Bringing eggs and bones to light

Carla W. van der Pol 2017

Bringing eggs and bones to light

Affecting leg bone development in broiler chickens through perinatal lighting schedules

Carla W. van der Pol

You are cordially invited to attend the public defence of my PhD thesis entitled:

Bringing eggs and bones to light
Affecting leg bone development in broiler chickens through perinatal lighting schedules

Carla van der Pol
cvdpol@hatchtech.nl

Paranymphs

Myrthe Gilbert
myrthe.gilbert@wur.nl

Mirjam Goedkoop
mirjam.goedkoop@gmail.com

Friday, 12 May 2017 at 16:00
Aula of Wageningen University
Generaal Foulkesweg 1
Wageningen

The ceremony will be followed by a reception and buffet at:
Café Loburg
Molenstraat 6
Wageningen

Please indicate if you would like to join the dinner before the 5th of May.

HATCHTECH
PROVIDING SUPERIOR CHICK QUALITY

Bringing eggs and bones to light

Affecting leg bone development in broiler chickens through perinatal lighting schedules

Carla W. van der Pol

Thesis committee

Promotor
Prof. Dr B. Kemp
Professor of Adaptation Physiology
Wageningen University & Research

Co-promotors
Dr H. van den Brand
Associate professor, Adaptation Physiology Group
Wageningen University & Research

Dr I. A. M. van Roovert-Reijrink
Manager Research
HatchTech B.V., Veenendaal

Other members
Prof. Dr P. W. G. Groot Koerkamp, Wageningen University & Research
Prof. Dr S. Özkan, Ege University, Turkey
Prof. Dr J. A. Stegeman, Utrecht University, the Netherlands
Dr I. C. de Jong, Wageningen University & Research

This research was conducted under the auspices of the Graduate School of Wageningen Institute of Animal Sciences (WIAS)

Bringing eggs and bones to light

Affecting leg bone development in broiler chickens through perinatal lighting schedules

Carla W. van der Pol

Thesis

submitted in fulfilment of the requirement of the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 12 May 2017
at 4 p.m. in the Aula.

Carla W. van der Pol
Bringing eggs and bones to light. Affecting leg bone development in broiler chickens through perinatal lighting schedules
214 pages

PhD thesis, Wageningen University, Wageningen, the Netherlands (2017)
With references, with summary in English

ISBN: 978-94-6343-142-2
DOI: 10.18174/410024

Abstract

Leg bone pathologies are a common problem in broiler chickens, and they can lead to decreased welfare and poor production performance. It can be speculated that the aetiology of some leg bone pathologies lies, to some extent, in suboptimal early life bone development. One factor that can be speculated to affect bone development, and consequently leg health, in broiler chickens is application of light. Light has several properties, such as light intensity, color, duration, and schedule. The present thesis focuses on lighting schedules. Aim was to investigate how lighting schedules applied during incubation and in the early post hatch period (the brooding period) affected leg bone development throughout a broiler's life and leg health at slaughter age.

In 4 studies, effects of eggshell temperature (EST) and lighting schedule during incubation and in the brooding period on leg bone development, leg health, and production parameters were explored. The first study found that an EST of 39.4°C led to lower bone dimensions at hatch than an EST between 37.8 and 38.6°C. It was then decided that incubation experiments on bone development would best be performed at a constant EST of 37.8°C, as this is also an EST that leads to good hatchability and chick quality. In two studies, the effects of circadian lighting schedules during incubation on leg bone development and leg health were investigated. Staining of the embryonic leg bones showed that applying a circadian lighting schedule of 12 hours of light, followed by 12 hours of darkness (12L:12D) resulted in an earlier onset of embryonic ossification of the tibia than continuous light (24L). Compared to 24L, 12L:12D furthermore resulted in higher tibia weight and length, and higher tibial cortical area, cortical thickness, and second moment of area around the minor axis at hatch as revealed by MicroCT scanning. It was furthermore found that 12L:12D resulted in a lower incidence of the leg pathology tibial dyschondroplasia. Continuous darkness (24D) was mostly intermediate. On the other hand, a circadian lighting schedule of 16 hours of light, followed by 8 hours of darkness (16L:8D) did not show the same stimulatory effect on leg bone development, as no differences in gene expression markers involved in embryonic ossification were found, leg bone dimensions at hatch were not increased, and bone mineral content as determined by DXA scanning was not higher for 16L:8D. It can therefore be speculated that the dark period should exceed 8 hours per day during incubation for increased bone dimensions and ossification. However, incidence and severity of the leg bone pathologies in the form of bacterial chondronecrosis with osteomyelitis and epiphyseal plate abnormalities were lowest for broilers exposed to 16L:8D during incubation, and tibial dyschondroplasia tended to be lower for 16L:8D than for 24D. Interactions between incubation and matching or mismatching post hatch lighting schedules were not found. It was speculated that the endocrine factors (pineal) melatonin, growth hormone, corticosterone, and IGF-1 were a pathway through which light affected leg bone development, but no evidence was found to support this hypothesis. Production performance was not greatly influenced by incubation lighting schedule, but 24L was found to result in higher body weights

at slaughter age than 16L:8D and 24D. In the final experiment, lighting schedules were applied during the brooding period from day 0 to 4 after hatching and leg bone development was measured at day 4 post hatch. 24L led to increased leg bone dimensions, but lower developmental stability of the leg bones than a lighting schedule with 1 or 6 hours of darkness after every 2 hours of light.

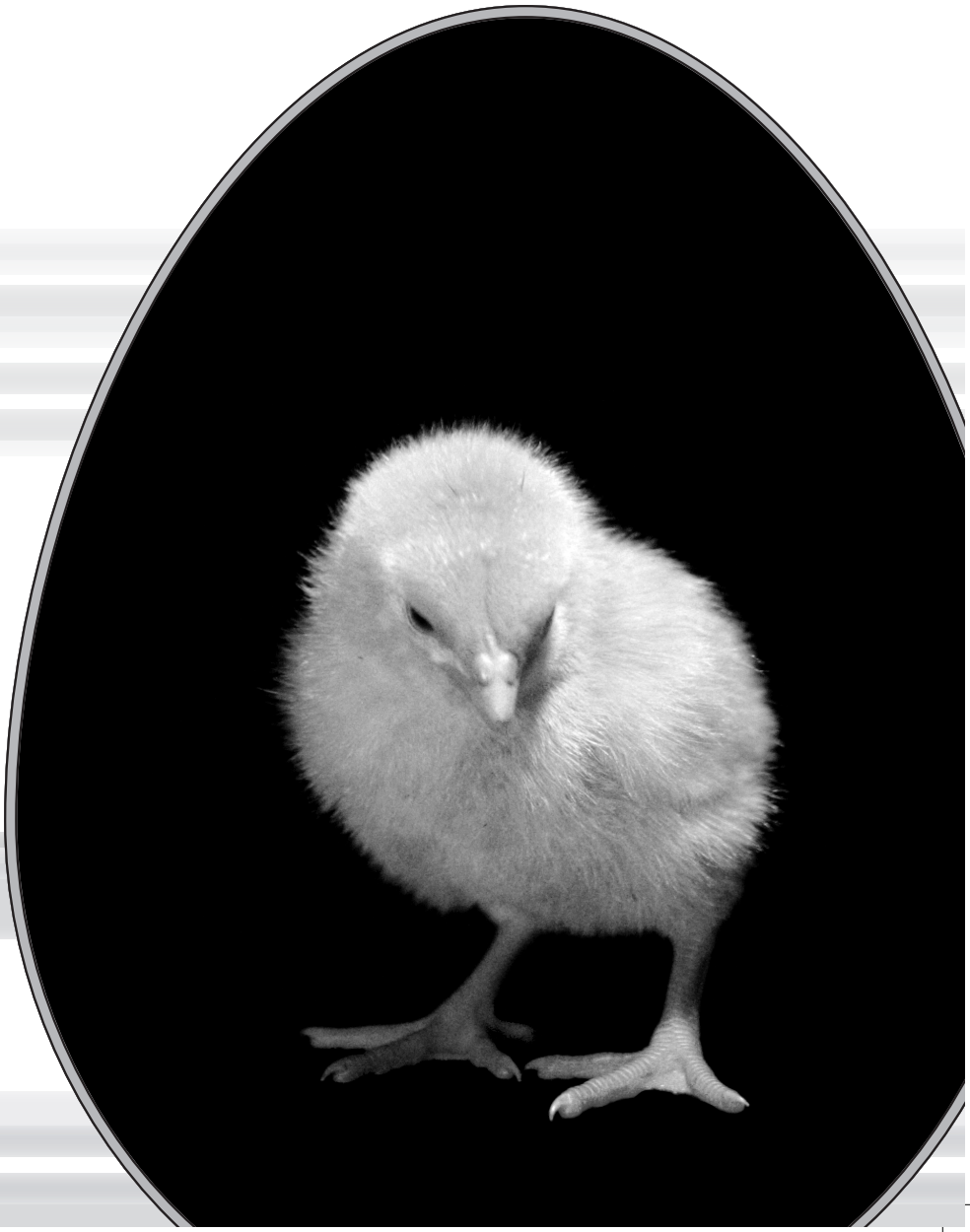
The overall findings of this thesis suggest that continuous light during incubation and in the brooding period had a detrimental effect on embryonic and early post hatch leg bone development and health. The involvement of endocrine factors was not clarified from the current results. Applying a light-dark rhythm during incubation may improve embryonic leg bone development and leg health at slaughter age compared to continuous light and continuous darkness, without affecting post hatch production performance, but it appears that the dark period should last longer than 8 hours per day for optimal leg bone development.

Table of contents

Chapter 1.	General introduction	10
Chapter 2.	Effect of eggshell temperature throughout incubation on broiler hatchling leg bone development	36
Chapter 3.	Circadian rhythms during incubation of broiler chicken embryos and their effects on embryonic and post hatch leg bone development	52
Chapter 4.	Lighting schedule throughout incubation: effects on broiler development and post hatch performance	78
Chapter 5.	Effects of lighting schedule during incubation of broiler chicken embryos on leg bone development at hatch and related physiological characteristics	98
Chapter 6.	Incubation lighting schedules and their interaction with matching or mismatching post hatch lighting schedules: effects on broiler bone development and leg health at slaughter age	120
Chapter 7.	Lighting schedule and dimming period in early life: consequences for broiler chicken leg bone development	144
Chapter 8.	General discussion	166
	Summary	196
	Dankwoord (Acknowledgements)	202
	About the author	206
	Training and Supervision Plan	212
	Colophon	214

Chapter 1

General introduction



1 | Leg bone pathologies in broilers

Broilers are a type of chicken bred and reared specifically for meat production. They are characterized by a high growth rate, that still increases as a result of genetic selection: a 28-day-old meat-type chicken weighed 316 grams in 1957, 632 grams in 1978, and 1,396 grams in 2005 (Zuidhof *et al.*, 2014). Slaughter weights are probably even higher nowadays: the 2014 Ross 308 growth chart for the Dutch market predicts a body weight of 1501 grams at 28 days of life (Aviagen, 2014). These high body weights at a young age can lead to leg problems, which are pathologies that result in impaired walking ability (Bradshaw *et al.*, 2002): body weight positively correlates with broiler lameness, irrespective of genotype, age, and feeding regime (Kestin *et al.*, 2001). Gait is often assessed on a 0-5 scale, with 0 as a gait without any identifiable abnormalities, and 5 as inability to stand (Kestin *et al.*, 1992). In the UK (Knowles *et al.*, 2008) and Denmark (Sanotra *et al.*, 2001), approximately 30% of all broiler chickens at slaughter age showed gait scores of >2 (an identifiable abnormality in the gait, with little impact on the chicken's function) (Kestin *et al.*, 1992). It is clear that these leg problems are painful; lame birds took 3 times as long as unimpaired birds to finish a feed-motivated obstacle course, but when an analgesic was administered, they completed it in the same time as unimpaired birds (McGeown *et al.*, 1999). The pain a broiler chicken experiences impairs its ability to express natural behaviours, such as preening while standing, walking, and standing idle (Weeks *et al.*, 2000). Leg problems, therefore, constitute a welfare problem.

Bradshaw *et al.* (2002) stated that leg problems can be hard to classify, because some pathologies show multifactorial causation (aetiology). This means that categories are often not mutually exclusive, and are arbitrarily chosen. The European Committee (2000) divided leg problems into being of developmental, degenerative, or infectious origin. Leg problems may include dermal, muscle, and tendon problems, but this thesis focuses on problems originating in the bones. Leg bone pathologies with a developmental origin are of particular interest in the current thesis, because it can be hypothesized that this category of leg bone pathologies, especially, can be improved through optimized long leg bone development during embryonic development and early life. The most well-known examples of developmental leg bone pathologies include tibial dyschondroplasia and distortions of the tibia and femur (Bradshaw *et al.*, 2002), such as valgus/varus deformity and rotated tibia (Bradshaw *et al.*, 2002; Bessei, 2006), and are described below. Bacterial chondronecrosis with osteomyelitis (BCO) is an example of a leg problem that is considered to be bacteriological in its origin (Bradshaw *et al.*, 2002), but mechanical damage through suboptimal early life vascularization may predispose bacteria to proliferate in the epiphysis (Figure 3) of bones (Wideman and Prisby, 2013). BCO is therefore described as well.

1.1 | Tibial dyschondroplasia

According to the European Committee (2000), tibial dyschondroplasia is the most common broiler leg bone lesion, with prevalence of 57.1% within Danish broiler flocks (Sanotra *et al.*, 2001). It often develops between week 2 and 5 post hatch. In tibial dyschondroplasia, prehypertrophying chondrocytes fail to progress into the next stage, where the tissue becomes vascularized and a calcified matrix surrounds it (Hargest *et al.*, 1984). Tibial dyschondroplasia consists of opaque, avascular cartilage lesions with necrotic chondrocytes, at the proximal end of the tibia (Figure 1). The cartilage masses can be found below the epiphyseal plate (also known growth plate), and extend into the metaphysis (Leach and Monsonego-Ornan, 2007). A large lesion may lead to a fractured epiphyseal plate (Bradshaw *et al.*, 2002), but it is more common for tibial dyschondroplasia to lead to bone deformity through tibial bowing, as evidenced by an abnormal tibial plateau angle (the angle of the tibia's proximal end relative to the tibia's long axis) (Lynch *et al.*, 1992). Not all cases of tibial dyschondroplasia will result in lameness: Lynch *et al.* (1992) found that 50% of their chickens with tibial dyschondroplasia were lame, all of which had an abnormal tibial plateau angle.

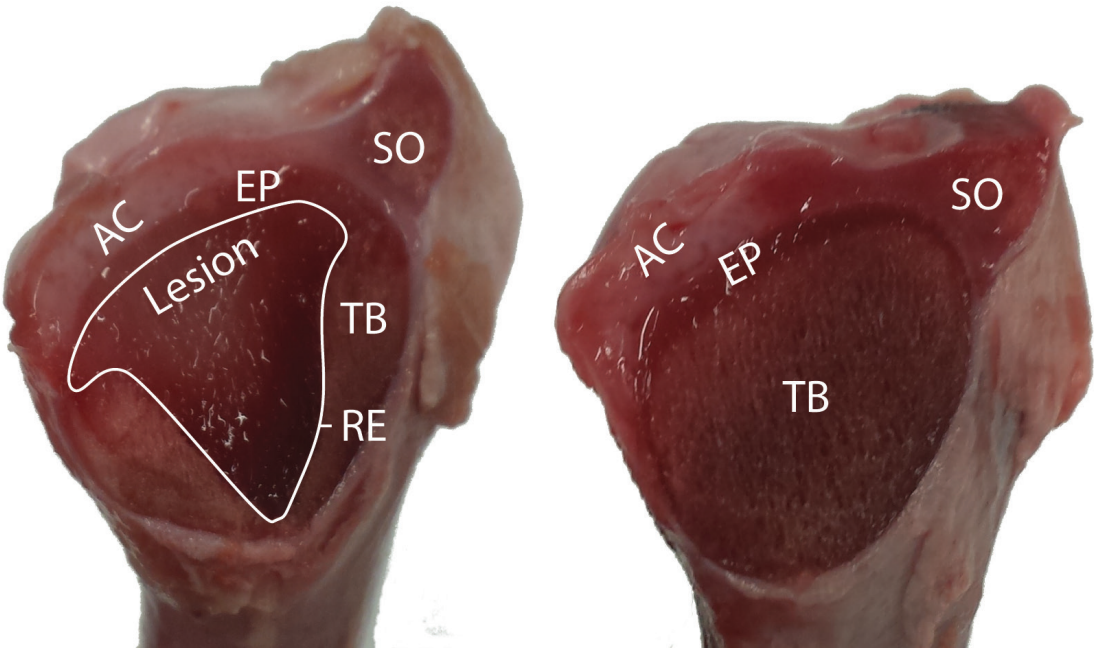


Figure 1. A broiler tibial head with an avascular cartilage lesion, indicating severe tibial dyschondroplasia (left), and a normal broiler chicken tibial head at D35 (right). AC = articular cartilage, EP = epiphyseal plate, TB = trabecular bone, separated by metaphyseal vessels, RE = resorbing edge, SO = secondary ossification centre. The cartilage lesion typical of tibial dyschondroplasia is circled. After Leach and Monsonego-Ornan (2007) and Hargets *et al.* (1984).

1.2 | Valgus/varus deformity

Valgus/varus deformity is characterized by abnormal angulation (>20 degrees) of the distal tibia at the intertarsal (hock) joint (Duff and Thorp, 1985). Severe cases can cause the gastrocnemius tendon to slip (Randall and Mills, 1981). In varus deformities, the hock rotates inward, and in the case of valgus deformities, it rotates outward (Figure 2) (Julian, 1984; Shim *et al.*, 2012). The deformity can affect one or both legs, and they are not necessarily affected to the same degree: one leg may even suffer from varus deformity, whereas the other suffers from valgus deformity (Julian, 1984). Prevalence of valgus/varus deformity was found to be 37.0% in Danish broiler flocks at market weight (Sanotra *et al.*, 2001). The aetiology of valgus/varus deformity is not well understood. In their review, Bradshaw *et al.* (2002) stated that immaturity of the joint's supporting tissue, suboptimal bone conformity, and poor ossification seem to play a role in the same condition in foals. Tibial dyschondroplasia is often found to occur along with valgus/varus deformity (Randall and Mills, 1981; Lynch *et al.*, 1992). Randall and Mills (1981) discussed that tibial dyschondroplasia may increase the likelihood of valgus/varus deformities, because it weakens the joint, but it does not seem to be a prerequisite as one can occur without the other.

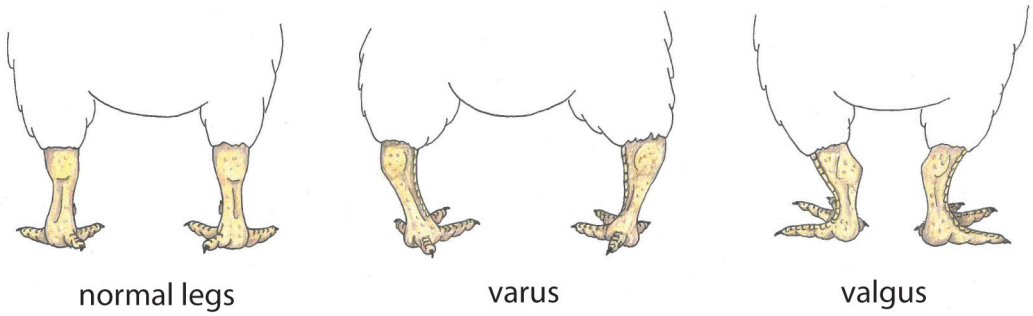


Figure 2. A broiler chicken viewed from the rear with normal posture, varus deformity in both legs, and valgus deformity in both legs.

1.3 | Rotated tibia

Valgus/varus deformities are also known as 'twisted leg', but that term is ambiguous as it seems to refer to torsional rather than angular deformities (Shim *et al.*, 2012). Rotated tibia is a torsional deformity of the tibia that is distinct from valgus/varus deformity, in that it has no angulation or gastrocnemius tendon displacement. Its aetiology is not well understood (Bradshaw *et al.*, 2002). Rotated tibia often occurs along with tibial dyschondroplasia (Lynch *et al.*, 1992). The prevalence of rotated tibia is unknown.

1.4 | Bacterial chondronecrosis with osteomyelitis and epiphyseolysis

Bacterial chondronecrosis with osteomyelitis (BCO) is another common leg problem. Dinev (2009) sampled clinically lame broilers on two Bulgarian farms and found that incidence of BCO was 91.2 to 95.5% in these lame birds. BCO presents itself as necrosis and bacterial infection, most often found in the proximal heads of bones (Wideman and Pevzner, 2012). It is also known as femoral head necrosis, but it may occur in any bone subjected to bending stresses (McNamee and Smyth, 2000; Wideman and Prisby, 2013). For BCO to appear, bacteria have to be present in the blood (McNamee and Smyth, 2000). Incidence of BCO may increase when broiler immunity is challenged by poultry diseases or environmental stressors (Wideman and Prisby, 2013). For example, Wideman and Pevzner (2012) found that injecting broilers with dexamethasone as an immunosuppressor raised the incidence of BCO from 0% in control, to 24 to 68% in treated groups. Although BCO is commonly described as being of infectious origin, some of the aetiology of BCO lies in early bone development, because development and vascularization of the epiphyseal plate affect susceptibility of the chicken to potential bacterial infection in the bone. Wideman and Prisby (2013) described how structural immaturity of the epiphyseal plate, and suboptimal blood supply to the epiphyseal plate, can lead to small lesions in the epiphyseal plate of the bone in which bacteria can colonize.

Epiphyseolysis is the phenomenon where the femoral head is separated from the articular cartilage. It can be a result of BCO, but it may also occur in the absence of bacteria (Thorp *et al*., 1993). Thorp *et al.* (1993) found that the histology of epiphyseolysis with and without bacterial infection was distinctly different. In non-infectious separations of the proximal cartilage, blockage of blood vessels and cartilage degeneration and disorganisation were visible, whereas infectious cases showed necrotic chondrocytes. Packialakshmi *et al.* (2015) distinguish traumatic and non-traumatic causes of femoral head problems. Trauma indicates physical injury as a result of environmental conditions, whereas non-traumatic causes include vascular occlusion (Packialakshmi *et al*., 2015); both may predispose a bone to BCO or epiphyseolysis. If part of the origin of BCO and epiphyseolysis lies in suboptimal vascularization and ossification of the articular cartilage, it can be speculated that stimulation of early life bone development may lower the incidence of BCO and epiphyseolysis.

2 | Early life bone development

To understand how prevalence of leg bone pathologies can be reduced by early life interventions, it is helpful to understand which processes take place during embryonic and perinatal life of a broiler chicken, and via which pathways.

2.1 | Morphology

The potential limb regions in the broiler embryo become first visible after approximately 50-55 hours of incubation, as condensations of mesenchymal cells commit to later become cartilage cells called chondrocytes (Liem *et al.*, 2001; Bellairs and Osmond, 2005; Gilbert, 2006). After 3 days of incubation (embryonic day 3, E3), each limb bud measures approximately 1 x 1 mm. A cartilaginous model of the hind limbs starts to appear at approximately E5 and the forelimbs appear at approximately E6 (Bellairs and Osmond, 2005). At approximately E7, the main skeletal components are fully laid out in a cartilage model (Kürtül *et al.*, 2009) and at approximately E7.5, chondrocytes become hypertrophic and produce cartilage matrix. A mineralized osteoid ring is formed around the cartilage core, expanding radially (Pechak *et al.*, 1986a,b). This can first be identified as a primary ossification centre from E9 onward (Kürtül *et al.*, 2009). The process of primary ossification in chickens differs from that in humans, where cartilage is replaced by bone tissue in a process called endochondral ossification (Gilbert, 2006). Instead, the cartilaginous part of the embryonic chicken bone is replaced by marrow in the medulla, while bone tissue is deposited in a collar around the cartilage core by bone cells derived directly from mesenchymal progrenitor cells. This type of ossification is similar to intramembranous osteogenesis as seen in, for example, the human skull (Pechak *et al.*, 1986a,b). From E9 onward, the cartilaginous core of the bone is replaced by bone marrow, consisting of fat and hemopoietic cells, and the marrow eventually expands into the original bone collar. Meanwhile, the first osteoid ring becomes well vascularized and forms the eventual trabecular channels in the trabecular bone. The bone collar continues to grow and increases in diameter (Pechak *et al.*, 1986b) and length (Kürtül *et al.*, 2009), as osteoblasts, which are bone forming cells, produce osteoid towards the bone core. Bone tissue girth increase is greater than marrow cavity increase by E14 (Pechak *et al.*, 1986b).

Length growth occurs near the bone's ends. Epiphyseal plate cartilage cells transition from resting to proliferating cells, become hypertrophic, and become apoptotic, or they differentiate into an osteoblast-like cell (Figure 3; Roach, 1997; Mackie *et al.*, 2008). Resorption of the cartilage cells occurs asymmetrically across the epiphyseal plate through vascular invasion at the tip of marrow canals. Because the cartilage initially remains mostly intact, the hypertrophic zone increases in thickness during embryonic development as length growth progresses (Roach, 1997). At approximately E17, all skeletal elements start to resemble the structural shape of a mature stage bone. The many processes that take place in bone development during incubation emphasize the importance of the incubation period for laying the fundaments of a correct bone.

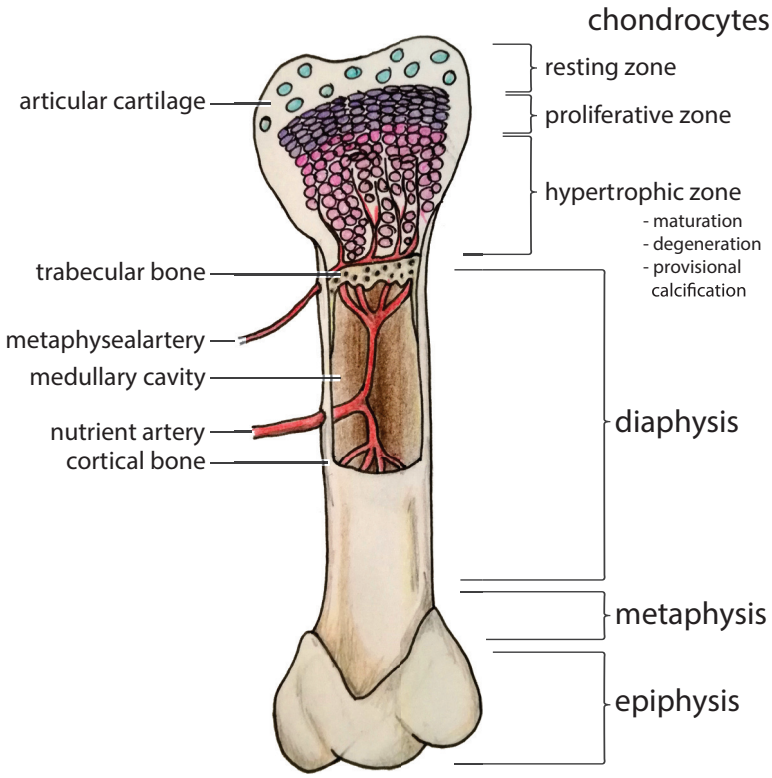


Figure 3. A tibia with simplified endochondral ossification at the epiphyseal plate, at approximately E18. After: Brighton (1978), Pines and Hurwitz (1991), Leach and Monsonego-Ornan (2007), and Herzog *et al.* (2011).

The secondary ossification centres, where endochondral ossification at the epiphyseal end of the bone takes place, do not appear until after hatching (Roach, 1997) (see Figure 1 for an example of tibiae with secondary ossification centres). In the secondary ossification centres, apoptotic cartilage cells are resorbed to allow invasion of the extracellular cartilage matrix by blood vessels and bone cells. Osteoblasts deposit bone matrix on the remnants of the cartilage matrix, using it as scaffolding. Osteoclasts are bone resorbing cells, and they assist in removal of cartilage matrix, as well as bone matrix for bone remodelling (Mackie *et al.*, 2008). Length growth continues from the epiphyseal plate, a distinct plate of cartilage cells located at the bone's epiphyseal end (Roach, 1997). Ossification continues after hatching (Kürtül *et al.*, 2009) and high relative length growth takes place in the first week post hatch (Applegate and Lilburn, 2002). Although bone length growth is not yet complete at slaughter age in broiler chickens, these findings suggest that particularly the embryonic and early post hatch period are of vital importance in laying the fundament of well-developed bones.

2.2 | Pathways

In the current thesis, light during incubation and in the early post hatch phase is used, aiming to stimulate early life bone development. Two pathways seem to be involved in the effect of light on bone development: the endocrine system and activity.

Endocrine system

The involvement of the endocrine system in embryonic bone development has been investigated in mammals in more detail than in chickens. In many cases, the regulatory mechanisms found in other species are presumed to apply to chickens as well (Leach and Monsonego-Ornan, 2007). A lot of the literature described here applies to post hatch animals. It is not known whether the same principles always apply during embryology.

In adult male rats, early life bone development is largely controlled by thyroid hormones, IGF-I, and GH (Figure 4) (Ostrowska *et al.*, 2002), which start to become functional in chicken embryos by the second half of incubation (Harvey *et al.*, 1998). In their review, Robson *et al.* (2002) suggest that insulin-like growth factor (IGF)-I is the most important growth factor in the regulation of longitudinal bone development (increase in length of the bone) during mammalian embryonic development (Robson *et al.*, 2002). In embryonic chickens, McQueeney and Dealy (2001) found high IGF-I expression in newly formed bone matrix, osteoblasts, and osteoclasts. It was furthermore found in high concentrations in hypertrophic chondrocytes undergoing programmed cell death at the tips of the epiphyseal plate, which could suggest that IGF-I is involved in maintaining homeostasis and delaying cell death in chondrocytes (McQueeney and Dealy, 2001). Leach *et al.* (2007) found that the role of IGF-I in postnatal chickens revolved mostly around the stimulation of collagen production. In postnatal chickens, IGF-I expression was increased in hypertrophic chondrocytes, although less so than IGF-II, and it is involved in epiphyseal chondrocyte matrix synthesis (Leach *et al.*, 2007).

Postnatally, pituitary growth hormone (GH) is an additional important modulator of bone development (Figure 4). GH and IGF-I play an important role in the hypothalamus-pituitary-epiphyseal plate axis of mammals (Van der Eerden *et al.*, 2003). Chicken chondrocytes express the GH receptor (Monsonego *et al.*, 1993, 1997), and GH in chickens inhibits differentiation of the chondrocytes at the epiphyseal plate, constraining the chondrocytes to remain in their proliferative state longer. When combined with epidermal growth factor in vitro, GH upregulated chondrocyte proliferation (Monsonego *et al.*, 1995). In mammals, GH has an indirect effect on bone growth through stimulation of IGF-I production in the liver (Robson *et al.*, 2002; Van der Eerden *et al.*, 2003) and in skeletal muscles (Velloso, 2008), and this pathway also exists in chickens, but not yet embryonically (reviewed by Kim, 2010). Hepatic IGF-I, in turn, provides feedback control on GH secretion in the mammalian pituitary (Robson *et al.*, 2002). GH secreting

cells are found in the anterior pituitaries of chicken embryos from E16 onward (Porter *et al.*, 1995).

The thyroid hormone T3 and its precursor T4 have a direct effect on embryonic and postnatal longitudinal bone growth through vascular invasion in mammals (Robson *et al.*, 2002) as well as stimulation of proliferation and differentiation of chondrocytes in the epiphyseal plate in chickens (Figure 4) (Van der Eerden *et al.*, 2003). In mammals, the thyroid hormones have an indirect effect on bone growth through their stimulatory effect on GH and IGF-I secretion (Van der Eerden *et al.*, 2003).

Another important hormone involved in bone formation is melatonin (Figure 4), which is secreted by the pineal gland. Melatonin production is photoperiod-dependent, and it peaks during dark periods (Özkan *et al.*, 2012a). Melatonin starts to show a clear circadian release rhythm by E16 to E18 (Csernus *et al.*, 2007). Providing a circadian rhythm through light may enhance the amplitude of melatonin release compared to the free running rhythm existing under continuous light or darkness (Lamošová *et al.*, 1995), and it can increase the overall melatonin levels over a whole lighting cycle; Archer and Mench (2014) incubated broilers under 12 hours of light, followed by 12 hours of darkness (12L:12D) and continuous darkness (24D), and found higher overall melatonin levels for broilers incubated under 12L:12D. In postnatal rats, melatonin promoted bone growth, expressed through gene expression of proteins involved in bone formation (Roth *et al.*, 1999). These proteins affect osteoblast formation and mineralization of the bone matrix. At low levels of melatonin, osteoclast function (which resorbs bone) is augmented, whereas osteoblast function (which forms new bone) is impaired (Cardinali *et al.*, 2003). Melatonin furthermore seems to play a regulatory role in the daily rhythm of release of GH and IGF-I, with GH and IGF-I peaking along with melatonin release in dark periods (Ostrowska *et al.*, 2002). It can be speculated that dark incubation leads to higher bone development than continuous light, because melatonin is released under dark periods. Furthermore, a circadian light-dark rhythm may be even more beneficial for bone development than continuous darkness, because total melatonin levels seem to be higher for applying a lighting schedule than for 24D.

Glucocorticoids are a negative regulator of bone growth in mammals (Figure 4). Glucocorticoid release is increased when an animal is in a stressful physiological state (Siegel, 1980; Hasan *et al.*, 2011). Glucocorticoids inhibit release of GH and IGF-I in mammals (Robson *et al.*, 2002; Van der Eerden *et al.*, 2003) and modulate local levels of thyroid hormones (Van der Eerden *et al.*, 2003). They furthermore directly increase collagen degradation (Hasan *et al.*, 2011), chondrocyte apoptosis, and reduce chondrocyte proliferation (Van der Eerden *et al.*, 2003) as well as osteoblast proliferation, absorption, and reabsorption of calcium in the gastrointestinal tract (Robson *et al.*, 2002) in mammals. Through these pathways, they enhance mammalian bone resorption and reduce bone matrix production (Robson *et al.*, 2002).

Activity

Activity is another pathway to influence bone development, and it can be stimulated through abiotic factors during incubation and in early life. Post hatch movements have a large impact on leg bone development of broilers (Bradshaw *et al.*, 2002; Bessei, 2006). The exact mechanisms and cells involved in the transduction of mechanical stimulation to bone cells have not been established yet. Differences in fluid flow inside the bone pores, hydrostatic pressure, direct cell strain, and electric fields as a result of bone deformation have been speculated to influence osteocyte and osteoblast functioning (Sikavitsas *et al.*, 2001). It is known that cartilage formation and ossification are stimulated by increased mechanical strain on the bone as a result of movement (Müller, 2003). Furthermore, mechanical loading of osteocytes increases IGF-I release (Sikavitsas *et al.*, 2001). The effect of mechanical loading on bone development is already present in the embryo: movement in the form of muscle contractions starts as early as E4, and peaks at E11 - E13 with nearly continuous movement (Bekoff, 1981). It has even been shown that several bones will show retarded growth (Bertram *et al.*, 1996) and joints will not form or they develop abnormally (Murray and Drachman, 1969) if movement of a chicken embryo is experimentally paralyzed using botulinum toxin. Applying light during incubation may affect movement in chicken embryos; Pollard *et al.* (2016) found increased movement of embryos in eggs that were windowed and exposed to an external light source compared to embryos that were kept in more dark conditions. This, in turn, may increase bone development.

3 | Lighting schedules and temperature

The aetiology of developmental leg bone pathologies, such as tibial dyschondroplasia and valgus/varus deformity, partly seems to lie in suboptimal embryonic or early life bone development, exacerbated with a disproportional weight load of the bone in later life as a result of the high growth rate of broilers. It can, therefore, be speculated that particularly the incidence of some developmental leg bone pathologies may be reduced through optimal circumstances for bone development during incubation (Yalçin *et al.*, 2007; Oviedo-Rondón *et al.*, 2008, 2009a) and in the post hatch brooding phase (Oviedo-Rondón *et al.*, 2013). In the current thesis, light and temperature during incubation and in the brooding phase (the first 0 to 4 days of a broiler chicken's life) in relation to bone development and leg health are investigated.

3.1 | Light during incubation

The sun emits a large spectrum of electromagnetic radiation ranging from radio waves to gamma waves. The wavelengths that can be perceived by the human eye are referred to

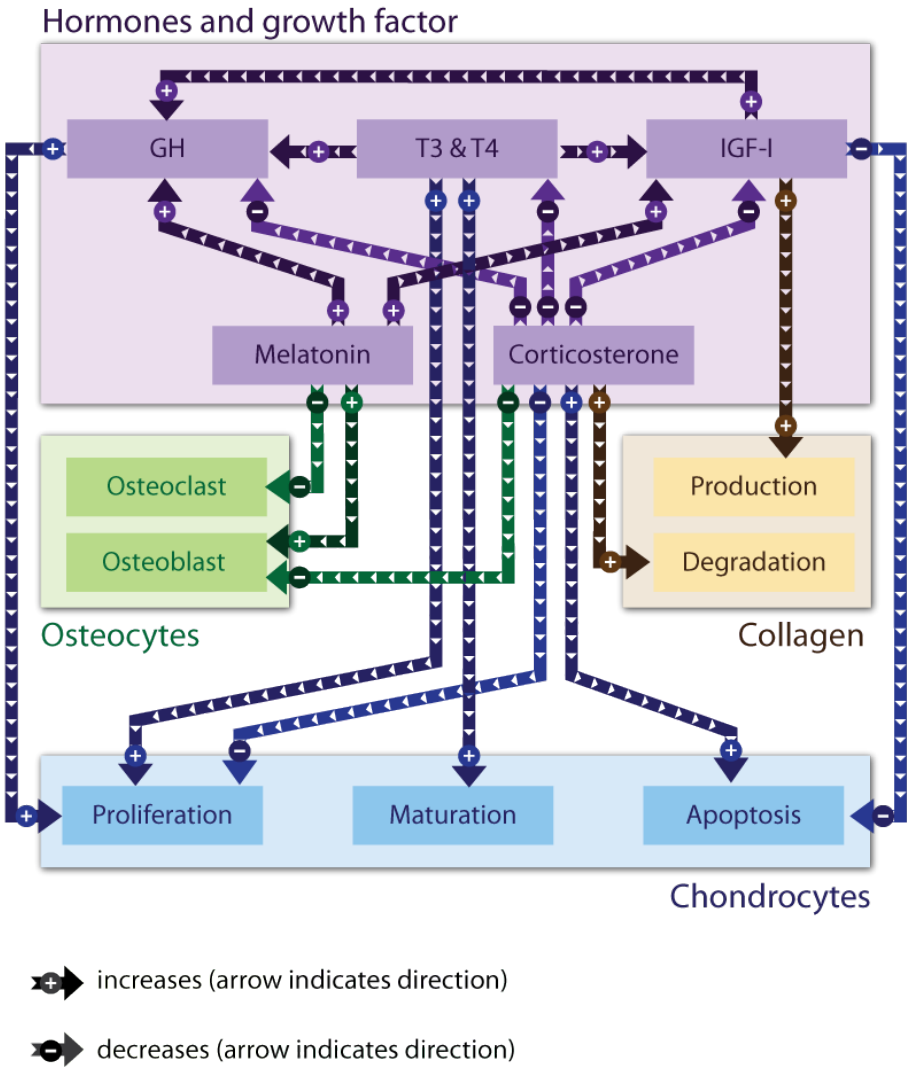


Figure 4. Suggested pathways through which growth hormone (GH), thyroid hormones (T3 and T4), insulin-like growth factor I (IGF-I), melatonin, and corticosterone may affect embryonic chicken bone development.

as 'visible light'. However, the chicken's eye differs in spectral sensitivity from the human eye (Prescott and Wathes, 1999), because chickens possess four types of cone pigments, instead of the three types in humans (Govardovskiĭ and Zueva, 1977). Colour vision in chickens differs from that of humans in two ways. Firstly, chickens are able to see a broader spectrum of light, including UV_A radiation. Secondly, they see a different colour balance than humans do: chickens show peak sensitivity to blue, green, and red light, whereas humans are primarily sensitive to blue and green light (Prescott and Wathes, 1999). Besides their retinal photoreceptors for image-forming vision, chickens can addi-

tionally directly sense light that enters their pineal gland through the skull. The pineal gland is a neuroendocrine gland, located directly under the skull, at the dorsal surface of the brain. It plays a role in seasonal and diurnal rhythmicity, by transforming photoperiodic information into hormonal output of melatonin (Skwarło-Sońta, 1996).

How much light reaches the embryo depends on characteristics of the light itself: transmission of light is minimal in the near-ultra violet range (200 to 300 nm) and maximal in the near-infrared range (1075 nm) (Shafey *et al.*, 2002). Furthermore, light absorption by the eggshell is dependent on its pigmentation and the region of the eggshell: dark pigmented Hybro eggs had higher absorption than light pigmented Hybro eggs, and absorption of light was higher on the egg's equator than at the small or large pole (Shafey *et al.*, 2004).

In a commercial situation, chicken embryos are incubated in complete darkness, except for the moment the eggs are candled for embryonic viability, and transferred from the setter incubator to the hatcher. However, there are indications that providing light during incubation has a positive effect on embryonic development. The pineal gland of chicken embryos becomes entrained to a circadian melatonin release pattern already during the last week of incubation. Embryos still showed a circadian release pattern even after 2 days of darkness when they were incubated under a 12L:12D schedule until E18 (Zeman *et al.*, 1999). Melatonin release is darkness dependent, but its release is stimulated even more when dark periods are alternated by light periods. Plasma melatonin levels at 6 days post hatch were higher for chickens incubated under a light-dark schedule of 16 hours of light, followed by 8 hours of darkness (16L:8D) compared to continuous dark incubation (Özkan *et al.*, 2012b). Because melatonin is involved in the development of new bone (paragraph 2.1) (Cardinali *et al.*, 2003), it can be speculated that bone development is stimulated in embryos incubated under a light-dark schedule compared to complete darkness.

Light during incubation furthermore has an effect on lateralization and brain development in the chicken embryo, dependent on the moment of incubation when embryos are exposed to light. Differences in brain development may have a long-lasting effect on post hatch chicken behaviour. Chickens illuminated on E19 showed less fearful behaviour, more approach behaviour to a novel stimulus, and less distress vocalizations 48 hours post hatch than chickens exposed to light on E15 or E17, probably because development of the visual system is not complete before E19 (Adam and Dimond, 1971). Archer and Mench (2017) incubated broiler embryos under continuous light (24L), 12L:12D, or 24D, and found less fear behaviour at 5 or 6 weeks post hatch during a tonic immobility and an inversion test for 12L:12D and 24L than for 24D. It can be hypothesized that light from E19 onwards can be used to create a less fearful chicken that will show more natural behaviour, and is more active, than a chicken that was exposed to darkness throughout incubation.

3.2 | Incubation temperature

When studying light during incubation, it is important to consider the potentially confounding effect of adding a light source on incubation temperature. Heat can be transferred from the light source through convection (as the light source heats up itself, and warm air blows over the eggs) or radiation (as light waves reach a mass). Modern LED systems have minimized heat production in the light source itself, and radiate little heat as the UV portion of the light is minimized. Even so, some heat transfer will always be present, which could heat up eggs or chickens and it is important to correct for this. Although some papers about light during incubation do not mention if and how heating of the eggs was prevented (Walter and Voitle, 1972; Zakaria, 1989; Shafey *et al.*, 2005), others have measured how incubator temperature was affected by lighted incubation (Garwood *et al.*, 1973; Isakson *et al.*, 1970; Tamimie and Fox, 1967). The development of an embryo is largely driven by its temperature, which is affected through incubator temperature, air velocity, and relative humidity, as well as eggshell conduction, heat loss, and embryonic heat production (Lourens *et al.*, 2011). As a result, incubator temperature does not always reflect the temperature an embryo experiences. As it is hard to measure embryo temperature without sacrificing the embryo, Lourens *et al.* (2011) recommended using eggshell temperature (EST) as a reflection of embryo temperature, as these are closely related. An eggshell temperature of +/- 37.5°C is considered to be optimal for highest hatchability and chick quality (Lourens *et al.*, 2005, 2007; Molenaar *et al.*, 2010, 2011) and is often used as the control treatment. Indeed, some previous studies on light during incubation measured EST to rule out temperature effects (Walter and Voitle, 1973; Özkan *et al.*, 2012a,b).

How temperature is affected by lighted treatments is relevant for the current thesis, as effects of incubator temperature on leg bone development have been found, although results are somewhat inconsistent. At high (>38.9°C) EST or incubator temperatures, both higher (Yalçin *et al.*, 2007; Oviedo-Rondón *et al.*, 2009b) and lower (Oviedo-Rondón *et al.*, 2008) relative leg bone weights have been found compared to control temperature treatment (37.8 to 38.0°C). Leg bone lengths have furthermore both been found to be shorter (Oviedo-Rondón *et al.*, 2008) and longer (Yalçin and Siegel, 2003; Hammond *et al.*, 2007) when temperatures deviated above or below the control temperature of 37.8 to 38.0°C. These effects may vary as a result of differences in the moment during the incubation period at which the treatments were applied, or differences between the use of incubator temperature or EST.

