test to quickly assess stress in the rearing farms.

CHAPTER 5

General discussion

Good management is essential in the farms of broiler breeders to give all the birds the same opportunities to consume. A flock not well managed, and thus with poor uniformity, may contain pullets under the advised standard body weight (**BW**) (birds underweight), not having received the nutrients required, and even lead to leg health and welfare issues.

Therefore, underweight pullets may have nutritional deficiencies which cause non-infectious leg health issues: varus&valgus, rickets or rupture of the gastrocnemius tendon (**RGT**). Varus&valgus appears late in rearing and affects mainly males, rickets happens mainly in the period from 5 to 15 wk of age, when the weekly feed increase is lower; and RGT affects females early in production. The three of them may cause important production and economic losses to the broiler breeder producers.

Likewise, pullets which do not receive the nutrients required perform stereotypic behavior, such as grasping feather pecking (**GFP**) or non-food object pecking (**NFOP**). These behaviors are not natural; they are a sign of stress and potential welfare issues. Feather pecking damages feather cover, which protects females from getting damaged during mating, and thus it has to be avoided to obtain correct hatchability, mainly late in production.

In order to avoid competition among the broiler breeder pullets and thus leg health and welfare issues, different management and nutritional strategies can be used.

Correct stocking density, enough feed and water space, uniform feed distribution and grading, are important management tools to reduce competition, to allow the birds to eat and drink at the same time, and to avoid nutritional deficiencies. Grading involves segregation of the pullets into pens or houses left empty at placement for this purpose. Birds are sorted into 2 or 3 sub-populations of different average weight. The objective is to feed the different graded populations to match their requirements and to maintain their coefficients of variations (**CV**) lower than10%. Therefore, as grading is important to allow the birds to consume uniformly and thus to avoid some of them going through under-consumption periods, in this thesis it was proposed to study how grading can influence leg health issues.

Other possible ways to prevent leg health and welfare issues could be the nutritional strategies. For this reason, it was decided to study how diet dilution, which is a strategy to increase daily feed allocation and to reduce feed competition, affects BW CV in order to avoid having underweight birds which could suffer nutritional deficiencies and stress. Likewise, due to the relevance of vitamin C in skeletal development (enhancement of 1,25-dihydroxyvitamin D (1,25-D) production) and due to its role in helping birds to cope with stressful situations (welfare involvement), it was tested as another nutritional strategy. The idea of the possible usefulness of vitamin C supplementation was supported by the fact that under stress, its endogenous synthesis might not be sufficient.

In this thesis, it was decided to carry out a field trial (**chapter 3**) with broiler breeders under commercial conditions. The objective was to study BW and BW CV evolution, and skeletal development of non-graded pullets. A second trial, with broiler breeders under experimental conditions (**chapter 4**), was designed to study the effect of diet dilution and vitamin C inclusion on pullet uniformity, carcass traits, skeletal strength and stereotypic behavior. Alkaline phosphatase (**ALP**) and osteocalcin (**OC**), serological markers of bone growth and mineralization, were proposed as tests to directly assess skeletal development; and wing and tail feather scores as tests to assess stress in the rearing farms.

5.1. Body weight, body weight uniformity and mortality

If flocks are not uniform, feed allocation might be excessive for the light birds with smaller skeletons (fed over their requirements) and insufficient for the heavy birds (fed under their requirements). Therefore, management to provide fair competition and reduce aggression among birds is essential to feed the birds evenly and thus reduce BW variability and mortality.

Since BW and uniformity are so important in broiler breeder production, the field trial (**chapter 3**), was designed to study the effect of low uniformity and thus underweight pullets on skeletal development. The trial showed some interesting results related to broiler breeder BW evolution. During the rearing and pre-breeder periods the lightest pullets had a BW significantly different from the heaviest, but at 30 wk of age their BWs almost matched and were not significantly different (light: 3943 and heavy: 3994 g, respectively). Likewise, the trial showed that from 10 to 25 wk of age the graded pullets had a CV lower than 10% (which is the target), whereas the non-graded pullets were over 10%; however, within the group of non-graded pullets the lightest were over 10% and the heaviest under 10%. It has to be highlighted that at 30 wk of age the CVs of the graded and non-graded birds were under 10%, even that of the lightest birds. The fact that at 30 wk of age BW and BW CV of the lightest and heaviest birds almost matched might be explained since at the onset of production breeders are moved to the production farm where stock density is lower, thus hens have more feed availability, more feed space and a longer clean-up time; therefore, competition among birds decreases and the lightest birds might obtain more feed. However, the results of this trial also showed that the lightest hens even being able to recover BW and BW CV were not able to recover tibia length (**TL**); hence, they had similar BW to the heaviest hens at 30 wk of age but their skeleton was smaller.

To avoid mortality is one of the main objectives of good broiler breeder management. The field trial showed lower mortality at the onset of production (25 wk) of the graded birds compared to the non-graded (8.1 and 10.6%, respectively). It is relevant that within the group of non-graded birds, the mortality of the heaviest was much lower than that of the lightest (2.5 and 20.0%, respectively); in fact, the heaviest pullets did not present mortality at all from 5 to 20 wk of age.

In the experimental trial (**chapter 4**), the standard BW profile was followed to avoid BW differences among treatments. **Figure 5.1** shows BW profiles of the four treatments (diluted and vitamin C supplemented) throughout rearing and pre-breeder periods; the figure shows that the objective of avoiding BW differences among treatments was achieved.

One of the objectives of the experimental trial was to study the effect of diet dilution on BW variation and mortality. Broiler breeders were fed diets with fiber raw materials; a forage pellet (67% ray grass and 33% barley straw) was used. Diet dilution increases feed allocation, birds fed diluted diets consumed 17.34% more feed than those of the control, and supposedly reduces competition among pullets. However, results showed that feed dilution did not modify BW CV throughout the trial, and at 22 wk of age the birds fed the diluted diet had similar BW CV than those fed the control (9.02 and 8.68%, respectively). Likewise, mortality was not significantly different, which is congruent with the result of de los Mozos et al. (2017) study, where fiber diluted rations did not affect broiler breeder mortality either.

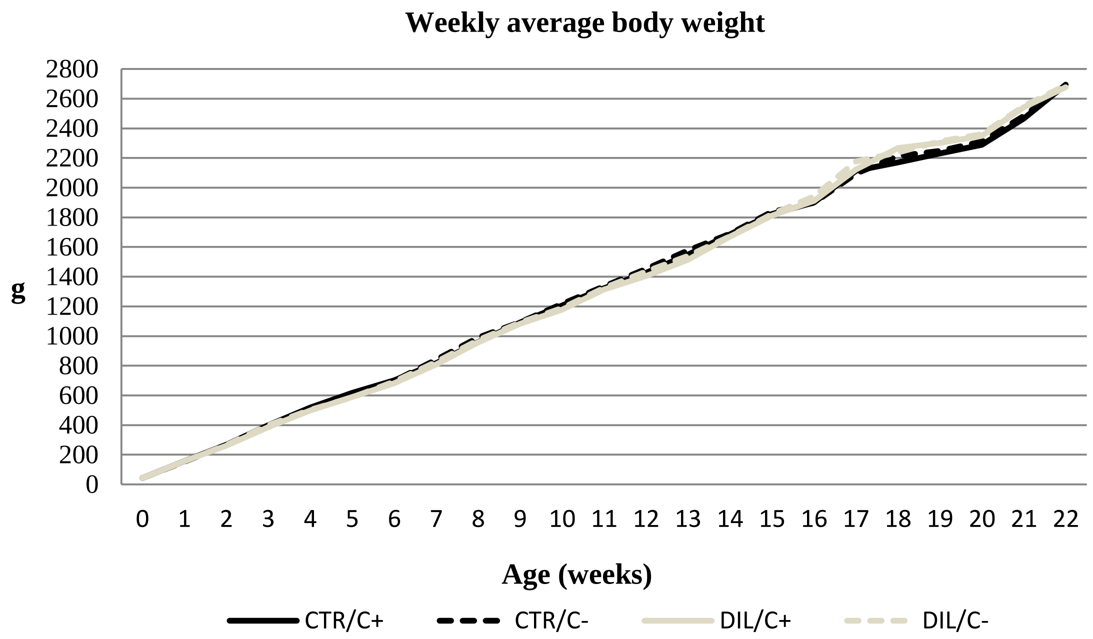


Figure 5.1. Weekly average body weight of the broiler breeder pullets from 0 to 22 wk of age depending on the treatment (means of 8 pens per treatment). Experimental trial (chapter 4).
CTR/C+: control diet vitamin C supplemented, CTR/C-: control diet not vitamin C supplemented, DIL/C+: diluted diet vitamin C supplemented and DIL/C-: diluted diet not vitamin C supplemented.

Therefore, in field conditions, grading reduces BW CV and mortality of the lightest birds of a flock. In the absence of grading in rearing, uniformity will be lower as probably the lightest pullets would consume below their requirements; and to obtain correct uniformity it would be only necessary to separate the lightest pullets, while the sub-populations with the heaviest and medium BW pullets may stay together. In experimental conditions, more feed allocation per pullet, which is supposed to reduce pullet competition, has no influence neither increasing flock uniformity, which is congruent with the study results from the authors Zuidhof et al. (2015) and de los Mozos et al. (2017), nor reducing mortality. After both trials, only grading was able to positively modify flock uniformity and to reduce mortality.