It can be speculated that bone development is inhibited under high incubation temperatures because of suppressed thyroid hormone concentrations. T3 and T4 concentrations are found to be decreased at high incubator temperatures from E17 onward (39°C compared to 36° or 37°C) (Wineland *et al.*, 2006). Corticosterone, the major glucocorticoid in chickens, was found to increase at high (>39°C) incubator temperatures for 6 hours a

day compared to control incubator temperature (37.8ºC) between E11 and E20 (Iqbal *et al.*, 1990). The same was found if this high temperature was applied from E7 until E16 for 12 hours a day (Piestun *et al.*, 2008). Decreased thyroid hormone concentrations and increased glucocorticoid levels can result in decreased proliferation and differentiation of chondrocytes (Robson *et al.*, 2002; Van der Eerden *et al.*, 2003) as well as decreased GH and IGF-I secretion (Van der Eerden *et al.*, 2003) (see 2.2: Pathways).

Changes in incubation temperature can furthermore be used to enhance pineal melatonin rhythmicity. Zeman *et al.* (2004) incubated eggs under a 12L:12D lighting cycle and reduced the incubator temperature to 4°C for 1 hour. When the temperature was dropped during the dark phase, pineal melatonin concentrations were higher than at the normal incubation temperature of 37.5°C. When it was dropped during the light phase, pineal melatonin concentrations did not change. These results suggest that an embryo can use both light and temperature cues to entrain the melatonin-generating system (Zeman *et al.*, 2004). It can therefore be speculated that cooling the eggs in a circadian fashion during the dark period in a lighted incubation cycle may further enhance embryonic bone development.

Although high EST inhibits release of several hormones involved in embryonic bone development, high EST may stimulate bone development through its effect on embryonic movement. Both very high (44.4°C) and very low (30.5°C) incubator temperatures for a period of less than 2 hours at E9 have been shown to increase neuromuscular activity compared to the control treatment (37.5°C incubator temperature throughout) (Oppenheim and Levin, 1975). Embryonic movement in response to high incubator temperatures may thereby have a stimulatory and long lasting effect on bone development (Hammond *et al.*, 2007).

To conclude, effects of incubation temperature on embryonic leg bone development are complicated and dependent on many aspects, such as incubation phase and duration of the treatments, severity of the temperature treatments, and interactions with possible light-dark schedules. It is therefore important to consider effects of heat transfer from the light source on bone development in any experiment on light during incubation.

3.3 | Lighting schedule post hatch

Effects of a variety of post hatch lighting schedules till slaughter age on bone development have been studied previously. However, effects of light have usually not been studied immediately post hatch. In previous studies, chickens were often kept under continuous light for at least 2 to 4 days before being subjected to experimental light-dark schedules to acclimatize them to their surroundings. It would be interesting to study effects of light-dark schedules on bone development in early life, because of the importance of the early post-natal phase in ossification and length growth (Applegate

and Lilburn, 2002; Kürtül *et al.*, 2009). Several pathways may play a role in this effect of light-dark schedule on bone growth. Firstly, effects are likely related to increased feed intake and body weight at longer light periods (Lewis *et al.*, 2009a). In the long term, high body mass inhibits broilers in showing active behaviour, thereby decreasing their bone development. Post hatch, low physical activity levels in heavy broiler chickens of several weeks of age are speculated to be the main cause of leg bone pathologies. Broilers are impaired in their activity by their weight, which increases with age (Bizeray *et al.*, 2000; Bokkers and Koene, 2003). Activity (standing behaviour and wing-flapping) was increased throughout the grow out period and sitting behaviour was decreased for broilers exposed to a light-dark schedule compared to broilers housed under continuous light (Sanotra *et al.*, 2010). Furthermore, post hatch bone development may be affected through the same endocrine pathway as described in paragraph 2.2.

4 | Aims and hypotheses

The area of light exposure during incubation has been largely unexplored so far, particularly with reference to bone development and leg bone pathologies. This is a promising field of research, as it has been proven previously that embryonic development is affected by light (Özkan *et al.*, 2009, 2012a,b; Rozenboim *et al.*, 2004). In the brooding phase (D0 till D4 post hatch), data on the influence of light on bone development and incidence of leg bone pathologies is lacking in literature. Light-dark schedules and colour have mostly been investigated when applied beyond several days post hatch and therefore effects of light-dark schedules and colours on bone development of a newly hatched broiler chicken are largely unknown.

The following objectives are formulated in the present thesis:

1. to investigate effects of lighting schedules during incubation and post hatch on bone development and underlying pathways of the endocrine system and embryonic activity

2. to investigate the long-term effect of these changes in bone development during incubation and the brooding phase on leg health (incidence of leg bone pathologies and gait scores) in later life.

The main hypothesis is that leg bone pathologies in broilers are in part a result of suboptimal bone development during embryonic development and in their early post hatch life. It is hypothesized that early life bone development can be stimulated in the incubation and brooding phase by providing a circadian lighting schedule during incubation and lighting schedules in the brooding phase. These factors may work mainly through two pathways: the endocrine system and embryonic activity, which are partly interrelated. In lighted incubation, temperature is taken into account, because EST will affect leg

embryonic bone development.

5 | Outline of the thesis

The magnitude of the effect of temperature on embryonic bone development was addressed in Chapter 2. It was investigated how bone morphology at hatch was affected by 4 different EST: Low (36.9°C), Normal (37.8°C), High (38.6°C), and Very high (39.4°C) throughout incubation.

Chapter 3 and 4 describe a first experiment with incubation lighting schedules. Ross 308 eggs were incubated under continuous light (24L), 12h of light, followed by 12h of darkness (12L:12D), or continuous darkness (24D) from set until hatch at a constant EST of 37.8°C. In Chapter 3, embryonic bone development, bone morphology and microstructure at hatch, D21, and D35 post hatch, and incidence of tibial dyschondroplasia at D35 were investigated. Plasma melatonin was measured between embryonic day (E)18.8 and E19.5, and growth hormone (GH), and IGF-I concentrations were measured at hatch. In Chapter 4, production results (chick quality and organ weights at hatch, and post hatch organ weights, body weight gain, feed intake, and feed conversion) are described.

In Chapter 5 and 6, a second experiment with light during incubation is described. Ross 308 eggs were again incubated at 1 of 3 light treatments: 24L, 16h of light, followed by 8h of darkness (16L:8D), or 24D. In Chapter 5, it is described how expression of genes involved in cartilage development and ossification were affected by light in tibias on E13, E17, and moment of hatch. Femur and tibia dimensions were measured at hatch. Pineal melatonin and plasma GH and corticosterone were measured between E18.8 and E19.5. Embryonic heart rate was measured from E12 till hatching as a reflection of embryonic activity. In Chapter 6, it is described how chickens incubated under the 3 lighting schedules were subsequently housed under 24L or 16L:8D from hatching till D35 post hatch. Gait was scored on D21, D28, and D34. Femur and tibia dimensions, mineral density, mineral content, and incidence and severity of several leg problems were scored on D35. Production performance data were furthermore recorded weekly.

Chapter 7 describes two experiments where newly hatched Ross 308 chickens were exposed to lighting schedules in the brooding phase. From D0.5 till D4 post hatch, chickens were kept under 24L, 2 hours of light, followed by 1 hour of darkness (2L:1D), or 2 hours of light, followed by 6 hours of darkness (2L:6D). Light periods transitioned to dark periods either abruptly or gradually through dimming. Dimming took 3 minutes in the first experiment, and 15 minutes in the second experiment. At D4, femur and tibia dimensions and relative asymmetry were measured. Chicken organ weights and production performance parameters were recorded.

In the general discussion in Chapter 8, results from the experiments are discussed and integrated to gain insight in the overall effect of incubation and post hatch lighting schedules on broiler chicken leg bone development and leg bone health. Several physiological pathways are discussed that might explain the results as found in the experiments.

6 | References

Adam, J., and S. J. Dimond. 1971. The effect of visual stimulation at different stages of embryonic development on approach behaviour. Anim. Behav. 19: 51-54.

Applegate, T. J., and M. S. Lilburn. 2002. Growth of the femur and tibia of a commercial broiler line. Poult. Sci. 81: 1289-1294.

Archer, G. S., and J. A. Mench. 2014. The effects of the duration and onset of light stimulation during incubation on the behavior, plasma melatonin levels, and productivity of broiler chickens. J. Anim. Sci. 92: 1753-1758.

Archer, G. S., and J. A. Mench. 2017. Exposing avian embryos to light affects post-hatch anti-predator fear responses. Appl. Anim. Behav. Sci. 186: 80-84.

Aviagen. 2014. Vleeskuiken standaarden en resultaten Ross 308. Published by Aviagen EPI NV, Arendonk, Belgium, and Aviagen EPI BV, Roermond, the Netherlands.

Bekoff, A. 1981 Embryonic development of chick motor behavior. Trends NeuroSci. 4: 181-184.

Bellairs, R., and M. Osmond. 2005. The atlas of chick development. Elsevier Academic Press, 84 Theobald's Road, London, WCIX 8RR, UK.

Bessei, W. 2006. Welfare of broilers: a review. World's Poult. Sci. J. 62: 455-466.

Bertram, J. E., L.S. Greenberg, T. Miyake, B.K. Hall. 1996. Paralysis and growth in the chick: growth shape trajectories of the pelvic limb. Growth Dev. Aging 61: 51-60.

Bizeray, D., C. Leterrier, P. Constantin, M. Picard, and J. M. Faure. 2000. Early locomotor behavior in genetic stocks of chickens with different growth rates. Appl. Anim. Behav. Sci. 68: 231-242.

Bokkers, E. A. M., and P. Koene. 2003. Behaviour of fast- and slow growing broilers to 12 weeks of age and the physical consequences. Appl. Anim. Behav. Sci. 81: 59-72.

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.

Brighton, C. T. 1978. Structure and function of the growth plate. Clin. Orthop. Relat. Res. 136: 22-32.

Cardinali, D. P., M. G. Ladizesky, V. Boggio, R. A. Cutrera, and C. Mautalen. 2003. Melatonin effects on bone: experimental facts and clinical perspectives. Pineal Res. 34: 81-87.

Csernus, V. J., A. D. Nagy, and N. Faluhelyi. 2007. Development of the rhythmic melatonin secretion in the embryonic chicken pineal gland. Gen. Comp. Endocrinol. 152: 148-153.

Duff, S. R. I., and B. H. Thorp. 1985. Patterns of physiological bone torsion in the pelvic appendicular skeletons of domestic fowl. Res. Vet. Sci. 40: 333-338.

European Committee. 2000. The welfare of chickens of chickens kept for meat production. Report of the Scientific Committee on Animal Health and

Animal Welfare. European Commission, Health and Consumer Protection Directorate-General (adopted 21 March 2000).

Garwood, V. A., E. J. Thornton, and P. C. Lowe. 1973. The effect of continuous illumination of incubating chicken eggs on embryonic development. Poult. Sci. 52: 337-340.

Gilbert, S. 2006. Developmental Biology, Eighth edition. Sinauer Associates, Inc. 23 Plumtree Road, Sunderland, MA 01375 USA.

Hammond, C. L., B. H. Simbi, and N. C. Stickland. 2007. In ovo temperature manipulation influences embryonic motility and growth of limb tissues in the chick (Gallus gallus). J. Exp. Biol. 210: 2667-2675.

Hargest, T. E., R. M. Leach, and C. V. Gay. 1985. Avian tibial dyschondroplasia. I. Ultrastructure. Am. J. Pathol. 119: 175.

Harvey, S., C. D. M. Johnson, P. Sharma, E. J. Sanders, and K. L. Hull. 1998. Growth hormone: a paracrine growth factor in embryonic development? Comp. Biochem. Physiol. 119: 305-315.

Hasan, K. M. M., Md. S. Rahman, K. M. T. Arif, and M. E. Sobhani. 2011. Physiological stress and aging: role of glucocorticoids (GCs). Age 34: 1421-1433.

Herzog, A., O. Genin, A. Hasdai, D. Shinder, and M. Pines. 2011. Hsp90 and angiogenesis in bone disorders—lessons from the avian growth plate. Am. J. Physiol. Regul. Integr. Comp. Physiol. 301: R140-R147.

Iqbal, A., E. Decuypere, A. A. El Azim, and E. R. Kühn. 1990. Pre-and post-hatch high temperature exposure affects the thyroid hormones and corticosterone response to acute heat stress in growing chicken (Gallus domesticus). J. Therm. Biol. 15: 149-153.

Isakson, S. T., B. J. Huffman, and P. B. Siegel. 1970. Intensities of incandescent light and the development of chick embryos in ovo and in vitro. Comp. Biochem. Physiol. 35: 229-235.

Julian, R. J. 1984. Valgus-varus deformity of the intertarsal joint in broiler chickens. Can. Vet. J. 25: 254-258.

Kestin, S. C, T. G. Knowles, A. E. Tinch, N. G. Gregory. 1992. Prevalence of leg weakness in broiler chickens and its relationship with genotype. Vet. Rec. 131: 190-194.

Kestin, S. C., S. Gordon, G. Su, and P. Sørensen. 2001. Relationships in broiler chickens between lameness, liveweight, growth rate and age. Vet. Rec. 148: 195-197.

Knowles, T. G., S. C. Kestin, S. M. Haslam, S. N. Brown, L. E. Green, A. Butterworth, S. J. Pope, D. Pfeiffer, and C. J. Nicol. 2008. Leg disorders in broiler chickens: prevalence, risk factors and prevention. PlosOne 3(2): e1545.

Kürtül, I., S. H. Atalgin, K. Aslan, and E. U. Bozkurt. 2009. Ossification and growth of the bones of the wings and legs in prehatching period of the Hubbert strain broiler. Kafkas Univ. Vet. Fak. Derg. 15: 869-874.

Lamošová, D., M. Zeman, M. Mackova, and E. Gwinner. 1995. Development of rhythmic melatonin synthesis in

cultured pineal glands and pineal cells isolated from chick embryo. Experientia 51: 970-975.

Leach, R. M. Jr., and E. Monsonego-Ornan. 2007. Tibial dyschondroplasia 40 years later. Poult. Sci. 86: 2053-2058.

Lewis, P. D., R. Danisman, and R. M. Gous. 2009. Photoperiodic responses of broilers. I. Growth, feeding behavior, breast meat yield, and testicular growth. Br. Poult. Sci. 50: 657-666.

Liem, K. F., W. E. Bemis, W. F. Walker Jr., and L. Grande. 2001. Functional Anatomy of the Vertebrates. Third edition. Brooks/Cole-Thomson Learning. 10 Davis Drive, Belmont, CA 94002-3098 USA.

Lourens, A., H. van den Brand, R. Meijerhof, and B. Kemp. 2005. Effect of eggshell temperature during incubation on embryonic development, hatchability, and post-hatch development. Poult. Sci. 84: 914-920.

Lourens, A., H. van den Brand, M. J. W. Heetkamp, R. Meijerhof, and B. Kemp. 2007. Effects of eggshell temperature and oxygen concentration on embryonic growth and metabolism during incubation. Poult. Sci. 86: 2194-2199.

Lourens, A., R. Meijerhof, B. Kemp, and H. van den Brand. 2011. Energy partitioning and consequences for embryo temperature: a theoretical approach. Poult. Sci. 90: 516-523.

Lynch, M., B. H. Thorp, and C. C. Whitehead. 1992. Avian tibial dyschondroplasia as a cause of bone deformity. Avian Pathol. 21: 275-285.

Mackie, E. J., Y. A. Ahmed, L. Tatarczuch, K. S. Chen, and M. Mirams. 2008. Review. Endochondral ossification: How cartilage is converted into bone in the developing skeleton. Int. J. Biochem. Cell Biol. 40: 46-62.

McQueeney, K., and C. N. Dealy. 2001. Roles of insulin-like growth factor-I (IGF-I) and IGF-I binding protein-2 (IGFBP2) and-5 (IGFBP5) in developing chick limbs. Growth Horm. IGF Res. 11: 346-363.

McGeown, D., T. C. Danbury, A. E. Waterman-Pearson, and S. C. Kestin. 1999. Effect of carprofen on lameness in broiler chickens. Vet. Rec. 144: 668-671.

Molenaar, R., R. Meijerhof, I. van den Anker, M. J. W. Heetkamp, J. J. G. C. van den Borne, B. Kemp, and H. van den Brand. 2010. Effect of eggshell temperature and oxygen concentration on survival rate and nutrient utilization in chicken embryos. Poult. Sci. 89: 2010-2021.

Molenaar, R., R. Hulet, R. Meijerhof, C. M. Maatjens, B. Kemp, and H. van den Brand. 2011. High eggshell temperatures during incubation decrease growth performance and increase the incidence of ascites in broiler chickens. Poult. Sci. 90: 624-632.

Monsonego, E., O. Halevy, A. Gertler, M. Volokita, M. Schickler, S. Hurwitz, and M. Pines. 1993. Growth hormone receptors in avian epiphyseal growth-plate chondrocytes. Gen. Comp. Endocrinol. 92: pp.179-188.

Monsonego, E., W. R. Baumbach, I. Lavelin, A. Gertler, S. Hurwitz, S., and M. Pines. 1997. Generation of growth hormone binding protein by avian

growth plate chondrocytes is dependent on cell differentiation. Mol. Cell. Endocrinol. 135: 1-10.

Müller, G. B. 2003. Embryonic motility: environmental influences and evolutionary innovation. Evol. Dev. 5: 56-60.

Murray, P. D. F., and B. Drachman. 1969. The role of movement in the development of joints and related structures: the head and neck in the chick embryo. J. Embryol. Exp. Morphol. 22: 349-371

Oppenheim, R. W., and H. L. Levin. 1975. Short-term changes in incubation temperature: Behavioral and physiological effects in the chick embryo from 6 to 20 days. Dev. Psychobiol. 8: 103-115.

Ostrowska, Z., B. Kos-Kudla, B. Marek, D. Kajdaniuk, and N. Ciesielska-Kopacz. 2002. The relationship between the daily profile of chosen biochemical markers of bone metabolism and melatonin and other hormone secretion in rats under physiological conditions. Neuroendocrinol. Lett. 23: 417-425.

Oviedo-Rondón, E. O., J. Small, M. J. Wineland, V. L. Christensen, P. S. Modziak, M. D. Koci, S. V. L. Funderburk, D. T. Ort, and K. M. Mann. 2008. Broiler embryo bone development is influenced by incubator temperature, oxygen concentration and eggshell conductance at the plateau stage in oxygen consumption. Br. Poult. Sci. 49: 666-676.

Oviedo-Rondón, E. O., M. J. Wineland, S. Funderburk, J. Small, H. Cutchin, and M. Mann. 2009a. Incubation conditions affect leg health in large, high-yield broilers. J. Appl. Poult. Res 18: 640-646.

Oviedo-Rondón, E. O., M. J. Wineland, J. Small, H. Cutchin, A. McElroy, A. Barri, and S. Martin. 2009b. Effect of incubation temperatures and chick transportation conditions on bone development and leg health. J. Appl. Poult. Res. 18: 671-678.

Oviedo-Rondón, E. O., M. Wineland, H. C. Evans, and J. Small. 2013. Effect of brooding temperatures under commercial conditions on broiler leg health and locomotion. Proceedings of the 2013 International Poultry Scientific Forum, Atlanta, Georgia, USA, pp 49.

Özkan, S., S. Yalçin, E. Babacanoğlu, H. Kozanoğlu, F. Karadaş, and S. Uysal. 2012a. Photoperiodic lighting (16 hours of light: 8 hours of dark) programs during incubation: 1. Effects on growth and circadian physiological traits of embryos and early stress response of broiler chickens. Poult. Sci. 91: 2912-2921.

Özkan, S., S. Yalçin, E. Babacanoğlu, H. Kozanoğlu, F. Karadaş, and S. Uysal. 2012b. Photoperiodic lighting (16 hours of light: 8 hours of dark) programs during incubation: 2. Effects on early posthatching growth, blood physiology, and production performance in broiler chickens in relation to posthatching lighting programs. Poult. Sci. 91: 2922-2930.

Packialakshmi, B., N. C. Rath, W. E. Huff, and G. R. Huff. 2015. Poultry femoral head separation and necrosis:

a review. Avian Dis. 59: 349-354.

Pechak, D. G., M. J. Kujawa, and A. I. Caplan. 1986a. Morphological and histochemical events during first bone formation in embryonic chick limbs. Bone 7: 441-458.

Pechak, D. G., M. J. Kujawa, and A. I. Caplan. 1986b. Morphology of bone development and bone remodelling in embryonic chick embryos. Bone 7: 459-472.

Piestun, Y., D. Shinder, M. Ruzal, O. Halevy, and S. Yahav. 2008. The effect of thermal manipulations during the development of the thyroid and adrenal axes on in-hatch and post-hatch thermoregulation. J. Therm. Biol. 33: 413-418.

Pines, M., and S. Hurwitz. 1991. The role of the growth plate in longitudinal bone growth. Poult. Sci. 70: 1806-1814.

Pollard, A. S., A. A. Pitsillides, and S. J. Portugal. 2016. Validating a Noninvasive Technique for Monitoring Embryo Movement In ovo. Physiol. Biochem. Zool. 89: 000-000.

Porter, T. E., G. S. Couger, C. E. Dean, and B. M. Hargis. 1995. Ontogeny of growth hormone (GH)-secreting cells during chicken embryonic development: initial somatotrophs are responsive to GH-releasing hormone. Endocrinology 136: 1850-1856.

Prescott, N. B., and C. M. Wathes. 1999. Spectral sensitivity of the domestic fowl (Gallus g. domesticus). Br. Poult. Sci. 40: 332-339.

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: 407-431.

Roach, H. I.. 1997. New aspects of endochondral ossification in the chick: chondrocyte apoptosis, bone formation by former chondrocytes, and acid phosphatase activity in the endochondral bone matrix. J. Bone Miner. Res. 12: 795-805.

Robson, H., T. Siebler, S. M. Shalet, and G. R. Williams. 2002. Interactions between GH, IGF-I, Glucocorticoids, and thyroid hormones during skeletal growth. Pediatr. Res. 52: 137-147.

Roth, J. A., B. Kim, W. Lin, M. Cho. 1999. Melatonin promotes osteoblast differentiation and bone formation. J. Biol. Chem. 31: 22041-22047.

Rozenboim, I., Y. Piestun, N. Mobarkey, M. Barak, A. Hoyzman, and O. Halevy. 2004. Monochromatic light stimuli during embryogenesis enhance embryo development and posthatch growth. Poult. Sci. 83: 1413-1419.

Sanotra, G. S., J. D. Lund, A. K. Ersbøll, J. S. Petersen, and K. S. Vestergaard. 2001. Monitoring leg problems in broilers: a survey of commercial broiler production in Denmark. Worlds Poult. Sci. J. 57: 55-69.

Sanotra, G. S., J. D. Lund, and K. S. Vestergaard. 2002. Influence of light-dark schedules and stocking density on behaviour, risk of leg problems and occurrence of chronic fear in broilers. Br. Poult. Sci. 43: 344-354.

Shafey, T. M., T. H. Al-Mohsen, A. A. Al-Sobayel, M. J. Al-Hassan, and M. M. Ghnnam. 2002. Effects of eggshell pigmentation and egg size on the spectral properties and characteristics

of eggshell of meat and layer breeders. Asian Australas. J. Anim. Sci. 15: 297-302.

Shafey, T. M., M. M. Ghannam, H. A. Al-Batshan, and M. S. Al-Ayed. 2004. Effect of pigment intensity and region of eggshell on the spectral transmission of light that passes the eggshell of chickens. Int. J. Poult. Sci. 3: 228-233.

Shafey, T. M., H. A. Al-Batshan, M. M. Ghannam, and M. S. Al-Ayed. 2005. Effect of intensity of eggshell pigment and illuminated incubation on hatchability of brown eggs. Br. Poult. Sci. 46: 190-198.

Shim, M. Y., A. B. Karnuah, N. B. Anthony, G. M. Pesti, and S. E. Aggrey. 2012. The effects of broiler chicken growth rate on valgus, varus, and tibial dyschondroplasia. Poult. Sci. 91: 62-65.

Siegel, H. S. 1980. Physiological stress in birds. Bioscience 30: 529-534.

Sikavitsas, V. I., J. S. Temenoff, and A. G. Mikos. 2001. Biomaterials and bone mechanotransduction. Biomaterials 22: 2581-2593.

Skwarło-Sońta, K. 1996. Functional connections between the pineal gland and immune system. Acta Neurobiologiae Experimentalis 56: 341-357.

Tamimie, H. S., and M. W. Fox. 1967. Effect of continuous and intermittent light exposure on the embryonic development of chicken eggs. Com. Biochem. Physiol. 20: 793-799.

Thorp, B. H., C. C. Whitehead, L. Dick, J. M. Bradbury, R. C. Jones, and A. Wood. 1993. Proximal femoral degeneration in growing broiler fowl. Avian Pathol. 22: 325-342.

Van der Eerden, B. C. J., M. Karperien, and J. M. Wit. 2003. Systemic and local regulation of the growth plate. Endocr. Rev. 24: 782-801.

Velloso, C. P. 2008. Review: Regulation of muscle mass by growth hormone and IGF-I. Br. J. Pharmacol. 154: 557-568.

Weeks, C. A., T. D. Danbury, H. C. Davies, P. Hunt, and S. C. Kestin. 2000. The behaviour of broiler chickens and its modifications by lameness. Appl. Anim. Behav. Sci. 67: 111-125.

Wineland, M. J., V. L. Christensen, I. Yildrum, B. D. Fairchild, D. T. Ort, and K. M. Mann. 2006. Incubator environment interacts with genetic line of broiler at the plateau stage to affect embryo plasma thyroxine and triiodothyronine concentrations. Int. J. Poult. Sci. 5: 714-722.

Yalçin, S., and P. B. Siegel. 2003. Exposure to cold or heat during incubation on developmental stability of broiler embryos. Poult. Sci. 82: 1388-1392.

Yalçin, S., H. B. Molayoglu, M. Baka, O. Genin, M. Pines. 2007. Effect of temperature during the incubation period on tibial growth plate chondrocyte differentiation and the incidence of tibial dyschondroplasia. Poult. Sci. 86: 1772-1783.

Zeman, M., E. Gwinner, I. Herichová, D. Lamošová, and L. Košťál. 1999. Perinatal development of circadian melatonin production in domestic chicks. J. Pineal Res. 26: 28-34.

Zeman, M., P. Pavlik, D. Lamošová, I. Herichová, and E. Gwinner. 2004. Entrainment of rhythmic melatonin

production by light and temperature in the chick embryo. Avian Poult. Biol. Rev. 15: 197-204.

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: 2970-2982.

Chapter 2

Effect of eggshell temperature throughout incubation on broiler hatchling leg bone development

C. W. van der Pol[ab], I. A. M. van Roovert-Reijrink[b], C. M. Maatjens[ab], I. van den Anker[b], B. Kemp[b], and H. van den Brand[b]

[a] HatchTech B.V., PO Box 256, 3900 AG Veenendaal, the Netherlands.

[b] Adaptation Physiology Group, Wageningen University, PO Box 338, 6700 AH Wageningen, the Netherlands.

Published: Poultry Science 4210 (2014)

Abstract

Leg problems in broiler chickens may partly be prevented by providing optimal circumstances for skeletal development during incubation. one of the factors demonstrated to affect bone development is eggshell temperature (EST), which provides a reliable reflection of embryo temperature. The present experiment aimed to investigate the effect of EST on development and asymmetry of the femur, tibia, and metatarsus in broiler chicken hatchlings. Eggs were incubated from d 0 until hatch at 1 of 4 EST: low (36.9°C), normal (37.8°C), high (38.6°C), and very high (39.4°C). At hatch, chick quality was determined in terms of chick length, yolk-free body mass, navel score, and organ weights. Tibia, femur, and metatarsus were weighed, their length and width (mediolateral diameter) and depth (craniocaudal diameter) at the middle of the shaft were measured, and their ash content was determined. Relative asymmetry of the leg bones was determined from their relative dimensions. Hatchability, chick quality, and organ development were lower for very high EST compared with all other treatments. Very high EST resulted in lowest tibia and metatarsus lengths (–3.1 to –8.4%) compared with all other treatments, and lower metatarsus weight (–9.1%) and femur length (–4.9%) compared with high EST. Relative asymmetry and ash content did not differ among treatments and no relation between EST and bone parameters was found. To conclude, very high EST resulted in lower bone development, hatchability, and chick quality. Few differences in bone development and chick quality were found between low, normal, and high EST.

1 | Introduction

At slaughter age, leg problems (pathologies that result in impaired walking ability), such as tibial dyschondroplasia, rickets, and femoral head necrosis, are highly prevalent in broiler chickens (reviewed by Dinev, 2012). These leg problems constitute a welfare problem and result in economic losses. Poor skeletal leg health in later life may be related to suboptimal leg bone development (defined here as bone weight, dimensions, and ash content) during incubation (Yalçin *et al.*, 2007; Oviedo-Rondón *et al.*, 2008, 2009a). It has therefore been suggested that leg problems can partly be prevented or reduced by providing a growing broiler embryo or hatchling with optimal circumstances for leg bone and muscle development.

Leg bone weights at hatch have been found to decrease at high (39.6°C) and low (36.9°C) incubator temperature when applied between embryonic d (E) 10 and E18 (Yalçin *et al.*, 2007), or at high (38.9°C) eggshell temperature (EST) applied between E18 and E21 (Oviedo-Rondón *et al.*, 2009b) compared with control (37.0 to 37.8°C throughout). However, Oviedo-Rondón *et al.* (2008) found higher femur weights at 36.0 and 39.0°C than at 37.0 and 38.0°C incubator temperature when applied from E17 till hatch. They furthermore found higher tibia length at 38.0°C than at 36.0 or 39.0°C incubator temperature (Oviedo-Rondón *et al.*, 2008), although others (Yalçin and Siegel, 2003; Hammond *et al.*, 2007) found that leg bone lengths increased at temperatures deviating from the control. Leg bone lengths have been shown to increase at high and low incubator temperatures when these were applied between E4 and E7 (Hammond *et al.*, 2007) or between E0 and E8, at E14, or between E10 and E18 (Yalçin and Siegel, 2003) compared with control (37.5°C throughout).

It is clear that incubation temperature can alter bone development, but results are ambiguous. Possibly, this is due to differences in the embryo temperature used. Because of differences in heat production or level of heat loss, embryo temperatures may vary greatly from incubator temperatures at different stages of incubation (Lourens *et al.*, 2011), and this could explain the discrepancies in the literature. Most previous studies on bone development did not measure embryo temperature or EST, but incubator temperature. To preserve the embryo, EST is measured as a reflection of embryo temperature. It has been shown that an EST of 37.5 to 38.0°C throughout incubation leads to highest yolk-free body mass (YFBM) and hatchability (Lourens *et al.*, 2005, 2007; Molenaar *et al.*, 2010, 2011a). Whether this holds for bone development as well is not known.

The aim of the present experiment is to investigate the effect of a constant low (36.9°C), normal (37.8°C), high (38.6°C), and very high (39.4°C) EST, applied from set until hatch, on the development of the femur, tibia, and metatarsus of a broiler chicken hatchling. Furthermore, the effect of EST on embryonic mortality, hatchability, and chick quality (chick length, YFBM, navel score, and organ development) will be investigated.

2 | Materials and Methods

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Wageningen University, Wageningen, the Netherlands.

2.1 | Experimental Setup

Storage and incubation of eggs took place at a commercial hatchery (Lagerwey, Lunteren, the Netherlands). Ross 308 hatching eggs (n = 223) from a parent stock aged 44 wk with an average egg weight of 65.0 g ± 3.6 were stored on setter trays for 2 d at 18°C. The number of eggs set was based on expected hatchability, and calculated to result in 37 hatchlings per treatment. Eggs were incubated from d 0 of incubation (E0) until hatch at 1 of 4 EST: low (36.9°C; n = 62), normal (37.8°C; n = 46), high (38.6°C; n = 53), and very high (39.4°C; n = 62). Each treatment was incubated in a HT-4,800 setter and hatcher (HatchTech B.V., Veenendaal, the Netherlands) with a capacity of 4,800 eggs.

Eggs were equally divided within the incubator on 24 trays to maintain constant EST. The EST was measured by 4 temperature sensors per treatment (NTC Thermistors: type DC 95; Thermometrics, Somerset, UK) attached to the equator of 4 individual eggs using tape and heat conducting paste (Dow Corning 340 Heat Sink Compound, Dow Corning GmbH, Wiesbaden, Germany). Eggs were warmed from 18.0°C storage temperature to their treatment EST in a time window of 10 h. Incubator temperature was adjusted automatically to maintain the treatment EST. Eggs were turned to an angle of 45° and then turned hourly by 90°. The RH was maintained between 45 and 60%, and CO_2 concentration was maintained between 0.25 and 0.35% throughout incubation.

At E18.5, eggs were transferred to hatcher baskets and placed in the hatcher. In the hatcher, eggshell sensors were attached to the eggs to determine EST. Incubator temperature was adjusted to maintain the treatment EST. At E19.5, the incubator temperature was fixed at the incubator temperature that corresponded with the treatment EST at that moment, and the EST was allowed to increase during the hatching process. The EST were thus maintained at contrasting levels until hatch. A difference in incubation time was expected for the various EST. Eggs were therefore allowed to hatch until E23.5. However, all viable eggs in normal, high, and very high EST had hatched by E21.5 and incubation was terminated at this point. All viable eggs in low EST had hatched by E22.5.

2.2 | Measurements

On E0 (day of set), each egg was numbered individually and weighed. Eggs were candled at E18.5. Clear eggs at E18.5 and unhatched eggs at E21.5 (for normal, high, and very high EST) or E22.5 (for low EST) were opened to determine infertility or stage of em-

bryonic mortality per week (Lourens *et al.*, 2005).

From E19.5 onward, hatched chicks (n = 133) were pulled every 12 h. Chicks were weighed, their length was recorded from the tip of the beak to the tip of the middle toe, excluding the nail (Molenaar *et al.*, 2010), and navel condition was scored as 1 (a clean and closed navel), 2 (a black button or gap of <2 mm), or 3 (a black button or gap of >2 mm; Molenaar *et al.*, 2010). Residual yolk was removed and weighed after cervical dislocation. Yolk-free body was frozen at –20°C. The YFBM was calculated as BW minus yolk weight. After thawing at room temperature for 12 h, heart, liver, stomach (gizzard and proventriculus), and intestines were weighed.

Both legs of each chick (n = 133) were removed at the hip joint and boiled in water for 5 min to allow easy removal of soft tissue. Tibia, femur, and metatarsus were cleaned of soft tissue and cartilage and weighed in grams to 4 decimals. Using a digital caliper (Skandia, Ridderkerk, the Netherlands), their length and the width (mediolateral diameter) and depth (craniocaudal diameter) at the middle of the shaft were measured twice in millimeters to 2 decimals. Relative asymmetry was calculated with the following formula (Møller *et al.*, 1999):

$$RA = \{|R - L|/[(R + L)/2]\} \times 100,$$

in which RA = relative asymmetry of the left and right bone (%), R = length, depth, or width of the right bone (mm), L = length, depth, or width of the left bone (mm), and |R - L| = absolute difference between R and L.

Tibia, femur, and metatarsus of the left leg (n = 133) were dried and ashed by the Animal Health Centre, Deventer, the Netherlands. Each bone was placed in a porcelain container and weighed to the nearest 0.1 mg. Bones were placed in a 550°C oven for 4 h and then placed in an desiccator for cooling. After cooling, bone ash was weighed to the nearest 0.1 mg. Ash content was calculated as a percentage of fresh bone weight.

2.3 | Statistical Analysis

The overall model used for all data was

$$Y_i = \mu + \text{Temperature}_i + \varepsilon_i, \quad [1]$$

where Y_i = the dependent variable, μ is the overall mean, Temperature_i = EST (i = low, normal, high, or very high), and ε_i = the residual error term.

Hatch time, YFBM, chick length, organ weights, relative asymmetry, and bone ash content were analyzed using the GLM procedure. Tibia, femur, and metatarsus weight, length, width, and depth were analyzed using the Mixed procedure with an auto-regressive covariance structure with side (left or right) as the repeated factor. Navel score,

fertility, embryonic mortality, and hatch of fertile were analyzed using the Logistics procedure. All data were analyzed in SAS (SAS Institute Inc., Cary, NC).

Individual eggs or chicks were considered the experimental unit. Model assumptions were verified by examination of the distributions of the means and residuals. Chick length data were sinus transformed to obtain normality. Relative asymmetry of tibia length was log transformed to obtain normality. Relative asymmetry of metatarsus length, width, and depth, and femur width and depth were square root transformed to obtain normality.

For bone dimensions (weight, length, depth, and width) and organ weights (stomach, liver, heart, and intestines), YFBM was added to model 1 as a covariable. To verify if the relation between EST and bone dimensions was linear or quadratic, EST was added as a fixed factor instead of a continuous factor in model 1. Least squares means were compared using Bonferroni adjustments for multiple comparisons. Data are presented as least squares means ± SEM. In the case of data transformation, least squares means and SEM are presented untransformed and P-values are presented as those of the transformed data. In all cases, differences were considered significant at $P \leq 0.05$.

3 | Results

3.1 | Bone Parameters

Metatarsus weight was higher for high EST than for very high EST (+0.012 g; $P = 0.044$; Table 1). Femur ($P = 0.06$) and tibia ($P = 0.15$) weights were not different among treatments. Femur length was higher for high EST than for low EST (+0.628 mm) and very high EST (+0.977 mm; $P = 0.001$). Tibia length was lower for very high EST than for low EST (–1.428 mm), normal EST (–1.768 mm), and high EST (–2.270 mm; $P < 0.001$). Metatarsus length was higher for high EST than for low EST (+0.748 mm) and very high EST (+1.344 mm) and it was higher for normal EST than for very high EST (+1.207 mm; $P < 0.001$). Femur ($P = 0.06$), tibia ($P = 0.14$), and metatarsus ($P = 0.46$) depth did not differ among treatments. Tibia width was higher for very high EST than for normal EST (+0.117 mm) and high EST (+0.086 mm; $P < 0.001$). Femur ($P = 0.18$) and metatarsus ($P = 0.08$) width did not differ among treatments. No linear or quadratic relation was found between EST and bone dimensions ($P \geq 0.06$).

Ash content and relative asymmetry of the tibia, femur, and metatarsus did not differ among treatments ($P \geq 0.16$, data not shown). Average ash contents were 12.7%±1.3 for the tibia, 13.0% ± 1.8 for the femur, and 12.3% ± 1.6 for the metatarsus. Relative asymmetry of the tibia was 0.51% for length, 1.00% for depth, and 0.99% for width.

Table 1. Femur, tibia, and metatarsus weight, length, depth, and width of chicks incubated at a Low (36.9°C), Normal (37.8°C), High (38.6°C), or Very high (39.4°C) eggshell temperature (EST) from set until hatch.

		Weight (g)			Length (mm)			Depth[1] (mm)			Width[2] (mm)		
EST	n	Femur	Tibia	Meta-tarsus	Femur	Tibia	Meta-tarsus	Femur	Tibia	Metat-arsus	Femur	Tibia	Meta-tarsus
Low	35	0.129	0.192	0.128^{ab}	19.484^{b}	26.191^{a}	19.364^{bc}	1.600	1.525	1.239	1.645	1.638^{ab}	2.274
Normal	38	0.121	0.183	0.129^{ab}	19.603^{ab}	26.531^{a}	19.975^{ab}	1.556	1.493	1.196	1.600	1.583^{b}	2.194
High	38	0.128	0.189	0.132^{a}	20.112^{a}	27.033^{a}	20.112^{a}	1.552	1.518	1.229	1.607	1.614^{b}	2.225
Very high	22	0.126	0.181	0.120^{b}	19.135^{b}	24.763^{b}	18.768^{c}	1.565	1.560	1.225	1.618	1.700^{a}	2.194
SEM		0.003	0.004	0.003	0.173	0.270	0.186	0.015	0.019	0.023	0.017	0.018	0.026
P-values[3]													
Linear		0.99	0.21	0.56	0.99	0.42	0.99	0.06	0.41	0.87	0.21	0.22	0.10
Quadratic		0.98	0.21	0.55	0.98	0.40	0.95	0.06	0.40	0.88	0.21	0.20	0.10
Overall		0.06	0.15	0.04	0.001	<0.001	<0.001	0.06	0.14	0.46	0.18	<0.001	0.08
YFBM[4]		<0.001	<0.001	<0.001	0.014	0.039	0.003	0.003	0.20	0.18	0.029	0.026	0.25

[a-c] Least squares means within a column lacking a common superscript differ (P ≤0.05).

[1] Craniocaudal diameter.

[2] Mediolateral diameter.

[3] Linear = EST added to the model as fixed factor, quadratic = EST x EST added as fixed factor, overall = EST added as continuous factor

[4] YFBM is used as a covariable.

Relative asymmetry of the femur was 0.70% for length, 0.96% for depth, and 1.04% for width. Relative asymmetry of the metatarsus was 0.67% for length, 2.27% for depth, and 0.96% for width.

3.2 | Hatchability

Fertility and first and second week embryonic mortality were not different between treatments (P ≥ 0.17; Table 2). Third week embryonic mortality was higher for low and very high EST than for normal (+29.7 and +26.3%, respectively) and high EST (+48.3 and +44.9%, respectively; P < 0.001). Hatchability of fertile eggs was higher for normal EST than for low (+35.7%) and very high EST (+57.7%), and it was higher for high (+41.1%) and low (+22.0%) than for very high EST (P < 0.001). Incubation duration was longer for low EST than for normal EST (+1.1 d), high EST (+1.7 d), and very high EST (+1.7 d). In addition, incubation duration was longer for normal EST than for high EST (+0.6 d) and very high EST (+0.6 d; P < 0.001).

Table 2. Fertility, embryonic mortality per week, hatchability of fertile eggs, and incubation duration of eggs incubated at a Low (36.9°C), Normal (37.8°C), High (38.6°C), or Very high (39.4°C) eggshell temperature (EST) from set until hatch.

			Embryonic mortality				
EST	n[1]	Fertility (% set)[2]	First week (% fertile)	Second week (% fertile)	Third week (% fertile)	Hatchability (% fertile)	Incubation duration (days)
Low	62	96.8	8.5	0.0	32.2[a]	59.3[b]	21.8[a]
Normal	46	87.0	2.5	0.0	2.5[b]	95.0[a]	20.7[b]
High	53	96.3	9.8	5.9	5.9[b]	78.4[ab]	20.1[c]
Very high	62	95.2	6.8	5.1	50.8[a]	37.3[c]	20.1[c]
P-value		0.17	0.62	0.99	<0.001	<0.001	<0.001

[a-c] Least squares means within a column lacking a common superscript differ (P ≤0.05).

[1] n = number of set eggs.

[2] Fertility = % of set eggs with good eggshell quality.

3.3 | Chick Quality

Navel score was higher (indicating lower quality) for very high EST than for low (+0.8 score), normal (+1.5 score), and high EST (+1.1 score). Navel score was furthermore higher for low EST than for normal EST (+0.7 score; P < 0.001; Table 3). Chick length (+6.6 to 7.1%) and YFBM (+9.6 to 13.4%) were higher for low, normal, and high EST than for very high EST (both P < 0.001). Body weight was higher for low (+7.4%) and

high EST (+6.2%) than for very high EST (P = 0.007). Residual yolk weight was higher for high and very high EST than for low and normal EST (+14.1 to 25.0%; P < 0.001). Stomach (corrected for YFBM; +10.6 to 16.1%) and liver (+19.3 to 25.0%) weight were higher for low, normal, and high EST than for very high EST (both P < 0.001). Heart weight differed between all treatments and decreased as EST increased (up to 41.9%; P < 0.001). Intestine weight did not differ among treatments (P = 0.11).

4 | Discussion

To the authors' knowledge, no previous experiments have been published where EST was maintained constant throughout incubation. The YFBM was decreased in chicks exposed to very high EST (39.4°C) throughout incubation compared with low (36.9°C), normal (37.8°C), or high EST (38.6°C). As a consequence, initial analysis showed absolute bone weight and length were lower for very high EST compared with all other treatments.

To correct for differences in BW, YFBM was added to the model as a covariable and it was found to explain a large proportion of the variation in bone weight and length. However, very high EST still resulted in lower metatarsus weight than high EST, lower femur length than low and very high EST, lower tibia length than all other EST, and lower metatarsus length than normal and high EST. This suggests that very high EST slows down not only absolute growth, but also relative bone growth. In any case, no linear or quadratic relation between EST and bone development was found in regression analysis. It appears that at the current state of the art it is not possible to appoint a single EST as the most optimal for bone development when applied continuously.

Previous studies applied their temperature treatments for shorter periods, but still found an effect on bone development. Yalçin *et al.* (2007) found decreased tibia weight at hatch for chicks exposed to cooling (36.9°C for 6 h per day) or heating (39.0°C for 6 h per day) from E0 to E8 or from E10 to E18 compared with chicks incubated at a 37.8°C incubator temperature. Oviedo-Rondón *et al.* (2009b) showed that particularly a high EST (39.0°C) during the third week of incubation resulted in lower leg bone weights compared with control (EST 37.0°C). This might suggest that continuous application of a specific EST, as performed in the current study, may not have created the largest differences in bone development. Hammond *et al.* (2007) suggested that high temperatures in the first week stimulate later bone development, and high temperatures during the last week decrease the rate of bone development.

Distinguished processes in bone development take place in various periods of incubation. The skeletal structures of the limbs start to appear around E5 (hind limbs) and E6 (forelimbs; Bellairs and Osmond, 2005). Initially, these skeletal structures are formed

Table 3. Navel score, chick length, yolk free body mass (YFBM), residual yolk, stomach (gizzard and proventriculus), liver, heart, and intestines weight of chicks incubated at a Low (36.9°C), Normal (37.8°C), High (38.6°C), or Very high (39.4°C) eggshell temperature (EST) from set until hatch. Navel score was analyzed as frequencies but is displayed as means.

EST	n[1]	Navel score	Chick length (cm)	YFBM (g)	Body weight (g)	Residual yolk (g)	Stomach (g)	Liver (g)	Heart (g)	Intestines (g)
Low	35	2.1[b]	19.5[a]	41.4[a]	47.8[a]	6.4[b]	2.20[a]	1.10[a]	0.43[a]	1.60
Normal	38	1.4[c]	19.6[a]	40.5[a]	46.9[ab]	6.4[b]	2.23[a]	1.08[a]	0.35[b]	1.60
High	38	1.8[bc]	19.6[a]	40.0[a]	47.3[a]	7.3[a]	2.31[a]	1.05[a]	0.29[c]	1.69
Very high	22	2.9[a]	18.3[b]	36.5[b]	44.5[b]	8.0[a]	1.99[b]	0.88[b]	0.25[d]	1.48
SEM			0.1	0.3	0.6	0.5	0.04	0.03	0.01	0.05
P-values										
EST		<0.001	<0.001	<0.001	0.007	<0.001	<0.001	<0.001	<0.001	0.11
YFBM[2]		n/a	n/a	n/a	n/a	n/a	<0.001	0.56	0.048	0.15

[a-d] Least squares means within a column lacking a common superscript differ ($P \leq 0.05$).

[1] n = # of chicks hatched.

[2] YFBM is used as a covariable.

by chondrocytes in cartilage. The cartilage appears fully formed around E6 to E7 for the main skeletal components (Kürtül *et al.*, 2009). It can be argued that the first week is the most sensitive period, as incubation temperatures affect chondrocyte proliferation and suboptimal temperatures will delay cell differentiation (Yalçin *et al.*, 2007). However, bones continue to ossify and the highest growth rate (Applegate and Lilburn, 2002) and increase of strength and stiffness (Yair *et al.*, 2012) of bones occur in the final week of incubation, suggesting an important role of the final week of incubation in bone development as well. It can be speculated that the first week of incubation was a programming phase for bone development. As incubation proceeded, the embryos might have become adapted to their incubation environment and were able to compensate bone development, resulting in similar bone development at hatch. Because leg bones were studied only at hatch in the present experiment, it is unknown when any of the found effects occurred, or if several events were compensated for by each other.

Contrary to the results on bone length, tibia width was highest for very high EST compared with normal and low EST. Tibia width has been associated with walking ability; Toscano *et al.* (2013) found that tibia width at 35 d of age was related to gait scores, suggesting more leg problems in broilers with wider leg bones. They speculated that this is due to greater tibial curving, which may result in suboptimal mechanical loading on the bone causing further leg problems (Toscano *et al.*, 2013). It can be speculated that the wider leg bones found for very high EST in the present study can increase the incidence of leg problems in later life.

Although EST had an effect on the dimensions of the leg bones, no effects on ash content were found. This is contrary to results of Yalçin *et al.* (2007), who found highest tibia ash content (% of bone weight) at hatch for chicks exposed to cooling (36.9°C for 6 h per day) and heating (39.0°C for 6 h per day) from E0 to E8 compared with 37.8°C incubator temperature throughout. However, they did not see an effect of heating or cooling applied from E10 to E18 on ash content at hatch. It appears that ash content, too, is influenced by the period during which the temperature treatment is applied and effects found at one moment may have been compensated for in another period of incubation.

The present experiment did not study leg health in later life, but literature suggests incubation temperature and bone development at hatch are related to leg problems at slaughter age. Previous studies have shown that high EST increased incidence of crooked toes and severe locomotion problems compared with control (EST 37.0°C; Oviedo-Rondón *et al.*, 2009a). Yalçin *et al.* (2007) found that cooling or heating from E0 to E8 increased the incidence of tibial dyschondroplasia compared with control, but later cooling and heating did not (Yalçin *et al.*, 2007). They demonstrated that this may be due to delayed chondrocyte cell differentiation, which was only observed when cooling or heating was applied from E0 to E8.

Fertility was numerically lowest in normal EST. This was likely due to the small sample

size of 46 to 62 eggs per treatment and because of that it appears not to be a treatment effect. Low and very high EST throughout incubation had a negative effect on hatchability compared with normal EST. This can largely be explained through increased third week embryonic mortality. Most of the unhatched chicks in very high EST were observed to be malpositioned, mainly with their head over their wing. Increased malpositions at high EST were previously found by Molenaar *et al.* (2010), who described increased numbers of head between legs and head over wing embryos when they were incubated at an EST of 38.9°C from E7 onward compared with normal EST (37.8°C throughout).

Very high EST furthermore led to poorer chick quality as evidenced by the shortest chick length, lowest YFBM, and worst navel scores compared with all other treatments. High EST (≥38.9°C) from E7, E9, or E14 onward have been shown previously to decrease chick length (Molenaar *et al.*, 2010), BW (Leksrisompong *et al.*, 2007), YFBM (Lourens *et al.*, 2007; Molenaar *et al.*, 2011a,b), and navel scores (Molenaar *et al.*, 2011b) compared with a constant EST of 37.8 to 38.2°C. High EST (38.9°C) have been found to decrease relative organ weights (applied from E7 onward, Molenaar *et al.*, 2011a), particularly heart weights (applied from E9 onward, Lourens *et al.*, 2007; and applied from E19 onward, Maatjens *et al.*, 2014). Indeed, stomach, liver, and heart weights (which were corrected for YFBM) were lower for very high than for all other treatments in the present experiment. It would appear from the present experiment that chick quality, organ development, and bone development are all negatively affected by very high EST.

In conclusion, a constant very high EST (39.4°C) throughout incubation led to decreased leg bone length, weight, and chick quality compared with an EST of 36.9, 37.8, or 38.6°C.

5 | Acknowledgements

The authors thank hatchery Lagerwey B.V. (Lunteren, the Netherlands) for donating eggs and their assistance during incubation, and Mariska Niesten (Wageningen University, Wageningen, the Netherlands) and Sascha Smits (Wageningen University) for their valuable input and help with the experiment.

6 | References

Applegate, T. J., and M. S. Lilburn. 2002. Growth of the femur and tibia of a commercial broiler line. Poult. Sci. 81: 1289–1294.

Bellairs, R., and M. Osmond. 2005. The atlas of chick development. Academic Press, London, UK.

Dinev, I. 2012. Review: Leg weakness pathology in broiler chickens. Jpn. Poult. Sci. 49: 63–67.

Hammond, C. L., B. H. Simby, and N. C. Stickland. 2007. In ovo temperature manipulation influences embryonic motility and growth of limb tissues in the chick (Gallus gallus). J. Exp. Biol. 210: 2667–2675.

Kürtül, I., S. H. Atalgin, K. Aslan, and E. U. Bozkurt. 2009. Ossification and growth of the bones of the wings and legs in prehatching period of the Hubbert strain broiler. Kafkas Univ. Vet. Fak. Derg. 15: 869–874.

Leksrisompong, N., H. Romero-Sanchez, P. W. Plumstead, K. E. Brannan, and J. Brake. 2007. Broiler incubation. 1. Effect of elevated temperature during late incubation on body weight and organs of chicks. Poult. Sci. 86: 2685–2691.

Lourens, A., R. Meijerhof, B. Kemp, and H. van den Brand. 2011. Energy partitioning and consequences for embryo temperature: A theoretical approach. Poult. Sci. 90: 516–523.

Lourens, A., H. van den Brand, M. J. W. Heetkamp, R. Meijerhof, and B. Kemp. 2007. Effects of eggshell temperature and oxygen concentration on embryonic growth and metabolism during incubation. Poult. Sci. 86: 2194–2199.

Lourens, A., H. van den Brand, R. Meijerhof, and B. Kemp. 2005. Effect of eggshell temperature during incubation on embryonic development, hatchability, and post-hatch development. Poult. Sci. 84: 914–920.

Maatjens, C. M., I. A. M. Reijrink, R. Molenaar, C. W. van der Pol, B. Kemp, and H. van den Brand. 2014. Temperature and CO2 during the hatching phase. I. Effects on chick quality and organ development. Poult. Sci. 93: 645–654.

Molenaar, R., S. de Vries, I. van den Anker, R. Meijerhof, B. Kemp, and H. van den Brand. 2011a. Effect of eggshell temperature and oxygen concentration during incubation on the developmental and physiological status of broiler hatchlings in the perinatal period. Poult. Sci. 90: 1257–1266.

Molenaar, R., R. Hulet, R. Meijerhof, C. M. Maatjens, B. Kemp, and H. van den Brand. 2011b. High eggshell temperatures during incubation decrease growth performance and increase the incidence of ascites in broiler chickens. Poult. Sci. 90: 624–632.

Molenaar, R., R. Meijerhof, I. van den Anker, M. J. W. Heetkamp, J. J. G. C. van den Borne, B. Kemp, and H. van den Brand. 2010. Effect of eggshell temperature and oxygen concentration on survival rate and nutrient utilization in chicken embryos. Poult. Sci. 89: 2010–2021.

Møller, A. P., G. S. Sanotra, and K. S. Vetergaard. 1999. Developmental instability and light regime in chickens

(Gallus gallus). Appl. Anim. Behav. Sci. 62: 57–71.

Oviedo-Rondón, E. O., J. Small, M. J. Wineland, V. L. Christensen, P. S. Modziak, M. D. Koci, S. V. L. Funderburk, D. T. Ort, and K. M. Mann. 2008. Broiler embryo bone development is influenced by incubator temperature, oxygen concentration and eggshell conductance at the plateau stage in oxygen consumption. Br. Poult. Sci. 49: 666–676.

Oviedo-Rondón, E. O., M. J. Wineland, S. Funderburk, J. Small, H. Cutchin, and M. Mann. 2009a. Incubation conditions affect leg health in large, high-yield broilers. J. Appl. Poult. Res. 18: 640–646.

Oviedo-Rondón, E. O., M. J. Wineland, J. Small, H. Cutchin, A. McElroy, A. Barri, and S. Martin. 2009b. Effect of incubation temperatures and chick transportation conditions on bone development and leg health. J. Appl. Poult. Res. 18: 671–687.

Toscano, M. J., M. A. F. Nasr, and B. Hothersall. 2013. Correlation between broiler lameness and anatomical measurements of bone using radiographical projections with assessments of consistency across and within radiographs. Poult. Sci. 92: 2251–2258.

Yair, R., Z. Uni, and R. Shahar. 2012. Bone characteristics of late term embryonic and hatchling broilers: Bone development under extreme growth rate. Poult. Sci. 91: 2614–2620.

Yalçin, S., H. B. Molayoglu, M. Baka, O. Genin, and M. Pines. 2007. Effect of temperature during the incubation period on tibial growth plate chondrocyte differentiation and the incidence of tibial dyschondroplasia. Poult. Sci. 86: 1772–1783.

Yalçin, S., and P. B. Siegel. 2003. Exposure to cold or heat during incubation on developmental stability of broiler embryos. Poult. Sci. 82: 1388–1392.

Chapter 3

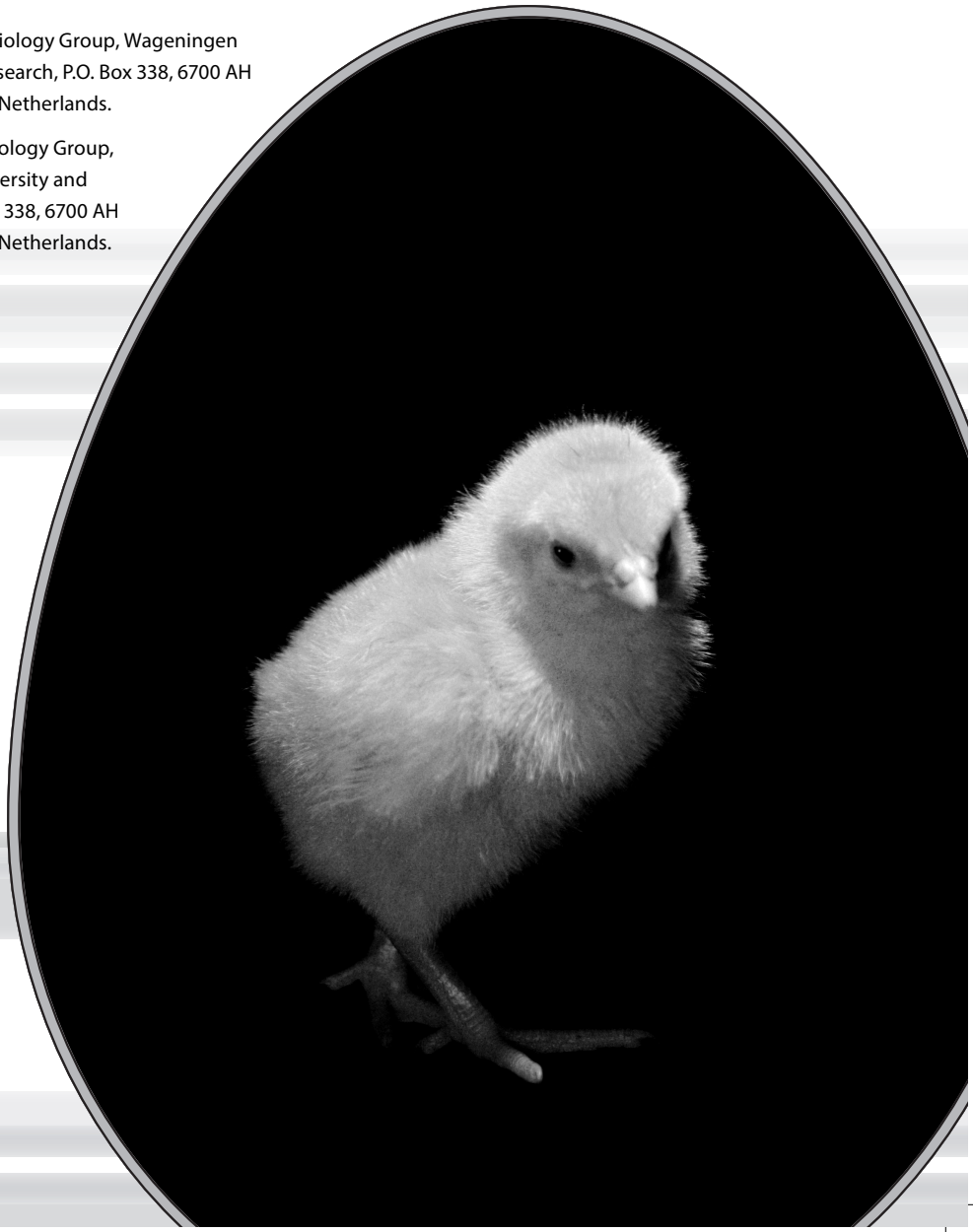
Circadian rhythms during incubation of broiler chicken embryos and their effects on embryonic and post hatch leg bone development

C. W. van der Pol[ab], I. A. M. van Roovert-Reijrink[b], G. Aalbers[b], C. M. Maatjens[ab], S. W. S. Gussekloo[c], S. Kranenbarg[c], J. Wijnen[ab], R. P. M. Pieters[c], H. Schipper[c], B. Kemp[b], and H. van den Brand[b]

[a] HatchTech B.V., P.O. Box 256, 3900 AG Veenendaal, the Netherlands.

[b] Adaptation Physiology Group, Wageningen University and Research, P.O. Box 338, 6700 AH Wageningen, the Netherlands.

[c] Experimental Zoology Group, Wageningen University and Research, P.O. Box 338, 6700 AH Wageningen, the Netherlands.



Abstract

Avian embryos provide an interesting model to study effects of circadian rhythmicity on embryonic development, as they develop in ovo and are not affected directly by maternal influences. The current experiment studied effects of lighting schedule: (24L, 12L:12D, or 24D) throughout incubation of broiler chicken eggs on the development and strength of leg bones, and the role of selected hormones in bone development. In the tibia and femur, growth and ossification during incubation and size and microstructure at D0, D21, and D35 post hatching were measured. Plasma melatonin, GH, and IGF-I were determined perinatally. Incidence of tibial dyschondroplasia, a leg pathology resulting from poor ossification at the bone's epiphyseal plates, was determined at slaughter on D35. 24L resulted in lower embryonic ossification at embryonic day (E)13 and E14, and lower femur length, and lower tibia weight, length, cortical area, second moment of area around the minor axis, and mean cortical thickness at hatching on D0 compared to 12L:12D especially. Results were long term, with lower femur weight and tibia length, cortical area, and second moment of area around the major axis, and a higher incidence of tibial dyschondroplasia for 24L compared to 12L:12D, with 24D intermediate. GH at D0 was higher for 24D than for 12L:12D, with 24L intermediate, but plasma melatonin and IGF-I did not differ between treatments, and the role of plasma melatonin, IGF-I, and GH in this process was therefore not clear. To conclude, in the current experiment, 24L during incubation of chicken eggs had a detrimental effect on embryonic leg bone development and later life leg bone strength compared to 24D and 12L:12D, while the circadian rhythm of 12L:12D may have a stimulating effect on leg health.

1 | Introduction

Circadian rhythm development is found in utero in primates (reviewed by Rivkees, 2003). Embryonic circadian rhythmicity seems to be mostly dependent on maternal melatonin rhythms of pineal origin, which crosses the placenta and provides the developing foetus with information about the circadian state (reviewed by Tamura *et al.*, 2008). In mature mammals, melatonin release is photoperiod dependent, and it is maximised during dark periods (Morin and Allen, 2006). Melatonin has many effects in mammalian foetuses, among which is regulation of foetal breathing movements (McMillen *et al.*, 1990), circadian regulation of synchrony within tissues before mature regulatory pathways are established (Johnston *et al.*, 2003), stimulation of adrenal gland growth (Torres-Farfan *et al.*, 2006), and a stimulatory effect of melatonin on skeletal development (Cardinali *et al.*, 2003), which is the focus of this study. In in vitro bone cells from mature humans, melatonin stimulates proliferation (Nakade *et al.*, 1999; Satomura *et al.*, 2007) and type I collagen synthesis of osteoblasts (Satomura *et al.*, 2007), the bone cells that realise bone formation through collagen matrix secretion and mineral deposition. In mice, melatonin was furthermore found to promote bone mineralization and differentiation of osteoblasts (Park *et al.*, 2011).

Birds provide an interesting model to study effects of circadian rhythmicity on embryonic development, because their embryonic development takes place mostly independent of maternal influences. Birds, too, know a darkness-dependent release of pineal melatonin (Özkan *et al.*, 2012). Pineal gland rhythmicity develops embryonically in precocial birds, and it can be influenced in ovo by exposing an egg to light (Zeman *et al.*, 1999a). Light is able to penetrate the eggshell (Shafey *et al.*, 2004), and creates the onset of a circadian release of melatonin in both the pineal gland (Zeman *et al.*, 1992) and the retina of chickens (Ivanova and Iuvone, 2003). When avian embryos are incubated under complete darkness, their internal circadian rhythm will likely show a random phase pattern. Entraining embryos through light-dark rhythms during incubation can possibly make their phases run synchronously, as is observed in other species (Takahashi and Zatz, 1982), and additionally might enhance the amplitude of oscillation. Indeed, when chicken embryo pineal glands were isolated and cultured in vitro under 12L:12D and then complete darkness, their amplitude of melatonin release decreased in darkness compared to when they were kept under 12L:12D (Lamošová *et al.*, 1995). In vivo, Özkan *et al.* (2012) found a tendency for higher blood melatonin levels during the dark period for chickens that had just hatched and had been exposed to 16L:8D or continuous darkness compared to samples taken in 16L:8D's light period. In nature, chicken eggs will be exposed to short bouts of light when the hen leaves the nest to eat and drink. These bouts off the nest were most frequent in the last week of incubation, when they lasted on average 15 minutes (Archer and Mench, 2014).

By providing incubating eggs with a light-dark rhythm, circadian melatonin release pat-

terns can be affected, which may influence bone development. In birds, too, melatonin levels affect skeletal development. Chickens that were pinealectomised at 3 days of age (eliminating circulating melatonin) all developed scoliosis within two weeks. Scoliosis incidence in pinealectomised chickens was reduced to 20% when melatonin injections were administered every other day (Machida *et al.*, 1995). Aota *et al.* (2013) found disrupted endochondral ossification at the epiphyseal plate for pinealectomised chickens. It can be speculated that, through the involvement of melatonin, bone development is influenced by day-night rhythms already during embryonic development. We speculate that because melatonin release is darkness dependent (Morin and Allen, 2006), dark incubation may stimulate bone development more than continuous light (reflected by heavier, longer, or thicker leg bones, and earlier onset or an increased rate of developmental processes such as ossification); and a light-dark rhythm may be more beneficial than continuous darkness, because applying a circadian rhythm may enhance the amplitude of melatonin release (Gonze and Goldbetter, 2000; Lamošová *et al.*, 1995).

Melatonin might have both a direct effect on bone development and an indirect effect by stimulation or inhibition of other hormones involved in bone development. In chickens, melatonin stimulates hypothalamic GH release (Zeman *et al.*, 1999b), and GH and IGF-I were observed to peak along with darkness-dependent melatonin release (Ostrowska *et al.*, 2002). GH and IGF-I both have a stimulatory effect on proliferation of cartilage cells (called chondrocytes) in the growth plates of long bones (Robson *et al.*, 2002; Van der Eerden *et al.*, 2003). Because of the stimulatory effect of melatonin on GH and IGF-I release, it can be expected that incubation with alternating light and dark periods accelerates embryonic development compared to continuous light and even continuous darkness.

In the current experiment, bone development is studied through embryonic ossification, embryonic and post hatch bone dimensions, and post hatch bone microstructure. These are used as a reflection of developmental processes in the bone. Additionally, bone dimensions and microstructure may provide insight into bone strength (Augat and Schorlemmer, 2006). Chickens bred for meat production ("broilers") are especially interesting in the study of bone development because the incidence of leg bone pathologies is higher than in other chicken populations as a result of their high growth rate (Kestin *et al.*, 1992; Bessei, 2006). Developmental leg bone pathologies, such as tibial dyschondroplasia (in which cartilage fails to mature and is not replaced by bone), can be prevalent in up to 57.1% of a flock (Sanotra *et al.*, 2001). We hypothesize that developmental leg abnormalities can partly be prevented by providing a growing broiler embryo with optimal circumstances for leg bone development. For example, incidence of tibial dyschondroplasia has been proven to be increased when incubation temperatures are above or below 37.8°C in the first week of incubation (Yalçin *et al.*, 2007). Since a high incidence of tibial dyschondroplasia may be considered to be an indicator of suboptimal embryonic leg bone development, it is used as a readout parameter in the current experiment.

The current experiment aims to investigate effects of circadian rhythms (24D, 24L, or 12L:12D) applied throughout incubation of broiler chicken embryos on embryonic plasma melatonin levels, GH and IGF-I at hatch, leg bone development during incubation, at hatch, and at slaughter age (5 week post hatch), and incidence of tibial dyschondroplasia at slaughter age.

2 | Materials and methods

The experimental design and protocol were approved by the Institutional Animal Care and Use Committee of Wageningen University, Wageningen, the Netherlands.

2.1 | Incubation

Experimental setup

Freshly laid Ross 308 broiler chicken eggs from a 40 week old parent flock were selected for egg weights between 62 and 65 grams. 744 embryos and chickens were used for the current experiment. Eggs were stored at 18°C for 3 days on egg trays in a commercial hatchery (Lagerwey BV, Lunteren, the Netherlands) before transportation to the experimental facilities of Wageningen University & Research (Wageningen, the Netherlands). Upon arrival, eggs were placed in climate respiration chambers (where climate conditions can be controlled strictly; Heetkamp *et al.*, 2015). Three regimes with 310 eggs each (to allow for an estimated hatchability of 80%) were used for incubation: continuous light (24L), an intermittent light-dark schedule (12L:12D) that started with the light period on embryonic day (E)0, or continuous darkness (24D). Lighting schedules were applied from E0 until hatch. Light was provided by 3 white light emitting diode (LED) strips per egg tray of approximately 500 lux at egg level and a colour temperature of 6,050K. Until E19 + 6h, eggshell temperature was measured using temperature sensors (NTC Thermistors: type DC 95; Thermometrics, Somerset, UK) attached to 5 randomly chosen eggs per treatment. Air temperature was adjusted automatically to maintain a constant eggshell temperature of 37.8°C throughout incubation to ensure that there was no temperature effect of the lighting treatments at embryo level. Sensors were attached to the equator of the egg using a small piece of duct tape and heat conducting paste (Dow Corning 340 Heat Sink Compound, Dow Corning GmbH, Wiesbaden, Germany) facing away from the light source. From E19 +6h onward, the climate respiration chamber's temperature was fixed at the current air temperature, and eggshell temperatures were allowed to increase during the hatching process. CO_2 concentration did not exceed 0.35%, and relative humidity was maintained between 45 and 55% throughout incubation. Until E18, eggs were incubated on egg trays. At the start of incubation, trays

were turned to an angle of 45° from the horizontal position, and trays were then turned hourly to an angle of 45° until E18. From E18 onward, eggs were placed in baskets for hatching, and no longer turned.

Measurements

From E8 until E14, 10 eggs per day, per treatment (N = 210) were removed from the incubator for measurement of ossification of the femur and tibia, using Alizarin red and Alcian blue staining in a procedure modified from Pothoff (1984). The time frame of E8 till E14 was chosen because Kürtül *et al.* (2009) demonstrated that ossification in the tibia and femur starts by E9, and we expected the largest portion of ossification to be completed by E14. Eggs were first sampled from the 12L:12D treatment, then 24L, then 24D. Total sampling time was approximately 20 min per treatment. Embryos were fixed in formalin for 24 h, and then stored for 10 months in 70% alcohol solution for later analysis. For the E13 and E14 embryos, most of the leg muscle and skin were removed before storing to facilitate penetration of the staining solutions. Embryos were placed in 96% alcohol for 24 h prior to the staining process. They were then transferred to a Alcian blue solution (80 mL 100% alcohol, 20 mL acetic acid, and 10 mg Alcian blue 8GS) for 24 h for embryos aged E8 to E10 or 48 h for embryos aged E11 to E14. Alcian blue staining was performed to provide a clear contrast between the cartilage and surrounding soft tissue. This cartilage staining did not take very well, but the difference between cartilage and surrounding tissue was still visible. Embryos were then placed in alcohol solutions that decreased every 2 h from 96% alcohol at the start to 100% MiliQ water after 10 h. Embryos were transferred to a Trypsin solution (30 mL saturated sodium borate water, 70 mL MiliQ water, and 1 g Trypsin) 2h. Embryos were then placed in an Alizarin red solution (100 mL 0.5% KOH and 10 mg Alizarin red S. (A16)) for 24 h, and then in the Trypsin solution until the stained bones were visible, which ranged from 11 days for E8 embryos to 20 days for E14 embryos. Embryos were then placed in a 0.5% KOH-Glycerine solution with 3-4 drops of 3% H_2O_2/100 mL solution in a KOH: Glycerine ratio of 3: 1 for 1 day, 1: 1 for 2 days, and 1: 3 for 3 days. Finally, all embryos were placed in 100% glycerine with a few thymol crystals. Embryos were placed in a petri dish and photographed through a Zeiss StemiSV 11 stereomicroscope (Jena, Germany) with an attached Olympus DP50 camera (Tokyo, Japan), and Olympus AnalySIS-FIVE software (Tokyo, Japan). Leg bones were photographed with a 0.63x objective, at a 6.6 x 10 magnification, with a 2.5x adapter. For examples of images of the stained bones, see Figure 3. The total length, ossified length, and width (at the middle of the diaphysis) of the femur and tibia of each left and right leg of the embryos were measured. Because bones were often not completely straight, the line for total length was drawn in three parts for standardisation; from the top of the bone's proximal end to the ossified zone, through the ossified zone, to the dent in the bone's distal end (Figure 3). Ossified percentage was calculated as ossified length / total length x 100.

On E18 +12h (444 h of incubation), E18 +18h (450 h of incubation), E19 (456 h of incubation), and E19 +6h (462 h of incubation), plasma melatonin was measured in 5 embryos per treatment (N = 60). Sampling took approximately 30 minutes per time point. The first measurement took place immediately prior to the start of 12L:12D's dark period, the second measurement in the middle of the dark period, the third measurement immediately prior to the start of the light period, and the fourth measurement in the middle of the light period. Embryos were removed from the eggshell first, and blood was collected from their jugular vein using a 1-mL syringe and 30-gauge needle into heparin coated tubes before decapitation. Blood was centrifuged for 10 min at 12,000 rpm. After centrifugation, plasma was collected and stored at -20°C. Melatonin was later analysed by a double antibody sandwich technique (Chicken melatonin ELISA, catalogue no. MBS262913, MybioSource, CA, USA).

At E19 +12h, the hatching process started, and hatchlings were checked every 3 h. Chickens that were assigned to be sampled immediately (50 per treatment; N = 150) were removed from the climate respiration chambers within 3 h post hatch. They were decapitated, and their blood was collected for IGF-I and GH analysis. Blood was centrifuged for 10 min, at 12,000 rpm. After centrifugation, plasma was collected and stored at -20°C. IGF-I was later analysed using a chicken enzyme-linked immunosorbent assay kit (SEA050Ga 96; Cloud-Clone Corp., Texas, USA), and growth hormone was later analysed using a chicken ELISA kit (CSB-E09866Ch; Cusabio, Maryland, USA). Non-bone tissue of the legs of the decapitated chickens was removed, and the femur and tibia were weighed. Bones were then wrapped in gauze soaked in saline, and stored in a freezer at -20°C for later analysis.

2.2 | Post hatch

Experimental setup

After 21 days and 12h of incubation, all hatched chickens that had been assigned to post hatching measurements (108 per treatment; N = 324) were transported to Coppens' Poultry Research Centre (Vlierden, the Netherlands) for 1h in a climate controlled car. They were housed in floor pens measuring 0.95 x 1.55 m with 9 pens per incubation treatment. From placement until D21 post hatch, 12 chickens were housed per pen. On D21, half of the chickens were removed for sampling, and 6 chickens per pen remained there until final sampling at D35. On D0, all chickens were exposed to 24 h of light before starting a lighting schedule of 16L:8D. The air temperature was maintained at 33°C at D0, decreasing linearly to 19°C at D35. Wood shavings were used as bedding of the pens, and water from nipple drinkers and a commercially available feed were provided ad libitum.

Measurements

At hatch, chicks designated for further growth (108 per treatment; N = 324) received an individual number on a neck label. At E21 +12h, all chicks designated for grow out were removed from the climate respiration chambers, sexed, and transported to the grow out facilities, where they were housed in mixed sex groups of 6 males and 6 females. Both at D21 and D35 post hatch, 54 chickens per treatment were slaughtered, and their tibias and femurs were weighed and stored as described previously.

After thawing, the length, width (mediolateral diameter), and depth (craniocaudal diameter) of all tibias and femurs were measured. The midpoint of 10 left sided tibias and femurs from D0, D21, and D35 (N = 90 tibias and femurs) were determined, and the bone was sawed on both sides 0.9 cm away from the midpoint using a hacksaw, resulting in a 1.8 cm long bone segment from the middle of each bone. These segments were scanned using a Skyscan 1072 MicroCT scanner (Skyscan n.v., Aartselaar, Belgium). The X-ray source was set at 100 µA and 79 kV. Images were acquired with a 1.13° rotation step over an angular range of 180°, and with an exposure time of 3920 ms. See Figure 4 for examples of the femur's and tibia's cross sections on various days. A region of interest of 100 slides around the midpoint was processed in NRecon (Bruker, Kontich, Belgium), and used for further analysis in ImageJ (Abramoff *et al.*, 2004). The plugin BoneJ (Doube *et al.*, 2010) was used to determine cortical and medullary area, second moment of area around the minor and major axis (abbreviated here to minor and major area moment), and maximal and mean cortical thickness per slice. CT scan measurements were then averaged over slides per bone.

D35 legs (both left and right sided) were examined by a veterinarian for incidence of tibial dyschondroplasia, scored as an abnormality in the metaphysis of tibiotarsus or tarsalmetatarsis, characterized by an avascular cartilage plug at the bone's proximal ends (Leach and Monsonego-Ornan, 2007).

2.3 | Statistical analysis

The statistical model used for analysis of the data of the current experiment primarily aimed to investigate the effect of incubation lighting schedules, taking time into account for pineal melatonin data. The overall model used was therefore:

$Y_i = \mu + \text{Incubation}_i + \varepsilon_i$, (1),

where Y_i = the dependent variable, µ is the overall mean, Incubation_i = Incubation lighting schedule (i = 24L, 12L:12D, or 24D), and ε_i = the residual error term. For the analysis of embryonic melatonin data, the model was extended with Time (444, 450, 456, and 462 h of incubation) and its interaction with Incubation treatment. GH, IGF-I, embryonic bone data, and bone dimensions on D0 and D21 were analysed using the

Mixed procedure (for fitting linear mixed models) in SAS (SAS Institute, Cary, NC, USA). Melatonin, bone dimensions on D35, and CT scan data were analysed using the Glimmix procedure (for fitting generalized linear mixed models), because not all data were normally distributed and the Glimmix procedure does not assume normality of residuals. Incidence of tibial dyschondroplasia was analysed using the Logistics procedure for logistic regression analysis of binary response data. Embryo or chicken was considered to be the experimental unit in the analysis of all data. Residuals were examined to verify model assumptions. Presented data are Least Square Means ±SEM. In all cases, differences were considered significant at $P \leq 0.05$.

3 | Results

3.1 | Plasma hormones

Plasma melatonin concentration between E18 +12h and E19 +6h was not affected by treatment ($F = 0.53$; P-value of the linear mixed model = 0.35), moment of sampling ($F = 0.66$; $P = 0.59$), or their interaction ($F = 0.89$; $P = 0.85$; Figure 1). GH at moment of hatching was higher for 24D than for 12L:12D (+21.8%) and 24L (+14.5%; $F = 7.17$; $P = 0.002$; Figure 2). IGF-I at moment of hatching was not affected by treatment ($F = 0.90$; $P = 0.41$).

3.2 | Embryonic bone development

Femur and tibia length were not affected by lighting treatment ($F < 2.25$; $P > 0.13$; Table 1). Femur width at E11 was higher for 24D than for 24L (+8.7%) and 12L:12D (+13.0%; $F = 6.36$; $P = 0.006$; Table 1). Tibia width at E8 was higher for 12L:12D than for 24L (+10.9%; $F = 6.98$; $P = 0.004$; Table 1). Tibia width at E11 was higher for 24D than for 24L (+2.3%) and 12L:12D (+6.1%; $F = 11.10$; $P < 0.001$). Femur and tibia width did not differ between treatments on other embryonic days ($F < 3.38$; $P > 0.053$).

The earliest ossification of the femur and tibia occurred on E11 (Figure 5). Of the 24D embryos, 6 out of 10 showed ossification while 0 out of 10 showed ossification of the 24L and 12L:12D embryos. On E12, all embryos had started ossification of the femur and tibia. Ossified percentage of the femur at E11 was higher for 24D than for 24L and 12L:12D (19.9% versus 0%; $F = 15.77$; $P < 0.001$). Ossified percentage of the femur did not differ between treatments on E12 ($F = 1.32$; $P = 0.29$), E13 ($F = 1.85$; $P = 0.18$), or E14 ($F = 3.24$; $P = 0.057$). Ossified percentage of the tibia at E11 was higher for 24D than for 24L and 12L:12D (+23.9% versus 0%; $F = 15.77$; $P < 0.001$). It did not differ between treatments on E12 ($F = 2.13$; $P = 0.14$). Ossified percentage was higher for 12L:12D than

for 24L at E13 (+2.83%; F = 3.76; P = 0.040) and E14 (+3.46%; F = 3.53; P = 0.045), with 24D intermediate (Figure 3).

3.3 | Post hatch bone development

On day of hatching (D0), femurs were longer for 12L:12D than for 24L (+1.6%) and 24D (+1.5%; F = 4.73; P = 0.010; Table 2). Tibia weight was higher for 12L:12D (+6.5%) and 24D (+3.3%) than for 24L (F = 7.31; P = 0.001). Tibias were longer for 12L:12D (+2.0%) and 24D (+1.2%) than for 24L (F = 6.17; P = 0.003). Femur weight, depth, and width, and tibia depth and width did not differ between treatments (F < 2.60; P > 0.08).

On D21 post hatch, femurs were longer for 12L:12D (+2.2%) and 24D (+1.8%) than for 24L (F = 4.77; P = 0.010). Tibias were longer for 12L:12D (+2.1%) and 24D (+1.6%) than for 24L (F = 4.05; P = 0.020). Tibia and femur weight, depth, and width did not differ between treatments (F < 2.13; P > 0.12).

On D35 post hatch, femur weight was higher for 12L:12D than for 24L (+5.7%; F = 3.85; P = 0.023), with 24D intermediate. Femur depth was higher for 24D than for 24L (3.3%) and 12L:12D (+2.5%; F = 6.00; P = 0.003). Tibias were longer for 12L:12D than for 24L (+1.5%), with 24D intermediate (F = 3.82; P = 0.023). Femur length and width, and tibia weight, depth, and width did not differ between treatments (F < 1.48; P > 0.23).

Incidence of tibial dyschondroplasia was higher for 24L (8.2% of all tibias) than for 12L:12D (1.1% of all tibias), with 24D intermediate (2.2% of all tibias; P = 0.046).

3.4 | CT scans

On D0 post hatch, the cortical area of the femur was not affected by treatment (F = 1.9; P = 0.17; Table 3). Cortical area of the tibia was higher for 12L:12D (+10.0%) and 24D (+13.6%) than for 24L (F = 4.3; P = 0.019). Medullary area of the femur and tibia were not affected by treatment (F < 2.87; P > 0.073). The minor area moment of the femur was higher for 24D than for 12L:12D (+21.6%; F = 3.48; P = 0.044). The minor area moment of the tibia was higher for 24D and 12L:12D than for 24L (both +19.4%; F = 4.34; P = 0.018). The major area moment of the femur and tibia, maximal cortical thickness of the femur and tibia, and mean cortical thickness of the femur did not differ between treatments (F < 2.15; P > 0.053). Mean cortical thickness of the tibia was higher for 12L:12D (+17.2%) and 24D (+14.3%) than for 24L (F = 4.92; P = 0.011).

On D21 post hatch, cortical and medullary area, minor and major area moments, and mean cortical thickness of the tibia and femur, as well as maximal cortical thickness of the femur were not affected by treatment (F < 1.66; P > 0.21). Maximal cortical thickness

of the tibia was higher for 12L:12D than for 24L (+8.8%) and 24D (+11.9%; $F = 3.93$; $P = 0.033$).

On D35 post hatch, cortical area of the tibia was 17.6% higher for 12L:12D than for 24L, with 24D intermediate ($F = 4.23$; $P = 0.027$). Major area moment of the tibia was 28.3% higher for 12L:12D than for 24L, with 24D intermediate ($F = 3.54$; $P = 0.045$). Maximal cortical thickness of the tibia was 14.7% higher for 24L and 11.2% higher for 12L:12D than for 24D ($F = 4.27$; $P = 0.026$). Cortical area of the femur, medullary area of the femur and tibia, minor and major area moments of the femur, minor area moment of the tibia, maximal and mean cortical thickness of the femur, and mean cortical thickness of the tibia did not differ between treatments ($F < 3.17$; $P > 0.060$).

An overview of all bone variables significantly affected by treatment can be seen in Table 4.

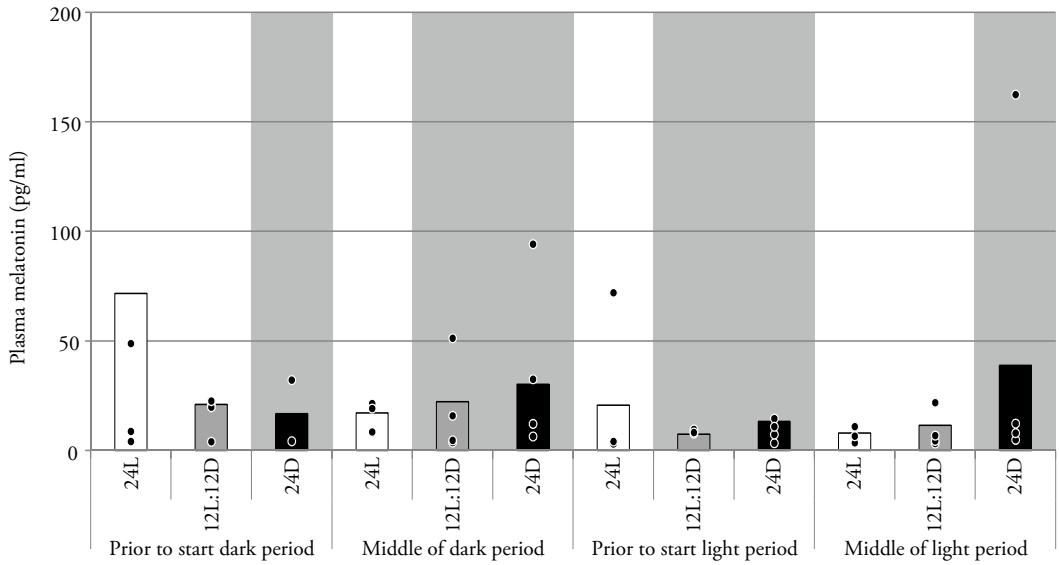


Figure 1. Plasma melatonin ±standard errors measured every 6 h between E18 +12h and E19 +6h in broiler embryos incubated under 24L, 12L:12D, or 24D of white LED light from set until hatch. Grey rectangle = dark period.

• Individual observations. n = 5 per treatment, per sampling moment. x = Observation with actual value = 495 pg/ml, value not within axis range.

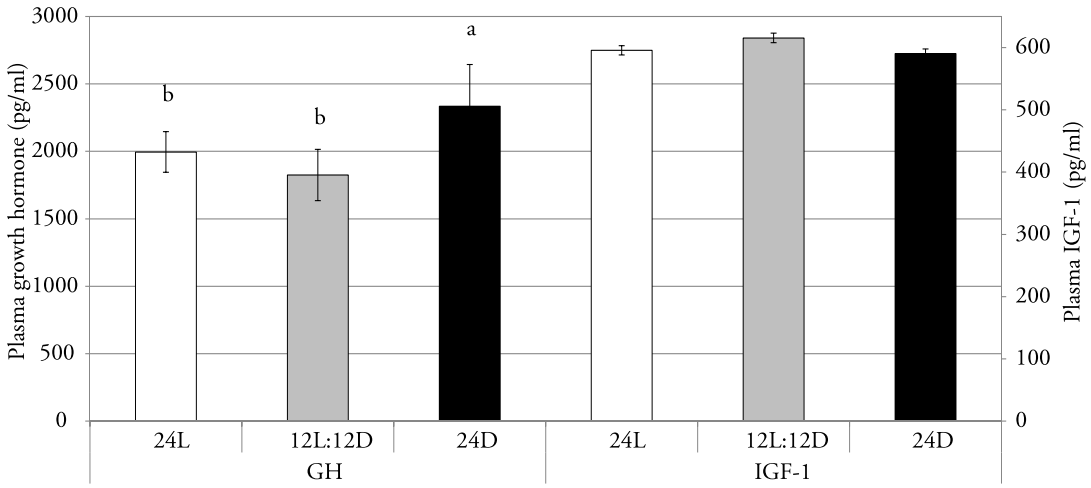


Figure 2. Plasma growth hormone (GH) and IGF-I ± standard errors at hatching in broiler chickens incubated under 24L, 12L:12D, or 24D of white LED light from day 0 until hatching

[a,b] Values within a hormone with different superscripts differ significantly at $P \leq 0.05$.
n = 50 chickens per treatment.

Table 1. Femur and tibia length and width from E8 till E14 in broiler embryos incubated under 24L, 12L:12D, or 24D of white LED light from day 0 until hatching

	n[1]	E8	E9	E10	E11	E12	E13	E14
					Femur length (mm)			
24L	10	4.33	5.45	7.16	8.21	9.90	11.33	12.83
12L:12D	10	4.40	5.27	6.92	8.39	10.02	11.26	12.57
24D	10	4.16	5.44	7.07	8.33	9.58	11.16	12.90
SEM		0.119	0.151	0.218	0.116	0.141	0.115	0.237
P-value		0.29	0.62	0.58	0.55	0.13	0.60	0.32
					Tibia length (mm)			
24L	10	4.73	6.53	8.84	10.34	12.82	14.86	17.07
12L:12D	10	4.71	6.58	8.49	10.50	12.92	14.83	17.01
24D	10	4.55	6.72	8.86	10.79	12.40	14.63	17.16
SEM		0.138	0.122	0.295	0.195	0.163	0.155	0.150
P-value		0.59	0.53	0.60	0.26	0.11	0.54	0.78
					Femur width (mm)			
24L	10	0.44	0.50	0.54	0.63[b]	0.83	1.01	1.23
12L:12D	10	0.44	0.50	0.53	0.60[b]	0.82	1.04	1.17
24D	10	0.44	0.50	0.54	0.69[a]	0.83	1.00	1.19
SEM		0.010	0.011	0.015	0.017	0.014	0.019	0.018
P-value		0.95	0.91	0.87	0.006	0.90	0.26	0.068
					Tibia width (mm)			
24L	10	0.41[b]	0.50	0.56	0.68[b]	0.82	1.00	1.32
12L:12D	10	0.46[a]	0.47	0.56	0.66[b]	0.81	1.06	1.29
24D	10	0.44[ab]	0.49	0.54	0.73[a]	0.84	1.01	1.24
SEM		0.014	0.008	0.019	0.011	0.019	0.020	0.031
P-value		0.004	0.064	0.75	<0.001	0.59	0.053	0.21

[a,b] Values within a day, within a measurement with different superscripts differ significantly at $P \leq 0.05$.