5.2. Carcass traits

It is relevant in the experimental trial (**chapter 4**) results, that pullets fed the diluted diet had at 22 wk of age, relative to BW, more caecum plus rectum (0.90

vs.0.64 %, *P* <.0001) and more proventricle plus gizzard (2.04 *vs*. 1.94 %, *P* = 0.035) than those fed the control. Regarding to caecum plus rectum results, it has to be taken into account that poultry gastrointestinal tract does not produce the necessary enzymes to digest fiber and thus, some of the water soluble particles including different fiber fractions enter into the caecum by antiperistaltic movements, in this organ a large bacterial community breaks down indigestible plant material. Therefore, it seems coherent that the birds fed diluted diets, with on average 37.5% more crude fiber (**CF**) than control diets, had more developed caecums plus rectums. In the case of proventicle plus gizzard, it has to be highlighted that diets including insoluble fiber sources can increase secretion of HCL in the proventricle, bile acids, and endogenous enzymes (Rogel et al., 1987; Svihus, 2011), which in turn would have an important role in the digestive processes. Likewise, several authors have reported that the high lignin content of most insoluble fiber sources leads to a longer retention of the feed in the gizzard, improving its muscular development and thus its function (Hetland and Svihus, 2001; Hetland et al., 2003; Jiménez-Moreno et al., 2010); and additionally, Mateos et al. (2012) results indicated that when broilers are fed with diets including low levels of fiber, gastrointestinal tract development can be penalized and mainly the gizzard, provoking a decrease in nutrient digestibility and feed efficiency.

Since the diluted diets contained a higher level of CF, and specifically 38.9% more acid detergent lignin, better digestibility of the birds fed these diets would be expected. However, the results of the experimental trial showed that pullets fed the diluted diet consumed more energy (10.8 *vs.* 10.4 kcal, *P* < .0001) and also more crude protein (0.58 *vs.* 0.55 g, *P* < .0001) per BW (g), compared to those fed the control diet.

Results from the experimental trial showed neither diet density nor vitamin C inclusion effect on the intestinal morphology. Therefore, the fact that the birds fed the diluted diets consumed more energy (kcal) and more crude protein (g) per BW (g) than the birds fed the control diets would not be related to a poorer intestinal mucosa morphometry.

Therefore, in broiler breeder pullets, the high level of insoluble fiber used in low-density diets may provoke a shorter mean retention time of the chyme in the gastrointestinal tract (Enting et al., 2007a), which might reduce the time that the enzymes have to act. Thus, even having a better proventricle plus gizzard development, the effect of the fiber diluted diets on digestibility may depend on the level and type of fiber inclusion.

Nowadays, to support broiler breeder production, the aim is to avoid too much breast meat deposition and to favor fad pad deposition during the rearing period; in fact, van Emous et al. (2015) found that fat content in rearing period is related to egg persistence in the second half of the production phase. Results from the experimental trial included in this thesis showed that pullets fed the diluted diet had at 22 wk of age, relative to BW, less breast meat (18.5 *vs.* 19.8%, *P* = 0.0003) and more abdominal fat pad deposition (1,14 *vs*. 0.87%, *P* = 0.0306), than those of the control.

Therefore, this feed strategy enhances some carcass traits in rearing which are related to better production results later.

5.3. Serological markers of bone formation

Serological markers are now an important tool in the assessment and differential diagnosis of metabolic bone diseases in humans. However, these markers have never been used in broiler breeder pullets to evaluate bone resorption and formation, and metabolic bone disorders.

Serological markets of bone formation, ALP and OC, were proposed to evaluate pullet development. The objective in the field trial (**chapter 3**) was to test these two markers as a system to directly assess skeletal development. **Figure 5.2** shows ALP serological levels depending on group of BW and wk of age. An interaction signal that the heaviest pullets at 5 wk had the higher level and the lower level was of the lightest pullets at 10 wk of age (3839 *vs*. 1798 UI, *P* = 0.023). In the case of OC, **Figure 5.3** shows that taking into account results from 5 and 10 wk of age, the heavy pullets presented a higher serological level than the lightest (1238 *vs.* 976 ng/ml, *P* = 0.029), and **Figure 5.4** that at 5 wk OC serological level is significantly higher than at 10 wk of age (1189 *vs*. 1025 ng/ml, *P* = 0.050). These results confirm that ALP and OC markers are able to detect pullet skeletal development differences between different ages and different groups of BW; and therefore, their usefulness as direct and practical serological markers has been demonstrated.

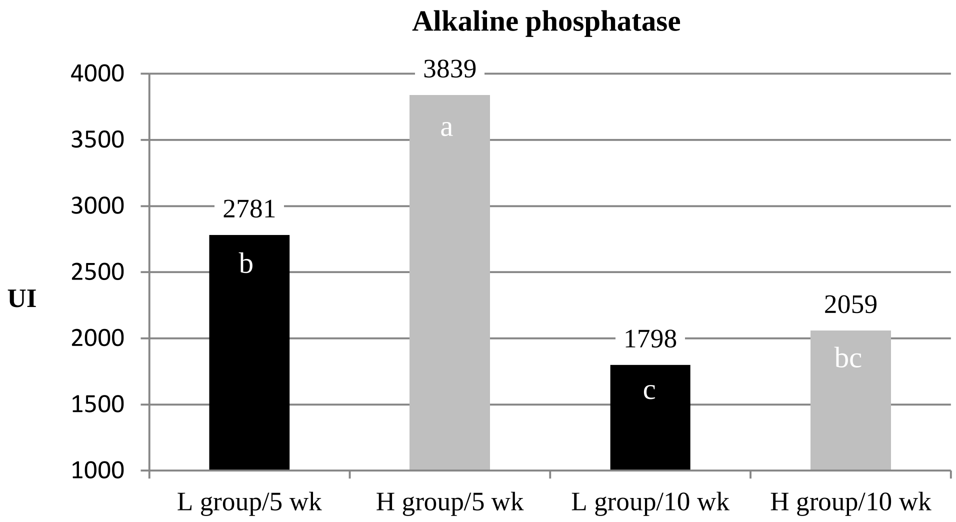


Figure 5.2. Alkaline phosphatase serological levels (UI) depending on body weight group and wk of age. Field trial (chapter 3).
L group: the lightest pullets and H group: the heaviest pullets.
P = 0.023

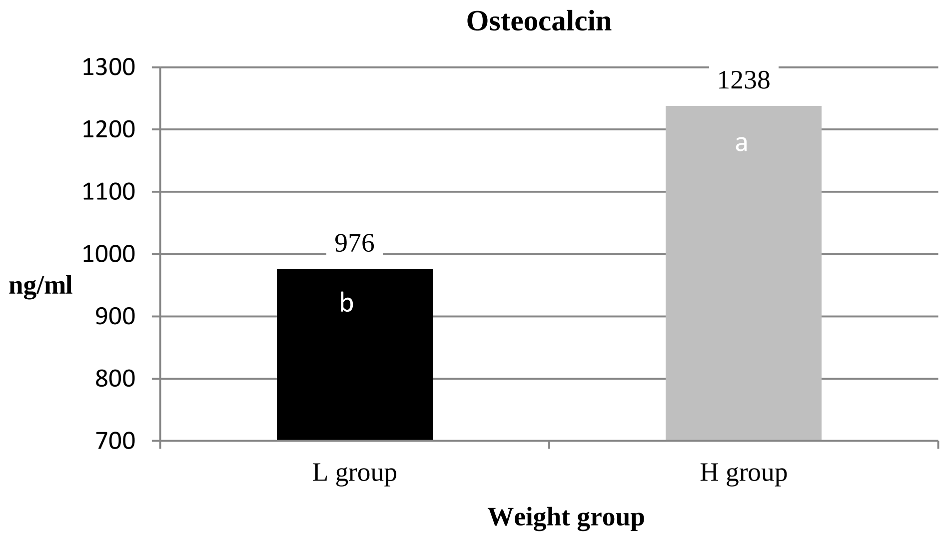


Figure 5.3. Osteocalcin serological levels (ng/ml) depending on body weight group (taking into account results from 5 and 10 wk of age). Field trial (chapter 3).
L group: the lightest pullets and H group: the heaviest pullets.
P = 0.029

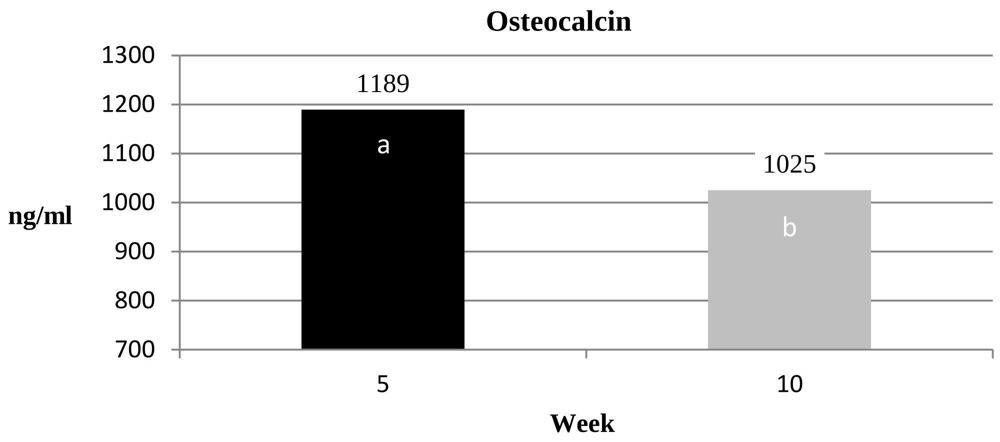


Figure 5.4. Osteocalcin serological levels (ng/ml) depending on week of age. Field trial (chapter 3). P = 0.050

ALP was used in the experimental trial (**chapter 4**) to study the effect of diet density and vitamin C inclusion on skeletal development. No effect was found from vitamin C inclusion, but results show that at 6 and 22 wk of age, the birds fed the fiber diluted diet had lower ALP serological level than those fed the control diet: 8042 *vs.* 10045 UI, P = 0.001 at 6 wk and 775 *vs.* 1107 UI, $P < .0001$ at 22 wk of age. Additionally, the lower ALP serological level of the pullets fed the diluted diet at 22 wk of age would discard the possibility of some compensatory growth and mineral deposition late in rearing, and would be non-congruent with Ekmay et al. (2012) study result; they used ALP serological levels to assess broiler breeder growth and bone mineral deposition during transition into sexual maturity, and found that at 24 and 26 wk of age some compensatory bone mineral deposition seemed possible.