[1] n = 10 chickens per treatment, per embryonic day

Table 2. Femur and tibia weight, length, width, and depth on D0, D21, and D35 post hatching in broilers incubated under 24L, 12L:12D, or 24D of white LED light from day 0 until hatching

		Femur				Tibia			
	n[1]	Weight (g)	Length (mm)	Depth (mm)	Width (mm)	Weight (g)	Length (mm)	Depth (mm)	Width (mm)
					Day 0 post hatch				
24L	50	0.197	21.91[b]	1.52	1.54	0.29[b]	30.07[b]	1.41	1.51
12L:12D	50	0.204	22.26[a]	1.53	1.55	0.31[a]	30.67[a]	1.43	1.54
24D	50	0.201	21.93[b]	1.54	1.55	0.30[a]	30.45[a]	1.44	1.53
SEM		0.002	0.09	0.009	0.01	0.004	0.12	0.01	0.01
P-value		0.11	0.010	0.25	0.92	0.001	0.003	0.08	0.22
					Day 21 posthatch				
24L	54	4.44	50.29[b]	6.27	5.85	6.35	72.00[b]	5.12	5.59
12L:12D	54	4.47	51.43[a]	6.27	5.79	6.47	73.57[a]	5.16	5.55
24D	54	4.52	51.20[a]	6.41	5.94	6.53	73.18[a]	5.14	5.63
SEM		0.08	0.28	0.06	0.05	0.12	0.40	0.06	0.05
P-value		0.79	0.010	0.18	0.12	0.53	0.020	0.92	0.52
					Day 35 post hatch				
24L	54	10.64[b]	68.62	9.18[b]	8.76	14.34	99.71[b]	7.07	8.44
12: 12D	54	11.28[a]	69.02	9.25[ab]	8.85	14.88	101.27[a]	7.12	8.51
24D	54	10.78[ab]	68.78	9.49[a]	8.85	14.71	100.69[ab]	6.96	8.46
SEM		0.175	0.299	0.066	0.063	0.225	0.404	0.072	0.077
P-value		0.023	0.61	0.003	0.52	0.23	0.023	0.26	0.81

[a,b] Values within a day, within a measurement with different superscripts differ significantly at $p \leq 0.05$.

[1] n = 50 chickens per treatment at hatching and 54 per treatment on day 21 and day 35 post hatch

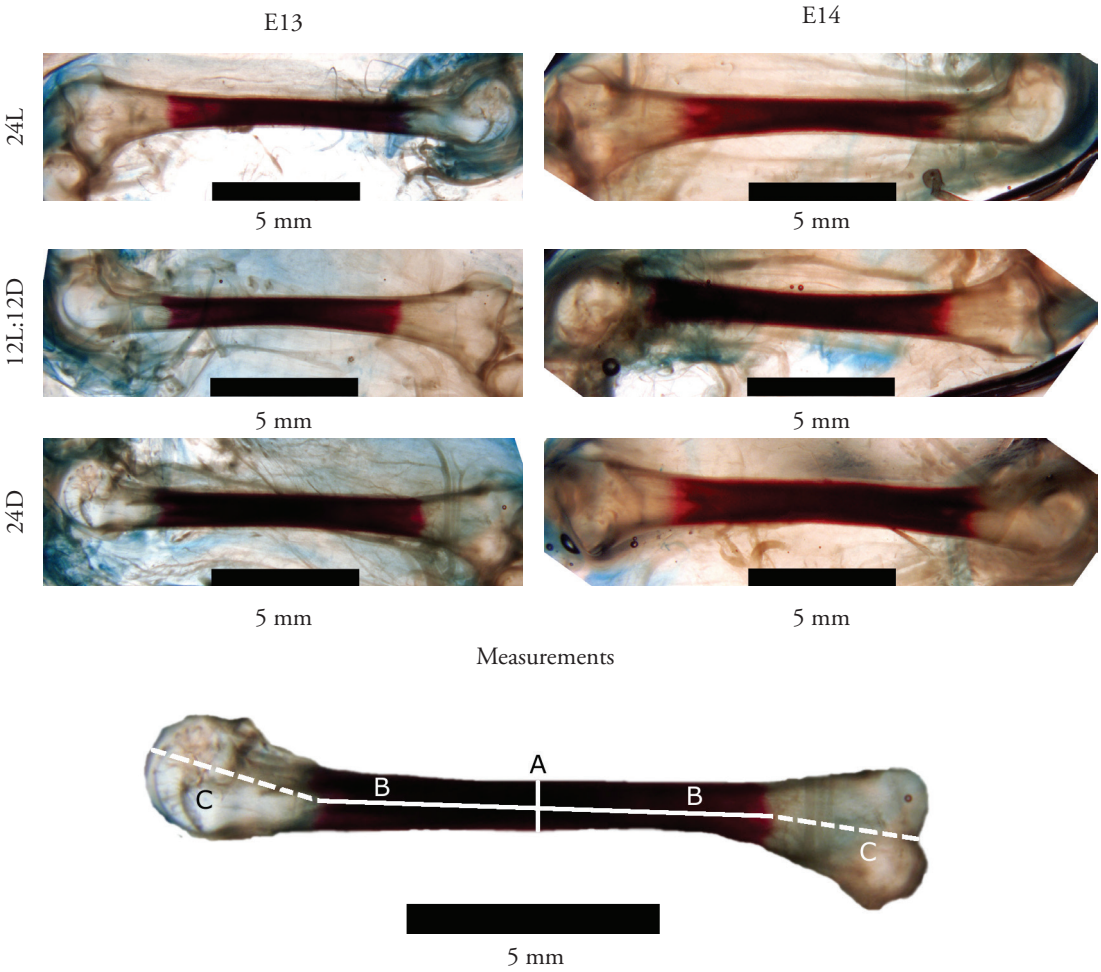


Figure 3. Examples of tibias stained with Alizarin red and Alcian blue staining in a procedure modified from Pothoff (1984) on embryonic day (E)13 and E14 in broiler embryos incubated under 24L, 12L:12D, or 24D of white LED light from day 0 until hatching.

A = bone width; B = ossified length; B + C = total length.
The ossified part of the bone is stained red; the cartilaginous part of the bone is uncoloured. The black bar indicates the scale (5 mm).

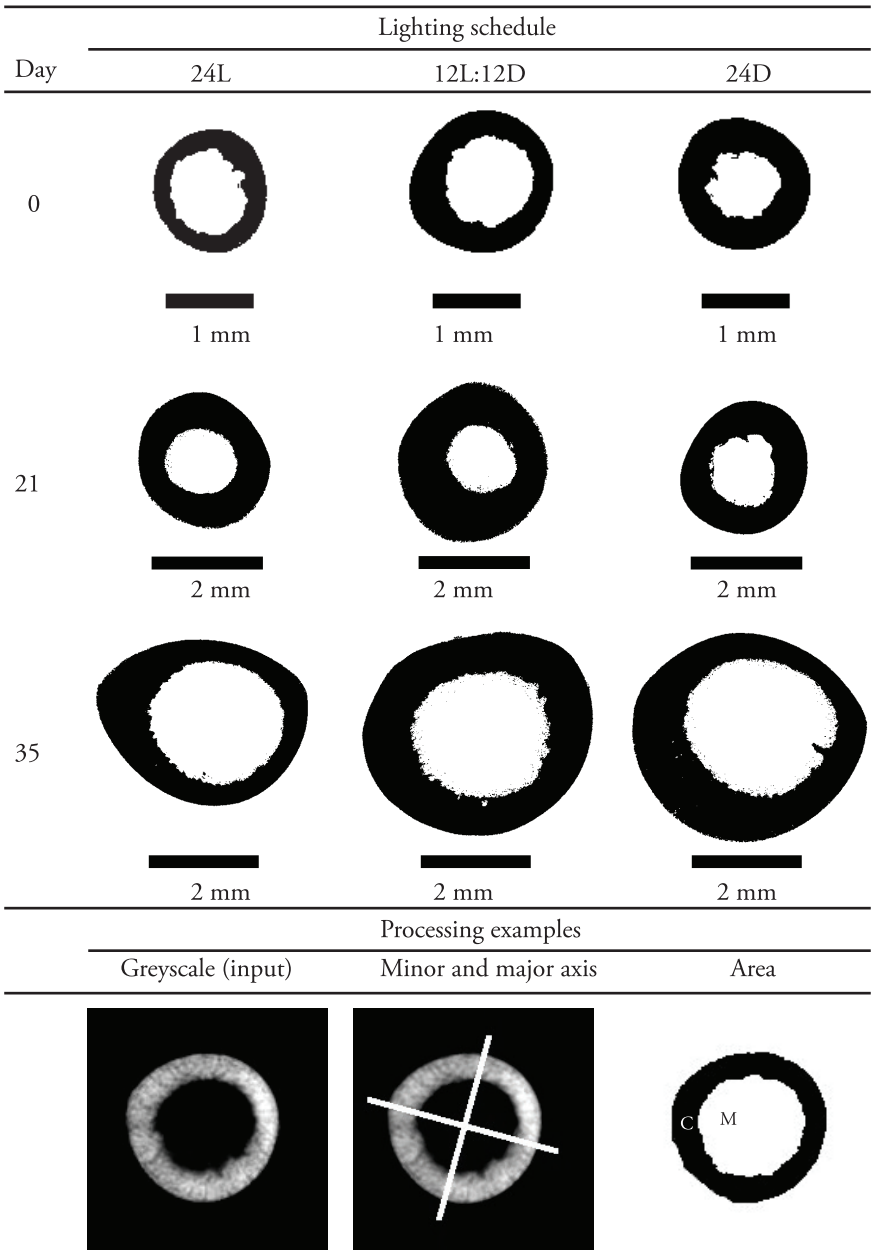


Figure 4. Examples of cross sections of tibiatarsus at the mid-diaphysis after reconstruction and thresholding of broilers incubated under 24L, 12L:12D, or 24D of white LED light from day 0 until hatching on D0, D21, and D35 post hatch.

White bars in the minor and major axis examples shows how BoneJ determined the minor and major axis within a slide.
C = cortical area, M = medullary area.

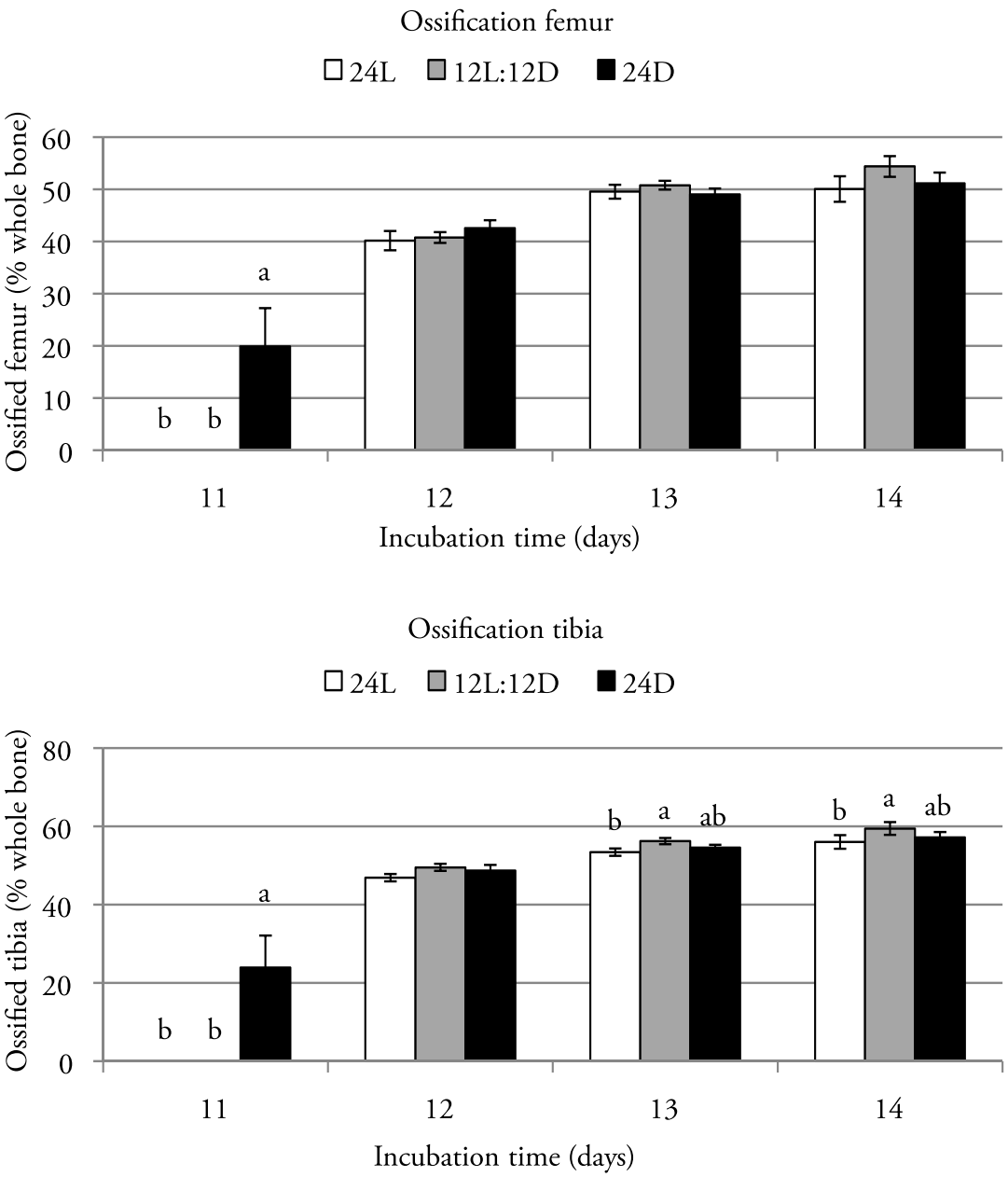


Figure 5. Ossified length of the femur and tibia as a percentage ±standard errors from E8 till E14 in broiler embryos incubated under 24L, 12L:12D, or 24D of white LED light from set until hatch

[a,b] Values within a day with different superscripts differ significantly at $p \leq 0.05$.

Table 3. Femur and tibia cortical and medullary area, minor and major area moment, and maximal and mean cortical thickness at D0, D21 and D35 post hatching in broilers incubated under 24L, 12L:12D, or 24D of white LED light from set until hatch

		Cortical area (mm^2)		Medullary area (mm^2)		Second moment of area - Minor axis (mm^4)		Second moment of area - Major axis (mm^4)		Maximal cortical thickness (mm)		Mean cortical thickness (mm)	
	n[1]	Femur	Tibia	Femur	Tibia	Femur	Tibia	Femur	Tibia	Femur	Tibia	Femur	Tibia
Day 0 post hatch													
24L	10	1.28	1.08[b]	2.31	2.14	0.31[ab]	0.25[b]	0.34	0.29	0.44	0.32	0.30	0.24[b]
12L:12D	10	1.21	1.20[a]	2.26	2.29	0.29[b]	0.31[a]	0.32	0.34	0.40	0.36	0.29	0.29[a]
24D	10	1.36	1.25[a]	2.47	2.28	0.37[a]	0.31[a]	0.39	0.35	0.44	0.35	0.31	0.28[a]
SEM		0.055	0.046	0.064	0.060	0.020	0.016	0.021	0.018	0.021	0.013	0.016	0.011
P-value		0.17	0.019	0.073	0.14	0.044	0.018	0.053	0.058	0.29	0.13	0.55	0.011
Day 21 post hatch													
24L	10	20.40	17.91	10.06	6.26	53.58	38.76	70.30	48.95	1.45	1.46[b]	0.81	1.05
12L:12D	10	19.36	18.05	9.91	6.92	49.35	40.40	65.78	51.48	1.43	1.60[a]	0.82	1.10
24D	10	18.47	16.07	9.88	6.69	46.09	33.43	60.00	43.18	1.51	1.41[b]	0.90	1.02
SEM		0.747	0.864	0.703	0.575	4.380	3.570	5.896	4.874	0.062	0.047	0.056	0.052
P-value		0.21	0.22	0.98	0.71	0.49	0.38	0.48	0.49	0.64	0.033	0.47	0.58
Day 35 post hatch													
24L	10	28.60	22.93[b]	28.37	18.07	162.35	86.82	219.10	129.83[b]	1.35	1.77[a]	0.82	1.12
12L:12D	10	26.29	27.84[a]	29.45	19.77	151.62	121.29	197.83	180.99[a]	1.32	1.70[a]	0.82	1.06
24D	10	29.95	25.93[ab]	31.00	20.58	182.63	116.38	250.46	164.88[ab]	1.28	1.51[b]	0.72	0.99
SEM		1.154	1.237	1.228	0.898	12.332	10.598	17.793	14.209	0.054	0.063	0.053	0.054
P-value		0.091	0.027	0.31	0.16	0.20	0.060	0.12	0.045	0.64	0.026	0.30	0.26

[a,b] Values within a day, within a measurement with different superscripts differ significantly at $P \leq 0.05$.

[1] n = 10 bones (tibia or femur) per treatment, per day

4 | Discussion

Ossification of embryonic chicken bones starts at the mid diaphysis of the bone in the primary ossification centre (Kürtül *et al.*, 2009). In the current experiment, ossification of the tibia and femur started approximately a day sooner for embryos exposed to 24D than to 12L:12D and 24L. For 6 out of 10 embryos from the 24D group, ossification of the tibia and femur started on E11. For all other 24D embryos, and all embryos from 12L:12D and 24L, it started on E12. Although the onset of ossification was earlier for 24D, the 12L:12D and 24L treatments no longer differed on E12. On E13 and E14, ossification of the tibia of 12L:12D was higher than that of 24L, suggesting that rate of ossification in the tibia had increased for 12L:12D compared to 24L from E13 onward. These results suggest that 24D accelerated onset of ossification, and an incubation lighting schedule of 12L:12D increased osteoblast activity by E13 in the tibia of broiler embryos more than continuous light.

By the time the chickens hatched, 12L:12D and 24D had longer and heavier tibias than 24L and 12L:12D had longer femurs than both 24L and 24D. Increase of bone length is mostly dependent on the rate with which hypertrophic cartilage cells are produced at the bone's epiphysis (Kronenberg, 2003). The cartilage is then replaced by bony tissue through endochondral ossification (Roach, 1997). It appears that 24L may decrease both ossification at the primary ossification centre, and bone length growth at the epiphyseal plates, compared to 12L:12D, with 24D intermediate.

Osteoblast formation is stimulated by melatonin, which is released in dark periods (Cardinali *et al.*, 2003); it can therefore be hypothesized that incubation under 24L results in lower basal melatonin levels than 24D or 12L:12D, which provide darkness. Melatonin release patterns start showing a clear circadian rhythm by E16 to E18 (Csernus *et al.*, 2007). Once a rhythm is present, plasma melatonin levels tend to be higher at internal pipping for embryos incubated under 16L:8D compared to 24D (Özkan *et al.*, 2012). However, in the current experiment, plasma melatonin concentrations between E18 +12h and E19 +6h did not differ between treatments or phase within the light-dark schedule. This is contrary to results found by Zeman *et al.* (1999a) and Özkan *et al.* (2012). Zeman *et al.* (1999a) incubated eggs under various lighting schedules, such as 16L:8D and 8L:16D, and found a clear dark-light dependent rhythm of plasma melatonin from E18 onward, but it was less pronounced than pineal melatonin concentrations. It is possible that a rhythm in melatonin release would have been observed if melatonin concentrations in the pineal gland had been measured in the current experiment. Growth hormone and IGF-I are known to stimulate chondrocyte proliferation in epiphyseal plates (Robson *et al.*, 2002; Van der Eerden *et al.*, 2003). Growth hormone level at hatching was found to be higher for 24D than for 12L:12D and 24L, and IGF-I level at moment of hatching was not affected by treatment. As bone dimensions at hatch were not higher for 24D than for 12L:12D, differences in leg bone development at hatch could not be

explained through the involvement of growth hormone. The results of this experiment do not clarify the endocrine basis of the stimulatory effect of 12L:12D and, to a lesser degree, 24D on embryonic bone development compared to 24L.

Table 4. Overview of femur and tibia measurements (embryonically, at hatching, or at D21 or D35 post hatch) found to be significantly decreased ($P < 0.05$) by treatment compared to another treatment for broilers incubated under 24L, 12L:12D, or 24D of white LED light throughout incubation.

Phase	Bone	Measurement	Decreased for	Compared to
Embryonic	Femur	Width E12	24D	12L:12D and 24L
	Tibia	Width E8	24L	12L:12D
		Width E11	24L and 12L:12D	24D
		Ossification E13	24L	12L:12D
		Ossification E14	24L	12L:12D
Hatching	Femur	Length	24L and 24D	12L:12D
		Minor area moment[1]	12L:12D	24D
	Tibia	Weight	24L	12L:12D and 24D
		Length	24L	12L:12D and 24D
		Cortical area	24L	12L:12D and 24D
		Minor area moment[1]	24L	12L:12D and 24D
		Mean cortical thickness	24L	12L:12D and 24D
D21	Femur	Length	24L	12L:12D and 24D
	Tibia	Length	24L and 24D	12L:12D
		Maximal cortical thickness	24L and 24D	12L:12D
D35	Femur	Weight	24L	12L:12D
		Depth	24L	24D
	Tibia	Length	24L	12L:12D
		Cortical area	24L	12L:12D
		Major area moment[1]	24L	12L:12D
		Maximal cortical thickness	24D	12L:12D
		No tibial dyschondroplasia	24L	12L:12D

[1] Minor or major area moment = second moments of area around the minor and major axis

Microstructure of the tibia differed between treatments at hatching of the chickens. 12L:12D and 24D both had a larger tibia cortical area, minor area moment, and mean cortical thickness than 24L. This effect was still present at slaughter for 12L:12D, but not for 24D. At slaughter on D35, a higher tibial cortical area and a higher tibial major area moment were found for 12L:12D compared to 24L, and higher maximal cortical thickness was found for 12L:12D and 24L than for 24D. Additionally, femur weight (but not length) was higher for 12L:12D than for 24L, and tibia length (but not weight)

was still higher for 12L:12D than for 24L at D35. According to Augat and Schorlemmer (2006), the bone cortical area, second moment of area, and bone size itself can predict up to 70 to 80% of whole bone strength. A higher second moment of area indicates greater resistance to bending. In adult humans, the risk of stress fractures in both the tibia and femur has been found to be increased with decreasing moments of inertia (Milgrom *et al.*, 1989). The increase in the values of the bone parameters in the 12L:12D incubated chickens therefore suggests that these bones were not only larger but also stronger at slaughter than those of 24L.

12L:12D did not only result in chicken leg bones that may have been more resistant to bending: the tibias also showed a lower incidence of tibial dyschondroplasia compared to 24L. Tibial dyschondroplasia's aetiology lies in poor ossification of the epiphyseal plates, characterized by an avascular lesion in the tibial head (Hargest *et al.*, 1984). It is a form of osteochondrosis, which is an abnormality in endochondral ossification (Leach and Monsonego-Ornan, 2007). It is not known how incubation under 12L:12D reduced incidence of tibial dyschondroplasia, but it can be speculated that applying a circadian lighting schedule stimulated vascularization (Herzog *et al.*, 2011) or ossification (Hargest *et al.*, 1984) at the epiphyseal plate.

The current experiment suggests that a circadian light schedule of 12L:12D from day 0 of incubation till hatching stimulates embryonic bone development, especially when compared to 24L. This effect is still present at slaughter, with larger bones and less tibial dyschondroplasia at D35 post hatch. 24D seems mostly intermediate, suggesting that darkness is essential for chicken embryonic bone development. The tibia seems especially susceptible to stimulation by incubation lighting schedules. The involvement of hormones in the stimulatory effect of a circadian rhythm during incubation, however, cannot be explained based on the current experiment. In chickens, we can conclude that a circadian rhythm during incubation stimulates embryonic bone development with possible long term effects on leg bone health.

5 | Acknowledgements

The authors would like to thank Marcel Heetkamp for his assistance with the climate respiration chambers, and Ilse Poolen and Loes Verrijt for their practical assistance during the experiment.

6 | References

Abramoff, M. D., P. J. Magalhaes, and S. J. Ram. 2004. Image Processing with ImageJ. Biophotonics International 11: 36-42.

Aota, Y., H. Terayama, T. Saito, and M. Itoh. 2013. Pinealectomy in a broiler chicken model impairs endochondral ossification and induces rapid cancellous bone loss. Spine J. 13: 1607-1616.

Augat, P., and S. Schorlemmer. 2006. The role of cortical bone and its microstructure in bone strength. Age Ageing 35 suppl 2: 27-31.

Bessei, W. 2006. Welfare of broilers: a review. World Poultry Sci J. 62: 455-466.

Cardinali, D. P., M. G. Ladizesky, V. Boggio, R. A. Cutrera, and C. Mautalen. 2003. J. Pineal Res. 34: 81-87.

Csernus, V. J., A. D. Nagy, and N. Faluhelyi. 2007. Development of the rhythmic melatonin secretion in the embryonic chicken pineal gland. Gen. Comp. Endocrinol. 152: 148-153.

Doube, M., M. M. Kłosowski, I. Arganda-Carreras, F. Cordeliéres, R. P. Dougherty, J. Jackson, B. Schmid, J. R. Hutchinson, and S. J. Shefelbine. 2010. BoneJ: free and extensible bone image analysis in ImageJ. Bone 47: 1076-1079.

Hargest, T. E., R. M. Leach, and C. V. Gay. 1985. Avian tibial dyschondroplasia. I. Ultrastructure. Am. J. Pathol. 119: 175.

Heetkamp, M. W. J., S. J. J. Alferink, T. Zandstra, P. Hendriks, H. van den Brand, and W. J. J. Gerrits. 2015. Design of climate respiration chambers, adjustable to the metabolic mass of subjects. In: W. Gerrits and E. Labussière, editors. Indirect calorimetry: techniques, computations and applications. pp 35-56.

Herzog, A., O. Genin, A. Hasdai, D. Shinder, and M. Pines. 2011. Hsp90 and angiogenesis in bone disorders—lessons from the avian growth plate. Am. J. Physiol. Regul. Integr. Comp. Physiol. 301: R140-R147.

Johnston, J. D., S. Messager, P. Barrett, and D. G. Hazlerigg. 2003. Melatonin action in the pituitary: neuroendocrine synchronizer and developmental modulator? J. Neuroendocrinol. 15: 405-408.

Kestin, S. C., T. G. Knowles, A. E. Tinch, N. G. Gregory. 1992. Prevalance of leg weakness in broiler chickens and its relationship with genotype. Vet. Rec. 131: 190-194.

Kronenberg, H. M. 2003. Developmental regulation of the growth plate. Nature 423: 332-336.

Kürtül, I., S. H. Atalgin, K. Aslan, and E. U. Bozkurt. 2009. Ossification and growth of the bones of the wings and legs in prehatching period of the Hubbert strain broiler. Kafkas Univ. Vet. Fak. Derg. 15: 869-874.

Lamošová, D., M. Zeman, M. Macková, and E. Gwinner. 1995. Development of rhythmic melatonin synthesis in cultured pineal glands and pineal cells isolated from chick embryo. Experientia 51: 970-975.

Leach, R. M. Jr, and E. Monsonego-Ornan. 2007. Tibial dyschondroplasia 40 years later. Poult. Sci. 86: 2053-

2058.

Machida, M., J. Dubousset, Y. Imamura, T. Iwaya, T. Yamada, and J. Kimura. 1995. Role of melatonin deficiency in the development of scoliosis in pinealectomised chickens. J. Bone Joint Sur. 77: 134-138.

McMillen, I. C., R. Nowak, D. W. Walker, and I. R. Young. 1990. Maternal pinealectomy alters the daily pattern of fetal breathing in sheep. Am. J. Physiol. 258: 284-287.

Milgrom, C., M. Gildadi, A. Simkin, N. Rand, R. Kedem, H. Kashtan, and M. Gomori. 1989. The second moment of area of the tibia: a risk factor for stress fractures. J. Biomech. 22: 1243-1248.

Morin, L. P., and C. N. Allen. 2006. The circadian visual system. Brain Res. Rev. 51: 1-60.

Nakade, O., H. Koyama, H. Ariji, A. Yajima, and T. Kaku. 1999. Melatonin stimulates proliferation and type I collagen synthesis in human bone cells in vitro. J. Pineal Res. 27: 106-110.

Ostrowska, Z., B. Kos-Kudla, B. Marek, D. Kajdaniuk, and N. Ciesielska-Kopacz. 2002. The relationship between the daily profile of chosen biochemical markers of bone metabolism and melatonin and other hormone secretion in rats under physiological conditions. Neuro Endocrinol. Lett. 23: 417-425.

Özkan, S., S. Yalçin, E. Babacanoğlu, H. Kozanoğlu, F. Karadaş, and S. Uysal. 2012. Photoperiodic lighting (16 h of light: 8 h of dark) programs during incubation: 1. Effects on growth and circadian physiological traits of embryos and early stress response of broiler chickens. Poult. Sci. 91: 2912-2921.

Park, K. H., J. W. Kang, E. M. Lee, J. S. Kim, Y. H. Rhee, M. Kim, S. J. Jeong, Y. G. Park, and S. H. Kim. 2011. Melatonin promotes osteoblastic differentiation through the BMP/ERK/Wnt signalling pathways. J. Pineal Res. 51: 187-194.

Pothoff, T. 1984. Clearing and staining techniques. Ontogeny and systematics of fishes, La Jolla, California, USA.

Rivkees, S. A. 2003. Developing circadian rhythmicity in infants. Pediatrics 112: 373-381.

Roach, H. I. 1997. New aspects of endochondral ossification in the chick: chondrocyte apoptosis, bone formation by former chondrocytes, and acid phosphatase activity in the endochondral bone matrix. J. Bone Miner. Res. 12: 795-805.

Robson, H., T. Siebler, S. M. Shalet, and G. R. Williams. 2002. Interactions between GH, IGF-1: Glucocorticoids, and thyroid hormones during skeletal growth. Pediatr. Res. 52: 137-147.

Sanotra, G. S., J. D. Lund, A. K. Ersboll, J. S. Petersen, and K. S. Vestergaard. 2001. Monitoring leg problems in broilers: a survey of commercial broiler production in Denmark. Worlds Poult.. Sci. J. 57: 55-69.

SAS Institute Inc. 2002-2010: SAS/STAT User's guide. Version 9.3. SAS Inst. Inc., Cary, NC.

Satomura, K., S. Tobiume, R. Tokuyama,

Y. Yamasaki, K. Kudoh, E. Maeda, and M. Nagayama. 2007. Melatonin at pharmacological doses enhances human osteoblastic differentiation in vitro and promotes mouse cortical bone formation in vivo. J. Pineal Res. 42: 231-239.

Shafey, T.M. 2004. Effect of lighted incubation on embryonic growth and hatchability performance of two strains of layer breeder egss. Br. Poult. Sci. 45: 223-229.

Takahashi, J. S., and M. Zatz. 1982. Regulation of circadian rhythmicity. Science 217: 1104-1111.

Tamura, H., Y. Nakamura, M. Terron, L. J. Flores, L. C. Manchester, D. Tan, N. Sugino, and R. J. Reiter. 2008. Melatonin and pregnancy in the human. Reprod. Toxicol. 25: 291-303.

Torres-Farfan, C., F. J. Valenzuela, A. M. Germain, M. L. Viale, C. Campino, F. Torrealba, G. J. Valenzuela, H. G. Richter, and M. Serón-Ferré. 2006. Maternal melatonin stimulates growth and prevents maturation of the capuchin monkey fetal adrenal gland. J. Pineal Res. 41: 58-66.

Van der Eerden, B. C. J., M. Karperien, and J. M. Wit. 2003. Systemic and local regulation of the growth plate. Endocr. Rev. 24: 782-801.

Yalçin, S., H. B. Molayoglu, M. Baka, O. Genin, and M. Pines. 2007. Effect of temperature during the incubation period on tibial growth plate chondrocyte differentiation and the incidence of tibial dyschondroplasia. Poult. Sci. 86: 1772-1783.

Zeman, M., E. Gwinner, and E. Somogyiová. 1992. Development of melatonin rhythm in the pineal gland and eyes of chick embryo. Experientia 48: 765-768.

Zeman, M., E. Gwinner, I. Herichová, D. Lamošová, and L. Koštál. 1999a. Perinatal development of circadian melatonin production in domestic chicks. J. Pineal Res. 26: 28-34.

Zeman, M., J. Buyse, D. Lamošová, I. Herichova, and E. Decuypere. 1999b. Role of melatonin in the control of growth and growth hormone secretion in poultry. Domest. Anim. Endocrinol. 17: 199-207.

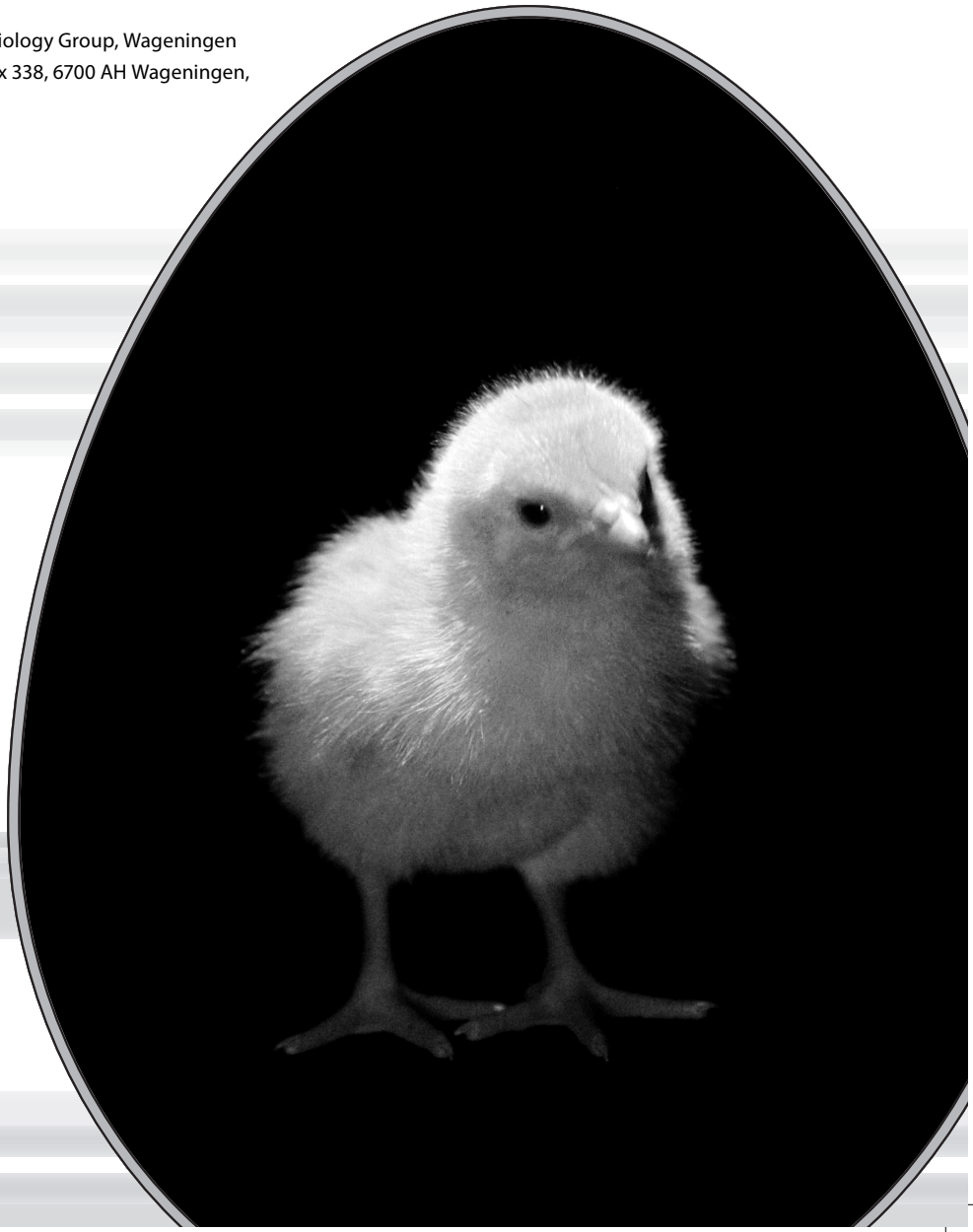
Chapter 4

Lighting schedule throughout incubation: effects on broiler development and post hatch performance

C. W. van der Pol[ab], L.P.G. Verrijt[b], I. A. M. van Roovert-Reijrink[a], C. M. Maatjens[ab], G. Aalbers[a]., B. Kemp[b], and H. van den Brand[b]

[a] HatchTech B.V., P.O. Box 256, 3900 AG Veenendaal, the Netherlands.

[b] Adaptation Physiology Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, the Netherlands.



Abstract

Commercial hatching eggs are incubated in complete darkness. In nature a hen will leave the nest intermittently, exposing the eggs to light. To test the effects of differing lighting schedules during incubation on chicken development, Ross 308 eggs (N = 1,005) from a 40 week old parent flock were incubated from embryonic day 0 (E0) until hatch using 1 of 3 lighting regimes: continuous light (24L), 12 hours of light, followed by 12 hours of darkness (12L:12D), or continuous darkness (24D). The type of lighting used was a 500 lux white light emitting diode (LED) with a colour temperature of 6,050K, and eggshell temperatures were maintained at 37.8°C. Chickens were grown out until day 35 post hatch. Hatch time and sex were recorded and chickens were sampled within 3 hours post hatch for body weight, length, navel score, and organ weights. Post hatch body weight, feed intake and growth:feed were determined weekly. At day 21 post hatch, organ weights were determined. Hamburger-Hamilton (H&H) embryonic stage, hatchability, and culls at hatch were not affected by treatment. Average hatch time was affected by a treatment x sex interaction, but the most conclusive finding was the main effect of treatment: overall incubation time was shorter for 24L (490 hours) than for 12L:12D and 24D (497 hours; $P < 0.001$). Yolk free body mass (YFBM), liver weight, and intestine weight at hatch were higher for 12L:12D than for 24L (+0.2 g, +0.13% of body weight, and +0.42% of body weight, respectively; $P < 0.031$). Intestinal weight at hatch was furthermore higher for 12L:12D than for 24D (+0.34% of body weight). Organ weights no longer differed at D21 post hatch. Body weight, feed intake, and growth:feed were not different between treatments. These results suggest that 12L:12D white LED light applied during incubation at a set eggshell temperature of 37.8°C stimulated chick development at hatch, independent of sex, when compared to a 24L regime. The stimulatory effect of 12L:12D was no longer observed during the grow out period when body weight, organ weights, feed intake, and growth:feed were investigated. To conclude, while a lighting schedule of 12L:12D during incubation seems to increase embryonic development, post hatch performance parameters such as growth and growth:feed were not improved when compared to using the industry standard of 24D.

1 | Implications

In commercial incubation, eggs are incubated in complete darkness. Light during incubation may be necessary for optimal embryonic development, which may be related to later life health and productivity. In the present study, providing continuous darkness, light, or a light-dark schedule during incubation was investigated in relation to hatchling quality and development. This is an area that is of particular interest, both for our scientific understanding of chicken embryo physiology, and as a potential tool utilised by hatcheries for improving long term chicken development and health.

2 | Introduction

In commercial practice, eggs are incubated in complete darkness, except for the moment the eggs are candled and transferred from the setter to the hatcher. However, there is evidence to suggest that by providing light during the incubation process a positive effect on embryonic development may be observed (see for example Özkan *et al.*, 2009, who used white fluorescent light; and Rozenboim *et al.*, 2004, Shafey and Al-Mohsen, 2002, and Shafey, 2004, who used monochromatic green light). In nature, a brooding hen will leave the nest intermittently, exposing her eggs to light (Archer and Mench, 2014). Although an eggshell absorbs about 99.8% of the light reaching its surface (Shafey *et al.*, 2002), some light is able to penetrate the egg and can be perceived by the embryo through its light-sensitive pineal gland (which is located directly under the skull) (Skwarło-Sońta, 1996) as well as through its developing eyes; the establishment of retinal vision is thought to be completely mature by E19 (Mey and Thanos, 2000).

Previous studies have shown that by providing a chicken embryo with light during incubation, compared with dark incubation, various aspects of the chicken's life are affected, such as embryonic brain development (reviewed by Tzschentke, 2012), post hatch fear behaviour (full spectrum fluorescent light; Archer and Mench, 2014), pectoralis muscle growth (green light, Rozenboim *et al.*, 2004; Halevy *et al.*, 2006; Zhang *et al.*, 2012 or fluorescent white light, Özkan *et al.*, 2012a), and corticosterone levels (fluorescent white light, Özkan *et al.*, 2012b). Lighted incubation applied from set till embryonic day (E)18 or hatch can influence subsequent embryonic development: it has been shown to increase embryo weight when it was applied as a 24L (white fluorescent, Garwood *et al.*, 1973; white incandescent, Lauber, 1975; green fluorescent, Shafey and Al-Mohsen, 2002; Shafey, 2004), 15 min L:15 min D (monochromatic green LED light, Rozenboim *et al.*, 2004) or 18L:6D (white fluorescent, Özkan *et al.*, 2009) lighting schedule. Incubation time furthermore decreased by 5 to 30 hours for 24L compared to 24D (white incandescent, Siegel *et al.*, 1969; Walter and Voitle, 1972, 1973; green fluorescent, Shafey and Al-Mohsen, 2002). This suggests that the application of a light-dark schedule and the provision of continuous light can have a stimulatory effect upon embryo develop-

ment when compared to dark incubation.

As demonstrated, several authors have investigated the effect of light during incubation on broiler embryo development. However, additional light sources also produce heat and this may have an influence upon the temperature the embryo experiences inside the egg. Initially, embryonic development may be accelerated by high incubation temperatures, but when applied throughout incubation they have a retarding effect on embryo development (Van der Pol *et al.*, 2014). This may have been the case for some authors who did not find an effect of lighted incubation on body weight at hatch (Tamimie, 1967; Walter and Voitle, 1972; Zakaria, 1989; Shafey *et al.*, 2005; Zhang *et al.*, 2012) but made no mention of measures to prevent overheating of the eggs. Other authors did take into account the difference in air temperature between lighted treatments. For example, Tamimie and Fox (1967) measured air temperature inside the incubator with and without bulbs before the start of the experiment, and Shafey (2004) performed a pilot study concluding that there were no air temperature differences between dark or lighted incubation. However, air temperature can differ greatly from the temperature an embryo experiences inside the egg. Embryo temperature can vary independently from air temperature because it is dependent upon both embryonic heat production and heat transfer from the embryo to its surroundings (Meijerhof and Van Beek, 1993). Heat transfer depends on factors such as egg characteristics, air velocity, and air temperature (Lourens *et al.*, 2011). Internal egg temperature is a more reliable readout for embryo temperature (Rozenboim *et al.*, 2004), but it is invasive and risks killing the embryo, eliminating its own heat production from the energy balance. Lourens *et al.* (2011) recommend using eggshell temperature (EST) as a non-invasive, reliable reflection of embryo temperature. In the present study, heat transferred by the LED lights was taken into account by continuously incubating at a set EST of 37.8°C for all treatments, meaning that air temperature was constantly adjusted to meet the EST requirement. A constant EST of 37.8°C is considered to be optimal, leading to the most optimal hatchability and yolk free body mass (YFBM), (Molenaar *et al.*, 2010, 2011a).

The present study aimed to investigate the effect of light during incubation on embryonic development and post hatch growth in broiler chickens, distinguishing between males and females. An EST of 37.8°C was maintained throughout incubation to eliminate embryo temperature as a confounding factor. We hypothesized that a lighting schedule of 12L:12D would stimulate embryonic development more than 24L or 24D, resulting in higher chick quality at hatch and better production performance up until slaughter age.

3 | Materials and methods

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Wageningen University, Wageningen, the Netherlands.

3.1 | Experimental Setup

Ross 308 eggs (N = 1,005) from a 40 week old parent flock with egg weights between 62 and 65 g were used. An excess of eggs were set, allowing for a hatchability of 80% to obtain the 804 hatchlings required, of which 474 were used in the present experiment and the rest were used for measurements outside of the scope of this paper. Eggs were stored on setter trays for 3 days at 18°C at a commercial hatchery (Lagerwey BV, Lunteren, the Netherlands), after which time they were transported to the experimental facility of Wageningen University (Wageningen, the Netherlands) for incubation.

Eggs were incubated from embryonic day (E)0 until hatch in 1 of 3 climate respiration chambers incorporating one of the following lighting regimes: continuous light (24L), 12 hours of light, followed by 12 hours of darkness (12L:12D), or continuous darkness (24D). The light treated groups, 24L and 12L:12D were provided with 3 strips of white 24V LED light of approximately 500 lux at egg level with a colour temperature of 6,050K which stretched over the full length of the tray or hatcher basket. At set, 12L:12D started the lighted period. The dark treated group 24D was incubated in complete darkness. No external light was allowed to enter the respiration chambers in any of the treatments. Eggs were not candled during incubation and any work undertaken inside the respiration chambers took place under low light intensity using a head torch of 40 lux at egg level.

Eggs were randomly divided over the treatments. EST was maintained at 37.8°C until E19.5. At E19.5, the air temperature within each climate respiration chamber was fixed at its current setting and the EST was allowed to increase during the hatching process. EST was measured using 5 temperature sensors (NTC Thermistors: type DC 95; Thermometrics, Somerset, UK) per treatment attached to the equator of 5 random eggs with heat conducting paste (Dow Corning 340 Heat Sink Compound, Dow Corning GmbH, Wiesbaden, Germany) and duct tape. Relative humidity was maintained between 45 and 55% and CO_2 concentration did not exceed 0.35% throughout incubation. For the first 18 days, eggs were placed on egg trays and turned every hour to an angle of 45° from a horizontal starting position. At E18, eggs were transferred to hatcher baskets and randomly divided within each treatment into 3 subgroups: to be sampled immediately, at day (D)21 post hatch, or at D35 post hatch.

After hatching, chickens assigned to the grow out group were housed at Coppens' Poultry Research Centre (Vlierden, the Netherlands). They were kept in 1.55 x 0.95 m floor pens holding 12 chickens per pen, resulting in 9 repetitions per treatment. At D21 post hatch, half of the chickens were removed for sampling, resulting in 6 chickens per pen until slaughter at D35. After an initial lighted period of 24 hours, chickens were held under an 18L:6D lighting schedule. Using ceiling ventilation air temperature was maintained at 33°C at D0 and then gradually decreased to 19°C at D35. The floor of the pens was covered with wood shavings and feed and water were available ad libitum. From D0 till D13, chickens were fed a starter diet containing 2,925 kcal ME, 210 g CP, and 11.3

g digestible lysine per kg of feed. From D14 till D27, chickens were fed a grower diet containing 3 040 kcal ME, 195 g CP, and 10.5 g digestible lysine per kg of feed. From D28 till D35, chickens were fed a finisher diet containing 3,080 kcal ME, 85 g CP, and 10.0 g digestible lysine per kg of feed.

3.2 | Measurements

From E6 until E14, 10 eggs per day, per treatment (N = 270) were opened to determine the stage of development of the live embryo according to Hamburger and Hamilton (1951). Between E19.5 and E21.5, the eggs were checked every 3 hours up until the moment of hatch as the number of hours since the start of incubation was being recorded for all chickens. Chickens assigned to grow out (108 per treatment; N = 324) received an individual number on a neck label and remained in the climate respiration chamber, while chickens assigned to be sampled at hatch (50 per treatment; N = 150) were taken from the climate respiration chamber and their sex, weight, length, and navel score on a 1 to 3 scale (Van der Pol *et al.*, 2013) were recorded within 3 hours after hatch. Thereafter, they were killed by decapitation and their residual yolk, heart, liver, stomach (gizzard and proventriculus), and intestines were removed and weighed. Yolk free body mass (YFBM) was calculated as body weight minus residual yolk. Organ weights were analysed as % of body weight.

At E21.5, culls were counted and unhatched eggs were opened to determine infertility or stage of embryonic mortality. Chickens allocated to the grow out group were then removed from the incubator and feather sexed. Their weight, length, and navel score were recorded. This ensured that these chickens were all measured for the first time at the moment of pulling, which is considered D0 in this experiment. The chickens were subsequently transported to the grow out facility, where males and females were equally divided over pens with one treatment per pen. Body weight (per chicken) and feed intake (per pen) were determined on a weekly basis. Body weight was averaged per pen for further analysis. Growth:feed ratio (G:F) was calculated per feed type (starter, grower, finisher) as g feed intake / g growth. At D21 post hatch, 53 chickens per treatment (1 chicken had died in each treatment; N = 159) were killed by cervical dislocation and heart, liver, and stomach were removed, the stomach was emptied, and the organs were weighed. At D35 post hatch, after the final weighing, all remaining chickens (53 for 12L:12D and 52 for 24L and 24D; N = 157) were slaughtered.

3.3 | Statistical Analysis

The overall model used for all data was:

$Y_i = \mu + \text{Treatment}_i + \varepsilon_i$,

where Y_i = the dependent variable, μ is the overall mean, $Treatment_i$ = Lighting treatment (i = 24L, 12L:12D, or 24D), and ε_i = the residual error term. For Hamburger-Hamilton (H&H) stage, the model was extended with incubation day and a Treatment x day interaction. For hatch time, chick length, YFBM, residual yolk weight, body weight at hatch, navel quality, and organ weights, the model was extended with sex (male or female) of the chick and a Treatment x sex interaction. In an additional analysis, for chick length, YFBM, residual yolk weight, body weight and hatch, and navel quality, the model was furthermore extended with hatch time as a covariable. For body weight in grow out, the model was extended with weeks post hatch and a Treatment x weeks post hatch interaction.

Hamburger-Hamilton (H&H) stage, hatchability, sex of the hatched chickens, culls at hatch, and navel quality were analysed using the Logistics procedure, hatch time was analysed using the Lifereg procedure, body weight in the grow out period was analysed using the Mixed procedure with week as the repeated factor, and all other data were analysed using the GLM procedure in SAS (SAS Institute, 2004). Pen was considered to be the experimental unit in analysis of body weight, feed intake, and G:F in the grow out period. Individual chickens were considered to be the experimental unit for all other analyses.

Model assumptions were verified by examination of the distributions of the means and residuals. Moment of hatch and body weight, residual yolk weight, YFBM, and liver weight at hatch were sinus transformed to obtain normality. Body weight in the grow out period was log transformed to obtain normality. Least square means were compared using Bonferroni adjustments for multiple comparisons. Data are presented as LSMeans ± SEM. In the case of data transformation, LSMeans and SEM are presented untransformed and P-values are presented as those of the transformed data. In all cases, differences were considered significant at $P \leq 0.05$.

4 | Results

4.1 | Incubation

No interaction between treatment and day of incubation was found for Hamburger-Hamilton stages (P = 0.38) or for treatment (P = 0.99; data not shown). Average hatch time was later for 12L:12D males than for 24L females (+9 hours) and 24L males (+7 h; P < 0.001; Table 1; Figure 1). Hatch time was furthermore later for 12L:12D females (+6 hours), 24D females (+7 hours), and 24D males (+8 hours) than for 24L females. Hatchability (average 94.6%; P = 0.67), culls at hatch (average 1.2%; P = 0.48), and sex of the hatched chickens (average 50.7% male; P = 0.69) were not affected by treatment.

Table 1. Hatch time, length at hatch and pulling, yolk free body mass (YFBM) and residual yolk weight at hatch, body weight at pulling, and navel quality at hatch and pulling for male and female chickens incubated under a lighting schedule of 24L, 12L:12D, or 24D from embryonic day 0 until hatch

		24L		12L:12D		24D			P-value		
	Moment	Female	Male	Female	Male	Female	Male	SEM	Light x Sex	Light	Sex
Hatch time (h)		489[c]	491[bc]	495[ab]	498[a]	496[ab]	497[ab]	0.6	<0.001	<0.001	<0.001
Length (cm)	Hatch[1]	19.3	19.2	19.0	19.1	19.1	19.1	0.04	0.78	0.15	0.78
Length (cm)	Pulling[2]	19.9[ab]	19.9[ab]	20.1[a]	19.9[ab]	19.9[ab]	19.8[b]	0.02	0.043	0.031	0.031
YFBM (g)	Hatch[1]	39.2	39.2	39.4	39.5	39.2	39.2	0.12	0.83	0.031	0.001
Residual yolk (g)	Hatch[1]	6.7	6.9	6.2	6.7	6.4	7.0	0.09	0.83	0.060	0.11
Body weight (g)	Pulling[2]	41.2	42.7	42.6	43.4	42.7	43.3	0.09	0.14	<0.001	<0.001
Navel score	Hatch[1]	1.5	1.6	1.6	1.6	1.5	1.5		0.51	0.88	0.44
Navel score	Pulling[2]	1.8	1.6	1.8	1.6	1.7	1.6		0.79	0.79	0.015

[1] n = 50

[2] n = 54

[a,b] Values within a row with different superscripts differ significantly at $P < 0.05$.

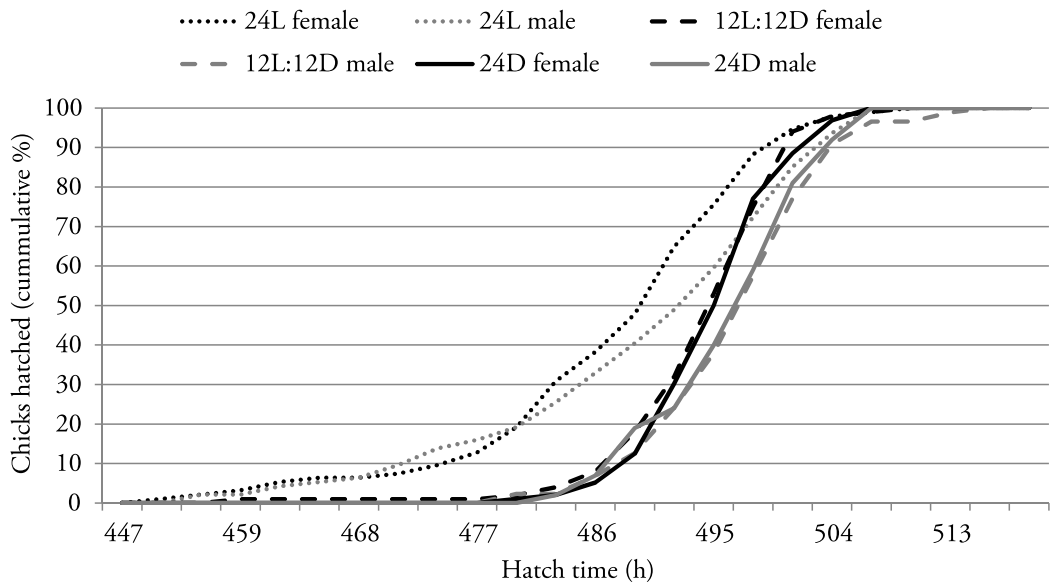


Figure 1. Hatch curve of female and male chickens incubated under 24L, 12L:12D, or 24D from embryonic day 0 until hatch.

4.2 | Chick Quality and Organ Development

At hatch, YFBM was higher for 12L:12D than for 24L (+0.2 g; P = 0.031) and higher for males than for females (+0.1 g; P = 0.001; Table 1). Residual yolk weight at hatch tended to be affected by treatment (P = 0.06). Chick length at hatch was not affected by treatment (P = 0.15), sex (P = 0.78), or their interaction (P = 0.78). Residual yolk weight at hatch and navel score at hatch were not affected by treatment x sex (P > 0.51) or sex (P = 0.11). Navel score at hatch was furthermore not affected by treatment (P = 0.88). When hatch time was added to the model as a covariable, no large differences in P-values were found.

Chick length at pulling was higher for 12L:12D females than for 24D males (+0.3 cm; P = 0.043), with the other treatment x sex interactions intermediate. Navel score at pulling was higher (indicating poor navel quality) for females than for males (+0.2 points; P = 0.015). Body weight at pulling was higher for 12L:12D and 24D (both +1.1 g) than for 24L (P < 0.001) and higher for males than for females (+0.9 g; P < 0.001). When hatch time was added to the model as a covariable, navel score was no longer significantly different between sexes (P = 0.11), and no large differences in P-values were found for the other measurements.

Liver weight at hatch was higher for 12L:12D than for 24L (0.13% of body weight; P = 0.004; Table 2) and it was higher for females than for males (+0.06% of body weight; P =

Table 2. Heart, liver, and stomach (gizzard and proventriculus) weight as a percentage of body weight (BW) at hatch and D21, and intestine weight at hatch, for male and female chickens incubated under a lighting schedule of 24L, 12L:12D, or 24D from embryonic day 0 until hatch

		24L		12L:12D		24D			P-value		
Organ (% BW)	Moment	Female	Male	Female	Male	Female	Male	SEM	Light x Sex	Light	Sex
Heart	Hatch[1]	0.72	0.71	0.71	0.73	0.71	0.68	0.007	0.28	0.21	0.83
Heart	D21[2]	0.62	0.65	0.64	0.69	0.65	0.67	0.006	0.57	0.16	0.009
Liver	Hatch[1]	2.00	1.88	2.08	2.07	2.03	1.98	0.016	0.36	0.004	0.048
Liver	D21[2]	3.14	3.26	3.22	3.39	3.37	3.33	0.031	0.34	0.13	0.17
Stomach	Hatch[1]	4.72	4.64	4.83	4.75	4.84	4.70	0.043	0.96	0.53	0.25
Stomach	D21[2]	2.68	2.61	2.69	2.64	2.78	2.55	0.027	0.29	0.93	0.037
Intestines	Hatch[1]	3.15	3.20	3.67	3.50	3.25	3.26	0.040	0.48	<0.001	0.60

[1] n = 50

[2] n = 54

0.048). Intestine weight at hatch was higher for 12L:12D than for 24D (+0.34% of body weight) and 24L (+0.42% of body weight; P < 0.001). Heart weight at D21 was higher for males than for females (+0.03% of body weight; P = 0.009). Stomach weight at D21 was higher for females than for males (+0.11% body weight; P = 0.037). Heart weight at hatch, stomach weight at hatch, and liver weight at D21 were not affected by treatment (P > 0.13), sex (P > 0.17), or their interaction (P > 0.28).

4.3 | Grow Out Performance

Body weight in the grow out period was not influenced by the treatment x weeks post hatch interaction (P = 0.44; Figure 2) or the main effect of treatment (P = 0.36). Average body weight at D35 was 2,095.9 g for 24L, 2,136.9 g for 12L:12D, and 2,161.3 g for 24D. Feed intake and G:F were not influenced by treatment (P > 0.14; Table 3).

Table 3. Feed intake and G:F in the starter, grower, and finisher phase for chickens incubated under a lighting schedule of 24L, 12L:12D, or 24D from embryonic day 0 until hatch

		Moment	24L	12L:12D	24D	SEM	P-value
Feed intake (g feed/chick)	Starter[1]	D0-D13	539	550	556	5.6	0.48
	Grower	D14-D27	1 549	1 527	1 578	10.6	0.14
	Finisher	D28-D35	1 015	1 026	1 021	14.0	0.95
	Overall	D0-D35	1 035	1 034	1 052	11.0	0.43
G:F ratio	Starter	D0-D13	0.55	0.56	0.56	0.019	0.67
	Grower	D14-D27	0.72	0.74	0.71	0.011	0.22
	Finisher	D28-D35	0.58	0.61	0.62	0.036	0.39
	Overall	D0-D35	0.61	0.63	0.63	0.024	0.28

[1] D0 till D20: n = 108 and D21 till D35: n = 54

5 | Discussion

The present study investigated the effects of lighted incubation on broiler chicken development and post hatch performance. Embryonic development was determined through Hamburger-Hamilton staging. Although Hamburger-Hamilton staging did not prove to differ between treatments from E6 till E14, many previous studies found increased chicken embryo weights for 24L from E5 till the end of the experiment at E14 (white fluorescent light; Garwood *et al.*, 1973), till E15 (green fluorescent light; Shafey and Al-Mohsen, 2002), or till E18 (white fluorescent light; Shafey, 2004) compared to 24D. Furthermore, increased embryo weight was found on E14, E15, E17, and E20 for embryos incubated under a 15 minutes L:15 minutes D regime (monochromatic green LED

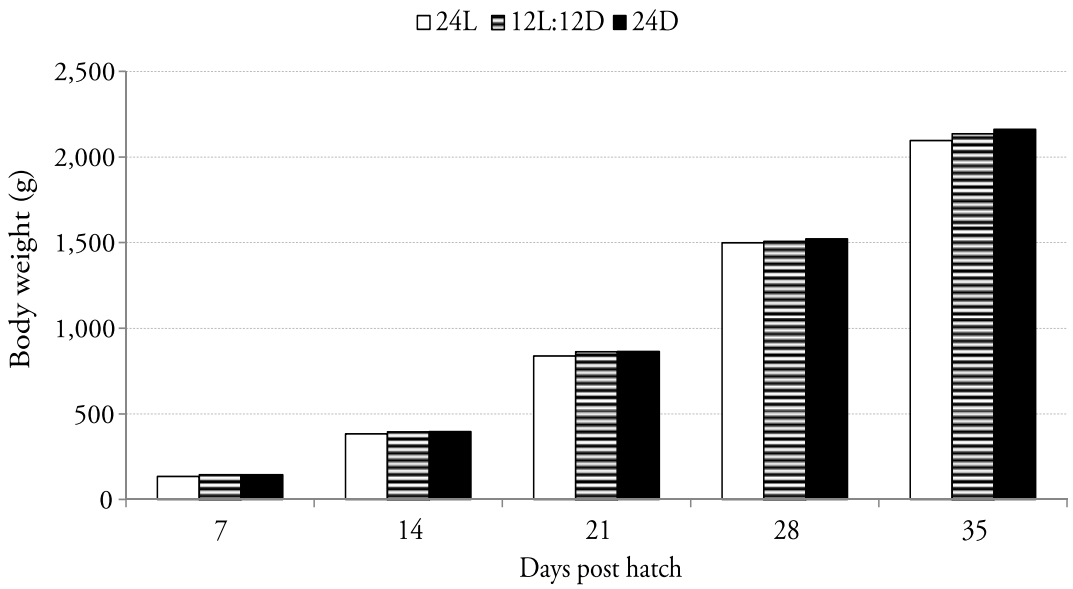


Figure 2. Body weights at 7 till 35 days post hatch of chickens incubated under 24L, 12L:12D, or 24D from embryonic day 0 until hatch.

light; Rozenboim *et al.*, 2004) and on E13 and E18 (the only sampling times) for embryos incubated under 18L:6D (white fluorescent; Özkan *et al.*, 2009) compared to 24D. Embryo weight was not measured in the current study; it is possible that the embryos were different in size within the same Hamburger-Hamilton stage.

It is possible that differences in embryonic development started to appear mostly after E14. Csernus *et al.* (2007) explanted pineal glands from dark incubated embryos on E13, E16, E17, and E18 and exposed them to a 12L:12D light-dark rhythm. For the E13 and E16 pineal glands, they observed an irregular pattern in blood melatonin (which stimulates release of GH and IGF-I; Zeman *et al.*, 1999; Ostrowska *et al.*, 2002). These levels were only slightly influenced by a light – dark rhythm, signifying a limited ability of pineal glands to synchronize to an external rhythm. From E17 onward, they observed a clear circadian melatonin release pattern that could be influenced by light (Csernus *et al.*, 2007). This would suggest that the main effects of light through the speculated pathway of pineal gland circadian rhythmicity do not occur until after about E17.

Light treatment had a very clear effect on hatch time. 24L females hatched earlier than all 12L:12D and 24D treatments, and 24L males hatched earlier than 12L:12D males. To illustrate, the 12L:12D and 24D chickens only began to hatch by the time 20% of the 24L chickens had emerged. This could be explained to some degree by increased hatching stress coinciding with higher corticosterone levels. Sui *et al.* (1997) suggested

that an interaction may exist between light exposure and corticosterone release around E19 and E20: they injected corticosterone into developing embryos at E18 and E20 and found that this resulted in behavioural responses similar to when embryos were treated with light on the corresponding days. Additionally Saito *et al.* (2005) found that increased corticosterone levels detected during an open field test in neonatal layers were completely suppressed by injecting melatonin. A repressing effect of melatonin on glucocorticoids was previously demonstrated in rats (Konakchieva *et al.*, 1998). These results suggest that melatonin release decreases corticosterone response. Corticosterone was not measured in the current experiment and a difference in hatch time of up to 9 hours is quite large, so it is possible that multiple mechanisms may be exerting an influence on this process.

While 24L chickens hatched earlier, 12L:12D chickens had higher YFBM at hatch. Not many studies compared 12L:12D to 24L and 24D, but those that did, did not see a difference between the treatments in body weight at hatch for White Leghorn (using incandescent bulbs; Walter and Voitle, 1972, 1973). Furthermore, liver weight (as a percentage of body weight) at hatch was higher for 12L:12D than for 24L and intestine weight was higher for both 12L:12D and 24D than for 24L. These results suggest that continuous light may speed up the hatching process, resulting in lower YFBM at hatch. It is feasible that the application of continuous light may retard embryonic development compared to where a light-dark rhythm is applied, with continuous darkness mostly exerting an intermediate effect.

The positive effect of 12L:12D and 24D on body weight did not last during the grow out period; like in the studies of Archer *et al.* (2009) and Archer and Mench (2014), who used full spectrum fluorescent light, body weight gain, feed intake, and feed conversion did not differ between treatments. Additionally, organ weights at D21 no longer differed between treatments. Possibly, this was the result of a mismatch between lighting conditions in the incubator and in the post hatch environment. Özkan *et al.* (2009, 2012b) found increased plasma corticosterone levels at D6 for chickens housed under a post hatch lighting schedule that did not match the light during incubation. Particularly 16L:8D incubated chickens showed low corticosterone levels when they were housed in a matching post hatch lighting schedule, while 24D-incubated chickens showed higher corticosterone levels when exposed to 16L:8D compared to 24L. In our experiment, all chickens were kept under 24L for the first day post hatch and then 16L:8D, which is out of phase with all incubation treatments. It is possible that the resulting stress from the mismatching environment reduced the positive effect of applying a lighting schedule during incubation.

Reports in the literature demonstrate ambiguous results regarding the application of light treatments. Besides lighting schedule, any differences found between studies may be caused by differences in the spectrum (Rozenboim *et al.*, 2003) or intensity (Shafey

et al., 2005) of the light, and the incubation phase in which it was applied (Özkan *et al.*, 2009). Furthermore, in some cases, the lamp's heat production may have been a confounding factor. Coleman *et al.* (1977) already demonstrated that many discrepancies between studies in hatchability, hatch time, and chick quality can be explained through differences in the type of light used and the effect the lamps have on temperature at the embryonic level. As mentioned previously, embryo temperature is a balance between heat production of the embryo and heat transfer to its surroundings (Meijerhof and Van Beek, 1993). LED light may increase the egg's temperature by heat transfer through radiation (although this is minimal compared to other types of illumination as the LED light used in this experiment has almost no infrared component) and through convection of air heated by an LED's inefficient semiconductors. Rozenboim *et al.* (2004) prevented overheating of the eggs by switching their LED light on and off every 15 minutes. In the present study, the confounding factor of LED heat transfer was eliminated by automatically and continuously adjusting the air temperature to maintain EST at 37.8°C for all treatments throughout incubation. Through this method, both heating of the air by the LED's semiconductors was prevented, and heat loss from the egg was encouraged by adjusting the air temperature when EST no longer equalled 37.8°C.

Sex of the hatched chickens was taken into account to discover a possible sex dependent effect of light during incubation on growth and development. Lighting schedule during incubation and sex interacted only for chick length at pulling time, with longer 12L:12D females than 24D males. As expected, it appears that the effect of light during incubation on chicken development is largely independent of sex. Several main effects of sex were found, among which was a difference in hatch curve between males and females. The statistical analysis was run additionally with hatch time as covariable and it was found that sex effects on navel score were mostly explained by differences in hatch time.

Noy and Sklan (2003) found a 10 gram decrease in body weight when chickens were held in the hatcher without feed and water for 48 hours post hatch. This body weight loss is mostly due to dehydration and the longer a chick spends without feed and water, the higher body weight loss will be. In the present trial, the difference in body weight between chickens measured at hatch or at pulling from the incubator was 8.9% for 24L, 6.3% for 12L:12D, and 6.5% for 24D. Since 24L hatched earlier than 12L:12D and 24D, this would suggest that the lower body weight at pulling for 24L was partly due to dehydration, and partly due to decreased development as reflected by lower YFBM at hatch. In an exploratory analysis, organ weights at hatch were corrected for hatch time, and not for body weight. Heart weight was then found to be higher for 24L than for 24D. Liver weight at hatch no longer differed between treatments and intestine weight was higher for 12L:12D than for 24D, with 24L intermediate. These findings show that the moment of emergence from the egg (and, thereby, total incubation time for that individual chick) is related to the development of a chick at hatch and at pulling time.

To conclude, 24L shortened the total incubation time compared to 12L:12D and 24D. 12L:12D white LED light applied throughout incubation at a set EST of 37.8°C had a stimulatory effect on chick development at hatch, independent of sex, when compared to 24L in particular. These effects were no longer visible in organ development and production parameters in the grow out phase.

6 | Acknowledgements

The authors would like to thank Marcel Heetkamp at the climate respiration chambers of Wageningen University (Wageningen, the Netherlands) for his assistance during incubation. We would furthermore like to thank Evelien Alderliesten and Theo and Karen Nooijen of Coppens' Poultry Research Centre (Vlierden, the Netherlands) for their assistance during the grow out period of the trial.

7 | References

Archer, G. S., and J. A. Mench. 2014. Natural incubation patterns and the effects of exposing eggs to light at various times during incubation on post-hatch fear and stress responses in broiler meat chickens. Appl. Anim. Behav. Sci. 152: 44-51.

Csernus, V. J., A. D. Nagy, and N. Faluhelyi. 2007. Development of the rhythmic melatonin secretion in the embryonic chicken pineal gland. Gen. Comp. Endocrinol. 152: 148-153.

Coleman, M. A., G. R. McDaniel, W. C. Neeley, and W. D. Ivey. 1977. Physical comparisons of lighted incubation in avian eggs. Poult. Sci. 56: 1421-1425.

Garwood, V. A., E. J. Thornton, and P. C. Lowe. 1973. The effect of continuous illumination of incubating chicken eggs on embryonic development. Poult. Sci. 52: 337-340.

Halevy, O., Y. Piestun, I. Rozenboim, and Z. Yablonka-Reuveni. 2006. In ovo exposure to monochromatic green light promotes skeletal muscle cell proliferation and affects myofiber growth in posthatch chicks. Am. J. Physiol. Regul. Integr. Comp. Physiol. 290: 1062-1070.

Hamburger, V., and H. L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. J. Morph. 88: 49-92.

Konakchieva, R., Y. Mitev, O. F. Almeida, and V. K. Patchev. 1998. Chronic melatonin treatment counteracts glucocorticoid-induced dysregulation of the hypothalamic-pituitary-adrenal axis in the rat. Neuroendocrinology 1998: 171–180.

Kürtül, I., S. H. Atalgin, K. Aslan, and E. U. Bozkurt. 2009. Ossification and growth of the bones of the wings and legs in prehatching period of the Hubbert strain broiler. Kafkas Univ. Vet. Fak. Derg. 15: 869-874.

Lauber, J. K. 1975. Photoacceleration of avian embryogenesis. Comp. Biochem. Physiol. 51: 903-907.

Lewis, P. D., and T. R. Morris. 2000. Poultry and coloured light. Worlds Poult. Sci. Journal 56: 189-207.

Lourens, A., R. Meijerhof, B. Kemp, and H. van den Brand. 2011. Energy partitioning and consequences for embryo temperature: A theoretical approach. Poult. Sci. 90: 516–523.

Meijerhof, R., and G. van Beek. 1993. Mathematical modelling of temperature and moisture loss of hatching eggs. J. Theor. Biol. 165: 27-41.

Mey, J., and S. Thanos. 1999. Development of the visual system of the chick I. Cell differentiation and histogenesis. Full-length review. Brain Res. Rev. 32: 343-379.

Molenaar, R., I. van den Anker, R. Meijerhof, B. Kemp, and H. van den Brand. 2011a. Effect of eggshell temperature and oxygen concentration during incubation on the development and physiological status of broiler hatchlings in the perinatal period. Poult. Sci. 90: 1257-1266.

Molenaar, R., R. Hulet, R. Meijerhof, C. M. Maatjens, B. Kemp, and H. van den Brand. 2011b. High eggshell temperatures during incubation decrease growth performance and incre-

ase the incidence of ascites in broiler chickens. Poult. Sci. 90: 624-632.

Noy, Y., A. Geyra, and D. Sklan. 2003. The effect of early feeding on growth and small intestinal development in the posthatch poult. Poult. Sci. 80: 912-919.

Ostrowska, Z., B. Kos-Kudla, B. Marek, D. Kajdaniuk, and N. Ciesielska-Kopacz. 2002. The relationship between the daily profile of chosen biochemical markers of bone metabolism and melatonin and other hormone secretion in rats under physiological conditions. Neuroendocrinol. Lett. 23: 417-425.

Özkan, S., S. Yalçin, E. Babacanoğlu, and H. Kozanoğlu. 2009. Effect of photoperiod on prenatal and early post hatch growth of broiler chickens. In Proceedings of the 2nd Mediterranean Summit of WPSA, 4-7 October 2009: Antalya, Turkey, pp. 263-268.

Özkan, S., S. Yalçin, E. Babacanoğlu, H. Kozanoğlu, F. Karadaş, and S. Uysal. 2012a. Photoperiodic lighting (16 hours of light: 8 hours of dark) programs during incubation: 1. Effects on growth and circadian physiological traits of embryos and early stress response of broiler chickens. Poult. Sci. 91: 2912-2921.

Özkan, S., S. Yalçin, E. Babacanoğlu, H. Kozanoğlu, F. Karadaş, and S. Uysal. 2012b. Photoperiodic lighting (16 hours of light: 8 hours of dark) programs during incubation: 2. Effects on early posthatching growth, blood physiology, and production performance in broiler chickens in relation to posthatching lighting programs. Poult. Sci. 91: 2922-2930.

Rozenboim, I., Y. Piestun, N. Mobarkey, M. Barak, A. Hoyzman, and O. Halevy. 2004. Monochromatic light stimuli during embryogenesis enhance embryo development and posthatch growth. Poult. Sci. 83: 1413-1419.

Shafey, T. M., and T. H. Al-Mohsen. 2002. Embryonic growth, hatching time and hatchability performance of meat breeder eggs incubated under continuous green light. Asian Australas. J. Anim. Sci. 15: 1702-1707.

Shafey, T. M., T. H. Al-Mohsen, A. A. Al-Sobayel, M. J. Al-Hassan, and M. M. Ghnnam. 2002. Effects of eggshell pigmentation and egg size on the spectral properties and characteristics of eggshell of meat and layer breeders. Asian Australas. J. Anim. Sci. 15: 297-302.

Shafey, T. M. 2004. Effect of lighted incubation on embryonic growth and hatchability performance of two strains of layer breeder egss. Br. Poult. Sci. 45: 223-229.

Shafey, T. M., H. A. Al-Batshan, M. M. Ghannam, and M. S. Al-Ayed. 2005. Effect of intensity of eggshell pigment and illuminated incubation on hatchability of brown eggs. Br. Poult. Sci. 46: 190-198.

Siegel, P. B., S. T. Isakson, F. N. Coleman, and B. J. Huffman. 1969. Photoacceleration of development in chick embryos. Comp. Biochem. Physiol. 28: 753-758.

Skwarło-Sońta, K. 1996. Functional connections between the pineal gland and immune system. Acta Neurobiol.

Exp. 56: 341-357.

Saito, S., T. Tachibana, Y. Choi, D. M. Denbow, and M. Furuse. 2005. ICV melatonin reduces acute stress responses in neonatal chicks. Behav. Brain Res. 165: 197-203.

Sui, N., C. Sandi, and S. P. R. Rose. 1997. Interactions of corticosterone and embryonic light deprivation on memory retention in day-old chicks. Dev. Brain Res. 101: 269-272.

Tamimie, H. S. 1967. Light exposure of incubating eggs and its influence on the growth of chicks – I. Brooding chicks under different light regimens. Comp. Biochem. Physiol. 21: 59-63.

Tamimie, H. S., and M. W. Fox. 1967. Effect of continuous and intermittent light exposure on the embryonic development of chicken eggs. Comp. Biochem. Physiol. 20: 793-799.

Tzschentke, B. 2012. Effect of hatching time on poultry behaviour: the impact of incubation environment during the last days of embryonic development. Paper presented at the 24th World Poultry Congress, 5-9 August 2012: Bahia, Brazil.

Van der Pol, C. W., I. A. M. van Roovert-Reijrink, C. M. Maatjens, H. van den Brand, and R. Molenaar. 2013. Effect of relative humidity during incubation at a set eggshell temperature and brooding temperature posthatch on embryonic mortality and chick quality. Poult. Sci. 92: 2145-2155.

Walter, J. H., and R. A. Voitle. 1972. Effects of photoperiod during incubation on embryonic and post-embryonic development of broilers. Poult. Sci. 51: 1122-1126.

Walter, J. H., and R. A. Voitle. 1973. Effects of photoperiod during incubation on embryonic and post-embryonic development of quail and chickens. Br. Poult. Sci. 14: 533-540.

Zakaria, A. H. 1989. Research note: effect of fluorescent light on hatchability of commercial broiler parent stock eggs and on body weight of chickens hatched under large-scale commercial conditions. Poult. Sci. 68: 1585-1587.

Zeman, M., J. Buyse, D. Lamošová, I. Herichova, and E. Decuypere. 1999. Role of melatonin in the control of growth and growth hormone secretion in poultry. Domest. Anim. Endocrinol. 17: 199-207.

Zhang, L., H. J. Zhang, X. Qiao, H. Y. Yue, S. G. Wu, J. H. Yao, and G. H. Qi. 2012. Effect of monochromatic light stimuli during embryogenesis on muscular growth, chemical composition, and meat quality of breast muscle in male broilers. Poult. Sci. 91: 1026-1031.

Chapter 5

Effects of lighting schedule during incubation of broiler chicken embryos on leg bone development at hatch and related physiological characteristics

C. W. van der Pol[ab], I. A. M. van Roovert-Reijrink[a], S. W. S. Gussekloo[c], S. Kranenbarg[c], K. M. Leon-Kloosterziel[c], M. H. Priester[ab], M. Zeman[de], B. Kemp[b], H. van den Brand[b]

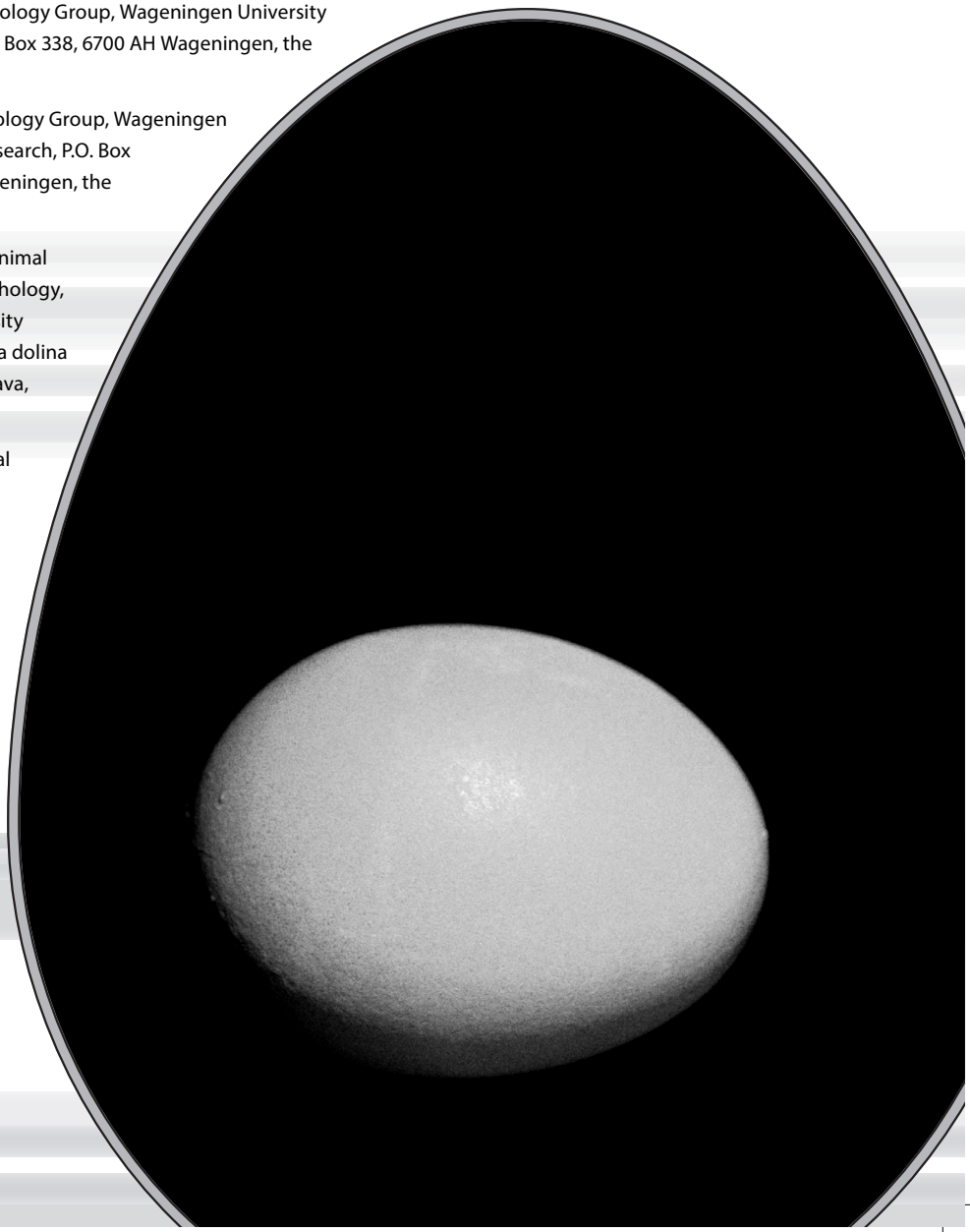
[a]HatchTech B.V., P.O. Box 256, 3900 AG Veenendaal, the Netherlands.

[b]Adaptation Physiology Group, Wageningen University and Research, P.O. Box 338, 6700 AH Wageningen, the Netherlands.

[c]Experimental Zoology Group, Wageningen University and Research, P.O. Box 338, 6700 AH Wageningen, the Netherlands.

[d]Department of Animal Physiology and Ethology, Comenius University Bratislava, Mlynska dolina B-2, 842 15 Bratislava, Slovak Republic.

[e]Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences, 900 28 Ivanka pri Dunaji, Slovak Republic.



Abstract

Providing a broiler chicken embryo with a lighting schedule during incubation may stimulate leg bone development. Bone development may be stimulated through melatonin, a hormone released in darkness that stimulates bone development, or increased activity in embryos exposed to a light-dark rhythm. Aim was to investigate lighting conditions during incubation and leg bone development in broiler embryos, and to reveal the involved mechanisms. Embryos were incubated under continuous cool white 500 lux LED light (24L), continuous darkness (24D), or 16h of light, followed by 8h of darkness (16L:8D) from the start of incubation until hatching. Embryonic bone development largely takes place through cartilage formation (of which collagen is an important component) and ossification. Expression of genes involved in cartilage formation (*col1α2*, *col2α1*, and *col10α1*) and ossification (*spp1*, *sparc*, *bglap*, and *alpl*) in the tibia on embryonic day (E)13, E17, and at hatching were measured through qPCR. Femur and tibia dimensions were determined at hatch. Plasma growth hormone and corticosterone and pineal melatonin concentrations were determined every 4h between E18.75 and E19.5. Embryonic heart rate was measured twice daily from E12 till E19 as a reflection of activity. No difference between lighting treatments on gene expression was found. 24D resulted in higher femur length and higher femur and tibia weight, width, and depth at hatch than 16L:8D. 24D furthermore resulted in higher femur length and width and tibia depth than 24L. Embryonic heart rate was higher for 24D and 16L:8D in both its light and dark period than for 24L, suggesting that 24L embryos may have been less active. Melatonin and growth hormone showed different release patterns between treatments, but the biological significance was hard to interpret. To conclude, 24D resulted in larger leg bones at hatch than light during incubation, but the underlying pathways were not clear from present data.

1 | Introduction

There are indications that providing a broiler embryo with a lighting schedule during incubation may stimulate leg bone development and leg health. Huth and Archer (2015) found 2.2% fewer chicks that were too weak to stand or suffered from leg abnormalities at hatch in embryos under 12h of light, followed by 12h of darkness (12L:12D) conditions compared to continuous darkness (24D) Furthermore, they found higher developmental instability, based on composite asymmetry of the leg bones, at day 14 post hatching for broilers incubated under 24D than under 12L:12D (Huth and Archer, 2015). Under 12L:12D conditions we previously found a higher rate of ossification of the tibia (tibiotarsus) between E12 and E14, higher cortical area, cortical thickness, and second moment of area of the tibia, and a longer tibia and femur at hatch compared to continuous light (24L), with 24D mostly intermediate (Van der Pol *et al.*, in preparation).

Bones with higher cortical thickness and a higher second moment of area, are probably stronger (Augat and Schorlemmer, 2006), and more resistant to stress fractures and bending than bones with lower cortical thickness (Milgrom *et al.*, 1989). This is relevant for broiler production, because the body conformation of broilers results in high body weight load on the legs. When body weight load is disproportionate to bone mass and strength, this may result in bones that are relatively not strong enough, and makes them more susceptible to leg pathologies, which are a major welfare problem in broiler chickens (reviewed by Bradshaw *et al.*, 2002). Stimulating embryonic leg bone development can therefore contribute to improved leg health in broiler chickens.

Chicken embryo bones are first formed as a cartilage model (Kürtül *et al.*, 2009), which later becomes ossified through a ring of bone material at the mid-diaphysis of the bone (Pechak *et al.*, 1986a,b). At the epiphyseal ends of the long bones, an epiphyseal or growth plate can be observed, where bone length is realized through production and ossification of cartilage. Cartilage cells called chondrocytes can be observed in resting, proliferating, and hypertrophic zones, after which they become apoptotic and their matrix becomes calcified by bone cells called osteoblasts to form bone material (Roach, 1997; Mackie *et al.*, 2008). It is not known which physiological pathways are involved in the relationship between lighting schedule and bone development. It was speculated that exposing embryos to a light-dark schedule would create a circadian rhythm in levels of melatonin (Özkan *et al.*, 2012), a hormone known to stimulate bone development in mammals (Cardinali *et al.*, 2003). For example, scoliosis was found to develop in all 30 chickens that were pinealectomized 3 days after hatching by Machida *et al.* (1995); but when these chickens received melatonin injections every other day, only 6 out of 30 chickens developed scoliosis. However, in a previous study, we did not find differences in plasma melatonin between lighting schedules in the period between embryonic day (E)18.8 and E19.5 (Van der Pol *et al.*, in preparation). Other authors did see a clear dark-light dependent rhythm of plasma melatonin from E18 onward for chickens incubated under

16L:8D (Zeman *et al.*, 1999a). Possibly, a circadian rhythm in melatonin levels can be discovered when the pineal gland, where the majority of melatonin is produced, is sampled directly. There are indications that growth hormone (GH) release is stimulated by melatonin, as GH was found to peak along with melatonin in male rats (Ostrowska *et al.*, 2002). In chickens, it appears that GH stimulates chondrocyte proliferation (Monsonego *et al.*, 1995). Corticosterone acts as a negative regulator of bone development through increasing collagen degradation (Hasan *et al.*, 2011) and decreasing chondrocyte (Van der Eerden *et al.*, 2003) and osteoblast proliferation (Robson *et al.*, 2002). Possibly, GH shows a similar circadian release pattern to what we expect in melatonin, while corticosterone could reflect a possible degree of stress from being under- or overexposed to light.

Activity is also involved in leg bone development in embryos. Post hatch, broiler activity is accepted to be of major importance in leg bone development (Bradshaw *et al.*, 2002). Embryonic activity may already be driving development of the broiler during incubation. The development of several bones (Bertram *et al.*, 1997) and joints (Murray and Drachman, 1969) has been shown to be retarded in chicken embryos that were experimentally paralyzed by botulin toxin. Chicken embryos normally show the first movement through muscle contractions by E4 (Bekoff, 1981). It has been suggested that heart rate reflects embryonic movement (Jackson and Rubel, 1978).

As we found increased ossification in 12L:12D in our previous research compared to 24L (Van der Pol *et al.*, in preparation), we were interested to see whether genes involved in cartilage and bone development would be up- or downregulated by light during incubation. First skeletal formation in the avian embryo takes place through the formation of a cartilage model, which becomes calcified from E9 onward (Pechak *et al.*, 1986a,b; Kürtül *et al.*, 2009). Collagen is the main component of cartilage, and several genes are associated with its formation, of which three are investigated in more detail. In chickens, collagen type I limits expansion of the diameter of the cartilage core (Roach, 1997; Pechak *et al.*, 1986a). The cartilage matrix becomes calcified, and the cartilage starts to change into a bone-forming surface. In chickens, type II collagen is primarily found in chondrocytes in the proliferative zone and in the articular zone of long bones (Yalçin *et al.*, 2007). Chicken collagen type X is synthesized by hypertrophic chondrocytes during endochondral ossification (Oviedo-Rondón *et al.*, 2008). This type of collagen facilitates endochondral ossification by regulation of the matrix mineralization, and it was found that collagen type X is a reliable marker for new bone formation (Shen, 2005).