It has to be highlighted that in the field trial at 5 wk of age serological ALP level was 3374 UI (average of the heavy and light pullets), while in the experimental trial at 6 wk of age was 9044 UI (average of the four treatments). A possible explanation would be that even describing the birds in the field trial as broiler breeders, they were grand parent stock (**GPS**), and in the experimental trial they were broiler breeders. Therefore, depending on the bird generation ALP serological levels might be different and thus the reference data to assess if skeletal development is correct. **Figure 5.5** shows average ALP serological levels of GPS found in the field trial at 5, 15 and 20 wk of age; and of broiler breeders found in the experimental trial at 6, 15 and 22 wk of age.

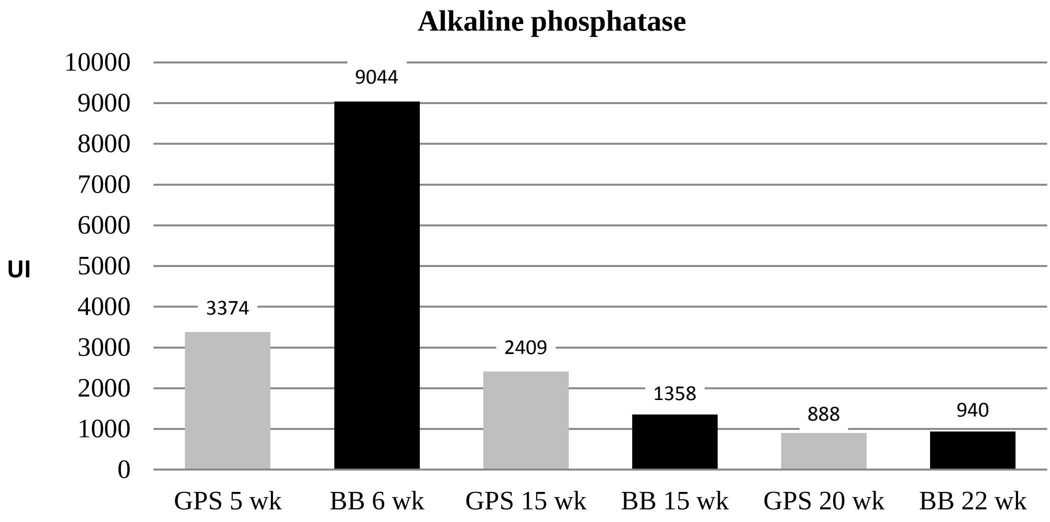


Figure 5.5. Average alkaline phosphatase serological levels (UI) of grand parent stock at 5, 15 and 20 wk and broiler breeders at 6, 15 and 22 wk of age.
GPS: grand parent stock. Field trial (chapter 3).
BB: broiler breeders. Experimental trial (chapter 4).

ALP and OC serological level reductions (5 to 10 wk of age) reinforces the importance of grading the pullets early in rearing, in order to recover the BW and bone development of the lightest birds before skeletal growth slows down and compensatory growth is no longer possible. This would be in line with the statement that 90% of the skeleton of the broiler breeders is already developed at 11-13 wk of age (Ross Parent Stock Management Handbook. Aviagen, 2013).

5.4. Tibia parameters and skeletal strength

To assess correct pullet development it was decided to take tibia measurements and to test their strength. These tests were chosen since they are a way to evaluate if the birds will be able, late in rearing and early in production, to endure the increase of BW without suffering injuries and the consequent leg health issues. Field trial (**chapter 3**) results of the tibias, according to age (wk), show that TL increased and differed significantly until 15 wk of age, within the period from 15 to 30 wk of age, TL was not significantly different; BS at 15 wk was lower than at 25 and 30 wk of age (38899 *vs.* 80255 and 80102 gf/mm^2; $P < 0.001$); and EM at 15 wk was lower than at 25 and 30 wk of age (37.9 *vs.* 75.6 and 78.2 Kgf/mm^2; $P < 0.001$); during the period between 15 and 25 wk of age tibias reached their maximum rigidity and strength.

Within the non-graded broiler breeders studied in the field trial, the lightest pullets had tibias with lower BS (64284 and 71373 gf/mm^2; $P = 0.085$), EM (61.5 and 68.9 Kgf/mm^2; $P = 0.072$) and TL (108 *vs.* 120 mm; $P < 0.001$) than the heaviest. Therefore, tibias from the lightest pullets have lower load endurance values and are more elastic, thus more vulnerable; this could be related to a bone with lower EM being less mineralized, as may also be expected of a rachitic bone (Turner and Burr, 1993). Likewise, their lower TL means that the skeletal development of the lightest pullets is poorer compared to the heaviest, which in turn also confirms that in the rearing period BW and skeletal growth are related as had been observed by other authors (Robinson et al., 2007).

Therefore, after these results, the importance of grading the pullets early in rearing in order to quickly recover BW and not to lose skeletal strength and development of the lightest birds permanently, has again to be highlighted. Likewise, the fact that the lightest pullets had lower tibia BS and EM, combined with their also lower ALP and OC serological levels early in rearing, confirms that their skeletal strength was impaired.

It was demonstrated in the field trial that underweight pullets within a flock have poorer skeletal strength and skeletal development permanently affected. Therefore, the objective of the experimental trial (**chapter 4**) was to study increased feed allocation and vitamin C inclusion as nutritional strategies to improve skeletal development. However, results show that birds fed the diluted diet, compared to those fed the control diet, had at 22 wk of age lower tibia BS values (41422 *vs.* 45138 gf/mm^2, $P = 0.009$), less rigid or more ductile tibias, their EM values were lower, (36.4 *vs.* 43.3 kgf/mm^2, $P = 0.001$), and lower tibia ash content (40.0 *vs.* 41.8%, $P = 0.001$). These results suggest that skeletal strength of the pullets fed fiber diluted diets was poorer and thus mineral Ca absorption could have been impaired; and they are congruent with the lower ALP serological levels at 6 wk of age of the pullets fed the diluted diet, which confirms that their bone mineral deposition was poorer.

As proposed in the paper of the experimental trial, a possible explanation for this impaired Ca absorption might be a shorter mean retention time in almost all sections of the gastrointestinal tract because of low-density diets (Enting et al., 2007a), and the consequent high insoluble fiber content (Leeson at al., 1991). However, the formation of Ca:phytate complexes reduces phytate hydrolysis and Ca and phosphorus digestibility (Sebastian et al., 1996; Tamim et al., 2004; Plumstead et al., 2008), and in this trial pullets fed the diluted diet consumed 19.2% more phytate throughout rearing and pre-breeder periods (up to 22 wk of age) than those of the control (diluted: 146 *vs*. control: 118 g, $P < .0001$). Phytate levels were not analyzed but are based on formulation levels and future research should consider the impact of phytate intake in diluted diet on bone mineralization.

5.5. Gastrocnemius tendon histopathology

In this thesis, the gastrocnemius muscle tendon was histologically assessed in both experiments to evaluate inflammation and fibrosis. Nowadays, the rupture of this tendon is one of the leg health issues reported by producers and due to its relevance it was decided to specifically study it. In the field trial (**chapter 3**), results showed that at 20 and 25 wk of age, the lightest within the group of non-graded pullets had more incidences of inflammation and fibrosis than the heaviest. **Figure 5.6** shows a histological section from a tendon without inflammation, and **Figure 5.7** shows a section with abundant edema, inflammatory cells and neovascularization in the tendon axis (black arrow).

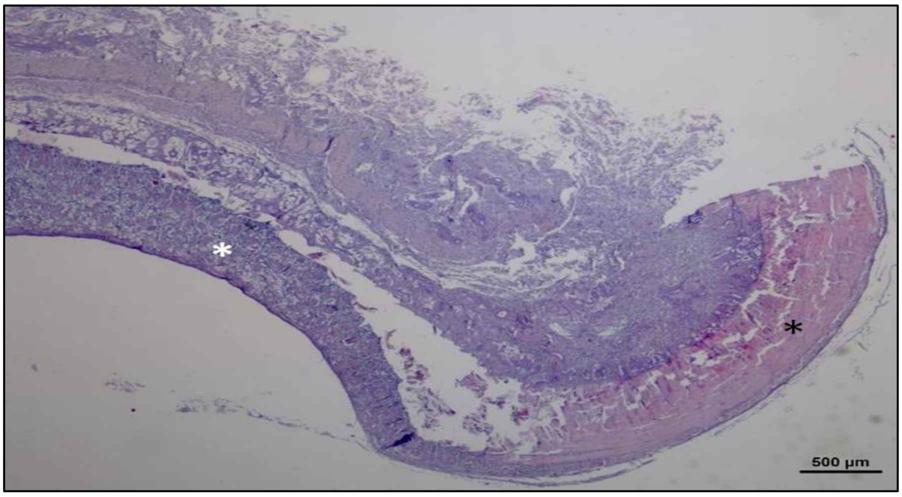


Figure 5.6. Normal gastrocnemius tendon from a pullet of the H group (the heaviest pullets). Field trial (chapter 3).

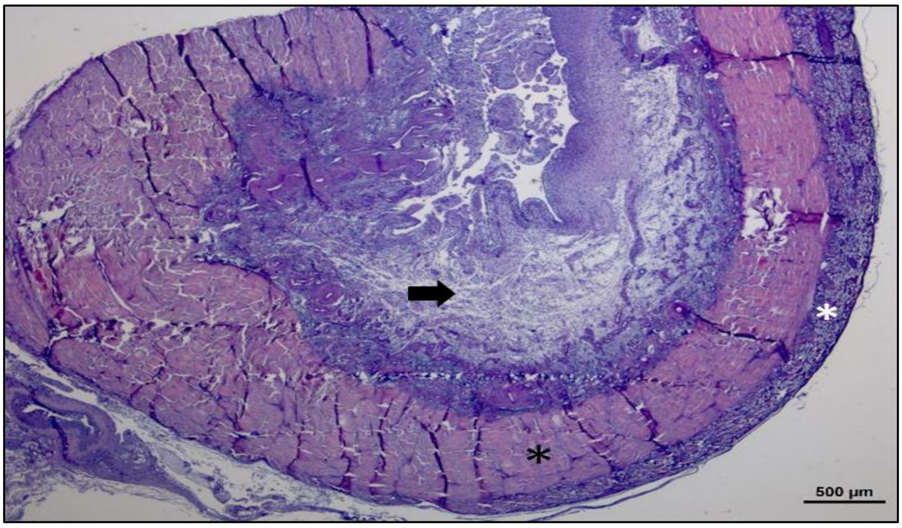


Figure 5.7. Gastrocnemius tendon with abundant edema, inflammatory cells and neovascularization in the tendon axis (black arrow). From a pullet of the L group (the lightest pullets).
Field trial (chapter 3).

The origin of damaged tendons of the lightest broiler breeder hens might come from feed under consumption, and thus from a lack of the necessary nutrients (deficiency states) to build a tendon able to endure strain and BW at the onset of production. In fact, in some investigations, the tendon rupture was associated with several non-infections factors such as deficiency states, variations in tensile strength, glucosaminoglycan content, etc. (van Walsum et al., 1981; Cook et al., 1983a, b; Bradshaw., 2002).

In the experimental trial (**chapter 4**) the results of the histological analysis at 22 wk of age show that there were no structural differences of the gastrocnemius tendons among treatments (diluted and vitamin C supplemented) and inflammation and fibrosis were almost absent.

Therefore, to grade the lightest pullets in order to allow them to consume more feed and to recover BW and thus their skeletal development; is likely to be important to avoid having RGT issues. Feed dilution with fiber raw materials and vitamin C inclusion did not modify tendon structure (mainly type I collagen), which was already correct in the birds fed the control diet and thus able to endure BW increase during rearing and pre-breeder phases.

5.6. Behavior, and wing and tail feather integrity

Since feather pecking results in damaged feather cover and may cause some welfare issues, in the experimental trial (**chapter 4**) of the present thesis was decided to study if diet dilution and vitamin C supplementation was able to affect pecking. Likewise, wing and tail feather scores to evaluate feather integrity, related to GFP, were proposed as a practical and quick system to assess stress in broiler breeder farms.

Pullets fed the fiber diluted diet showed a reduction in GFP and NFOP at 15 and 22 wk of age (**Table 4.9, chapter 4**). GFP reduction is related to wing and tail feather integrity and, in fact, birds fed the diluted diet showed lower GFP behavior and also lower wing and tail feather scores and thus a better wing and tail feather integrity. Feather pecking reduction related to diet dilution found in this study is in agreement with Morrissey et al. (2014) results; they used feather pecking to assess welfare, and their study showed that broiler breeders fed fiber diluted diets feather pecked significantly less often. Vitamin C inclusion did not reduce GFP or NFOP; however, reduced wing feather score and thus enhanced feather integrity (**Table 5.1**); and an interaction showed that vitamin C inclusion in the control diet was able to reduce tail feather score and thus to enhance tail feather integrity at the same level as the diluted diet (**Table 4.9**, **chapter 4**). In fact, several studies show that vitamin C could help birds to cope with stressful situations (Satterlee et al., 1993, 1994; Jones et al., 1996, 1999).

The results of wing feather score were not included in the paper of the experimental trial, since it was considered that the integrity of the wing feathers could not be only affected by stress and thus pecking, but also by the damage pullets caused to each other with their claws when they are moved because of management tasks (weighing, vaccination or topping up the litter). Taking into account that tail feather score, and thus tail feather integrity, is only related to GFP, we consider that it may be a practical and quick way to assess stress in the rearing farms by technicians and veterinarians, and if it is necessary to apply corrective measures.

Table 5.1. Effects of diet density and vitamin C inclusion on wing feather score at 22 wk of age. Experimental trial (chapter 4).

Effects	Wing feather score
Diet density	
CTR	1.12
DIL	0.73
n	192
Vitamin C	
C-	1.01
C+	0.83
n	192
P-values	
Diet	<.0001
Vitamin C	0.036
Interaction	0.183

Wing feather score: (0) fully feathered, (1) rough, (2) some broken feathers and small bald areas, (3) heavily broken feathers and some bald areas, (4) almost bald/large bald areas and (5) bald (no feather cover).

CTR: control diet, and DIL: diluted diet.

C-: not vitamin C supplemented diet, and C+: vitamin C supplemented diet.

$P < 0.05$ was considered significant.

5.7. Final considerations

After analyzing the results of this thesis there are some considerations to be reflected on.

It was hypothesized that feed supplementation with vitamin C might increase skeletal strength and reduce stereotypic behavior, and after the obtained results it has not been accomplished. It was initially designed to supplement 200 mg/ kg of vitamin C but the average of the different diets was 138 mg/kg. The cause of this reduction could have been the heat treatment of the feed. After literature review we still think that vitamin C can help to improve skeletal strength and reduce stereotypic behavior of the broiler breeders during rearing, thus in future investigations it would be interesting to test several and higher levels of vitamin C supplementations.

Uniformity and mortality were not positively affected by diet dilution in experimental conditions. It is important to remark that the effect of diet dilution should be studied in field conditions, where stock density is higher and feed distribution is not so uniform compared to experimental conditions. Therefore, to study diet dilution and thus higher feed allocation under commercial conditions, where sometimes incorrect management results in not all the birds being able to consume at the same time, would be an interesting investigation line in the future.

Diluted diets reduced skeletal strength of the pullets, because of poorer bone mineralization. This finding has to be further investigated since it may cause a major problem. After literature review, the possible formation of Ca:phytate complexes, which could impair Ca and phosphorus digestibility, would be a line of research to follow in the future.

Serological markers of bone formation were proposed as a direct way to assess skeletal development. The results obtained in this thesis show that they are a real tool to be used to evaluate skeletal development. However, depending on the bird generation ALP and OC serological levels might be different; therefore, it would be interesting to study their levels in females and males of grand parent stock, broiler breeders and broilers.

CHAPTER 6

Conclusions

From the results presented in this thesis, the following conclusions can be drawn:

1. Broiler breeder pullets with body weight below standard at 5 wk of age, and never graded in rearing, have shorter, less resistant and more ductile tibias, along with weak tendons once their skeletal development is completed.
2. To obtain correct uniformity and reduce mortality in rearing it would be necessary to grade the lightest broiler breeder pullets, while the sub-populations with the heaviest and medium body weight pullets can stay together.
3. Low-density diets, diluted with raw materials source of insoluble fiber, allow higher daily feed allocation, but do not modify either broiler breeder pullet uniformity or mortality.
4. Broiler breeder pullets fed a diet diluted with insoluble fiber have less breast meat yield and more abdominal fat pad; however, they have poorer bone mineral deposition and thus skeletal strength.
5. Diet diluted with insoluble fiber reduces stereotypic behavior of the broiler breeder pullets, such as grasping feather pecking and non-food object pecking, and thus enhances wing and tail feather integrity.
6. Vitamin C inclusion (200 mg/kg) does not affect skeletal strength of the broiler breeder pullets, but it is effective in enhancing wing feather integrity.
7. Alkaline phosphatase and osteocalcin might be a direct way to assess skeletal development and bone mineral deposition in broiler breeder pullets.
8. Tail feather score could be a practical test to quickly assess good management and well-being in the broiler breeder rearing farms.

CHAPTER 7

References

Adedokun, S. A., and O. Adeola. 2013. Calcium and phosphorus digestibility: Metabolic limits. J. Appl. Poult. Res. **22**:600–8.