Ossification of the cartilage model takes place through the involvement of several bone cell related proteins. In chickens, osteopontin and osteocalcin play a role in extracellular matrix formation (McKee *et al.*, 1992). Osteopontin was found in the columns of chondrocytes at the bone's epiphyseal plate (Yalçin *et al.*, 2007), where it marked the onset of differentiation and calcification of the chondrocytes. Osteocalcin is considered to be a phenotypic marker of osteoblasts, which are the bone building cells (Gerstenfeld

et al., 1987), and it has high binding affinity with calcium (Hauschka and Carr, 1982). Osteocalcin seems to appear during new bone formation when new osteoblasts begin to proliferate (Gerstenfeld *et al.*, 1987). Osteonectin is produced by chondrocytes (Pacifici *et al.*, 1990) and osteoblasts (Jundt *et al.*, 1987), and it accumulates in the mineralizing zone in bone formation (Pacifici *et al.*, 1990). Alkaline phosphatase is another phenotypic marker of bone formation (Gerstenfeld *et al.*, 1987). It is found in the hypertrophic zone of the epiphyseal plate (Väänänen, 1980), where it is produced exclusively by mature, differentiated chondrocytes (Yalçin *et al.*, 2007). A reduction in alkaline phosphatase could therefore be a sign of delayed chondrocyte differentiation (Yalçin *et al.*, 2007), which could indicate a delay in the development of the bone.

Aim of this study was to investigate effects of lighting conditions on leg bone development in broiler chicken embryos, and to reveal the mechanisms involved in differences in bone development. We used a schedule of 16L:8D, which is similar to the post hatch broiler house lighting conditions, and the two extremes of 24L and 24D. We hypothesized that 16L:8D would result in upregulation of genes involved in cartilage and bone development, a clear circadian rhythm in pineal melatonin and plasma GH release, lower heart rate as a result of increased embryonic activity, and increased bone development at hatch compared to 24L in particular with 24D intermediate, as the strongest contrast in embryonic bone development was previously found between 24L and a circadian rhythm in light during incubation.

We did find an effect of lighting conditions, but the involved mechanisms and pathways have not yet completely been revealed by the current experiment.

2 | Materials and methods

The Institutional Animal Care and Use Committee of Wageningen University & Research (Wageningen, the Netherlands) approved the experimental design and protocols.

2.1 | Experimental setup

Ross 308 broiler eggs from a 42-week-old parent flock were selected for egg weight between 62.0 and 65.0 g (to reduce variation among eggs). Assuming a hatchability of 80%, 561 eggs were selected to obtain the 447 embryos and chickens required in the experiment. They were then stored at a commercial hatchery (Lagerwey BV, Lunteren, the Netherlands) for 3 days at 18°C before being transported to the climate respiration chambers (CRCs; Heetkamp *et al.*, 2015) at the experimental facility of Wageningen University & Research (Wageningen, the Netherlands). At the experimental facilities, eggs were allocated to one of three CRCs, each containing 187 eggs and programmed

for one of the following treatments: incubation under continuous light (24L), incubation under 16 h of light, followed by 8 h of darkness (16L:8D), or incubation under continuous darkness (24D).

Light was provided by cool white light emitting diode (LED) strips with a colour temperature of 6,050 K, and an intensity of approximately 500 lux at eggshell level. LED strips were attached to the egg tray above the experimental eggs. A head torch with low light intensity of approximately 10 lux at eggshell level was used in dark conditions for checks and during measurements. No other light was allowed to enter the CRCs. 16L:8D's lighting treatment started with 12 h of light, followed by the first dark period of 8 h.

At the start of incubation, eggs were randomly divided over 3 setter trays per treatment, and turned to an initial angle of 45°. Eggs were then turned by 90° hourly. Eggs were heated from storage temperature to incubation temperature over a time frame of 10 h. From then on, eggs were incubated at a set eggshell temperature of 37.8°C till E19.5, measured through 5 temperature sensors (NTC Thermistors: type DC 95; 142 Thermometrics, Somerset, UK) attached to the equator of 5 random eggs per treatment using heat conducting paste (Dow Corning 340 Heat Sink Compound, Dow 144 Corning GmbH, Wiesbaden, Germany) and a small piece of tape. An eggshell temperature of 37.8°C was found to result in the most optimal balance between bone development, hatchability, and chick quality (Van der Pol *et al.*, 2014). The median of the 5 temperature measurements from the sensors was used to automatically adjust the CRCs air temperature. CO_2 levels were not allowed to rise above 0.35%, and RH was maintained between 45 and 55% throughout incubation.

On E19.5, eggs were transferred to hatching baskets by hand. Eggs were not candled for embryonic mortality or fertility to exclude the influence of light on 24D and 16L:8D's dark period as much as possible. At E19.5, the machine's air temperature was fixed at its current setting, since hatching could start from that point onward, and eggshell temperatures were no longer reliable once the chicken has emerged from the egg. At 510 h of incubation or E21.25, the experiment was ended.

2.2 | Embryonic heart rate

Heart rate was measured for the first time on E12, and for the last time on E19 at 20:00. It was measured using a Buddy Mk2 digital egg monitor (Avitronics, Truro, United Kingdom) in 10 randomly chosen eggs twice a day: at 8:00, during 16L:8D's dark period, and at 20:00, during 16L:8D's light period.

2.3 | Quantitative real-time PCR (qRT-PCR)

On E13, E17, and at hatch, 15 randomly selected eggs or chickens per treatment (N = 135) were sampled for tibial gene expression relevant to bone development. Eggs and chickens were kept in their treatment lighting conditions until sampling. Embryos or chickens were sacrificed by cervical dislocation, and the left leg was removed at the femoral head. The tibia was chosen for gene expression measurements, because a previous study showed differences in ossification in the tibia between incubation lighting treatments from E13 onwards (Van der Pol *et al.*, in preparation). The tibia was separated from the leg, the fibula was removed, and soft tissue was removed using dry paper. Immediately after dissection, tibias were placed in tubes, snap frozen in liquid nitrogen, and stored at -80°C.

RNA was obtained from the complete tibia using the RNeasy fibrous tissue mini kit (Qiagen, Germany). The bones were homogenized in RLT buffer using a TissueLyser II (Qiagen, Germany). RNA concentration was measured with a NanoDrop 1000 (ThermoFisher Scientific, USA). From each treatment, a few samples were randomly chosen to evaluate the integrity of extracted RNA in gel electrophoresis. cDNA was synthesized from 1 µg total RNA using the Quantitect Reverse Transcription Kit (Qiagen) Gene-specific primers were designed using primerBLAST (www.ncbi.nlm.nih.gov) (Table 1).

The qRT-PCR reactions were performed in a 72-well Rotor-Gene Q using the Rotor-Gene SYBR Green PCR kit according to the manufacturers protocol. Samples were randomly chosen between different time points and treatments for each run to avoid run effects. The Ct value and amplification efficiency for each sample was obtained with the Comparative Quantitation Analysis from the Rotor-Gene Q software. Four different putative reference genes, *Tuba1C*, *ELF1*, *28S*, and *β-actin* were tested for their suitability using the BestKeeper software tool (Pfaffl *et al.*, 2004). As *β-actin* (Table 1) gave the most stable results, it was chosen as the reference gene and was included in every run. The amplification efficiency for each gene was determined by taking the average of the amplification efficiency of all samples. The expression of the genes relative to *β-actin* was calculated according to the Pfaffl method (Pfaffl, 2001).

2.4 | Melatonin, GH, and corticosterone analysis

On 450, 454, 458, 462, 466, and 470h of incubation (E18.75 till E19.5), 15 embryos per treatment, per time point were sacrificed for blood samples and pineal gland removal (N=270). Time points were chosen to reflect the lighting cycle; 458h was at the start of 16L8D's dark period, 462h was in the middle of the dark period, 466h was at the start of the light period, 470h was 4 h into the light period of 16L:8D, 450h was 8 h into the light period, and 454h was 12 h into the light period (Figure 1). Blood samples

were collected in heparin coated tubes on ice. After centrifugation, plasma was stored at -20°C for analysis of GH and corticosterone. Corticosterone was analysed with a double antibody corticosterone 125 RIA kit for rats and mice (ImmuChem Corticosterone DA, Catalog no. 07-120102; MP Biomedicals, LCC, New York, USA). To be able to use the kit for our chicken samples, it was determined in a preliminary run that the sample had to be diluted from 200x to 5x. GH was analysed with a chicken GH ELISA kit (CSB-E09866Ch; Cusabio, Maryland, USA) following the kit's instructions. Chickens were decapitated, and pineal glands were removed from the brain, and submerged in an Eppendorf in liquid nitrogen before being stored at -80°C. Pineal melatonin was determined by radio-immunoassay, extracting the pineal glands with absolute methanol prior to the essay, according to Zeman *et al.* (1999a). The detection limit of the assay was 1.2 pg/tube and intra- and inter-assay coefficients of variation for a pooled night time plasma were 7.2 and 9.9, respectively.

Table 1. Proteins and their encoding genes, sequences, codes, and primers.

Protein	Gene symbol	Sequence	Primer sequence
Collagen type I, pro-alpha 2 chain.	*col1α2*	NM_001079714.2	F: AAACCAGGCGAAAGGGGTCT R: GATGGACCACGGCTTCCAAT
Collagen type II, alpha 1 chain	*col2α1*	NM_204426.1	F: GACCGCGACCTCCGACAA R: CCTCGGGGTCCTACAACATC
Collagen type X, alpha 1 chain	*col10α1*	XM_003641007.3	F: CCACAACATTTGAGGACGGA R: CCCCTTGATGCTGGACTGTT
Osteopontin	*spp1*	NM_204535	GAAAAATACGACCCCAGGAGC TGCTGAAGTGAAGCCAGGTC
Osteonectin	*sparc*	NM_204410.1	ACTGCACCACTCGCTTCTTT ATGTCCTGCTCCTTAATGCCAA
Osteocalcin	*bglap*	NM_205387.1	TAAAGCCTTCATCTCCCACCG TCAGCTCACACACCTCTCGT
Alkaline Phosphatase	*alpl*	NM_205360.1	GTCAAAGCCAACGAGGGGAC TTCATCCTTAGCCCAGCGGA
ß-actin	*bact*	NM_205518	TGATATTGCTGCGCTCGTTG ATACCAACCATCACACCCTGA

2.5 | Hatch data

From E19.75 (474 h of incubation) onward, eggs were checked every 4h, and all hatched chickens were feather sexed, weighed, and numbered individually with a neck label. Their moment of hatch was written down for calculation of total incubation time. Starting at the 12th hatched chicken, every 5th hatched chicken per treatment was sampled for its leg bones until 29 chickens were sampled per treatment (N = 87) to ensure an even spread over the middle of the hatch window. Tibia and femur weight, length, depth,

and width were determined in these 87 chickens. The left tibia of every odd numbered chicken was sampled for analysis of gene expression as described previously (n = 15 per treatment). At 510h incubation time, all unhatched eggs were counted to determine hatchability (as a percentage of set eggs), and the experiment was terminated.

2.6 | Statistical analysis

The overall model used for all data was:

$Y_i = \mu + \text{Lighting schedule}_i + \varepsilon_i$, (1),

where Y_i = the dependent variable, μ is the overall mean, $\text{Lighting schedule}_i$ = Incubation lighting schedule (i = 24L, 16L:8D, or 24D), and ε_i = the residual error term.

For the analysis of heart rate, 16L:8D data were split up to measurements in its light period (16L:8D light) and in its dark period (16L:8D dark). Incubation time (E12, E12.5, E13 etc. up to E19.5) was added as a covariable. For the analysis of gene expression, the model was extended with Day (E13, E17, or hatch), and its interaction with Lighting schedule. Heart rate, pineal melatonin, plasma corticosterone, and plasma GH data were analysed both per day or time point and overall (averaged over day or time point). All data were analysed with the Glimmix procedure (for fitting generalized linear mixed models) in SAS (SAS Institute, Cary, NC, USA), with the exception of hatchability and percentage females, which were analysed using the Logistics procedure for logistic regression analysis of binary response data. The individual embryo or chicken was considered to be the experimental unit. Data are presented as least square means (LSMeans) ±SEM. Differences between treatments were considered to be significant at $P \leq 0.05$.

3 | Results

3.1 | Heart rate and hatch data

Heart rate between E12 and E19 was higher for 16L:8D in the light period (283 beats/minute), 16L:8D in the dark period (279 beats/minute), and 24D (279 beats/minute) than for 24L (274 beats/minute) (SEM = 1.6; F = 107.4; P < 0.001). Total incubation time till hatch (hatch time) was shorter for 16L:8D than for 24L (-0.6h) and 24D (-2.4h; F = 8.9; P < 0.001; Table 2). Body weight at the end of incubation was higher for 24D than for 24L (+1.3%) and 16L:8D (+1.1%; F = 9.4; P < 0.001). Hatch of fertile eggs did not differ between treatments (P = 0.15). The hatchling sex ratio, tested as percentage of females, did not differ between treatments (P = 0.50).

Table 2. Hatch time, body weight at hatch, hatch of fertile, and percentage females of hatched (as a measure of sex distribution) for broiler eggs and chickens incubated under continuous light (24L), 16h of light, followed by 8h of darkness (16L:8D), or continuous darkness (24D).

	n[1]	Hatch time (h)	Body weight (g)	Hatch of fertile (%)	Females (%)
24L	329	497.9[a]	46.5[b]	85.7	46.5
16L:8D	329	496.1[b]	46.6[b]	89.8	51.9
24D	329	498.5[a]	47.1[a]	91.2	47.5
SEM		0.41	0.10		
P-value		<0.001	<0.001	0.15	0.50

[1] Chicken or egg was the experimental unit.

[a,b] Values within a column with different superscripts differ significantly at P≤0.05.

3.2 | Gene expression

No differences were found between 24L, 16L:8D, or 24D in expression of the genes *col1α2*, *col2α1*, *col10α1*, *spp1*, *sparc*, *bglap*, and *alpl*, relative to the reference gene *bact*, in the overall studied period ($F < 3.0$; $P > 0.062$) or on any of the measured time points ($F < 2.1$; $P > 0.10$; Table 3) or lighting. Irrespective of incubation lighting schedule, *col10α1* expression relative to *bact* was 32.5 to 54.3% higher at hatching than on E13 and E17 ($F = 7.7$; $P = 0.001$). Relative expression of other genes did not differ between days of incubation ($F < 2.6$; $P > 0.089$).

3.3 | Hormones E18.75 – E19.5

Pineal melatonin levels differed between incubation lighting schedules on several of the studied time points. At 458h of incubation, which was at the start of 16L:8D's dark period, pineal melatonin was higher for 16L:8D than for 24L (+20.7%) and 24D (+80.3%), and it was 75.2% higher for 24L than for 24D ($F = 63.4$; $P < 0.001$; Figure 1A). At 462h of incubation, which was in the middle of 16L:8D's dark period, pineal melatonin was higher for 24L than for 16L:8D (+43.9%) and 24D (+51.3%; $F = 10.7$; $P < 0.001$). At 470h of incubation, which was 6h into 16L:8D's light period, pineal melatonin was 76.1% higher for 16L:8D, and 79.7% higher for 24D than for 24L ($F = 34.2$; $P < 0.001$). Lighting schedule did not affect pineal melatonin content on any other moment ($F < 3.4$; $P > 0.05$). When data were averaged over all time points, because fluctuations in 24L and 24D were expected to be rhythmic for individuals, but not for the whole group, pineal melatonin did not differ between treatments ($F = 1.2$; $P = 0.30$).

Table 3. Gene expression values of collagen type I (*col1α2*), collagen type II (*col2α1*), collagen type X (*col10α1*), osteopontin (*spp1*), osteonectin (*sparc*), osteocalcin (*bglap*), and alkaline phosphatase (*alpl*) relative to β-actin (*bact*) on embryonic day (E)13, E17, and at hatching for broiler embryos and chickens incubated under continuous light (24L), 16h of light, followed by 8h of darkness (16L:8D), or continuous darkness (24D).

	n[1]	*col1α2*	*col2α1*	*col10α1*	*spp1*	*sparc*	*bglap*	*alpl*
Treatment								
24L	45	1.77	1.64	1.42	1.11	1.28	1.00	1.46
16L:8D	45	1.29	1.16	1.24	1.05	1.32	1.31	1.25
24D	45	1.10	1.08	1.54	1.14	1.07	1.15	1.15
SEM		0.264	0.174	0.193	0.173	0.153	0.144	0.241
Day								
E13	45	1.66	1.36	0.90[b]	1.42	1.43	1.11	1.47
E17	45	1.36	1.33	1.33[b]	0.95	1.16	1.34	1.13
Hatching	45	1.14	1.19	1.97[a]	0.92	1.08	1.01	1.25
SEM		0.264	0.174	0.193	0.173	0.153	0.144	0.241
Treatment x day								
24L - E13	15	2.46	2.19	0.83	1.52	1.93	0.81	2.20
24L - E17	15	1.78	1.66	1.54	0.99	0.99	1.39	1.15
24L – Hatching	15	1.06	1.07	2.27	0.82	0.93	0.81	1.02
16L:8D - E13	15	1.34	0.81	0.56	1.44	1.33	1.25	0.98
16L:8D - E17	15	1.29	1.30	1.41	0.85	1.45	1.60	1.22
16L:8D – Hatching	15	1.25	1.36	2.29	0.85	1.19	1.08	1.57
24D - E13	15	1.18	1.08	1.32	1.30	1.03	1.27	1.23
24D - E17	15	1.01	1.03	1.05	1.03	1.05	1.04	1.03
24D – Hatching	15	1.11	1.15	1.35	1.09	1.13	1.13	1.18
SEM		0.457	0.302	0.334	0.300	0.265	0.250	0.417
P-values								
Treatment		0.20	0.062	0.55	0.93	0.44	0.34	0.66
Day		0.39	0.78	0.001	0.089	0.26	0.25	0.61
Treatment x Day		0.61	0.10	0.12	0.94	0.20	0.50	0.34

[1] Embryo was the experimental unit.

[a,b] Values within a factor, within a column with different superscripts differ significantly at P≤0.05.

Corticosterone was not affected by incubation lighting schedule on any sampling point ($F < 2.3$; $P > 0.12$; Figure 1B) or when averaged over sampling points ($F = 0.6$; $P = 0.53$). At 450h of incubation, 8h into 16L:8D's light period, GH was higher for 24L than for 16L:8D (+37.4%) and 24D (+44.6%; $F = 23.2$; $P < 0.001$; Figure 1C). At 458h of incubation, at the start of 16L:8D's dark period, GH was 17.4% higher for 24D than for 24L, with 16L:8D intermediate ($F = 6.1$; $P = 0.006$). GH was not affected by treatment on any

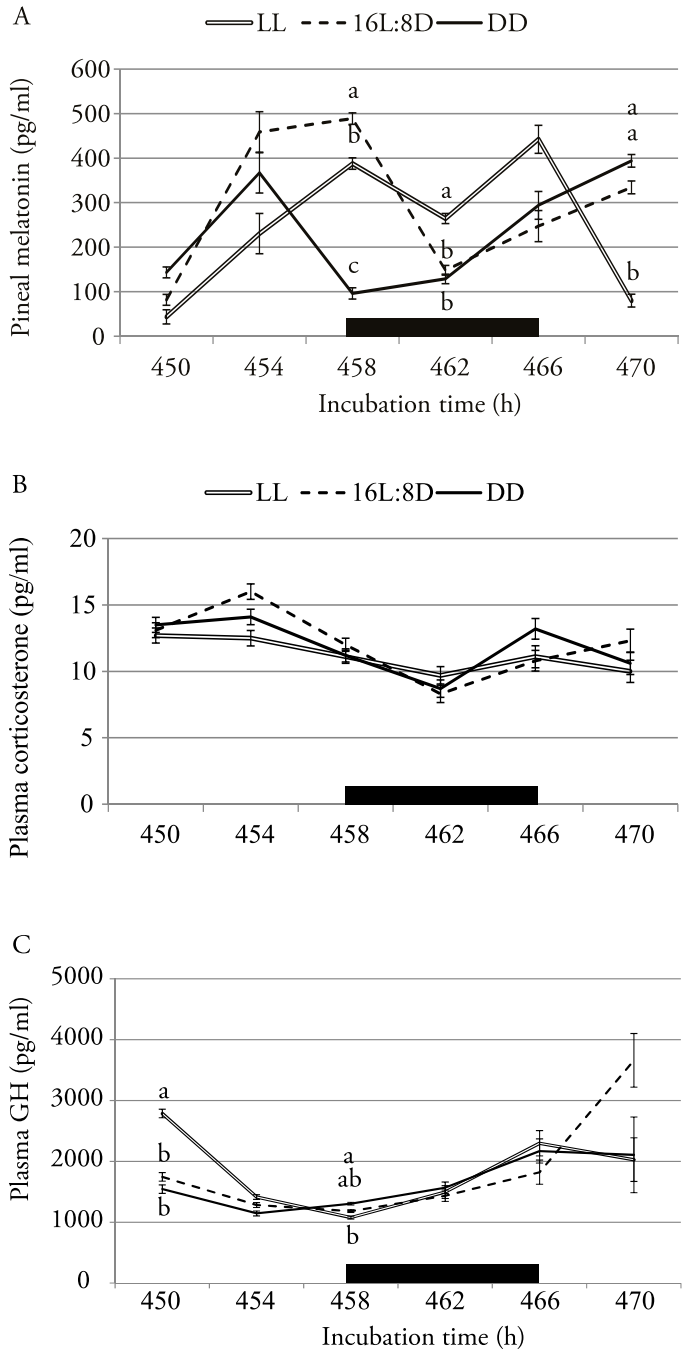


Figure 1. Pineal melatonin (A), plasma corticosterone (B), and plasma growth hormone (C) between 450 and 470h of incubation, corresponding with light or dark periods for the 16L:8D treatment, in broiler chicken embryos incubated under continuous light (24L), 16h of light, followed by 8h of darkness (16L:8D), or continuous darkness (24D). The black box indicates 16L:8D's dark period.

other sampling time (F < 2.9; P > 0.073). When data were averaged over all time points, plasma GH did not differ between treatments (F = 1.2; P = 0.30).

3.4 | Bone dimensions at hatch

Both femur and tibia weight were higher for 24D than for 16L:8D. Femur weight at hatch was 8.0% higher for 24D than for 16L:8D, with 24L intermediate (F = 4.6; P = 0.013; Table 4). Tibia weight at hatch was 4.5% higher for 24D than for 16L:8D, with 24L intermediate (F = 3.1; P = 0.049). Femur length at hatch was 3.0 to 3.2% higher for 24D than for 16L:8D and 24L (F = 22.0; P = 0.013), but tibia length was not affected by incubation lighting schedule (F = 1.1; P = 0.35). Both femur and tibia depth resulted in lowest values for 16L:8D. Femur depth was higher for 24L (+3.2%) and 24D (+5.7%) than for 16L:8D (F= 10.3; P < 0.001). Tibia depth was higher for 24D than for 24L (+2.3%) and 16L:8D (+6.3%), and it was higher for 24L than for 16L:8D (+4.1%; F = 13.4; P < 0.001). Femur width was higher for 24D than for 24L (+3.7%) and 16L:8D (+4.2%; F = 8.8; P < 0.001). Tibia width was higher for 24L (+3.8%) and 24D (+5.9%) than for 16L:8D (F = 13.1; P < 0.001).

Table 4. Weight, length, depth, and width of the femur and tibia at hatch of broiler chickens incubated under continuous light (24L), 16h of light, followed by 8h of darkness (16L:8D), or continuous darkness (24D).

		Weight (g)		Length (mm)		Depth (mm)		Width (mm)	
Treatment	n[1]	Femur	Tibia	Femur	Tibia	Femur	Tibia	Femur	Tibia
24L	29	0.217^{ab}	0.331^{ab}	21.94^{b}	30.93	1.87^{a}	1.71^{b}	1.82^{b}	1.82^{a}
16L:8D	29	0.207^{b}	0.321^{b}	21.99^{b}	31.00	1.81^{b}	1.64^{c}	1.81^{b}	1.75^{b}
24D	29	0.225^{a}	0.336^{a}	22.67^{a}	31.17	1.92^{a}	1.75^{a}	1.89^{a}	1.86^{a}
SEM		0.0044	0.0043	0.089	0.120	0.017	0.016	0.015	0.016
P-value		0.013	0.049	<0.001	0.35	<0.001	<0.001	<0.001	<0.001

[1] Chicken was the experimental unit.

[a,b] Values within a column with different superscripts differ significantly at P ≤ 0.05.

4 | Discussion

In a previous study, we found a longer ossified portion of the tibia for broiler embryos incubated under 12L:12D than for embryos incubated under 24L on E13 and E14 (Van der Pol *et al.*, in preparation). First ossification of chicken leg bones occurs from approximately E9 onward in primary ossification centres in the middle of the bone's diaphysis (Kürtül *et al.*, 2009). In the primary ossification centres, cartilage is replaced by bone marrow, while collagen type 1 rich osteoid forms a bone collar around the cartilage mo-

del. The osteoid later becomes mineralized (Pechak *et al.*, 1986a,b). Sampling of bones for ossification measurements stopped at E14 in the previous study (Van der Pol *et al.*, in preparation), so it is not known how ossification continued for the various lighting schedules post E14, but ossification is known to be incomplete at hatch (Kürtül *et al.*, 2009). We expected to find higher expression of genes related to cartilage (*col1α2*, *col2α1*, *col10α1*) and bone development (*spp1*, *sparc*, *bglap*, and *alpl*) for tibias of broiler embryos incubated under 16L:8D. However, we did not see any differences between incubation lighting treatments in expression of these genes on E13, E17, or at moment of hatch. Whole tibias were sampled in the present experiment, but it is possible that expression of the genes measured in this study was localized, and sampling the whole bone did not reveal localized upregulation. The epiphyseal plate seems to be a relevant region, as osteopontin (Yalçin *et al.*, 2007; McKee *et al.*, 1992), osteocalcin (McKee *et al.*, 1992), osteonectin (Pacifici *et al.*, 1990), and alkaline phosphatase (Väänänen, 1980; Yalçin *et al.*, 2007) are all found in the hypertrophic or mineralizing zones of the epiphyseal plate. Possibly, sampling only the primary ossification centres, or only the epiphyseal plates, may reveal differences in gene expression.

At hatch, femur and tibia weight, depth, width, and femur length were higher for 24D than for 16L:8D. 24L also showed smaller bone dimensions than 24D, with lower femur length, tibia depth, and femur width. The same held when body weight was added to the statistical model as a covariable in an exploratory analysis to correct for the fact that larger chickens may have larger bones. The reducing effect of 16L:8D on bone dimensions was unexpected, considering our previous research where 12L:12D resulted in a longer femur at hatch than both 24D and 24L, and 24D and 12L:12D resulted in a longer and heavier tibia than 24L (Van der Pol *et al.*, in preparation). The lighting source in the present experiment was the same as in the previous experiment, and eggs were of the same breed and a similar parent flock age. It can be hypothesized that the length of the dark period should be more than 8 hours, as that seems to be the largest difference between the 16L:8D and 12L:12D treatments. 16L:8D was chosen as a lighting schedule comparable to what a chicken might experience post hatch. However, during incubation, chicken embryos are exposed to fewer hours of light. In nature, hens will not leave the nest daily, and when they do, they are off the nest for 15 minutes to 1,5 hours (Archer and Mench, 2014). Combined with the fact that 24L was found to result in reduced bone dimensions at hatch compared to 24D, it can be speculated that exposure to too many hours of light (in this case, 16 or 24 hours per day) is detrimental for leg bone development.

One pathway that can affect bone development at hatch is embryonic activity, as movement stimulates bone development in chicken embryos (Murray and Drachman, 1969; Bertram *et al.*, 1996). Heart rate readings were used as a non-invasive measurement of activity in the present experiment. In human foetuses, higher activity is positively related with heart rate (DiPietro *et al.*, 2001). In chicken embryos, Noiva *et al.* (2014)

found higher heart rate for embryos when they were inactive than for moving embryos. However, this was likely a response to overheating. When incubated under high air temperatures (38.9°C), Noiva *et al.* (2014) found increased heart rate, but decreased movements in broiler embryos compared to incubation at an air temperature of 37.8°C. In the present experiment, overheating of the embryos was prevented by incubating the eggs at an eggshell temperature, as a reliable reflection of embryo temperature (Lourens *et al.*, 2011), of 37.8°C throughout incubation. Heart rate in the present experiment was lower for 24L than for 16L:8D, both in its dark and light period, and 24D. This would suggest that 24L incubated broiler embryos were less active than 16L:8D and 24D incubated embryos. Because leg bone development was advanced for 24D compared to 16L:8D, it seems that the relation between heart rate as a reflection of embryonic activity and bone development at hatch is not completely clear from the present experiment.

In literature, a clear pattern in pineal melatonin concentration for chicken embryos exposed to a lighting schedule during incubation is described, with peaks in the dark period (Zeman *et al.*, 1999a; Zawilska *et al.*, 2007). The differences in pineal melatonin concentrations found in the present experiment did not follow a logical pattern over 16L:8D's light and dark periods, or overall between treatments. Pineal melatonin in 16L:8D appears to increase 4 hours before the start of the dark period, which could have been suggestive of synthesis and storage of pineal melatonin, which was later released into the plasma when the dark period started. However, literature does not support this, as pineal melatonin was not found to start increasing already 4 hours before the onset of darkness (Zeman *et al.*, 2004), and concentration of plasma melatonin closely follows pineal melatonin's rhythm (Zeman *et al.*, 1999a). Possibly, we did not find a rhythm in pineal melatonin because embryos were exposed to light for too long during blood sampling before pineal extraction; it is not known how quickly melatonin levels deteriorate in the chicken pineal gland in vivo after light exposure. Plasma GH was expected to follow a rhythm closely resembling that of pineal melatonin, as GH has been shown to peak along with melatonin release (Zeman *et al.*, 1999b; Ostrowska *et al.*, 2002). However, results showed that the plasma GH pattern was very different from the pattern of pineal melatonin concentration. Corticosterone, which was measured a as a possible indicator of stress, did not differ between treatments. Overall, the endocrine pathway did not provide a clear explanation for the differences between incubation lighting treatments in leg bone development that we found at hatch.

In our previous study, lighting schedule during incubation had a long lasting effect on leg bone morphology (Van der Pol *et al.*, in preparation). At day 35 post hatch, 12L:12D still showed the largest leg bones, with higher femur weight and tibia length than 24L. This could be relevant for broiler chicken leg health, as bone size is an important predictor of bone strength (Augat and Schorlemmer, 2006). On the other hand, Toscano *et al.* (2013) found worse gait scores in broilers with wider tibias, possibly because of greater tibial curving. The wider, deeper bones found for 24D compared to 16L:8D might therefore

in the long term be a negative predictor of leg health.

5 | Conclusions

To conclude, applying light during incubation appeared to reduce bone dimensions at hatch compared to dark incubation, but which physiological pathways are involved is not clear.

6 | Acknowledgements

The authors would like to thank Lagerwey hatchery (Lunteren, the Netherlands) for providing the eggs used in this experiment. We would furthermore like to thank Marcel Heetkamp, Anne-Lise Mary, and Francisca Scholl for their help and assistance during the whole experiment.

7 | References

Archer, G. S., and J. A. Mench. 2014. Natural incubation patterns and the effects of exposing eggs to light at various times during incubation on post-hatch fear and stress responses in broiler (meat) chickens. Appl. Anim. Behav. Sci. 152: 44-51.

Augat, P., and S. Schorlemmer. 2006. The role of cortical bone and its microstructure in bone strength. Age Ageing 35(suppl 2): ii27-ii31.

Bekoff, A. 1981 Embryonic development of chick motor behavior. Trends NeuroSci. 4: 181-184.

Bertram, J. E., L. S. Greenberg, T. Miyake, and B. K. Hall. 1997. Paralysis and long bone growth in the chick: growth shape trajectories of the pelvic limb. Growth Dev. Aging 1997;61: 51-60.

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.

Cardinali, D. P., M. G. Ladizesky, V. Boggio, R. A. Cutrera, and C. J. Mautalen. 2003. Melatonin effects on bone: experimental facts and clinical perspectives. Pineal Res. 34: 81-87.

DiPietro, J. A., R. A. Irizarry, M. Hawkins, K. A. Costigan, and E. K. Pressman. 2001. Cross-correlation of fetal cardiac and somatic activity as an indicator of antenatal neural development. Am. J. Obstet. Gynecol. 185: 1421-1428.

Gerstenfeld, L. C., S. D. Chipman, J. Glowacki, and J. B. Lian. 1987. Expression of differentiated function by mineralizing cultures of chicken osteoblasts. Dev. Biol. 122: 49-60.

Hasan, K. M. M., MdS. Rahman, K. M. T. Arif, and M. E. Sobhani. 2001. Physiological stress and aging: role of glucocorticoids (GCs). Age 34: 1421-1433.

Hauschka, P. V., and S. A. Carr. 1982. Calcium-dependent. alpha.-helical structure in osteocalcin. Biochemistry 21: 2538-2547.

Heetkamp, M. W. J., S. J. J. Alferink, T. Zandstra, P. Hendriks, H. van den Brand, and W. J. J. Gerrits. 2015. Design of climate respiration chambers, adjustable to the metabolic mass of subjects. In: W. Gerrits and E. Labussière, editors. Indirect calorimetry: techniques, computations and applications. pp 35-56.

Huth, J. C., and G. S. Archer. 2015. Effects of LED lighting during incubation on layer and broiler hatchability, chick quality, stress susceptibility and post-hatch growth. Poult. Sci. pev298.

Jackson, H., and E. W. Rubel. 1987. Ontogeny of behavioral responsiveness to sound in the chick embryo as indicated by electrical recordings of motility. J. Comp. Physiol. Psych. 92: 682.

Jundt, G., K. H. Berghäuser, J. D. Termine, and A. Schulz. 1987. Osteonectin—a differentiation marker of bone cells. Cell Tissue Res. 248: 409-415.

Kürtül, I., S. H. Atalgin, K. Aslan, and E. U. Bozkurt. 2009. Ossification and growth of the bones of the wings and legs in prehatching period of the Hubbert strain broiler. Kafkas Univ.

Vet. Fak. Derg. 15: 869-874.

Lourens, A., R. Meijerhof, B. Kemp, and H. van den Brand. 2011. Energy partitioning and consequences for embryo temperature: A theoretical approach. Poult. Sci. 90: 516–523.

Machida, M., J. Dubousset, Y. Imamura, T. Iwaya, T. Yamada, and J. 1995. Kimura. Role of melatonin deficiency in the development of scoliosis in pinealectomised chickens. J. Bone Joint Surg. 77: 134-138.

Mackie, E. J., Y. A. Ahmed, L. Tatarczuch, K. S. Chen, and M. Mirams. 2008. Review. Endochondal ossification: How cartilage is converted into bone in the developing skeleton. Int. J. Biochem. Cell Biol. 40: 46-62.

McKee, M. D., M. J. Glimcher, and A. Nanci. 1992. High-resolution immunolocalization of osteopontin and osteocalcin in bone and cartilage during endochondral ossification in the chicken tibia. Anat. Rec. 234: 479-492.

Milgrom, C., M. Gildadi, A. Simkin, N. Rand, R. Kedem, H. Kashtan, M. Stein, and M. Gomori. The area moment of inertia of the tibia: a risk factor for stress fractures. 1989. J. Biochem. 22: 1243-1248.

Monsonego, E., O. Halevy, A. Gertler, S. Hurwitz, and M. Pines. Growth hormone inhibits differentiation of avian epiphyseal growth-plate chondrocytes. Mol. Cell. Endocrinol. 114: 35-42.

Murray, P. D. F., and B. Drachman. 1969. The role of movement in the development of joints and related structures: the head and neck in the chick embryo. J. Embryol. Exp. Morph. 22: 349-371.

Noiva, R. M., A. C. Menezes, M. C. Peleteiro. 2014. Influence of temperature and humidity manipulation on chicken embryonic development. BMC Vet. Res. 10: 1.

Ostrowska, Z., B. Kos-Kudla, B. Marek, D. Kajdaniuk, and N. Ciesielska-Kopacz. 2002. The relationship between the daily profile of chosen biochemical markers of bone metabolism and melatonin and other hormone secretion in rats under physiological conditions. Neuro Endocrinol. Lett. 23: 417-425.

Oviedo-Rondón, E. O., J. Small, M. J. Wineland, V. L. Christensen, P. S. Modziak, M. D. Koci, S. V. L. Funderburk, D. T. Ort, and K. M. Mann. 2008. Broiler embryo bone development is influenced by incubator temperature, oxygen concentration and eggshell conductance at the plateau stage in oxygen consumption. Br. Poult. Sci. 49: 666-676.

Özkan, S., S. Yalçin, E. Babacanoğlu, H. Kozanoğlu, F. Karadaş, and S. Uysal. 2012. Photoperiodic lighting (16 hours of light: 8 hours of dark) programs during incubation: 1. Effects on growth and circadian physiological traits of embryos and early stress response of broiler chickens. Poult. Sci. 91: 2912-2921.

Pacifici, M., O. Oshima, L. W. Fisher, M. F. Young, I. M. Shapiro, and P. S. Leboy. 1990. Changes in osteonectin distribution and levels are associated with mineralization of the chicken tibial growth cartilage. Calcif. Tissue

Int. 47: 51-61.

Pechak, D. G., M. J. Kujawa, A. I. Caplan. 1986a. Morphological and histochemical events during first bone formation in embryonic chick limbs. Bone 7: 441-458.

Pechak, D. G., M. J. Kujawa, and A. I. Caplan. 1986b. Morphology of bone development and bone remodelling in embryonic chick embryos. Bone 7: 459-472.

Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29: p. e45

Pfaffl, M. W., A. Tichopad, C. Prgomet, and T. P. Neuvians. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. Biotech. Lett. 26: 509-515.

Roach, H. I.. 1997. New aspects of endochondral ossification in the chick: chondrocyte apoptosis, bone formation by former chondrocytes, and acid phosphatase activity in the endochondral bone matrix. J. Bone Miner. Res. 12: 795-805.

Robson, H., T. Siebler, S. M. Shalet, and G. R. Williams. 2002. Interactions between GH, IGF-1: Glucocorticoids, and thyroid hormones during skeletal growth. Pediatr. Res. 52: 137-147.

SAS Institute Inc. 2002-2010: SAS/STAT User's guide. Version 9.3. SAS Inst. Inc., Cary, NC.

Shen, G. 2005. The role of type X collagen in facilitating and regulating endochondral ossification of articular cartilage. Orthod. Craniofac. Res. 8: 11-17.

Toscano, M. J., M. A. F. Nasr, and B. Hothersall. 2013. Correlation between broiler lameness and anatomical measurements of bone using radiographical projections with assessments of consistency across and within radiographs. Poult. Sci. 92: 2251-2258.

Väänänen, H. K. 1980. Immunohistochemical localization of alkaline phosphatase in the chicken epiphyseal growth cartilage. Histochemistry 65: 143-148.

Van der Eerden, B. C. J., M. Karperien, and J. M. Wit. 2003. Systemic and local regulation of the growth plate. Endocr. Rev. 24: 782-801.

Van der Pol, C. W., I. A. M. van Roovert-Reijrink, C. M. Maatjens, I. Van den Anker, B. Kemp, and H. Van den Brand. 2014. Effect of eggshell temperature throughout incubation on broiler hatchling leg bone development. Poult. Sci. PS4210.

Van der Pol, C. W., I. A. M. van Roovert-Reijrink, G. Aalbers, C. M. Maatjens, S. W. S. Gussekloo, S. Kranenbarg, J. Wijnen, B. Kemp, and H. van den Brand. Unpublished. Circadian rhythms during incubation of broiler chicken embryos and their effects on embryonic and post hatch leg bone development.

Yalçin, S., H. B. Molayoglu, M. Baka, O. Genin, and M. Pines. 2007. Effect of temperature during the incubation period on tibial growth plate chondrocyte differentiation and the incidence of tibial dyschondroplasia.

Poult. Sci. 86: 1772-1783.

Zawilska, J. B., A. Lorenc, M. Berezińska, B. Vivien-Roels, P. Pevet, and D. J. Skene. 2007. Photoperiod-dependent changes in melatonin synthesis in the turkey pineal gland and retina. Poult. Sci. 86: 1397-1405.

Zeman, M., E. Gwinner, I. Herichová, D. Lamošová, L. Košťál. 1999a. Perinatal development of circadian melatonin production in domestic chicks. J. Pineal Res. 26: 28-34.

Zeman, M., J. Buyse, D. Lamošová, I. Herichova, and E. Decuypere. 1999b. Role of melatonin in the control of growth and growth hormone secretion in poultry. Domest. Anim. Endocrinol. 17: 199-207.

Zeman, M., P. Pavlik, D. Lamošová, I. Herichová, and E. Gwinner. 2004. Entrainment of rhythmic melatonin production by light and temperature in the chick embryo. Avian Poulty Biol Rev. 2004;15: 197-204.

Chapter 6

Incubation lighting schedules and their interaction with matching or mismatching post hatch lighting schedules: effects on broiler bone development and leg health at slaughter age

C. W. van der Pol[ab], I. A. M. van Roovert-Reijrink[a], G. Aalbers[a], B. Kemp[b], H. van den Brand[b]

[a] HatchTech B.V., P.O. Box 256, 3900 AG Veenendaal, the Netherlands.

[b] Adaptation Physiology Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, the Netherlands.

Abstract

The incidence of leg pathologies with a developmental origin may be decreased through optimal early life bone development. There are indications that lighting schedules during incubation can stimulate bone development, but long term effects may depend on post hatch lighting conditions. Aim of this study was to investigate how lighting schedules during incubation and their interactions with matching or mismatching lighting schedules post hatch affected bone development and leg health at slaughter age. Eggs from a prime Ross 308 parent flock were incubated under 3 different lighting schedules: continuous light (Inc24L), 16h of light, followed by 8h of darkness (Inc16L:8D), or continuous darkness (Inc24D) from set till hatch, under LED light with a colour temperature of 6,050K. After hatch, broilers were housed under continuous light (PH24L, to match Inc24L and Inc24D) or 16h of light, followed by 8h of darkness (PH16L:8D, to match Inc16L:8D), provided by white TL light with a colour temperature of 4,000K. Gait scores were determined on D21, D28, and D34. After slaughter on D35, legs were scored for varus-valgus deformities, rotated tibia, tibial dyschondroplasia, bacterial chondronecrosis with osteomyelitis (BCO), epiphyseolysis, and epiphyseal plate abnormalities from 1 = absent to 4 = severe. Femur and tibia weight, length, and width were determined, and bones were scanned using a DXA for mineral density and content. Inc24L led to a higher epiphyseal plate abnormality score than Inc16L:8D or Inc24D. Inc24D led to a higher BCO score than Inc16L:8D. Gait scores on D21, D28, and D34, and femur or tibia length, weight, or width were not affected by the incubation lighting schedule or the post hatch interaction. Inc24L led to higher femur mineral density than Inc24D with Inc16L:8D intermediate. Providing a chicken with a post hatch lighting schedule that matched the incubation schedule did not affect most measurements of bone development and leg health. It can be concluded that applying a circadian incubation lighting schedule may improve leg health in broiler chickens at slaughter age.

1 | Introduction

Fast growing broiler chickens can reach a marketable slaughter weight in as little as 5 weeks post hatch, but this fast growth rate may have a negative effect on leg health (which is expressed as a poor walking ability and a high incidence of leg pathologies), because of the imbalance between body load and bone development (Shim *et al.*, 2012). The exact incidence of leg pathologies in broiler chickens is not known, but it is clear that suboptimal leg health is a widespread phenomenon. For example, in an assessment of 51,000 UK broilers from 176 flocks, Knowles *et al.* (2008) found that only 2.2% of the chickens scored a 0 ("completely normal gait"), and 71.2% of the chickens scored > 2 on a 0 to 5 scale, which means that their gaits ranged from a mild walking disability to being unable to stand.

The aetiology of some leg pathologies, such as varus-valgus deformities, rotated tibia, tibial dyschondroplasia, and chondrodystrophy, lies in the development of a chicken's leg bone (Bradshaw *et al.*, 2002). The largest part of leg bone development takes place during the embryonic phase, when the skeleton is laid out in a cartilage model by the first week of incubation, and ossification is started a few days later (Kürtül *et al.*, 2009). By the time a chicken hatches, the skeleton is complete and functional, although ossification will continue (Kürtül *et al.*, 2009), and the highest rate of bone length growth will take place in the first week post hatch (Applegate and Lilburn, 2002). This emphasizes the potential importance of the embryonic period for optimal leg bone development, which might have carry-over effects on later life.

Light during incubation may be one of the factors that could affect embryonic bone development. For example, Huth and Archer (2015) found more chickens that suffered from leg weakness or abnormalities at hatch, and more asymmetry of the leg bones at D14 post hatch, when broiler eggs were incubated under continuous darkness (Inc24D) than under 12h of light, followed by 12h of darkness (Inc12L:12D). The effects of a lighting schedule during incubation on leg bones might be long term and affect leg health at slaughter age.