Allard, J. P. 2005. Broiler nutrition in the United States. A brief overview. Pages 1–5 in Arkansas Annual Animal Nutrition Conference. Rogers, AR, US.

AOAC International. 2005. Official Methods of Analysis of AOAC International, 18th ed. AOAC International, Gaithersburg, MD, US.

ARC. 1975. The Nutrient Requirements of Farm Livestock, No1: Poultry. Technical Reviews and Summaries. Agricultural Research Council. Hatfield, Pretoria, ZA.

Aviagen. 2010. Protocol for Scoring the Feather Cover of Broiler Breeders during the Laying Period.

Aviagen. 2012. Ross 308 Parent Stock Performance Objectives.

Aviagen. 2013. Ross Parent Stock Management Handbook.

Aviagen. 2014. Ross 308 Parent Stock Nutrition Specifications.

Aviagen. 2016. Ross 308 Parent Stock Nutrition Specifications.

Aviagen. 2016. Ross 308 Parent Stock Performance Objectives.

Aviagen. 2016. Uniformity of Female Broiler Breeders.

Bach Knudsen, K. E. 2001. The nutritional significance of "dietary fiber" analysis. Anim. Feed Sci. Technol. **90**:3–20.

Bailey, R. A. 2013. Gut Health in Poultry – The World Within. Aviagen, Ross Tech Note.

Bataille, R., P. Delmas, and J. Sany. 1987. Serum bone gla-protein in multiple myeloma. Cancer **59**:329–34.

Benevides, G. P., E. R. Pimentel, M. H. Toyama, J. C. Novello, S. Marangoni, and L. Gomes. 2004. Biochemical and biomechanical analysis of tendons of caged and penned chickens. Connect. Tissue Res. **45**:206–15.

Berge, Vanden. J. C. 1981. "Miologia das aves". Pages 1691–1728 in Anatomia dos Animais Domésticos. Sisson/Grossman, R. Getty, ed. Interamericana, Rio de Janeiro, BR.

Beuving, G., and G. M. A. Vonder. 1978. Effect of stressing factors on corticosterone levels in plasma of laying hens. Gen. Comp. Endocrinol. **35**:153–9.

Bouillon, R., D. Vanderschueren, E. Van Herck, H. K. Nielsen, M. Bex, W. Heyns, and H. Van Baelen. 1992. Homologous radioimmunoassay of human osteocalcin. Clin. Chem. **38**:2055–60.

Bradshaw, R. H., R. D. Kirkden, and D. M. Broom. 2002. A Review of the Aetiology and Pathology of Leg Weakness in Broilers in Relation to Welfare. Avian Poult. Biol. Rev. **13**:45–103.

Brown, J. P., P. D. Delmas, L. Malaval, C. Edouard, M. C. Chapuy, and P. J. Meunier. 1984. Serum bone Gla-protein: a specific marker for bone formation in postmenopausal osteoporosis. Lancet (London, England) **1**:1091–3.

Charles, P., J. W. Poser, L. Mosekilde, and F.T. Jenson. 1985. Estimation of bone turnover evaluated by 47 calcium kinetics: efficiency of serum bone gamma-carboxy-glutamic acid containing protein, serum alkaline phosphatase and urinary hydroxyproline excretion. J. Clin. Invest. **76**:2254–8.

Chen, J.-T., K. Hosoda, K. Hasumi, E. Ogata, and M. Shiraki. 2009. Serum N-terminal osteocalcin is a good indicator for estimating responders to hormone replacement therapy in postmenopausal women. J. Bone Miner. Res. **11**:1784–92.

Cherbut, C., E. Albina, M. Champ, J. L. Doublier, and G. Lecannu. 1990. Action of guar gums on the viscosity of digestive contents and on the gastrointestinal motor function in pigs. Digestion. **46**:205–13.

Christenson, R. H., K. Panigrahi, J. C. Chapman, and L. M. Silverman. 1996. Isoenzymes and isoforms. Pages 1077–85 in Clinical Chemistry Theory, Analysis, Correlation. 3rd ed. L. A. Kaplan, A. J. Pesce, eds. Mosby-Year Book, St. Louis, MO, US.

Christenson, R. H. 1997. Biochemical markers of bone metabolism: an overview. Clin. Biochem. **30**:573–93.

Clemens, E. T., C. E. Stevens, and M. Southworth. 1975. Sites of Organic Acid Production and Pattern of Digesta Movement in the Gastrointestinal Tract of Geese. J. Nutr. **105**:1341–50.

Coelho, M. 2002. Vitamin Stability in Premixes and Feeds. A practical Approach in Ruminant Diets. Pages 127–145 in Proceedings of the 13th Annual Florida Ruminant Symposium. FL, US.

Cook, M. E., W. T. Springer, and J. A. Hebert. 1983a. Enhanced incidence of leg abnormalities in reovirus WVU 2937-infected chickens fed various dietary levels of selected vitamins. Avian Dis. **28**:548–61.

Cook, M. E., W. T. Springer, K. M. Kerr, and J. A. Hebert. 1983b. Severity of tenosynovitis in reovirus-infected chickens fed various dietary levels of choline, folic acid, manganese, biotin, or niacin. Avian Dis. **28**:562–73.

Curwin, S. 1997. Biomechanics of tendon and the effects of immobilization. Foot Ankle Clin. **2**:371–89.

Daghir, N. J. 1995. Poultry Production in Hot Climates. Second Edition. N. J. Daghir, ed. Cromwell Press, Trowbridge, UK.

Dawkins, M. S., and R. Layton. 2012. Breeding for better welfare: genetic goals for broiler chickens and their parents. Animal Welfare. **21**:147–55.

de Jong, I. C., S. van Voorst, D. A. Ehlhardt, and H. J. Blokhuis. 2002. Effects of restricted feeding on physiological stress parameters in growing broiler breeders. Br. Poult. Sci. **43**:157–68.

de Jong, I. C., H. Enting, A. van Voorst, and H. J. Blokhuis. 2005. Do low-density diets improve broiler breeder welfare during rearing and laying? Poult. Sci. **84**:194–203.

de Los Mozos, J., A. I. García-Ruiz, L. A. den Hartog, and M. J. Villamide. 2017. Growth curve and diet density affect eating motivation, behavior, and body composition of broiler breeders during rearing. Poult. Sci. **96**:2708–17.

Delmas, P. D., D. M. Wilson, K. G. Mann, and B. L. Riggs. 1983a. Effect of Renal Function on Plasma Levels of Bone Gla-Protein*. J. Clin. Endocrinol. Metab. **57**:1028–30.

Delmas, P. D., D. Stenner, H. W. Wahner, K. G. Mann, and B. L. Riggs. 1983b. Increase in serum bone gamma-carboxyglutamic acid protein with aging in women. Implications for the mechanism of age-related bone loss. J. Clin. Invest. **71**:1316–21.

Dinev, I., M. Lutzkanov, D. Stoikov, and V. Grozeva. 1995. Clinical-epizootological and histological investigations in staphylococcal infection in birds. I. Pages 137–144 in Proceedings of the 6th National Conference of the Union of the Bulgarian Scientists, Stara Zagora, BG.

Dinev, I. 2008. Clinical and morphological studies on spontaneous rupture of the gastrocnemius tendon in broiler breeders. Br. Poult. Sci. **49**:7–11.

Doan, B. H. 2000. Effects of different levels of dietary calcium and supplemental vitamin C on growth, survivability, leg abnormalities, total ash in the tibia, serum calcium and phosphorus in 0-4 week old chicks under tropical conditions. Livestock Research for Rural Development. **12**:1–4.

DSM OVN. 2012. Optimum Vitamin Nutrition (in the production of quality animal foods). 5M Publishing, Sheffield, UK.

Duff, S. R., and C. J. Randall. 1986. Tendon lesions in broiler fowls. Res. Vet. Sci. **40**:333–8.

Duke, G. E. 1986. Alimetary canal: Anatomy, regulation of feeding, and motility. Pages 269–288 in Avian Physiology. P.D. Sturkie, ed. Springer-Verlag, NY, US.

Ekmay, R. D., C. Salas, J. England, S. Cerrate, and C. N. Coon. 2012. The effects of pullet body weight, dietary nonpyhtate phosphorus intake, and breeder feeding regimen on production performance, chick quality, and bone remodeling in broiler breeders. Poult. Sci. **91**:948–64.

Enting, H., A. Veldman, M. W. A. Verstegen, and P. J. van der Aar. 2007a. The effect of low-density diets on broiler breeder development and nutrient digestibility during the rearing period. Poult. Sci. **86**:720–6.

Enting, H., T. A. Kruip, M. W. A. Verstegen, and P. J. van der Aar. 2007b. The effect of low-density diets on broiler breeder performance during the laying period and on embryonic development of their offspring. Poult. Sci. **86**:850–6.

Fancher, B. 2015. What is the Upper Limit to Commercially Relevant Body Weight in Modern Broilers? In Proceedings New Zealand Poultry Beyond 2020 Conference, NZ.

Farquharson, C., and C. C. Whitehead. 1995. Differentiation and mineralization in chick chondrocytes maintained in a high cell density culture: a model for endochondral ossification. In Vitro Cell. Dev. Biol. Anim. **31**:288–94.

FEDNA norms. 2008. Necesidades nutricionales para avicultura: pollos de carne y aves de puesta. Fundación Española para el Desarrollo de la Nutrición Animal.

Fleming, R. H., H. A. McCormack, and C. C. Whitehead. 1998a. Bone structure and strength at different ages in laying hens and effects of dietary particulate limestone, vitamin K and ascorbic acid. Br. Poult. Sci. **39**:434–40.

Fleming, R. H., H. A. McCormack, L. McTeir, and C. C. Whitehead. 1998b. Medullary bone and humeral breaking strength in laying hens. Res. Vet. Sci. **64**:63–7.

Fleming, E. C., C. Fisher, and J. McAdam. 2007a. Genetic progress in broiler traits – implications for welfare. Abstract No 050 in Proceedings of the British Society of Animal Science 2007. UK.

Fleming, E. C., C. Fisher, and J. McAdam. 2007b. Genetic progress in broiler traits – implications for body composition. Abstract No 067 in Proceedings of the British Society of Animal Science 2007. UK.

Fleming, R. H. 2008. Nutritional factors affecting poultry bone health. Proc. Nutr. Soc. **67**:177–83.

Franchini, A., A. Meluzzi, G. Manfreda, and C. Tosarelli. 1994. Vitamin C and bone tissue in broiler. Archiv für Geflügelkunde. **54**:161–5.

Fujita, H., K. Sugimoto, S. Inatomi, T. Maeda, M. Osanai, Y. Uchiyama, Y. Yamamoto, T. Wada, T. Kojima, H. Yokozaki, T. Yamashita, S. Kato, N. Sawada, and H. Chiba. 2008. Tight junction proteins claudin-2 and -12 are critical for vitamin D-dependent Ca2+ absorption between enterocytes. (A Nusrat, Ed.). Mol. Biol. Cell **19**:1912–21.

Ghetie, V., S. T. Chitescu, V. Cotofan, and A. Hillebrand. 1981. Pages 18–137 in Atlas de Anatomía de las Aves Domésticas. Acribia-Paraninfo, Zaragoza and Madrid, ES.

Gonzalez-Alvarado, J. M., E. Jimenez-Moreno, R. Lazaro, and G. G. Mateos. 2007. Effect of Type of Cereal, Heat Processing of the Cereal, and Inclusion of Fiber in the Diet on Productive Performance and Digestive Traits of Broilers. Poult. Sci. **86**:1705–15.

Gous, R. M., and T. R. Morris. 2005. Nutritional interventions in alleviating the effects of high temperatures in broiler production. Worlds. Poult. Sc. J. **61**:463–75.

Gross, W. B., and H. S. Siegel. 1983. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. Avian Dis. **27**:972–9.

Gundberg, C. M., P. S. Wilson, P. M. Gallop, and A. M. Parfitt. 1985. Determination of osteocalcin in human serum: results with two kits compared with those by a well-characterized assay. Clin. Chem. **31**:1720–3.

Hampson, D. J. 1986. Alterations in piglet small intestinal structure at weaning. Res. Vet. Sci. **40**:32–40.

Harris, H. 1990. The human alkaline phosphatases: what we know and what we don't know. Clin. Chim. Acta. **186**:133–50.

Havenstein, G. B., P. R. Ferket, and M. A. Qureshi. 2003a. Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult. Sci. **82**:1500–8.

Havenstein, G. B., P. R. Ferket, and M. A. Qureshi. 2003b. Carcass composition and yield of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult. Sci. **82**:1509–18.

Hess, G. P., W. L. Cappiello, R. M. Poole, and S. C. Hunter. 1989. Prevention and Treatment of Overuse Tendon Injuries. Sport. Med. **8**:371–84.

Hetland, H., and B. Svihus. 2001. Effect of oat hulls on performance, gut capacity and feed passage time in broiler chickens. Br. Poult. Sci. **42**:354–61.

Hetland, H., B. Svihus, and V. Olaisen. 2002. Effect of feeding whole cereals on performance, starch digestibility and duodenal particle size distribution in broiler chickens. Br. Poult. Sci. **43**:416–23.

Hetland, H., B. Svihus, and A. Krogdahl. 2003. Effects of oat hulls and wood shavings on digestion in broilers and layers fed diets based on whole or ground wheat. Br. Poult. Sci. **44**:275–82.

Hetland, H., B. Svihus, and M. Choct. 2005. Role of insoluble fiber on gizzard activity in layers. J. Appl. Poult. Res. **14**:38–46.

Hetland, H., and B. Svihus. 2007. Inclusion of dust bathing materials affects nutrient digestion and gut physiology of layers. J. App. Poult. Res. **16**:22–6.

Hiemstra, S. J., and J. Ten Napel. 2013. Study of the impact of genetic selection on the welfare of chickens bred and kept for meat production, DG SANCO 2011/12254. IBF International Consulting, Brussels, BE.

Hocking, P. M., M. H. Maxwell, and M. A. Mitchell. 1993. Welfare assessment of broiler breeder and layer females subjected to food restriction and limited access to water during rearing. Br. Poult. Sci. **34**:443–58.

Hocking, P. M., M. H. Maxwell, and M. A. Mitchell. 1996. Relationships between the degree of food restriction and welfare indices in broiler breeder females. Br. Poult. Sci. **37**:263–78.

Hocking, P. M., M. H. Maxwell, M. A. Mitchell, and G. W. Robertson. 1998. Welfare and productivity of restricted broiler breeder females fed ad libitum or restricted after the peak of egg production. Br. Poult. Sci. **39**:S16–7.

Hocking, P. M., M. H. Maxwell, and M. A. Mitchell. 1999. Welfare of food restricted male and female turkeys. Br. Poult. Sci. **40**:19–29.

Hocking, P. M., V. Zaczek, E. K. M. Jones, and M. G. Macleod. 2004. Different concentrations and sources of dietary fibre may improve the welfare of female broiler breeders. Br. Poult. Sci. **45**:9–19.

Hocking, P.M. 2006. High-fibre pelleted rations decrease water intake but do not improve physiological indexes of welfare in foodrestricted female broiler breeders. Br. Poult. Sci. **47**: 19–23.

Hocking, P. M. 2014. Unexpected consequences of genetic selection in broilers and turkeys: problems and solutions. Br. Poult. Sci. **55**:1–12.

INRA. 1984. L'alimentation des animaux monogastriques: porc, lapin et volailles. IRNA publications. Paris. FR.

Jiménez-Moreno, E., J. M. González-Alvarado, D. González-Sánchez, R. Lázaro, and G. G. Mateos. 2010. Effects of type and particle size of dietary fiber on growth performance and digestive traits of broilers from 1 to 21 days of age. Poult. Sci. **89**:2197–212.

Johnston, L. J., S. Noll, A. Renteria, and J. Shurson. 2003. Feeding by-products high in concentration of fiber to nonruminants. Pages 1–26 in Proceedings of the 3rd National Alternative Feeds Symposium Western Regional Coordinating Committee, Kansas City, MO, US.

Jones, R. C., J. R. Guneratne, and K. Georgiou. 1981. Isolation of viruses from outbreaks of suspected tenosynovitis (viral arthritis) in chickens. Res. Vet. Sci. **31**:100–3.

Jones, R. C., and F. S. B. Kibenge. 1984. Reovirus induced tenosynovitis in chickens: The effect of breed. Avian Pathol. **13**:511–28.

Jones, R. B., D. G. Satterlee, J. Moreau, and D. Waddington. 1996. Vitamin C supplementation and fear-reduction in Japanese quail: short-term cumulative effects. Br. Poult. Sci. **37**:33–42.

Jones, R. B., D. G. Satterlee, and G. G. Cadd. 1999. Timidity in Japanese quail: effects of vitamin C and divergent selection for adrenocortical response. Physiol. Behav. **67**:117–20.

Jozsa, L., M. Lehto, M. Kvist, B. J. Balint, and A. Reffy. 1989. Alterations in dry mass content of collagen fibers in degenerative tendinopathy and tendonrupture. Matrix. **9**:140–6.

Julian, R. J. 1984. Valgus-varus deformity of the intertarsal joint in broiler chickens. Can. Vet. J. = La Rev. Vet. Can. **25**:254–8.

Kannus, P. 2000. Structure of the tendon connective tissue. Scand. J. Med. Sci. Sports. **10**:312–20.

Kapell, D. N. R. G., W. G. Hill, A. M. Neeteson, J. McAdam, A. N. M. Koerhuis, and S. Avendaño. 2012. Twenty-five years of selection for improved leg health in purebred broiler lines and underlying genetic parameters. Poult. Sci. **91**:3032–43.

Kibenge, F. S. B., M. D. Robertson, G. E. Wilcox, and D. A. Pass. 1982a. Bacterial and viral agents associated with tenosynovitis in broiler breeders in Western Australia. Avian Pathol. **11**:351–9.

Kibenge, F. S., M. D. Robertson, and G. E. Wilcox. 1982b. Staphylococcus aureus isolated from poultry in Australia. II. Epidemiology of strains associated with tenosynovitis. Vet. Microbiol. **7**:485–91.

Kirkendall, D. T., and W. E. Garrett. 1997. Function and biomechanics of tendons. Scand. J. Med. Sci. Sports. **7**:62–6.

Kleerekoper, M. D., and G. W. Edelson. 1996. Biochemical studies in the evaluation and management of osteoporosis: current status and future prospects. Endocr. Pract. **2**:13–9.

Kleven, S. H. 2003. Mycoplasma synoviae infection. Pages 756–62 in Diseases of Poultry. Y. M. Saif, ed. Iowa State University Press, Ames, IA, US.