In addition, Özkan *et al.* (2012) demonstrated that prenatal exposure to photoperiods may adapt an embryo to a comparable post hatch photoperiodic environment. They found differences in blood melatonin and corticosterone levels for chickens exposed to an incubation lighting schedule of 16h of light, followed by 8h of darkness (Inc16L:8D) or continuous darkness (Inc24D) followed by a post hatch lighting schedule of 16L:8D (PH16L:8D) or 24L (PH24L). In mammals, melatonin is known to stimulate bone development (Robson *et al.*, 2002; Van der Eerden *et al.*, 2003). Possibly, exposing hatchlings to a lighting schedule that matches the lighting schedule they experienced during incubation may lead to enhanced leg bone development and improved leg health through the involvement of the endocrine system.

Aim of the present experiment was to investigate how lighting schedule during incubation and its interaction with a matching or mismatching lighting schedule post hatch affected bone development and leg health during grow out. Leg health is known to be related strongly to body weight, as evidenced by worse gait scores with increasing body weights (Kestin *et al.*, 2001). Therefore, growth and production performance of the broilers were taken into account until slaughter age. In the current experiment, Inc16L:8D was applied to simulate potential post hatch lighting conditions in practice. For embryos exposed to Inc16L:8D, PH16L:8D was considered a match; for embryos exposed to Inc24L and Inc24D, which were expected to lead to a free running circadian rhythm in a random phase pattern in the embryos (Takahashi and Zatz, 1982), PH24L was considered the matching situation. We hypothesize that exposing broiler embryos to an incubation lighting schedule followed by a matching post hatch lighting schedule will result in the highest bone development and the lowest incidence of leg pathologies compared to continuous light or darkness during incubation, followed by a mismatching lighting schedule.

2 | Materials and methods

The experimental design and protocol were approved by the Institutional Animal Care and Use Committee of Wageningen University, Wageningen, the Netherlands. Eggs were incubated under 3 different lighting treatments and exposed to 2 different post hatch lighting treatments in a 3 x 2 factorial arrangement.

2.1 | Experimental Setup

Incubation

Hatching eggs (N = 987) from a 42-week-old Ross 308 parent flock were selected for egg weights between 62.0 and 65.0 g. Eggs were stored on setter trays at 18°C for 3 days at a commercial hatchery (Lagerwey BV, Lunteren, the Netherlands) before transport to the experimental facilities of Wageningen University & Research (Wageningen, the Netherlands), where they were allocated to 1 of 3 climate respiration chambers (CRC) (Heetkamp *et al.*, 2015) for incubation. Eggs were randomly divided over 1 of 3 incubation lighting schedules: continuous light (Inc24L), 16 h of light, followed by 8 h of darkness (Inc16L:8D), or continuous darkness (Inc24D) with 329 eggs per treatment.

Incubation lighting schedules were applied per CRC from embryonic day (E)0 until the end of the incubation period at 510 h of incubation. In the lighted treatments, light was provided by cool white LED light strips equally distributed over the eggs with a colour

temperature of 6,050 K and an intensity of 500 lux at egg level. In Inc16L:8D, incubation started with a light period of 12 h, followed by the first 8 h dark period at 3:00 a.m. No other light was allowed to enter the CRCs, except when eggs or chickens were checked using a head torch of low light intensity (10 lux).

Eggshell temperatures (EST) were measured through eggshell sensors (NTC Thermistors: type DC 95; 142 Thermometrics, Somerset, UK) per treatment attached with heat conducting paste (Dow Corning 340 Heat Sink Compound, Dow 144 Corning GmbH, Wiesbaden, Germany) and a small piece of duct tape to the equator of 5 random eggs distributed over the egg trays. EST was increased from storage temperature to incubation temperature over a period of 10 h. EST was constantly monitored and maintained at a median of 37.8°C for the 5 eggs per treatment by automatically adjusting the air temperature of the CRCs. From E19.5 onward, air temperature was fixed at its current value and EST was allowed to increase during the hatching process.

From E0 till E19.5, eggs were incubated on egg trays with approximately 110 eggs per egg tray. Trays were turned to an angle of 45° at the start of incubation and then turned to an angle of 90° every hour. On E19.5, eggs were transferred to hatching baskets, where eggs were allowed to hatch until 510 h of incubation. Eggs were not candled at transfer to eliminate the effect of a light impulse on the synchronization of circadian rhythmicity in the embryos. Relative humidity was maintained between 45 and 55% and CO_2 levels did not exceed 0.35% throughout incubation. From 474 h of incubation onwards, eggs were checked every 4 h to monitor the hatch window. Newly hatched chickens remained in the CRC.

Post Hatch

At 510 h of incubation, the incubation process was terminated. Hatched chickens were randomly distributed over post hatch lighting schedules in a 3 by 2 factorial arrangement: incubation lighting schedule (Inc24L, Inc16L:8D, or Inc24D) x post hatch lighting schedule (continuous light; PH24L or 16 h of light, followed by 8 h of darkness; PH16L:8D), with 8 pens containing 9 chickens per incubation x post hatch treatment combination (432 chickens in total; other eggs were used for embryonic measurements outside of the scope of this paper). In total, 4 CRCs were used in the post hatch phase, and the pens were divided over 1 of 2 CRCs per post hatch lighting schedule, with 12 1.10 x 1.80 m floor pens per CRC (N = 48 pens). Each pen contained alternately 4 males and 5 females or 5 males and 4 females from the same incubation lighting schedule. Incubation treatments were randomly distributed over pens within a CRC, but not allowing 2 pens of the same incubation lighting schedule to be adjacent to each other.

Light post hatch was provided by 18W Philips Master TL-D Super 80 high frequency TL light (Philips Lighting, Eindhoven, the Netherlands) with a colour temperature of

4,000K. The dark period of PH16L:8D started at 3:00 a.m., which was timed to run in synchrony with Inc16L:8D. Light was applied from day of hatch till slaughter at D35. Bedding was provided as a highly absorbent pelleted combination of cellulose and wood shavings (SoftCell, Ten Damme, Groenlo, the Netherlands). Bedding was checked for wet spots daily and refreshed when necessary to prevent the formation of skin dermatitis, which would affect gait scoring (De Jong *et al.*, 2013). Chickens were fed a starter diet from D0 till D7 containing 2,849 kcal/kg ME, 211.2 g CP, and 12.2 g digestible lysine per kg of feed, and a grower diet from D8 till D35 containing 2,949 kcal/kg ME, 200.6 g CP, and 11.4 g digestible lysine per kg of feed. They had continuous access to water from 3 drinking nipples per pen.

2.2 | Measurements

Newly hatched chickens were feather sexed, weighed, and numbered individually with a neck label. Individual body weights and average daily feed intake (ADFI) per pen were measured weekly. Gain:feed was calculated per pen as average body weight gain (g) / ADFI (g) per week. Mortality was determined daily and computed per week per pen. Gait scores were determined on D21, D28, and D34, scoring from 0 (flawless gait) to 5 (unable to take a single step) according to Kestin *et al.* (1992) by separating a chicken from the group at the far end of the home pen and allowing it to walk back to the group.

On D35, blood was collected from the jugular vein of 23 chickens per treatment randomly chosen from all pens, evenly balancing males and females. Samples were collected in heparin coated syringes. Samples were centrifuged and plasma was stored at -20°C for later analysis of growth hormone and corticosterone. Growth hormone was analysed using a chicken growth hormone ELISA kit (CSB-E09866Ch; Cusabio, College Park, MD), following the kit's instructions. Corticosterone was analysed using a double antibody corticosterone 125 RIA kit for rats and mice (ImmuChem Corticosterone DA, Catalog no. 07-120102; MP Biomedicals, LCC, New York, NY), adapted for chickens by diluting samples 5x instead of the prescribed 200x.

On D35, chickens were anesthetized via a ketamine injection in the breast muscle followed by cervical dislocation. After slaughter, all chickens were assessed for varus-valgus deformities in both legs, which presents as an abnormal angulation at the tibial hock joint. All tibiae were examined macroscopically for a rotational deformity (rotated tibia), and the proximal epiphysis of all tibiae was examined for tibial dyschondroplasia, which is characterized by a plug of avascular cartilage under the epiphyseal plate, and epiphyseal plate abnormalities, such as an irregular shape or thickening. The proximal epiphysis of all femurs was examined for bacterial chondronecrosis with osteomyelitis (BCO), which is characterized by a bacterial infection of the epiphysis, causing degeneration and lesions, and epiphyseolysis, in which the epiphysis is separated from the bone

at the epiphyseal plate. All leg pathologies were scored per leg as either 1 = absent, 2 = mild, 3 = moderate, or 4 = severe. Leg problem scores per chicken or leg were averaged per pen. The femurs and tibias of the same 23 chickens per treatment that were used for blood analysis were examined in more detail by removing soft tissue, weighing them, and measuring the length and width of the bones in the middle of the diaphysis using a digital calliper. The tibias and femurs from each pen were scanned simultaneously for bone mineral density (g/cm^2) and bone mineral content (g) of whole bones using dual-energy X-ray absorptiometry in a Hologic Horizion DXA system (Hologic, Inc., Marlborough, MA) using the small animal research package.

2.3 | Statistical Analysis

The overall model used was:

$$Y_i = \mu + Incubation_i + Post\ hatch_j + Interaction_k + \varepsilon_{ijk}, (1),$$

where Y_i = the dependent variable, μ is the overall mean, $Incubation_i$ = Incubation lighting schedule (i = Inc24L, Inc16L:8D, or Inc24D), $Post\ hatch_j$ = Post hatch lighting schedule (j = PH24L or PH16L:8D), $Interaction_k$ = Incubation lighting schedule x Post hatch lighting schedule (k = Inc24L – PH24L, Inc16L:8D - PH24L, Inc24D - PH24L, Inc24L – PH16L:8D, Inc16L:8D - PH16L:8D, or Inc24D - PH16L:8D), and ε_{ijk} = the residual error term. Data were analysed using the Glimmix procedure in SAS per measured time point (SAS Institute, Cary, NC), with the exception of mortality, which was analysed as count data per week in the Logistics procedure, as an ordinal response. Gait scores (D34), leg problem scores (D35), and body weight (D35) D34 or D35 were furthermore correlated in SAS to produce Pearson correlation coefficients between variables.

Pen was considered to be the experimental unit in the analysis of all data. Presented data are LSMeans. In all cases, differences were considered significant at $P \leq 0.05$.

3 | Results

3.1 | Performance data

Body weight was not affected by an incubation x post hatch lighting schedule interaction ($P \geq 0.33$; Table 1). Body weight on D21 was 2.7 and 2.3% higher for Inc24L than for Inc16L:8D and Inc24D, respectively ($P = 0.044$). Body weight on D35 was 2.6 and 3.3% higher for Inc24L than for Inc16L:8D and Inc24D, respectively ($P = 0.013$). Body weight was affected by post hatch lighting schedule from D7 till D28 ($P \leq 0.015$), with 2.4 to 10.6% higher body weight for PH24L than for PH16L:8D, but not on D35 (P =

0.50).

ADFI was not affected by an incubation x post hatch lighting schedule interaction (P ≥ 0.13; Table 2). ADFI in week 4 was affected by incubation lighting schedule, with 4.3 and 5.1% higher ADFI for Inc24L than for Inc16L:8D or Inc24D, respectively (P = 0.020). ADFI was affected by post hatch lighting schedule, with 3.4 to 10.9% higher ADFI for PH24L than PH16L:8D until week 3 (P ≤ 0.006), no effect in week 4 (P = 0.087), and 3.0% lower ADFI for PH24L than for PH16L:8D in week 5 (P = 0.041).

Table 1. Body weights from D0 till D35 of broiler chickens incubated under Inc24L, Inc16L:8D, or Inc24D, and housed under PH24L or PH16L:8D during grow out.

		Body weight (g)					
	n[1]	D0	D7	D14	D21	D28	D35
Incubation							
Inc24L	16	46.5[b]	191	513	1047[a]	1707	2534[a]
Inc16L:8D	16	46.6[b]	188	503	1019[b]	1669	2467[b]
Inc24D	16	47.1[a]	190	509	1024[b]	1661	2449[b]
SEM		0.10	1.7	4.5	8.2	14.0	20.2
Post hatch							
PH24L	24	-[2]	196[a]	536[a]	1054[a]	1700[a]	2475
PH16L:8D	24	-[2]	183[b]	479[b]	1007[b]	1659[b]	2491
SEM		-[2]	1.4	3.6	6.7	11.4	16.5
Incubation x Post hatch							
Inc24L - PH24L	8	-[2]	196	538	1062	1716	2506
Inc16L:8D - PH24L	8	-[2]	196	535	1053	1703	2475
Inc24D - PH24L	8	-[2]	197	536	1046	1680	2445
Inc24L - PH16L:8D	8	-[2]	187	487	1032	1699	2561
Inc16L:8D - PH16L:8D	8	-[2]	180	470	985	1634	2460
Inc24D - PH16L:8D	8	-[2]	183	481	1002	1643	2453
SEM		-[2]	2.4	6.3	11.6	19.8	28.5
P-values							
Incubation		<0.001	0.39	0.29	0.044	0.054	0.013
Post hatch		-[2]	<0.001	<0.001	<0.001	0.015	0.50
Incubation x Post hatch		-[2]	0.33	0.54	0.27	0.41	0.46

[1] pen is the experimental unit. For D0, chicken is the experimental unit, and n = 209.

[2] No post hatch treatment applied yet on D0

24L = continuous light, 16L:8D = 16 h of light, followed by 8 h of darkness, and 24D = continuous darkness

[a,b] Values within a column and factor lacking a common superscript differ (P ≤ 0.05).

Table 2. ADFI and G:F from week 1 till week 5 of broiler chickens incubated under Inc24L, Inc16L:8D, or Inc24D, and housed under PH24L or PH16L:8D during grow out.

		ADFI (g/day)					Gain:feed				
	n[1]	Week 1	Week 2	Week 3	Week 4	Week 5	Week 1	Week 2	Week 3	Week 4	Week 5
Incubation											
Inc24L	16	16.6	54.8	100.6	134.5[a]	180.0	0.605	0.742	0.679	0.551	0.499
Inc16L:8D	16	16.7	53.4	99.2	128.7[b]	177.2	0.620	0.746	0.688	0.541	0.507
Inc24D	16	16.9	53.8	98.8	127.6[b]	174.6	0.623	0.742	0.673	0.540	0.502
SEM		2.25	4.60	6.98	12.49	15.73	0.0243	0.013	0.0144	0.0153	0.0181
Post hatch											
PH24L	24	17.3[a]	57.1[a]	101.3[a]	128.5	174.5[b]	0.617	0.744	0.669[b]	0.527[b]	0.498
PH16L:8D	24	16.2[b]	50.9[b]	97.8[b]	132.1	180.0[a]	0.615	0.742	0.691[a]	0.562[a]	0.508
SEM		1.83	3.76	5.70	10.20	12.84	0.0199	0.0106	0.0117	0.0125	0.0148
Incubation x Post hatch											
Inc24L - PH24L	8	17.1	57.2	102.2	130.6	176.1	0.608	0.742[ab]	0.673	0.530	0.494
Inc16L:8D - PH24L	8	17.1	57.6	101.1	128.6	173.6	0.607	0.764[a]	0.673	0.527	0.498
Inc24D - PH24L	8	17.7	56.5	100.4	126.2	173.8	0.635	0.728[b]	0.662	0.523	0.501
Inc24L - PH16L:8D	8	16.1	52.3	99.0	138.3	183.9	0.602	0.742[ab]	0.686	0.573	0.503
Inc16L:8D - PH16L:8D	8	16.4	49.2	97.2	128.9	180.7	0.633	0.728[b]	0.702	0.556	0.517
Inc24D - PH16L:8D	8	16.1	51.1	97.2	129.0	175.3	0.611	0.756[ab]	0.685	0.556	0.504
SEM		3.18	6.51	9.87	17.67	22.25	0.0135	0.0103	0.0092	0.0069	0.0066
P-values											
Incubation		0.77	0.33	0.40	0.020	0.24	0.36	0.90	0.32	0.19	0.45
Post hatch		0.006	<0.001	0.005	0.087	0.041	0.87	0.75	0.005	<0.001	0.065
Incubation x Post hatch		0.60	0.13	0.95	0.34	0.55	0.19	0.014	0.67	0.60	0.46

[1] pen is the experimental unit

24L = continuous light, 16L:8D = 16 h of light, followed by 8 h of darkness, and 24D = continuous darkness

[a,b] Values within a column and factor lacking a common superscript differ ($P \leq 0.05$).

In week 2, an interaction was found between incubation and post hatch lighting schedule for G:F, with a lower G:F for Inc16L:8D – PH16L:8D and Inc24D – PH24L (both 0.73 g gain / g feed) than for Inc16L:8D – PH24L (0.76 g gain / g feed; P = 0.014) and all other treatment combinations intermediate. No interaction was found for any other week (P ≥ 0.46). G:F was never affected by incubation lighting schedule (P ≥ 0.19). In week 3 (P = 0.005) and 4 (P < 0.001), G:F was affected by post hatch lighting schedule, with 0.02 to 0.03 g lower gain / g feed (indicating less efficient feed conversion) for PH24L than for PH16L:8D.

Total mortality from D1 till D35 was 2.8%, and it did not differ between treatments overall (P = 0.41) or for separate weeks (P ≥ 0.95; data not shown).

3.2 | Leg pathologies

None of the leg pathologies were affected by an incubation x post hatch lighting schedule interaction (P ≥ 0.081), Tibial dyschondroplasia tended to be affected by incubation lighting schedule, with a higher score for Inc24D than for Inc16L:8D with Inc24L intermediate (P = 0.052; Table 3, Figure 1). The score for epiphyseal plate abnormality was affected by incubation lighting schedule, with a higher score for Inc24L than for Inc16L:8D or Inc24D, respectively (P = 0.027; Figure 2). The score for BCO was affected by incubation lighting schedule (P = 0.025; figure 3), with a higher score for Inc24D than for Inc16L:8D, and Inc24L intermediate. The scores for rotated tibia (P ≥ 0.50), varus-valgus deformity (P ≥ 0.17), and epiphyseolysis (P ≥ 0.084) were not affected by incubation or post hatch lighting schedule. Post hatch lighting schedule affected tibial dyschondroplasia (P = 0.041) and epiphyseal plate abnormalities (P = 0.038), with higher scores for PH16L:8D than for PH24L.

3.3 | Gait scores

Gait scores on D21, D28, and D34 were not affected by incubation lighting schedule or its interaction with post hatch lighting schedule (P ≥ 0.24; Table 4). PH24L resulted in higher gait scores, indicating worse walking ability, on D21 (+0.14 point; P = 0.011) and D28 (+0.19 points; P = 0.004), but lower gait scores on D34 (-0.22 points; P = 0.002). Gait scores positively correlated with body weight (P = 0.023; R = 0.33), indicating worse gait scores with increasing body weight. Gait scores did not correlate with leg pathologies (P ≥ 0.060).

Table 3. Tibial dyschondroplasia (TD), rotated tibia (RT), varus-valgus deformities (VV), epiphyseal plate abnormalities (EPA), bacterial chondronecrosis with osteomyelitis (BCO), and epiphyseolysis (EPI) scored for severity (1 = absent to 4 = severe) at D35 in broiler chickens incubated under Inc24L, Inc16L:8D, or Inc24D, and housed under PH24L or PH16L:8D during grow out.

	n[1]	TD score	RT score	VV score	EPA score	BCO score	EPI score
Incubation							
Inc24L	16	1.29	1.35	1.50	1.34[a]	1.05[ab]	1.71
Inc16L:8D	16	1.15	1.32	1.50	1.17[b]	1.02[b]	1.59
Inc24D	16	1.35	1.28	1.58	1.13[b]	1.12[a]	1.54
SEM		0.058	0.047	0.085	0.055	0.025	0.099
Post hatch							
PH24L	24	1.19[b]	1.34	1.52	1.14[b]	1.08	1.71
PH16L:8D	24	1.33[a]	1.28	1.54	1.28[a]	1.04	1.51
SEM		0.047	0.038	0.070	0.045	0.080	0.045
Incubation x Post hatch							
Inc24L - PH24L	8	1.22	1.33	1.36	1.17	1.06	1.83
Inc16L:8D - PH24L	8	1.14	1.37	1.53	1.17	1.04	1.64
Inc24D - PH24L	8	1.22	1.33	1.65	1.10	1.15	1.67
Inc24L - PH16L:8D	8	1.36	1.36	1.64	1.51	1.04	1.58
Inc16L:8D - PH16L:8D	8	1.16	1.26	1.47	1.17	1.00	1.55
Inc24D - PH16L:8D	8	1.48	1.22	1.50	1.16	1.09	1.41
SEM		0.078	0.036	0.066	0.082	0.121	0.139
P-values							
Incubation		0.052	0.57	0.77	0.027	0.025	0.48
Post hatch		0.041	0.24	0.83	0.038	0.19	0.084
Incubation x Post hatch		0.35	0.50	0.17	0.081	0.82	0.78

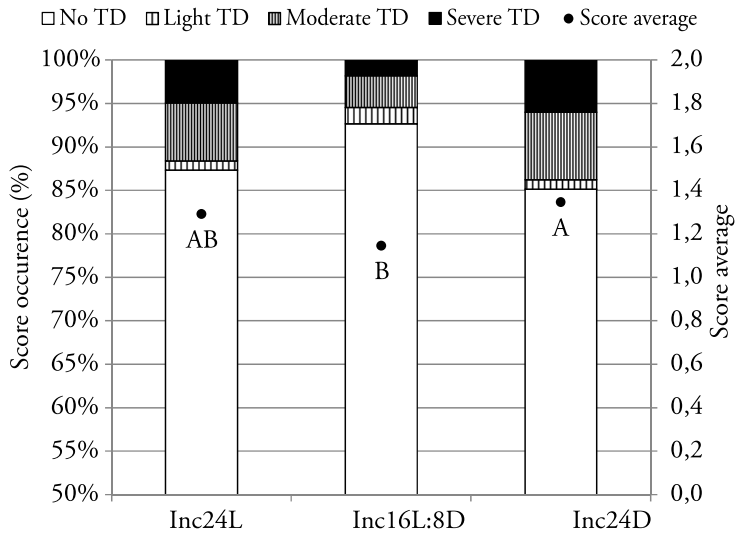
[1] Pen is the experimental unit

24L = continuous light, 16L:8D = 16 h of light, followed by 8 h of darkness, and 24D = continuous darkness

[a,b] Values within a column and factor lacking a common superscript differ ($P \leq 0.05$).

3.4 | Bone morphology

Incubation lighting schedule ($P \geq 0.30$; Table 5) and the incubation x post hatch lighting schedule interaction ($P \geq 0.21$) did not affect femur or tibia length, weight, or width on D35. Post hatch lighting schedule tended to affect femur length, with a 1.1% longer femur for PH24L than for PH16L:8D ($P = 0.057$; Table 5). The incubation x post hatch lighting schedule did not affect bone mineral density or content of the femur or tibia on D35 ($P \geq 0.24$; Table 5). Incubation lighting schedule affected femur mineral density,



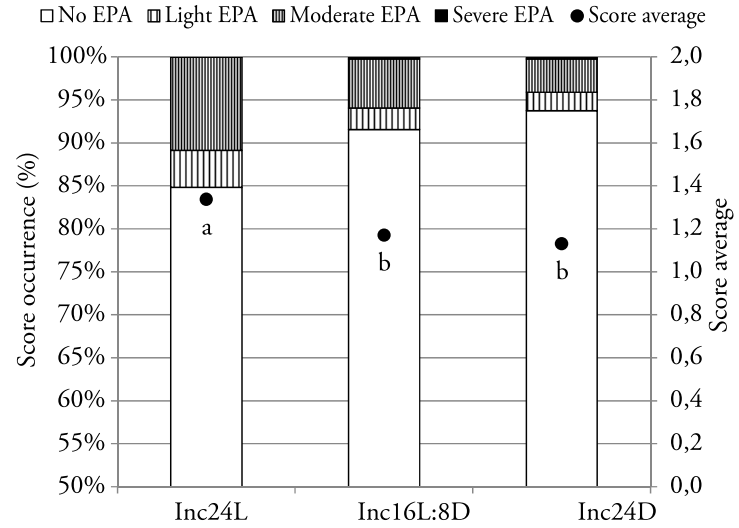


Figure 1. Average tibial dyschondroplasia (TD) score and occurrence per score (none, mild, moderate, or severe TD) in broiler chickens incubated under Inc24L, Inc16L:8D, or Inc24D, and housed under PH24L or PH16L:8D during grow out.

Figure 2. Average epiphyseal plate abnormalities (EPA) score and occurrence per score (none, mild, moderate, or severe EPA) in broiler chickens incubated under Inc24L, Inc16L:8D, or Inc24D, and housed under PH24L or PH16L:8D during grow out.

24L = continuous light, 16L:8D = 16 h of light, followed by 8 h of darkness, and 24D = continuous darkness.

[A,B] Average scores lacking a common superscript tend to differ (P = 0.053).

[a,b] Average scores lacking a common superscript differ (P = 0.027).

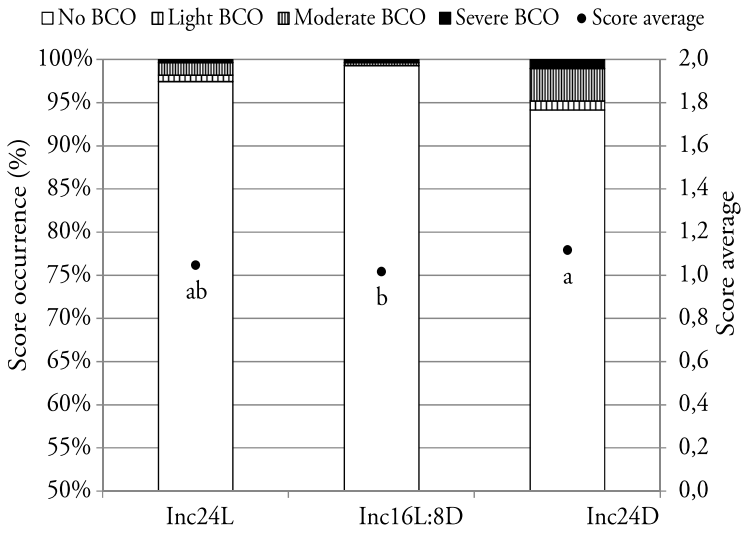


Figure 3. Average bacterial chondronecrosis with osteomyelitis (BCO) score and occurrence per score (none, mild, moderate, or severe BCO) in broiler chickens incubated under Inc24L, Inc16L:8D, or Inc24D, and housed under PH24L or PH16L:8D during grow out.

24L = continuous light, 16L:8D = 16 h of light, followed by 8 h of darkness, and 24D = continuous darkness.

[a,b] Average scores lacking a common superscript differ (P = 0.025).

with higher density for Inc24L than for Inc24D (+12.4%; P = 0.043) and Inc16L:8D intermediate. Incubation lighting schedule did not affect tibia mineral density or femur and tibia mineral content (P ≥ 0.31). Post hatch lighting schedule affected both femur and tibia mineral density and content, with 11.2 to 15.5% higher values for PH16L:8D than for PH24L (P ≤ 0.034).

3.5 | Plasma hormones

Plasma corticosterone levels on D35 did not differ between incubation lighting schedule (P = 0.76), post hatch lighting schedule (P = 0.26), or their interaction (P = 0.53; data not shown). Plasma corticosterone was on average 5.099 ng/ml for Inc24L, 4.422 ng/ml for Inc16L:8D, and 5.384 ng/ml for Inc24D. Plasma growth hormone levels on D35 did not differ between incubation lighting schedule (P = 0.24), post hatch lighting schedule (P = 0.094), or their interaction (P = 0.30; data not shown). Plasma growth hormone was on average 3.148 ng/ml for Inc24L, 3.522 ng/ml for Inc16L:8D, and 3.351 ng/ml for Inc24D.

Table 4. Gait scores at D21, D28, and D34, from 0 (flawless gait) to 5 (unable to take a single step) in broiler chickens incubated under Inc24L, Inc16L:8D, or Inc24D, and housed under PH24L or PH16L:8D during grow out.

		Gait score		
	n[1]	D21	D28	D34
Incubation				
Inc24L	16	1.49	1.95	2.85
Inc16L:8D	16	1.38	1.92	2.78
Inc24D	16	1.47	1.89	2.82
SEM		0.047	0.054	0.059
Post hatch				
PH24L	24	1.52[a]	2.02[a]	2.70[b]
PH16L:8D	24	1.38[b]	1.82[b]	2.92[a]
SEM		0.038	0.044	0.048
Incubation x Post hatch				
Inc24L - PH24L	8	1.59	2.07	2.73
Inc16L:8D - PH24L	8	1.45	2.04	2.67
Inc24D - PH24L	8	1.52	1.94	2.71
Inc24L - PH16L:8D	8	1.40	1.84	2.96
Inc16L:8D - PH16L:8D	8	1.32	1.79	2.88
Inc24D - PH16L:8D	8	1.41	1.85	2.93
SEM		0.066	0.076	0.083
P-values				
Incubation		0.24	0.74	0.72
Post hatch		0.011	0.004	0.002
Incubation x Post hatch		0.87	0.55	0.99

[1] Pen is the experimental unit

24L = continuous light, 16L:8D = 16 h of light, followed by 8 h of darkness, and 24D = continuous darkness

[a,b] Values within a column and factor lacking a common superscript differ ($P \leq 0.05$).

Table 5. Femur and tibia length, weight, and width, and mineral density and content on D35 of broiler chickens incubated under Inc24L, Inc16L:8D, or Inc24D, and housed under PH24L or PH16L:8D during grow out.

	n[1]	Length (mm)		Weight (g)		Width (mm)		Mineral density (g/cm^2)		Mineral content (g)	
		Femur	Tibia	Femur	Tibia	Femur	Tibia	Femur	Tibia	Femur	Tibia
Incubation											
Inc24L	16	68.73	100.07	12.04	17.38	12.31	12.75	0.153[a]	0.175	0.932	1.405
Inc16L:8D	16	68.95	100.74	12.10	17.05	11.89	12.27	0.143[ab]	0.183	0.939	1.462
Inc24D	16	68.99	99.95	11.94	17.08	12.18	12.49	0.134[b]	0.177	0.848	1.458
SEM		0.321	0.531	0.217	0.344	0.204	0.206	0.0052	0.0037	0.0602	0.0662
Post hatch											
PH24L	24	68.53	100.20	11.96	16.91	12.20	12.58	0.135[b]	0.168[b]	0.830[b]	1.341[b]
PH16L:8D	24	69.26	100.31	12.09	17.44	12.06	12.42	0.152[a]	0.189[a]	0.982[a]	1.542[a]
SEM		0.262	0.433	0.177	0.281	0.166	0.168	0.0042	0.003	0.0491	0.054
Incubation x Post hatch											
Inc24L - PH24L	8	67.98	99.74	11.83	16.94	12.33	12.75	0.150	0.170	0.923	1.342
Inc16L:8D - PH24L	8	69.01	101.22	12.15	16.91	11.90	12.23	0.132	0.172	0.814	1.370
Inc24D - PH24L	8	68.60	99.63	11.91	16.87	12.36	12.77	0.123	0.163	0.754	1.311
Inc24L - PH16L:8D	8	69.49	100.40	12.25	17.83	12.29	12.74	0.156	0.181	0.941	1.469
Inc16L:8D - PH16L:8D	8	68.89	100.26	12.05	17.20	11.88	12.31	0.154	0.194	1.064	1.553
Inc24D - PH16L:8D	8	69.38	100.27	11.97	17.29	12.00	12.20	0.145	0.192	0.942	1.605
SEM		0.454	0.751	0.307	0.486	0.288	0.291	0.0925	0.0073	0.0052	0.0851
P-values											
Incubation		0.83	0.53	0.87	0.75	0.36	0.30	0.043	0.31	0.49	0.79
Post hatch		0.057	0.86	0.62	0.19	0.55	0.48	0.008	<0.001	0.034	0.012
Incubation x Post hatch		0.21	0.47	0.68	0.81	0.80	0.48	0.47	0.24	0.38	0.67

[1] Pen is the experimental unit

24L = continuous light, 16L:8D = 16 h of light, followed by 8 h of darkness, and 24D = continuous darkness

[a,b] Values within a column and factor lacking a common superscript differ ($P \leq 0.05$).

4 | Discussion

4.1 | Incubation x post hatch lighting schedule

The effect of incubation lighting schedule followed by a matching or mismatching post hatch lighting schedule on leg health and bone development was very limited. The epiphyseal plate abnormalities score tended to be higher for Inc24L – PH16L:8D than for all other treatment combinations. Hypothetically, Inc24L – PH16L:8D group moved from a free running circadian rhythm at hatch to a fixed lighting schedule post hatch, which may have caused some degree of stress and effort for the chickens to adapt. However, other mismatching post hatch lighting schedules did not result in an increased score for epiphyseal plate deformities. It appears that the main effects of incubation and post hatch lighting schedule had more impact on leg health and bone development than their interaction.

4.2 | Incubation lighting schedule

Incubation lighting schedule did not affect tibia or femur length, width, or weight at slaughter age. Femur bone mineral density, however, was higher for Inc24L than for Inc24D. In live chickens, bone mineral density measurements obtained from DXA scanning have been found to correlate with bone breaking force and bone ash content (Hester *et al.*, 2004), suggesting that the bones of Inc24L broilers may be more resistant against breaking forces. In the present experiment, higher mineralization of the femur for Inc24L was not reflected in better gait scores; these did not differ between incubation lighting schedules. Inc24L broilers were heavier at slaughter than Inc16L:8D or Inc24D broilers; this may have been correlated with their higher bone mineralization. Inc16L:8D and Inc24D resulted in a lower epiphyseal plate abnormality score (indicating better leg health) compared to Inc24L. We furthermore found that chickens exposed to Inc16L:8D showed lower BCO scores and numerically lower tibial dyschondroplasia scores than chickens exposed to Inc24D. Tibial dyschondroplasia cannot be seen independently from epiphyseal plate abnormalities. Tibial dyschondroplasia originates in the epiphyseal plate, where articular cartilage fails to become absorbed, vascularized, and ossified, resulting in an abnormal cartilage plug below the epiphyseal plate (Rath *et al.*, 1998; Leach and Monsonego-Ornan, 2007). Epiphyseal plate abnormalities as described in this study are a more general description of all malformations in the epiphyseal plate, ranging from abnormal thickening to irregular thickness, excluding tibias that had the avascular cartilage plug typical for tibial dyschondroplasia. It was unexpected that lighting schedule during incubation had an effect on the occurrence of BCO. BCO, also (but inaccurately) known as femoral head necrosis, is considered to have an infectious aetiology; it is thought to be caused by blood-borne bacteria that adhere to blood vessels

at the metaphyseal cartilage, causing lesions in the epiphyseal plates of the long bones (McNamee and Smyth, 2000). However, structural immaturity in the form of poor mineralization of chondrocyte columns in the proximal epiphyseal plates can lead to small fractures and impaired blood flow, which may encourage the colonization of bacteria in the blood vessels (Wideman and Prisby, 2013). This suggests that poor early development and maturation of the epiphyseal plate may contribute to the incidence of BCO. Possibly, Inc16L:8D stimulated epiphyseal plate development or chondrocyte mineralization in a way that reduced the opportunity for BCO to manifest, and additionally reduced incidence of epiphyseal plate abnormalities. To our knowledge, no evidence exists that light during incubation affects vasculogenesis, but this might have been a pathway. It appears that abnormalities with a common origin in abnormal epiphyseal plate development are decreased when hatchlings are exposed to a lighting schedule during incubation compared to continuous light or darkness.

The mechanisms that explain the effect of incubation lighting schedule on long term leg bone development and health are not well understood. Plasma corticosterone and growth hormone are known to be involved in bone development in mammals, with a direct stimulating effect of growth hormone on chondrocyte proliferation (Robson *et al.*, 2002), whereas corticosterone increases degradation of collagen (Hasan *et al.*, 2011) and reduces proliferation and increases apoptosis of chondrocytes (Van der Eerden *et al.*, 2003) and osteoblasts (Robson *et al.*, 2002). Plasma growth hormone and corticosterone levels were not found to differ between treatments at slaughter age, which may have been too long after the incubation treatments to still observe an effect. Differences between incubation lighting schedules were probably created in early life as the highest rate of length growth and ossification of leg bones takes place perinatally (Applegate and Lilburn, 2002; Kürtül *et al.*, 2009), and the effects we see at slaughter age may be a result of this differing development in early life.

Gait scores did not differ between incubation lighting schedules or their interaction with post hatch lighting schedule, and gait scores did not correlate with the frequency or severity of leg pathologies. Generally, it can be expected that a more abnormal walking pattern is found in chickens with leg pathologies than those without (Reiter and Bessei, 1997), but we found only a weak correlation with body weight. It is known that leg pathologies and gait scores often worsen at increasing body weights; in fact, when Kestin *et al.* (2001) added body weight into their model as a covariable for lameness, their previously found effects of genotype and feed type nearly all disappeared. This emphasizes the impact of body weight on gait scores. At slaughter, we found higher body weights for Inc24L than for Inc16L:8D, and higher body weights for Inc24L than for Inc24D. In exploratory statistical analysis, the data from the current experiment were therefore analyzed with body weight as a covariable, and although it explained some of the variation, the effects of treatments on leg health still stood. Overall, light during incubation did not impact production negatively, with higher body weights at slaughter for Inc24L than for

Inc24D, and higher ADFI in week 4.

4.3 | Post hatch lighting schedule

Near-continuous light regimes of 23L:1D (23 h of light followed by 1 h of darkness per day) or 24L allow for uninterrupted ADFI, and are therefore sometimes considered as a way to increase production (Classen and Riddell, 1989). However, EU welfare regulations require that chickens are exposed to periods of darkness of at least 6 h per day from 3 days post hatch onwards, of which at least 1 dark period has to last for 4 uninterrupted h (Commission of the European Communities, 2007). Generally, it is found that continuous (Sanotra *et al.*, 2010) or prolonged (>20 h photoperiod; Brickett *et al.*, 2007) light periods are detrimental for occurrence of leg disorders (Buckland *et al.*, 1976; Wilson *et al.*, 1984; Classen and Riddell, 1989; Renden *et al.*, 1991; Renden *et al.*, 1992; Renden *et al.*, 1996), tibial dyschondroplasia scores (Renden *et al.*, 1991; Sanotra *et al.*, 2010), gait scores, and bone ash content (Brickett *et al.*, 2007). We found varying results of post hatch lighting schedule on leg health. PH16L:8D resulted in higher mineralization of the femur and tibia and better gait scores on D21 and D28, but, unexpectedly, worse gait scores on D34 compared to PH24L. On day 35, PH16L:8D also resulted in higher epiphyseal plate abnormalities and tibial dyschondroplasia scores, indicating poorer leg health. This is in disagreement with most literature. In most previous studies, light regimes were not applied immediately post hatch. Chickens were often kept under continuous light for 2 to 4 days before being subjected to experimental light regimes to acclimatize them to their surroundings. However, it was discovered that lighting schedule in the first 4 days of a broiler chicken's life can already affect leg bone growth, with increased growth, but also higher developmental instability for broilers exposed to 24L compared to 2L:1D (Van der Pol *et al.*, 2015). In the present experiment, lighting conditions were applied from the day of placement onwards to avoid interruption of the circadian rhythm already present in the hatchlings, but this does not seem to have resulted in better leg health at slaughter age.

It seems that providing a chicken with a post hatch lighting schedule that matches the one experienced during incubation is not very effective to stimulate leg bone development, and increase leg health. However, incubating broiler eggs under a lighting schedule of Inc16L:8D may improve leg health compared to continuous light or darkness.

5 | Acknowledgements

The authors would like to thank Lagerwey hatchery (Lunteren, the Netherlands) for providing hatching eggs. We would furthermore like to thank Martijn Nieuwenhout at Tromp Medical B.V. (Castricum, the Netherlands) for allowing us to use their DXA

machine, and assisting us with the scans. We would like to thank Stefan Veenstra for his veterinary assistance with classification of leg pathologies, and Anne-Lise Mary, Francisca Scholl, and Marieke Priester for their assistance during the experiment.

6 | References

Applegate, T. J., and M. S. Lilburn. 2002. Growth of the femur and tibia of a commercial broiler line. Poult. Sci. 81: 1289-1294.

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.

Brickett, K. E., J. P. Dahiya, H. L. Classen, C. B. Annett, and S. Gomis. 2007. The impact of nutrient density, feed form, and photoperiod on the walking ability and skeletal quality of broiler chickens. Poult. Sci. 86: 2117-2125.

Buckland, R. B., D. E. Bernon, and A. Goldrosen. 1976. Effect of four lighting regimes on broiler performance, leg abnormalities and plasma corticoid levels. Poult. Sci. 55: 1072-1076.

Classen, H. L., and C. Riddell. 1989. Photoperiodic effects on performance and leg abnormalities in broiler chickens. Poult. Sci. 68: 873-879.

Commission of the European Communities. 2007. Laying down minimum rules for the protection of chickens kept for meat production. Council Directive 2007/43/EC.

De Jong, I. C., H. Gunnink, and J. van Harn. 2013. Wet litter not only induces footpad dermatitis but also reduces overall welfare, technical performance, and carcass yield in broiler chickens. J. Appl. Poult. Res. 23: 51-58.

Hasan, K. M., M. S. Rahman, K. M. T. Arif, and M. E. Sobhani. 2012. Psychological stress and aging: role of glucocorticoids (GCs). Age 34: 1421-1433.

Heetkamp, M. W. J., S. J. J. Alferink, T. Zandstra, P. Hendriks, H. van den Brand, and W. J. J. Gerrits. 2015. Design of climate respiration chambers, adjustable to the metabolic mass of subjects. In: W. Gerrits and E. Labussière, editors. Indirect calorimetry: techniques, computations and applications. pp 35-56.

Hester, P. Y., M. A. Schreiweis, J. I. Orban, H. Mazzuco, M. N. Kopka, M. C. Ledur, and D. E. Moody. 2004. Assessing bone mineral density in vivo: dual energy X-ray absorptiometry. Poult. Sci. 83: 215-221.

Huth, J. C., and G. S. Archer. 2015. Effects of LED lighting during incubation on layer and broiler hatchability, chick quality, stress susceptibility and post-hatch growth. Poult. Sci. pev298.

Kestin, S.C, T.G. Knowles, A.E. Tinch, N.G. Gregory. 1992. Prevalence of leg weakness in broiler chickens and its relationship with genotype. Vet. Rec. 131: 190-194.

Kestin, S. C., S. Gordon, G. Su, and P. Sørensen. 2001. Relationships in broiler chickens between lameness, liveweight, growth rate and age. Vet. Rec. 148: 195-197.

Knowles, T. G., S. C. Kestin, S. M. Haslam, S. N. Brown, L. E. Green, A. Butterworth, S. J. Pope, D. Pfeiffer, and C.J. Nicol. 2008. Leg disorders in broiler chickens: prevalence, risk factors and prevention. PlosOne 3(2):

e1545.

Kürtül I., S. H. Atalgin, K. Aslan, and E. U. Bozkurt. 2009. Ossification and growth of the bones of the wings and legs in prehatching period of the Hubbert strain broiler. Kafkas Univ. Vet. Fak. 15: 869-874.

Leach, R. M. Jr., and E. Monsonego-Ornan. 2007. Tibial dyschondroplasia 40 years later. Poult. Sci. 86: 2053-2058.

McNamee, P. T., and J.A. Smyth. 2000. Bacterial chondronecrosis with osteomyelitis ('femoral head necrosis') of broiler chickens: a review. Avian Pathol. 29: 477-495.

Özkan, S., S. Yalçin, E. Babacanoğlu, H. Kozanoğlu, F. Karadaş, and S. Uysal. 2012. Photoperiodic lighting (16 hours of light: 8 hours of dark) programs during incubation: 2. Effects on early posthatching growth, blood physiology, and production performance in broiler chickens in relation to posthatching lighting programs. Poult. Sci. 91: 2922-2930.

Rath N. C., W. E. Huff, G. R. Bayyari, and J. M. Balog. 1998. Cell death in avian tibial dyschondroplasia. Avian Dis. 42: 72-79.

Reiter, K., and W. Bessei. 1997. Gait analysis in laying hens and broilers with and without leg disorders. Equine Vet. J., Supplement 23: 110-112.

Renden, J. A., S. F. Bilgili, R. J. Lien, and S. A. Kincaid. 1991. Live performance and yields of broilers provided various lighting schedules. Poult. Sci. 70: 2055-2062.

Renden, J. A., S. F. Bilgili, and S. A. Kincaid. 1992. Effects of photoschedule and strain cross on broiler performance and carcass yield. Poult. Sci. 71: 1417-1426.

Renden, J. A., E. T. Moran Jr., and S. A. Kincaid. 1996. Lighting programs for broilers that reduce leg problems without loss of performance or yield. Poult. Sci. 75: 1345-1350.

Robson, H., T. Siebler, S. M. Shalet, and G. R. Williams. 2002. Interactions between GH, IGF-I, Glucocorticoids, and thyroid hormones during skeletal growth. Pediatr. Res. 52: 137-147.