Kleyn, R. 2013a. Broiler breeder nutrition. Pages 191–208 in Chicken nutrition. A guide for nutritionists and poultry professionals. Context Publications, Packington, UK.

Kleyn, R. 2013b. Typical breeder diets. Page 321 in Chicken nutrition. A guide for nutritionists and poultry professionals. Context Publications, Packington, UK.

Leboy, P. S., L. Vaias, B. Uschmann, E. Golub, S. L. Adams, and M. Pacifici. 1989. Ascorbic acid induces alkaline phosphatase, type X collagen, and calcium deposition in cultured chick chondrocytes. J. Biol. Chem. **264**:17281–6.

Leeson, S., J. D. Summers, and L. J. Caston. 1991. Diet Dilution and Compensatory Growth in Broilers. Poult. Sci. **70**:867–73.

Leeson and Summers. 2005. Commercial Poultry Nutrition. 3rd edition. Nottingham University Press. Notttingham. UK.

Leterrier, C., and Y. Nys. 1992. Clinical and anatomical differences in varus and valgus deformities of chick limbs suggest different aetiopathogenesis. Avian Pathol. **21**:429–42.

Lewis, P. D., M. Ciacciariello, D. Backhouse, and R. M. Gous. 2007. Effect of age and body weight at photostimulation on the sexual maturation of broiler breeder pullets transferred from 8L:16D to 16L:8D. Br. Poult. Sci. **48**:601–8.

Lian, J. B., and G. S. Stein. 1999. The cells of bone. Pages 165–186 in Dynamics of bone and cartilage metabolism. M. J. Seibel, S. P. Robins, J. P. Bilezikian, eds. Academic Press, San Diego, CA, US.

Lumeij, J. T., and I. Westerhof. 1987. Blood chemistry for the diagnosis of hepatobiliary disease in birds. A review. Vet. Q. **9**:255–61.

Mateos, G. G., and J. Piquer. 1994. Fundamentos nutricionales y diseño de programas de alimentación para reproductoras pesadas. Fedna. **10**:65–83.

Mateos, G. G., D. G. Valencia, and E. Jiménez-Moreno. 2004. Microminerales en alimentación de monogástricos: aspectos técnicos y consideraciones legales. Fedna. **20**:275–323.

Mateos, G. G., E. Jiménez-Moreno, M. P. Serrano, and R. P. Lázaro. 2012. Poultry response to high levels of dietary fiber sources varying in physical and chemical characteristics. J. Appl. Poult. Res. **21**:156–74.

Maxwell, M.H. 1993. Avian blood leucocyte responses to stress. World. Poult. Sci. J. **49**:34–43.

McCormack, H. A., R. H. Fleming, L. McTeir, and C. C. Whitehead. 2001. Bone development up to 6 weeks of age in feed restricted broiler breeders fed diets supplemented with different concentrations of ascorbic acid. Br. Poult. Sci. **42**:S91–S92.

McNab, J. M., and R. R. Smithard. 1992. Barley β-glucan: An antinutritional factor in poultry feeding. Nutr. Res. Rev. **5**:45–60.

Mench, J. A. 2002. Broiler breeders: feed restriction and welfare. World. Poult. Sci. J. **58**:23–9.

Mertens, D. R. 2003. Challenges in measuring insoluble dietary fiber. J. Anim. Sci. **81**:3233–49.

Monaghan, D. A., M. J. Power, and P. F. Fottrell. 1993. Sandwich enzyme immunoassay of osteocalcin in serum with use of an antibody against human osteocalcin. Clin. Chem. **39**:942–7.

Moore, S. J. 1999. Food breakdown in an avian herbivore; who needs teeth? Aust. J. Zool. **47**:625–32.

Morel, P .C. H., T. S. Lee, and P. J. Moughan. 2006. Effect of feeding level, live weight and genotype on the apparent faecal digestibility of energy and organic matter in the growing pig. Anim. Feed Sci. Technol. **126**:63–74.

Morrissey, K. L. H., T. Widowski, S. Leeson, V. Sandilands, A. Arnone, and S. Torrey. 2014. The effect of dietary alterations during rearing on growth, productivity, and behavior in broiler breeder females. Poult. Sci. **93**:285–95.

Mosenthin, R., E. Hambrecht, and W. C. Sauer. 2001. Utilisation of different fibres in piglet feeds. Pages 300–320 in Recent Development in Pig Nutrition 3. P. C. Gransworthy, J. Wiseman, eds. Notthingham University Press, Notthingham, UK.

Mussini, F. J. 2012. Comparative response of different broiler genotypes to dietary nutrient levels. Dissertation, University of Arkansas, AR, US.

Nakagaki, W. R., A. Biancalana, G. P. Benevides, and L. Gomes. 2007. Biomechanical and Biochemical Properties of Chicken Calcaneal Tendon Under Effect of Age and Nonforced Active Exercise. Connect. Tissue Res. **48**:219–28.

National Chicken Council, 2016. https://www.nationalchickencouncil.org/about-the-industry/statistics/u-s-broiler-performance/.

National Research Council. 1994. Nutritional Requirements of Poultry. 9th rev. ed. National Academy Press, Washington, DC, US.

Neeteson-van Nieuwenhoven, A. M., P. Knap, and S. Avendaño. 2013. The Role of Commercial Pig and Poultry Breeding for Food Security. Animal Frontiers, 3, 1, 52–57. http://www.animalfrontiers.org/content/3/1/52.full.pdf+html.

Nielsen, B. L., K. Thodberg, J. Malmkvist, and S. Steenfeldt. 2011. Proportion of insoluble fibre in the diet affects behaviour and hunger in broiler breeders growing at similar rates. Animal **5**:1247–58.

Noblet, J., and G. Le Goff. 2001. Effect of dietary fibre on the energy value of feeds for pigs. Anim. Feed Sci. Technol. **90**:35–52.

NRC. 1994. Nutrient Requirements of Poultry. Ninth Revised Edition. National Research Council. National Academy Press. WA, US.

NRC. 2012. Nutrient Requirements of Pigs. Eleventh Revised Edition. National Research Council. National Academy Press. WA, US.

O'Brien, M. 1997. Structure and metabolism of tendons. Scand. J. Med. Sci. Sports. **7**:55–61.

Pardue, S. L., J. P. Thaxton, and J. Brake. 1985. Role of ascorbic acid in chicks exposed to high environmental temperature. J. Appl. Physiol. **58**:1511–6.

Pardue, S. L., and J. P. Thaxton. 1986. Ascorbic acid in poultry: a review. World. Poult. Sci. J. **42**:107–23.

Parviainen, M., I. Kuronen, H. Kokko, M. Lakaniemi, K. Savolainen, and I. Mononen. 2009. Two-site enzyme immunoassay for measuring intact human osteocalcin in serum. J. Bone Miner. Res. **9**:347–54.

Perry, R. W., G. N. Rowland, T. L. Foutz, and J. R. Glisson. 1991. Poult malabsorption syndrome. III. Skeletal lesions in market-age turkeys. Avian Dis. **35**:707–13.

Pinchasov, Y., D. Galili, N. Yonash, and H. Klandorf. 1993. Effect of feed restriction using self-restricting diets on subsequent performance of broiler breeder females. Poult. Sci. **72**:613–19.

Pines, M. 2007. Poultry bone disorders. Pages 110–121 in Aust. Poult. Sci. Symp., Sydney, Univ. Sydney, School of Veterinary Science. Sydney, AU.

Pishnamazi, A., R. A. Renema, M. J. Zuidhof, and F. E. Robinson. 2008. Effect of initial feeding of broiler breeder pullets on carcass development and body weight variation. J. Appl. Poult. Res. **17**:505–14.

Pishnamazi, A., R. A. Renema, M. J. Zuidhof, and F. Robinson. 2014. Effect of age at photostimulation on sexual maturation in broiler breeder pullets. Poult. Sci. **93**:1274–81.

Plumstead, P. W., A. B. Leytem, R. O. Maguire, J. W. Spears, P. Kwanyuen, and J. Brake. 2008. Interaction of calcium and phytate in broiler diets. 1. Effects on apparent prececal digestibility and retention of phosphorus. Poult. Sci. **87**:449–58.

Power, M. J., and P. F. Fottrell. 1991. Osteocalcin: Diagnostic Methods and Clinical Applications. Crit. Rev. Clin. Lab. Sci. **28**:287–335.

Price, P. A., M. K. Williamson, and J. W. Lothringer. 1981. Origin of the vitamin K-dependent bone protein found in plasma and its clearance by kidney and bone. J. Biol. Chem. **256**:12760–6.

Price, P. A. 1987. Vitamin K-dependent bone protein. Pages 419–426 in Calcium regulation and bone metabolism: basic and clinical aspects. D. V. Cohn, T. J. Martin, P. J. Meunier, eds. Elsevier, Amsterdam, NL.

Rajkhowa, T. K., A. K. Katiyar, and J. L. Vegad. 1996. Effect of ascorbic acid on the inflammatory-reparative response in the punched wounds of the chicken skin. Indian J. Anim. Sci. **66**:120–5.

Randall, C. J., and C. P. J. Mills. 1981. Observations on leg deformity in broilers with particular reference to the intertarsal joint. Avian Pathol. **10**:407–31.

Randall, C. J. 1991. Miscellaneous diseases. Page 158 in Diseases and Disorders of the Domestic Fowl and Turkey. C. J. Randall, ed. Iowa State University Press, Ames, IA, US.

Rath, N. C., G. R. Huff, W. E. Huff, and J. M. Balog. 2000. Factors regulating bone maturity and strength in poultry. Poult. Sci. **79**:1024–32.

Renema, R., F. Robinson, M. Newcombe, and R. McKay. 1999. Effects of body weight and feed allocation during sexual maturation in broiler breeder hens. 1. Growth and carcass characteristics. Poult. Sci. **78**:619–28.

Renema, R. A., and F. E. Robinson. 2004. Defining normal: comparison of feed restriction and full feeding of female broiler breeders. World. Poult. Sci. J. **60**:508–22.

Renema, R. A., M. E. Rustad, and F. E. Robinson. 2007. Implications of changes to commercial broiler and broiler breeder body weight targets over the past 30 years. Worlds. Poult. Sci. J. **63**:457–72.

Richardson, A. J. 1970. The role of the crop in the feeding behaviour of the domestic chicken. Anim. Behav. **18**:633–9.

Rizzoli, R., and J. P. Bonjour. 1999. Physiology of calcium and phosphate homeostasis. Pages 247–260 in Dynamics of bone and cartilage metabolism. M. J. Seibel, S. P. Robins, J. P. Bilezikian, eds. Academic Press, San Diego, CA, US.

Robert, S., J. Rushen, and C. Farmer. 1997. Both energy content and bulk of food affect stereotypic behaviour, heart rate and feeding motivation of female pigs. Appl. Anim. Behav. Sci. **54**:161–71.

Robinson, F. E., M. J. Zuidhof, and R. A. Renema. 2007. Reproductive Efficiency and Metabolism of Female Broiler Breeders as Affected by Genotype, Feed Allocation, and Age at Photostimulation. 1. Pullet Growth and Development. Poult. Sci. **86**:2256–66.

Rogel, A. M., D. Balnave, W. L. Bryden, and E. F. Annison. 1987. Improvement of raw potato starch digestion in chickens by feeding oat hulls and other fibrous feedstuffs. Aust. J. Agric. Res. **38**:629–37.

Rostagno. 2005. Tablas Brasileñas para Aves y Cerdos. Composición de Alimentos y Requerimientos Nutricioinales. 2ª edición. H. S. Rostagno, ed. Universidad Federal de Viçosa. Viçosa, MG, BR.

Sacranie, A., B. Svihus, V. Denstadli, B. Moen, P. A. Iji, and M. Choct. 2012. The effect of insoluble fiber and intermittent feeding on gizzard development, gut motility, and performance of broiler chickens. Poult. Sci. **91**:693–700.

Sandilands, V., B. J. Tolkamp, and I. Kyriazakis. 2005. Behaviour of food restricted broilers during rearing and lay - effects of an alternative feeding method. Physiol. Behav. **85**:115–23.

Sandilands, V., B. J. Tolkamp, C. J. Savory, and I. Kyriazakis. 2006. Behaviour and welfare of broiler breeders fed qualitatively restricted diets during rearing: Are there viable alternatives to quantitative restriction? Appl. Anim. Behav. Sci. **96**: 53–67.

Sapolsky, R.M., L. M. Romero, and A.U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. **21**:55–89.

Satterlee, D. G., R. B. Jones, and F. H. Ryder. 1993. Short-Latency Stressor Effects on Tonic Immobility Fear Reactions of Japanese Quail Divergently Selected for Adrenocortical Responsiveness to Immobilization. Poult. Sci. **72**:1132–6.

Satterlee, D. G., R. B. Jones, and F. H. Ryder. 1994. Effects of ascorbyl-2-polyphosphate on adrenocortical activation and fear-related behavior in broiler chickens. Poult. Sci. **73**:194–201

Savory, C. J., P. M. Hocking, J. S. Mann, and M. H. Maxwell. 1996. Is broiler breeder welfare improved by using qualitative rather than quantitative food restriction to limit growth rate? Anim. Welf. **5**:105–27.

Savory, C. J., and J. M. Lariviere. 2000. Effects of qualitative and quantitative food restriction treatments on feeding motivational state and general activity level of growing broiler breeders. Appl. Anim. Behav. Sci. **69**:135–47.

Sebastian, S., S. P. Touchburn, E. R. Chavez, and P. C. Laguë. 1996. Efficacy of supplemental microbial phytase at different dietary calcium levels on growth performance and mineral utilization of broiler chickens. Poult. Sci. **75**:1516–23.

Seibel, M. J. 2005. Biochemical markers of bone turnover: part I: biochemistry and variability. Clin. Biochem. Rev. **26**:97–122.

Sklan, D., B. Shachaf, J. Baron, and S. Hurwitz. 1978. Retrograde movement of digesta in the duodenum of the chick: extent, frequency, and nutritional implications. J. Nutr. **108**:1485–90.

Stinson, R. A., and B. A. Hamilton. 1994. Human liver plasma membranes contain an enzyme activity that removes membrane anchor from alkaline phosphatase and converts it to a plasma-like form. Clin. Biochem. **27**:49–55.

Sullivan, T. W. 1994. Skeletal Problems in Poultry: Estimated Annual Cost and Descriptions. Poult. Sci. **73**:879–82.

Svihus, B. 2011. The gizzard: function, influence of diet structure and effects on nutrient availability. World Poult. Sci. J. **67**:207–24.

Takahashi, K., Y. Akiba, and M. Horiguchi. 1991. Effects of supplemental ascorbic acid on performance, organ weight and plasma cholesterol concentration in broilers treated with propylthiouracil. Br. Poult. Sci. **32**:545–54.

Tamim, N. M., R. Angel, and M. Christman. 2004. Influence of dietary calcium and phytase on phytate phosphorus hydrolysis in broiler chickens. Poult. Sci. **83**:1358–67.

Taylor, A. K., S. G. Linkhart, S. Mohan, and D. J. Baylink. 1988. Development of a new radioimmunoassay for human osteocalcin: evidence for a midmolecule epitope. Metabolism. **37**:872–7.

Tipton, C. M., R. D. Matthes, J. A. Maynard, and R. A. Carey. 1975. The influence of physical activity on ligaments and tendons. Med. Sci. Sports Exerc. **7**:165–75.

Turner, C. H., and D. B. Burr. 1993. Basic biomechanical measurements of bone: a tutorial. Bone **14**:595–608.

van Emous, R. A., R. P. Kwakkel, M. M. van Krimpen, and W. H. Hendriks. 2015. Effects of dietary protein levels during rearing and dietary energy levels during lay on body composition and reproduction in broiler breeder females. Poult. Sci. **94**:1030–42.

van Walsum, J., M. H. Vertommen, and A. vd Laan. 1981. Contribution to the aetiology of synovitis in chickens, with special reference to non-infective factors. V. Extractability of glucosaminoglycans/mucoproteins from tendon tissue. Vet. Q. **3**:111–7

Vergara, P., C. Ferrando, M. Jiménez, E. Fernández, and E. Goñalons. 1989. Factors determining gastrointestinal transit time of several markers in the domestic fowl. Q. J. Exp. Physiol. **74**:867–74.

Villamide, M. J., and M. J. Fraga. 1999. Composition of vitamin supplements in Spanish poultry diets. Br. Poult. Sci. **40**:644–52.

Ward, N. E. 1993. Vitamin supplementation rates for US commercial broilers, turkeys and layers. J. Appl. Poult. Res. **2**:286–96.

Weber, P. 1999. The role of vitamins in the prevention of osteoporosis--a brief status report. Int. J. Vitam. Nutr. Res. **69**:194–7.

Weiser, H., M. Schlachter, and H . Bachmann. 1988. The importance of vitamin C for hydroxylation of Vitamin D 3 to 1 a, 25 (OH)z DJ and of 24R, 25 (OH)2 D3 to a more active metabolite. Pages 644-653 in Proceedings of the Seventh Workshop on Vitamin D, Rancho Mirage, California, US. Walter de Gruyter, Berlin, DE.

Whitehead, C. C., J. S. Rennie, H. A. McCormack, and P. M. Hocking. 1993. Defective down syndrome in chicks is not caused by riboflavin deficiency in breeders. Br. Poult. Sci. **34**:619–23.

Whitehead, C. C., and T. Keller. 2003. An update on ascorbic acid in poultry. Worlds. Poult. Sci. J. **59**:161–84.

Whittow, G. C. 2000. Page 685 in Sturkie's Avian Physiology. 5th Edition. G. C. Whittow, ed. Academic Press, San Diego, CA, US.

Wynn, T. A. 2007. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. J. Clin. Invest. **117**:524–9.

Zuidhof, M. J., F. E. Robinson, J. J. R. Feddes, R. T. Hardin, J. L. Wilson, R. I. McKay, and M. Newcombe. 1995. The effects of nutrient dilution on the well-being and performance of female broiler breeders. Poult. Sci. **74**:441–56.

Zuidhof, M. J., B. L. Schneider, V. L. Carney, D. R. Korver, and F. E. Robinson. 2014. Growth, efficiency, and yield of commercial broilers from 1957, 1978, and 2005. Poult. Sci. **93**:2970–82.

Zuidhof, M. J., D. E. Holm, R. A. Renema, M. A. Jalal, and F. E. Robinson. 2015. Effects of broiler breeder management on pullet body weight and carcass uniformity. Poult. Sci. **94**:1389–97.