Sanotra, G. S., J. D. Lund, and K. S. Vestergaard. 2010. Influence of light-dark schedules and stocking density on behavior, risk of leg problems and occurrence of chronic fear in broilers. Br. Poult. Sci. 43: 344-354.

SAS Institute Inc. 2002-2010, SAS/STAT User's guide. Version 9.3. SAS Inst. Inc., Cary, NC.

Shim, M. Y., A. B. Karnuah, N. B. Anthony, G. M. Pesti, and S. E. Aggrey. 2012. The effects of broiler chicken growth rate on valgus, varus, and tibial dyschondroplasia. Poult. Sci. 91: 62-65.

Takahashi, J. S., and M. Zatz. 1982. Regulation of circadian rhythmicity. Science 217: 1104-1111.

Van der Eerden, B. C. J., M. Karperien, and J. M. Wit. 2003. Systemic and local regulation of the growth plate. Endocr. Rev. 24: 782-801.

Van der Pol, C. W., R. Molenaar, C. J. Buitink, I. A. M. van Roovert-Reijrink, C. M. Maatjens, H. van den Brand, and B. Kemp. 2015. Lighting schedule and dimming period in early life: consequences for broiler chic-

ken leg bone development. Poult. Sci. 94: 2980-2988.

Wideman, R. F., and R. D. Prisby. 2013. Bone circulatory disturbances in the development of spontaneous bacterial chondronecrosis with osteomyelitis: a translational model for the pathogenesis of femoral head necrosis. Front. Endocrinol. 3: 1-14.

Wilson, J. L., W. D. Weaver Jr., W. L. Beane, and J. A. Cherry. 1984. Effects of light and feeding space on leg abnormalities in broilers. Poult. Sci. 63: 565-567.

Chapter 7

Lighting schedule and dimming period in early life: consequences for broiler chicken leg bone development

Carla W. van der Pol[ab], Roos Molenaar[a], Christiaan J. Buitink[b], Inge A. M. van Roovert-Reijrink[a], Conny M. Maatjens[ab], Henry van den Brand[b], and Bas Kemp[b]

[a] HatchTech B.V., P.O. Box 256, 3900 AG Veenendaal, the Netherlands.

[b] Adaptation Physiology Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, the Netherlands.

Published: Poultry Science 94: 2980-2988 (2015)

Abstract

Prolonged (>20 h) light periods during grow-out of broiler chickens have been shown to increase the occurrence of skeletal abnormalities, but the effects of early life light-dark schedules are not well known. The present experiment investigated the effect of lighting schedule and light-dark transition during the first days of a broiler chicken's life on leg bone development. In 2 experiments, Ross-308 broiler chicks (n = 2,500 per experiment) were subjected to 1 of 5 treatments for 4 d: 24L; 2L:1D lighting schedule with either an abrupt or gradual light-dark transition ("dimming"); and a 2L:6D lighting schedule with an abrupt transition or dimming. At D4, tibia and femur weight, length, and diameter, yolk free body mass, organ weights, realized weight gain, feed intake, feed conversion ratio, and mortality were determined. In Experiment 2, chick length and relative asymmetry of the femur and tibia were determined additionally. Data were analyzed using orthogonal contrasts. 24L resulted in higher femur diameter ($P < 0.028$; both experiments), tibia diameter ($P < 0.001$; Experiment 1), relative asymmetry of tibia length ($P = 0.002$; Experiment 2), and relative asymmetry of femur length ($P = 0.003$) than applying a light-dark schedule. A 2L:1D lighting schedule resulted in higher femur length ($P = 0.039$; Experiment 1) and relative asymmetry of tibia length ($P = 0.032$; Experiment 2) and lower relative asymmetry of tibia diameter ($P = 0.016$) than a 2L:6D lighting schedule. An abrupt light-dark transition resulted in higher relative asymmetry of tibia length ($P = 0.004$; Experiment 2) and relative asymmetry of tibia diameter ($P = 0.018$) than dimming. To conclude, leg bone development in the first 4 d of a broiler chicken's life was higher for 24L than when a lighting schedule was applied, but relative asymmetry was higher as well, suggesting developmental instability. The effect of dimming on leg bone development was less pronounced, but the decreased relative asymmetry levels in the dimming treatment suggested lower environmental stress than for the abrupt light-dark transition.

1 | Introduction

Intensive selection for high growth rate in broiler chickens over the past decades has led to an increase in various leg problems (Kestin *et al.*, 1992; Bessei, 2006). It is estimated that 3% (based on UK data; EU, 2000) to 30% (based on Danish data; Sanotra *et al.*, 2001) of all commercial broiler chickens suffer from lameness, ranging from an identifiable abnormality that impairs the ability of the bird to walk normally to incapability of sustained walking. The difference in occurrence of leg problems among countries are not easy to explain, but it can be speculated that management conditions, including stocking density, and methods of measuring between investigations might play a role. Another potential explanation might be the difference in antimicrobial use to reduce incidence of infectious leg problems; in the UK, total veterinary microbial sale in 2005 was 72 mg/population correction unit (PCU), while it was 46 mg/PCU in Denmark (European Medicines Agency, 2011). Among other factors, leg problems are related to lighting schedule in the broiler house. It was found that >20 h of light periods per day applied from D4 or later onward until slaughter age increased the occurrence of skeletal abnormalities (Classen *et al.*, 1991), leg problems (Buckland *et al.*, 1976; Wilson *et al.*, 1984; Classen and Riddell, 1989; Renden *et al.*, 1991, 1992), tibial dyschondroplasia scores (Renden *et al.*, 1991; Sanotra *et al.*, 2002), and gait scores at slaughter age compared to light-dark schedules with dark periods of >4 h per day.

The highest relative growth rate of the tibia and femur occurs in the first week post-hatch (Applegate and Lilburn, 2002). It can be speculated that the first few days of a broiler chicken's life may have a profound impact when it comes to changes in leg bone development (expressed here as the weight, length, depth, and width of the femur and tibia). This may be related to later life leg problems, but how early life bone development relates to later life leg problems is currently not well understood. Most previous studies were aimed at effects of light on skeletal problems at slaughter age and started with a 23L:1D lighting schedule or 24L for the first 3 or 4 days (Classen *et al.*, 1991; Sanotra *et al.*, 2002; Brickett *et al.*, 2007), followed by their treatment lighting schedules. Although some authors did apply light-dark schedules from D1 post-hatch onwards (Lewis *et al.*, 2009), they continued these schedules throughout the growing period. In the present study, leg bone development was investigated in a brooding system, called HatchBrood (Van der Pol *et al.*, 2013). This system is specially designed for the first 4 d of a broiler chicken life, because it is assumed that this period is the most sensitive period for a chicken's life, including bone development. To the authors' knowledge, the solitary effect of lighting schedule during the first days of a broiler chicken's life on early life bone development has not yet been investigated.

A schedule with both light and dark periods may promote bone development through the involvement of 2 main mechanisms. Firstly, during dark periods, melatonin release maximizes. Melatonin is known to promote bone development throughout life directly

(Roth *et al.*, 1999; Cardinali *et al.*, 2003) and indirectly through release of other factors or hormones involved in bone development (Ostrowska *et al.*, 2002; Robson *et al.*, 2002; Van der Eerden *et al.*, 2003). Secondly, animal locomotion increases during light periods. Locomotion is known to promote bone development through increases of mechanical strain on the bone, which increases transcription of genes that stimulate long bone development (Bradshaw *et al.*, 2002; Müller, 2003; Bessei, 2006). Applying a dark period during the first few days of a chicken's life may therefore promote bone development compared to continuous light.

In addition to the lighting schedule, the transition from light to dark might have an impact on bone development. In practice, newly hatched broiler chickens are exposed to light for the first time when they are pulled from the dark hatching environment. In the broiler house, light is often switched on and off abruptly. It may be beneficial for chickens to make these transitions gradual. Providing laying hens with dimming (a gradual transition from light to dark and vice versa) has been shown to create a behavioural and physiological circadian rhythm in light-dark periods, reduce stress, and increase feeding behaviour prior to the dark period (reviewed by Kristensen, 2008). Glucocorticoids are released when an animal is in a stressful physiological state (Hasan *et al.*, 2011) and they impair bone growth (Robson *et al.*, 2002; Van der Eerden *et al.*, 2003) through enhancement of bone resorption and reduction of bone matrix production (Robson *et al.*, 2002). Furthermore, light-dark transitions have been shown to affect the melatonin release patterns in rats (Laakso *et al.*, 1992). It can, therefore, be speculated that the stress associated with an abrupt light-dark transition has a negative effect on bone development.

The aim of the current study was to investigate the effect of a light-dark schedule or continuous light and dimming or an abrupt transition from light to dark and vice versa from D0 until D4 post-hatch on leg bone development and developmental stability, expressed as relative asymmetry (RA) (Møller *et al.*, 1999) at D4. In order to be able to relate leg bone development to chick development, weight gain, feed intake, feed conversion ratio (FCR), and organ development on D4 were also studied. Two experiments with small differences in the setup were conducted.

2 | Materials and methods

2.1 | Animals and Treatment

Experiment 1

Newly hatched Ross-308 broiler chicks (n = 2,500) from a 27-week-old parent flock were used. A 27-week-old parent flock was chosen because Torres (2013) demonstrated that tibia and femur breaking strength at hatch were lower for young (32 to 33 wk) flocks than for prime (45 to 46 wk) and old (59 to 60 wk) flocks, suggesting that bones of chicks from a young parent flock can be improved most. They were subjected to 1 of 5 lighting treatments for 4 d in an augmented factorial arrangement: 24L was used as a control group, added to a 2 × 2 factorial arrangement with light-dark schedule (2L:1D or 2L:6D lighting schedule) and transition (dimming or abrupt) as experimental treatments. 24L was chosen as a control group with the largest contrast compared to applying a light-dark schedule. A 2L:1D lighting schedule was chosen as it is the recommended light-dark schedule in HatchBrood (see paragraph "Housing") and a 2L:6D lighting schedule was chosen as a light-dark schedule with a contrasting prolonged dark period. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Wageningen University, Wageningen, the Netherlands.

Experiment 2

Animals and treatment were similar to Experiment 1, except that chicks from a 29-week old parent flock were used (n = 2,500) and dimming duration was prolonged to 15 min.

2.2 | Housing

Experiment 1

Newly hatched chicks were randomly divided over the 5 treatments and then placed in 72.7 × 79.0 × 19.0 cm (width x depth x height) plastic baskets, called cradles. Each cradle contained 50 chicks. Ten cradles per treatment (n = 500 chicks) were placed in 1 section of a HatchBrood unit from D0 till D4. HatchBrood is a brooding system for the first 4 d of a broiler chicken's life in which temperature, relative humidity, and CO_2 concentration are controlled. For full details of the HatchBrood system, see Van der Pol *et al.* (2013). Water and a commercially available prestarter feed (ME = 3,100 kcal/kg, CP = 23.5%, vitamin D = 5,000 IU/kg, calcium = 0.9%, phosphorus = 0.66%) were

available ad libitum throughout the 4 d of brooding. Both sexes were used and chick sex was determined at dissection on D4.

Light was provided by strips of cool-white light emitting diode (LED) lights above the drinking gutter of each cradle. Light intensity within the cradle closest to the LED lights was 306 lux and intensity farthest from the LED lights was 17 lux. In all treatments, an initial lighting schedule of 12 h was given to familiarize the chicks with their surroundings. Thereafter, chicks were kept under continuous light (24L), 2 h of light followed by 1 h of dark (2L:1D lighting schedule), or 2 h of light followed by 6 h of dark (2L:6D lighting schedule). In the treatments with dark period, light was either turned on and off abruptly (2L:1D abrupt lighting schedule and 2L:6D abrupt lighting schedule) or it was turned on and off gradually by dimming the light from full luminescence to full dark in a time period of 3 min (2L:1D dimming lighting schedule and 2L:6D dimming lighting schedule). Air temperature decreased from 35.3°C at D0 to 26.1°C at D4 to maintain rectal temperatures between 40.0 and 40.6°C. Relative humidity was maintained between 50 and 65%. CO_2 concentration did not exceed 0.25%.

Experiment 2

Housing was the same as in Experiment 1, except that chicks were feather-sexed at D0 and only male chicks were used in the experiment to create a more uniform sample. To see whether sex affected bone development in 4-day-old broiler chickens, Experiment 1 data were subjected to preliminary analysis. This revealed that tibia width, tibia depth, and femur depth were significantly larger in males compared to females ($P < 0.05$). Therefore, in Experiment 2, it was decided to only use male broiler chicks to rule out a potential interfering effect of sex. The dimming period was extended to 15 min, because it was speculated that the 3 min dimming period in Experiment 1 was too abrupt.

2.3 | Measurements

Experiment 1

Chicks and feed were weighed per cradle on D0 and D4 to determine weight gain and feed intake. Feed intake was corrected for mortality by assuming that dead chicks had eaten for 2 d. FCR was calculated as total feed intake (g) divided by total weight gain (g). Rectal temperatures of 5 randomly chosen chicks per treatment were determined daily to confirm that they remained between 40.0 and 40.6°C. On D0, 35 chicks per treatment were randomly marked with a black marker as chicks to be used for bone development and organ weight analysis on D4. All marked chicks (5 x 35; n = 175) were weighed individually and decapitated on D4. Their left legs were separated from the body at the

hip joint and the bodies and legs were stored at -20°C.

Frozen legs were boiled in tap water for 5 min to remove soft tissue easily without damaging the bones. It was found that the cartilage, particularly at the rostral end of the tibia, came loose easily, and it was therefore removed from the bones and not taken into account during measurements. The cleaned tibia and femur were weighed in grams to 4 decimals. The length and diameter of the tibia and femur (average of 2 measurements perpendicular to each other) at the middle of the shaft were measured twice using a digital caliper in millimeters to 2 decimals.

After thawing the bodies of the chicks in plastic bags in 40°C tap water for 15 min, sex and weight of residual yolk, heart, liver, stomach (gizzard plus proventriculus), and intestines were determined. Intestines were emptied by gently squeezing out the contents. Yolk free body mass (YFBM) was calculated as body weight minus residual yolk.

Experiment 2

Measurements were the same as in Experiment 1, with a few exceptions. On D0 and D4, all marked chicks (n = 175) were weighed individually and their body length was determined from the tip of the beak till the tip of the middle toe, excluding the nail, as a measure of frame development (Molenaar *et al.*, 2010). Both legs were used for bone measurements (weight, length, and diameter) as described for Experiment 1. RA was calculated as follows (Møller *et al.*, 1999):

$$RA = \{|L - R|/[(L + R)/2]\} \times 100,$$

in which RA = relative asymmetry of the left and right bone (%), L = length or diameter of the left bone (mm), R = length or diameter of the right bone (mm), and $|L - R|$ = absolute difference between L and R.

2.4 | Statistics

Experiment 1

The overall model used for all data were:

$$Y_i = \mu + Treatment_i + \varepsilon_i, \quad (1)$$

where Y_i = the dependent variable, μ is the overall mean, $Treatment_i$ = Lighting treatment (i = 24L, 2L:1D dimming lighting schedule, 2L:1D abrupt lighting schedule, 2L:6D dimming lighting schedule, or 2L:6D abrupt lighting schedule), and ε_i = the residual error term. In a preliminary analysis, the interaction of sex (male or female) and treatment

was added to model [1] to test for sex effects. However, none were found, and sex was eliminated from the model. Mortality was analyzed using the Logistics procedure and all other data were analyzed using the GLM procedure in SAS (SAS Institute, 2004). To analyze the main effects of hours of dark and dimming, 4 orthogonal contrasts were analyzed through the Estimate statement. These contrasts were the interaction between light-dark schedule and dimming, excluding the 24L control, 24L versus a light-dark schedule (2L:1D lighting schedule and 2L:6D lighting schedule), 2L:1D lighting schedule versus 2L:6D lighting schedule, and abrupt versus dimming.

Individual chicks were considered to be the experimental unit in analysis of bones, YFBM, and organ weights. Cradles were considered to be the experimental unit in analysis of weight gain, feed intake, FCR, and mortality. Model assumptions were verified by examination of the distributions of the means and residuals. Residual yolk weights were log transformed to obtain normality. Least square means were compared using Bonferroni adjustments for multiple comparisons. Data are presented as LSMeans ± SEM. In the case of data transformation, LSMeans and SEM are presented untransformed and P-values are presented as those of the transformed data. When a light-dark schedule – dimming interaction was found, the main effects of a 2L:1D period vs a 2L:6D lighting schedule and abrupt vs dimming were not discussed in the results section. In all cases, differences were considered significant at $P \leq 0.05$.

Experiment 2

For Experiment 2, the same statistical approach was used as in Experiment 1. Additionally, body weight at D0, RA, and chick length were analyzed using the GLM procedure. Bone weight, length, and diameter were analyzed using the Mixed procedure with an auto-regressive covariance structure with leg side (left or right) as the repeated factor. For body weight at D0, RA, and chick length, individual chicks were considered the experimental unit. For bone weight, length, diameter, and RA, YFBM, chick length, and organ weights, model [1] was extended with body weight at D0 (which was not measured in Experiment 1) as a covariable to take chick development at the start of the experiment into account. Femur diameter RA and weight gain were sinus transformed to obtain normality.

3 | Results

3.1 | Bone Measurements

In Experiment 1, tibia length was higher for the 2L:1D dimming lighting schedule (+0.88 mm) and the 2L:1D abrupt lighting schedule (+1.00 mm) than for the 2L:6D dimming lighting schedule, with 2L:6D abrupt lighting schedule intermediate (P = 0.008; Table 1). Tibia diameter was lower for the 2L:6D abrupt lighting schedule than for the 2L:1D dimming lighting schedule (-0.04 mm), 2L:1D abrupt lighting schedule (-0.08 mm), and 2L:6D dimming lighting schedule (-0.11 mm; P < 0.001). Femur diameter was lower for the 2L:6D abrupt lighting schedule than for the 2L:1D abrupt lighting schedule and the 2L:6D dimming lighting schedule (both -0.08 mm) with the 2L:1D lighting schedule intermediate (P = 0.008). 24L resulted in higher tibia diameter (+0.08 mm; P <

Table 1. Experiment 1. Tibia and femur weight, length, and diameter of 4-day-old broiler chickens exposed to a light-dark schedule of 24L, 2L:1D dimming or abrupt, or 2L:6D dimming or abrupt from 0.5 until 4 days post-hatch.

		Tibia			Femur		
Contrast	n[1]	Weight (g)	Length (mm)	Diameter (mm)	Weight (g)	Length (mm)	Diameter (mm)
24L	35	0.311	32.11	1.90	0.224	23.99	2.06
2L:1D dimming	35	0.315	32.64[a]	1.80[a]	0.211	24.24	1.94[ab]
2L:1D abrupt	35	0.329	32.76[a]	1.84[a]	0.223	24.26	1.98[a]
2L:6D dimming	35	0.315	31.76[b]	1.87[a]	0.230	23.65	1.98[a]
2L:6D abrupt	35	0.306	32.18[ab]	1.76[b]	0.213	24.16	1.90[b]
P interaction2		0.09	0.008	<0.001	0.25	0.15	0.008
24L	35	0.311	32.11	1.90[a]	0.224	23.99	2.06[a]
Light-dark schedule	140	0.316	32.33	1.82[b]	0.219	24.08	1.95[b]
P 24L vs light-dark		0.47	0.060	<0.001	0.49	0.63	<0.001
2L:1D	70	0.322	32.70[a]	1.82	0.217	24.25[a]	1.96
2L:6D	70	0.310	31.97[b]	1.81	0.222	23.91[b]	1.94
P 2L:1D vs 2L:6D		0.10	0.004	0.67	0.41	0.039	0.40
Abrupt	70	0.318	32.47[a]	1.80	0.218	24.21	1.94
Dimming	70	0.315	32.20[b]	1.83	0.220	23.95	1.96
P abrupt vs dimming		0.69	0.048	0.09	0.87	0.12	0.50
SEM		0.003	0.09	0.01	0.002	0.07	0.01

[a,b] Least squares means lacking a common superscript within a contrast, within a column differ (P ≤ 0.05).

[1] Chick is the experimental unit.

0.001) and femur diameter (+0.11 mm; P < 0.001) than applying a light-dark schedule. The 2L:1D lighting schedule resulted in higher femur length (+0.34 mm; P = 0.039) than 2L:6D. None of the treatments affected tibia and femur weight.

In Experiment 2, 24L resulted in higher RA of tibia length (+1.32%; P = 0.002; Table 2), and of femur length (+1.77%; P = 0.003), and higher femur diameter (+0.05 mm; P = 0.028) than applying a light-dark schedule. A 2L:1D lighting schedule resulted in higher RA of tibia length (+0.70%; P = 0.032) and lower RA of tibia diameter (-0.98%; P = 0.016) than the 2L:6D lighting schedule. The abrupt lighting schedule resulted in higher RA of tibia length (+0.94%; P = 0.004) and higher RA of tibia diameter (+0.96%; P = 0.018) than dimming. None of the treatments affected tibia weight, length, or diameter, femur weight, length, and RA of femur diameter.

3.2 | Chick Development

In Experiment 1, 24L resulted in lower YFBM (-4.44 g; P = 0.003), stomach weight (-0.34 g; P < 0.001), and intestine weight (-0.48 g; P < 0.001) than applying a light-dark schedule. The 2L:1D lighting schedule resulted in higher YFBM (+3.07 g; P = 0.019), stomach weight (+0.19 g; P = 0.021), and intestine weight (+0.80 g; P < 0.001) than the 2L:6D lighting schedule. The 2L:1D lighting schedule resulted in lower residual yolk weight (-0.27 g; P < 0.001) than the 2L:6D lighting schedule. Heart weight was higher for the 2L:1D lighting schedule than for the 2L:6D lighting schedule in Experiment 1 (+0.04 g; P = 0.003). Liver weight was higher for the 2L:1D lighting schedule than for the 2L:6D lighting schedule (+0.43 g; P < 0.001).

In Experiment 2, body weight at the start of the experiment (D0) did not differ between treatments. Heart weight was higher for the 2L:1D abrupt lighting schedule than for the 2L:6D abrupt lighting schedule (+0.07 g) with 2L:1D dimming lighting schedule and 2L:6D dimming intermediate lighting schedule (P = 0.023; Table 3). Intestine weight was higher for the 2L:6D abrupt lighting schedule than for the 2L:1D dimming lighting schedule (+0.98 g), the 2L:1D abrupt lighting schedule (+0.69g), and the 2L:6D dimming lighting schedule (+1.35 g; P < 0.001). Intestine weight was furthermore higher for the 2L:1D abrupt lighting schedule than for the 2L:6D dimming lighting schedule (+0.66 g). 24L resulted in higher liver weight (+0.46 g; P < 0.001), and stomach weight (+0.33 g; P = 0.006) than applying a light-dark schedule. Liver weight was higher for the 2L:1D lighting schedule than for the 2L:6D lighting schedule (+0.26 g; P = 0.006). None of the treatments affected YFBM, residual yolk weight, or chick length.

Table 2. Experiment 2. BW at D0, tibia and femur weight, length, diameter, length, and diameter relative asymmetry (RA) of 4-day-old broiler chickens exposed to a light-dark schedule of 24L, 2L:1D dimming or abrupt, or 2L:6D dimming or abrupt from 0.5 until 4 days post-hatch.

			Tibia					Femur				
Contrast	n[1]	BW D0 (g)	Weight (g)	Length (mm)	RA length (%)	Diame-ter (mm)	RA diameter (%)	Weight (g)	Length (mm)	RA length (%)	Diame-ter (mm)	RA diameter (%)
24L	35	37.25	0.376	32.48	4.36	1.85	2.67	0.257	24.26	4.67	2.01	4.93
2L:1D dimming	35	37.46	0.368	32.60	2.89	1.82	2.51	0.263	24.32	2.71	1.97	3.77
2L:1D abrupt	35	37.50	0.373	32.55	3.89	1.86	3.07	0.265	24.44	3.21	1.99	3.62
2L:6D dimming	35	37.57	0.374	32.65	2.25	1.83	3.09	0.255	24.22	2.79	1.93	3.36
2L:6D abrupt	35	36.74	0.372	32.96	3.13	1.84	4.45	0.257	24.59	2.9	1.96	4.69
P interaction[2]		0.39	0.55	0.24	0.86	0.36	0.32	0.99	0.31	0.66	0.89	0.36
24L	35	37.25	0.376	32.48	4.36[a]	1.85	2.67	0.257	24.26	4.67[a]	2.01[a]	4.93
Light-dark schedule	140	37.32	0.372	32.69	3.04[b]	1.84	3.28	0.260	24.39	2.90[b]	1.96[b]	3.86
P 24L vs light-dark		0.90	0.54	0.25	0.002	0.52	0.24	0.56	0.34	0.003	0.028	0.28
2L:1D	70	37.48	0.370	32.57	3.39[a]	1.84	2.79[b]	0.264	24.38	2.96	1.98	3.69
2L:6D	70	37.16	0.373	32.81	2.69[b]	1.83	3.77[a]	0.256	24.41	2.84	1.94	4.02
P 2L:1D vs 2L:6D		0.53	0.69	0.13	0.032	0.79	0.016	0.055	0.83	0.79	0.070	0.89
Abrupt	70	37.12	0.372	32.76	3.51[a]	1.85	3.76[a]	0.261	24.51	3.06	1.98	4.15
Dimming	70	37.52	0.371	32.62	2.57[b]	1.82	2.80[b]	0.259	24.27	2.75	1.95	3.56
P abrupt vs dimming		0.44	0.77	0.37	0.004	0.12	0.018	0.64	0.053	0.49	0.14	0.51
SEM		0.51	0.002	0.06	0.35	0.01	0.43	0.002	0.05	0.47	0.01	0.61

[a,b] Least squares means lacking a common superscript within a contrast, within a column differ ($P \leq 0.05$).

[1] Chick is the experimental unit.

[2] Interaction light-dark schedule and dimming.

Table 3. Experiment 1 and 2. Chick length, yolk free body mass (YFBM), residual yolk weight, length (only for Experiment 2), heart, liver, stomach, and intestines weight of 4-day-old broiler chickens exposed to a light-dark schedule of 24L, 2L:1D dimming or abrupt, or 2L:6D dimming or abrupt from 0.5 until 4 days post-hatch.

		YFBM (g)		Residual yolk (g)		Length (cm)	Heart (g)		Liver (g)		Stomach (g)		Intestines (g)	
Contrast	n[1]	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
24L	35	84.95	95.59	0.59	0.42	23.3	0.64	0.76	3.49	3.94	4.05	4.97	4.72	5.62
2L:1D dimming	35	91.10	93.58	0.49	0.48	23.3	0.65	0.71^{ab}	3.75	3.49	4.41	4.68	5.49	5.57^{bc}
2L:1D abrupt	35	90.75	93.97	0.49	0.37	23.3	0.65	0.77^{a}	3.82	3.73	4.54	4.58	5.71	5.86^{b}
2L:6D dimming	35	86.60	91.54	0.71	0.42	23.3	0.60	0.72^{ab}	3.42	3.31	4.30	4.64	4.75	5.20^{c}
2L:6D abrupt	35	89.12	93.60	0.81	0.34	23.4	0.62	0.70^{b}	3.30	3.40	4.28	4.65	4.85	6.55^{a}
P interaction[2]		0.27	0.60	0.81	0.80	0.68	0.50	0.023	0.22	0.41	0.35	0.62	0.61	<0.001
24L	35	84.95^{b}	95.59	0.59	0.42	23.3	0.64	0.76	3.49	3.94^{a}	4.05^{b}	4.97^{a}	4.72^{b}	5.62
Light-dark schedule	140	89.39^{a}	93.17	0.62	0.40	23.3	0.63	0.73	3.57	3.48^{b}	4.39^{a}	4.64^{b}	5.20^{a}	5.79
P 24L vs light-dark		0.003	0.18	0.94	0.76	0.52	0.63	0.064	0.40	<0.001	<0.001	0.006	<0.001	0.23
2L:1D	70	90.93^{a}	93.77	0.49^{a}	0.43	23.3	0.65^{a}	0.74	3.79^{a}	3.61^{a}	4.48^{a}	4.63	5.60^{a}	5.71
2L:6D	70	87.86^{b}	92.57	0.76^{b}	0.38	23.4	0.61^{b}	0.71	3.36^{b}	3.35^{b}	4.29^{b}	4.65	4.80^{b}	5.87
P 2L:1D vs 2L:6D		0.019	0.45	<0.001	0.39	0.30	0.003	0.082	<0.001	0.005	0.021	0.90	<0.001	0.24
Abrupt	70	89.93	93.79	0.65	0.35	23.3	0.64	0.73	3.56	3.57	4.41	4.62	5.28	6.20^{a}
Dimming	70	88.85	92.56	0.60	0.45	23.3	0.63	0.72	3.59	3.4.0	4.36	4.66	5.12	5.38^{b}
P abrupt vs dimming		0.40	0.44	0.43	0.053	0.90	0.51	0.24	0.76	0.065	0.50	0.69	0.16	<0.001
SEM		0.60	0.71	0.03	0.02	0.04	0.01	0.01	0.04	0.04	0.04	0.05	0.06	0.07

[a,b,c] Least squares means lacking a common superscript within a contrast, within a column differ ($P \leq 0.05$).

[1] Chick is the experimental unit.

[2] Interaction light-dark schedule and dimming.

3.3 | Post-hatch Performance

24L resulted in higher feed intake from D0 till D4 (+2.80 g/chick; P < 0.001; Table 4) and higher FCR (+0.05 FCR (+0.05 g feed/g weight gain; P < 0.001) than applying a light-dark schedule. The 2L:1D lighting schedule resulted in higher weight gain from D0 till D4 (+2.00 g; P < 0.001), higher feed intake from D0 till D4 (+4.59 g/chick; P < 0.001), and higher FCR (+0.06 g feed/g weight gain; P < 0.001) than the 2L:6D lighting schedule. An abrupt lighting schedule resulted in higher feed intake (+0.70 g; P = 0.013) than a dimming lighting schedule. None of the treatments affected mortality.

In Experiment 2, weight gain was higher for the 2L:1D abrupt lighting schedule than for the 2L:1D dimming lighting schedule (+2.18 g) and the 2L:6D abrupt lighting schedule (+5.42 g; P < 0.001; Table 4). Weight gain was furthermore higher for the 2L:2D dimming lighting schedule than for the 2L:6D abrupt lighting schedule (+3.24 g). FCR was higher for the 2L:1D dimming lighting schedule than for the 2L:1D abrupt lighting schedule (+0.02 g feed/g weight gain) and the 2L:6D abrupt lighting schedule (+0.03 g feed/g weight gain; P < 0.001). FCR was furthermore higher for the 2L:1D abrupt lighting schedule (+0.04 g feed/g weight gain) and the 2L:6D abrupt lighting schedule (+0.03 g feed/g weight gain) than the 2L:6D dimming lighting schedule. 24L resulted in higher weight gain (+1.45 g; P = 0.004), higher feed intake from D0 till D4 (+2.38 g/chick; P <0.001), and higher FCR (+0.03 g feed/g weight gain; P < 0.001) than applying a light-dark schedule. The 2L:1D lighting schedule resulted in higher feed intake from D0 till D4 (+3.23 g/chick; P < 0.001) than the 2L:6D lighting schedule. None of the treatments affected mortality.

4 | Discussion

In practice, continuous light (24L) is normally used during the first 4 days of a broiler chicken's life, the brooding period. The present experiment aimed to investigate the effect of lighting schedules and dimming during the brooding period on leg bone development. It was found that 24L resulted in the overall highest tibia diameter (in Experiment 1) and femur diameter (in both experiments) at D4 post-hatch compared to applying a light-dark schedule. Within the light-dark schedules, the 2L:6D lighting schedule (total light period per day of 6 h) resulted in a shorter tibia and femur in Experiment 1 compared to the 2L:1D lighting schedule (total light period per day of 16 h). At the start of the experiment, it was hypothesized that lighting schedules that included a (prolonged) dark period would result in the highest leg bone development through the involvement of melatonin, which has a darkness-dependent release already during embryonic development of the chicken (Csernus *et al.*, 2007), and it is known to stimulate bone development (Cardinali *et al.*, 2003). However, leg bone development was not found to be increased for the 2L:1D lighting schedule or the 2L:6D lighting schedule over 24L. It is possible

Table 4. Experiment 1 and 2. Weight gain, feed intake, and feed conversion ratio (FCR), and mortality of 4-day-old broiler chickens exposed to a light-dark schedule of 24L, 2L:1D dimming or abrupt, or 2L:6D dimming or abrupt from 0.5 until 4 days post-hatch.

		Weight gain D0-D4 (g)		Feed intake D0-D4 (g/chick)		FCR (g feed/g weight gain)		Mortality D0-D4 (%)	
Contrast	n[1]	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
24L	10	55.49	58.10	44.50	46.44	0.80	0.80[a]	1.0	0.2
2L:1D dimming	10	55.95	56.85[b]	43.42	45.47	0.78	0.80[a]	1.0	0.0
2L:1D abrupt	10	57.11	59.03[a]	44.56	45.90	0.78	0.78[b]	1.8	0.0
2L:6D dimming	10	54.54	57.21[ab]	39.29	43.07	0.72	0.74[c]	0.4	0.6
2L:6D abrupt	10	54.52	53.61[c]	39.55	41.82	0.73	0.77[b]	0.8	1.2
P interaction[2]		0.20	<0.001	0.11	0.051	0.96	<0.001	0.35	0.99
24L	10	55.49	58.10[a]	44.50[a]	46.44[a]	0.80[a]	0.80[a]	1.0	0.0
Light-dark schedule	40	55.53	56.68[b]	41.70[b]	44.06[b]	0.75[b]	0.77[b]	1.0	0.5
P 24L vs light-dark		0.95	0.004	<0.001	<0.001	<0.001	<0.001	0.17	0.99
2L:1D	20	56.53[a]	57.94[a]	43.99[a]	45.68[a]	0.78[a]	0.79[a]	1.4	0.1
2L:6D	20	54.53[b]	55.41[b]	39.40[b]	42.45[b]	0.72[b]	0.76[b]	0.6	0.9
P 2L:1D vs 2L:6D		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.35	0.99
Abrupt	20	55.81	56.32	42.05[a]	43.86	0.75	0.77	1.3	0.6
Dimming	20	55.24	57.03	41.35[b]	44.27	0.75	0.77	0.7	0.4
P abrupt vs dimming		0.22	0.099	0.013	0.33	0.32	0.85	0.67	0.99
SEM		0.45	0.32	0.27	0.31	0.01	0.01		

[a,b,c] Least squares means lacking a common superscript within a contrast, within a column differ ($P \leq 0.05$).

[1] Cradle (with 50 chicks per cradle) is the experimental unit.

[2] Interaction light-dark schedule and dimming.

that another mechanism has overruled the effect of melatonin.

It can be speculated that the retarded bone development for treatments with longer dark periods was mostly due to decreased duration of activity in those groups. Movement is known to be essential for bone development throughout life as mechanical strain on the bone increases cartilage formation and ossification (Müller, 2003). During dark periods, chicks show more resting behaviour than during light periods (Yeates, 1963; Coenen *et al.*, 1988), and it can be speculated that the lowest activity is found in treatments with a prolonged dark period such as the 2L:6D lighting schedule. In Experiment 2, chicks were weighed individually on D0 and BW was added as a covariable. In a preliminary analysis, it was found that femur weight and length at D4 positively correlated with BW at D0 (data not shown). This suggests that the weight of the day-old-chick may influence subsequent bone development as well.

Increased activity would also explain why FCR was highest, indicating less efficient conversion from feed to body mass, in animals kept in continuous (24L) light compared to a lighting schedule or with long total exposure to light per day (2L:1D lighting schedule) compared to the 2L:6D lighting schedule. More energy may have been spent on activity in the treatment groups with prolonged light period. This is supported by findings of Apeldoorn *et al.* (1999), who found that broiler FCR was less efficient, and ME: GE ratio tended to be lower for their 23L:1D lighting schedule compared to their 1L:3D lighting schedule when applied from D0 till D20. They found that this improved efficiency was due to higher metabolization of the diet through improved digestion and a lower amount of energy spent on activity (Apeldoorn *et al.*, 1999). This improved metabolization of the diet might be reflected in the organ weights found in Experiment 1: intestine weight was higher for treatments with a dark period than for 24L, suggesting development of organs involved in the gastro-intestinal tract was prioritized when dark periods were applied.

Another explanation for the difference in bone development between 24L and particularly the 2L:6D lighting schedule are the effects on feed intake. Feed intake was higher for 24L compared to applying a lighting schedule and higher for the 2L:1D lighting schedule than for the 2L:6D lighting schedule in both experiments. Lewis *et al.* (2009) demonstrated that broilers will learn to eat in dark when they are kept under prolonged (> 16 h) dark periods in a lightproof room, similar to the present study. However, they subjected broilers to an uninterrupted dark period (Lewis *et al.*, 2009), whereas the dark period in the present study lasts a maximum 6 h at a time. This may have decreased the broiler's incentive to start eating in the dark compared to a continuous long dark period, and resulted in lower overall feed intake for the 2L:6D lighting schedule compared to the other treatments. The higher nutrient availability through increased feed intake in 24L may have facilitated maximal bone development. Bruno *et al.* (2007) exposed 7-day-old broilers to qualitative feed restriction by lowering protein or energy levels compared to ad libitum feeding. Both protein and energy restriction reduced femur width and humerus

weight, indicating that lower nutrient availability may retard bone development (Bruno *et al.*, 2007).

Although 24L led to increased leg bone diameter compared to applying a lighting schedule, it also resulted in increased RA of tibia and femur length. RA is regarded a measure of developmental stability, with higher asymmetry values resulting in worse functional morphology. It is thought to be the result of stressful or unfit environmental conditions (Møller *et al.*, 1999). It is therefore not unexpected that the current study as well as and those of Møller *et al.* (1999) and Campo *et al.* (2007) found higher mean RA values for chickens exposed to 24L compared to those exposed to a lighting schedule of 16L:8D (applied from D3 till slaughter in broilers; Møller *et al.*, 1999) or 14L:10D (applied from 20 to 36 wk of age in various chicken breeds; Campo *et al.*, 2007). It was furthermore found that a 2L:1D lighting schedule increased RA of tibia length, but decreased RA of tibia diameter compared to a 2L:6D lighting schedule. The effect of dark period length on developmental instability therefore seems less clear.

The 3 min dimming period in Experiment 1 was possibly too short to show differences between dimming and abrupt light-dark transition. It was therefore extended to a 15 min period in Experiment 2. Dimming did not have a large effect on bone development, but it decreased tibia length in Experiment 1 as well as femur length, although only numerically, in Experiment 2. This contradicts our hypothesis that dimming would increase bone development, although the fact that RA of tibia length and diameter was higher in the abrupt treatment may suggest this treatment was indeed more stressful and resulted in more developmental instability (Møller *et al.*, 1999). The retarding effect of dimming on bone development found in the present study might again be explained by different activity levels between dimming treatments. Sherlock *et al.* (2010) observed a tendency for higher activity in a treatment with an abrupt light-dark transition compared to their treatment with a step-up light transition. In that experiment, an abrupt or step-up transition did not result in differences in leg health at slaughter age (Sherlock *et al.*, 2010). Possibly, our gradual dimming provided a larger contrast from the abrupt transition than a step-up schedule, but long-term effects were not investigated in the present experiment.

How leg bone development found in the present study at D4 post-hatch relates to leg problems in later life is unknown. As mentioned previously, prolonged (>20 h) light periods, when maintained until slaughter age, have been associated with increased incidence of leg problems and lower walking ability, even when body weights were not different from other treatments (Buckland *et al.*, 1976; Renden *et al.*, 1996; Sanotra *et al.*, 2002; Brickett *et al.*, 2007). Furthermore, high RA (as found in the 24L treatment compared to applying a lighting schedule) at slaughter has been related to tibial dyschondroplasia and poor gait scores (Møller *et al.*, 1999; Sanotra *et al.*, 2001). It can therefore be speculated that 24L may accelerate leg bone development, but does not lead to improved leg health.

To conclude, leg bone length at D4 post-hatch was higher when a light-dark schedule

with a shorter dark period of the 2L:1D lighting schedule was applied compared to the 2L:6D lighting schedule, and leg bone diameter was highest for chicks exposed to 24L for 4 days post-hatch compared to applying a lighting schedule. However, RA, as an indicator of developmental instability, increased for 24L compared to applying a lighting schedule or providing an abrupt light-dark transition compared to dimming. How this relates to later life leg problems and performance is unknown.

5 | Acknowledgements

The authors wish to thank hatchery Probroed & Sloot Langenboom (Langenboom, the Netherlands) for donating chickens and their assistance with the experiment.

6 | References

Apeldoorn, E. J., J. W. Schrama, M. M. Mashaly, and H. K. Parmentier. 1999. Effect of melatonin and lighting schedule on energy metabolism in broiler chickens. Poult. Sci. 78: 223–229.

Applegate, T. J., and M. S. Lilburn. 2002. Growth of the femur and tibia of a commercial broiler line. Poult. Sci. 81: 1289–1294.

Bessei, W. 2006. Welfare of broilers: a review. Worlds Poult. Sci. J. 62: 455–466.

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 Biol. Res. 13: 45–103.

Brickett, K. E., J. P. Dahiya, H. L. Classen, C. B. Annett, and S. Gomis. 2007. The impact of nutrient density, feed form, and photoperiod on the walking ability and skeletal quality of broiler chickens. Poult. Sci. 86: 2117–2125.

Bruno, L. D. G., B. C. Luquetti, R. L. Furlan, and M. Macari. 2007. Influence of early qualitative feed restriction and environmental temperature on long bone development of broiler chickens. J. Therm. Biol. 32: 349–354.

Buckland, R. B., D. E. Bernon, and A. Goldrosen. 1976. Effect of four lighting regimes on broiler performance, leg abnormalities and plasma corticoid levels. Poult. Sci. 55: 1072–1076.

Campo, J. L., M. G. Gil, S. G. D´avila, and I. Muñoz. 2007. Effect of lighting stress on fluctuating asymmetry, heterophil-tolymphocyte ratio, and tonic immobility duration in eleven breeds of chickens. Poult. Sci. 86: 37–45.

Cardinali, D. P., M. G. Ladizesky, V. Boggio, R. A. Cutrera, and C. Mautalen. 2003. Melatonin effects on bone: experimental facts and clinical perspectives. J. Pineal Res. 34: 81–87.

Classen, H. L., and C. Riddell. 1989. Photoperiodic effects on performance and leg abnormalities in broiler chickens. Poult. Sci. 68: 873–879.

Classen, H. L., C. Riddell, and F. E. Robinson. 1991. Effects of increasing photoperiod length on performance and health of broiler chickens. Br. Poult. Sci. 32: 21–29.

Coenen, A. M. L., E. M. T. J. Wolters, E. L. J. M. Vanluijtelaar, and J. H. Blokhuis. 1988. Effects of intermittent lighting on sleep and activity in the domestic hen. Appl. Anim. Behav. Sci. 20: 309–318.

Csernus, V. J., A. D. Nagy, and N. Faluhelyi. 2007. Development of the rhythmic melatonin secretion in the embryonic chicken pineal gland. Gen. Comp. Endocrinol. 152: 148–153.

EU. 2000. The welfare of chickens kept for meat production. European Commission, Health and Consumer Protection Directorate-General. Report of the Scientific Committee on Animal Health and Animal Welfare, adopted 21 March 2000.

European Medicines Agency. 2011. Trends in the sales of veterinary antimicrobial agents in nine European countries (2005–2009). EMA

238630/2011.

Hasan, K. M. M., Md.S. Rahman, K. M. T. Arif, and M. E. Sobhani. 2011. Physiological stress and aging: role of glucocorticoids (GCs). AGE 34: 1421–1433.

Kestin, S.C, T. G. Knowles, A. E. Tinch, and N. G. Gregory. 1992. Prevalence of leg weakness in broiler chickens and its relationship with genotype. Vet. Rec. 131: 190–194.

Kristensen, H. H. 2008. The effects of light intensity, gradual changes between light and dark and definition of darkness for the behaviour and welfare of broiler chickens, laying hens, pullets and turkeys. Scientific Report for the Norwegian Scientific Committee for Food Safety.

Laakso, M., L. Leinonen, S. Joutsiniemi, T. Porkka-Heiskanen, and D. Stenberg. 1992. Pineal melatonin and locomotor activity of rats under gradual illuminance transitions. Physiol. Behav. 52: 621–628.

Lewis, P. D., R. Danisman, and R. M. Gous. 2009. Photoperiodic responses of broilers. III. Tibial breaking strength and ash content. Br. Poult. Sci. 50: 673–679.

Molenaar, R., R. Meijerhof, I. van den Anker, M. J. W. Heetkamp, J. J. G. C. van den Borne, B. Kemp, and H. van den Brand. 2010. Effect of eggshell temperature and oxygen concentration on survival rate and nutrient utilization in chicken embryos. Poult. Sci. 89: 2010–2021.

Møller, A. P., G. S. Sanotra, and K. S. Vetergaard. 1999. Developmental instability and light regime in chickens (Gallus gallus). Appl. Anim. Behav. Sci. 62: 57–71.

Müller, G. B. 2003. Embryonic motility: environmental influences and evolutionary innovation. Evol. Dev. 5: 56–60.

Ostrowska, Z., B. Kos-Kudla, B. Marek, D. Kajdaniuk, and N. Ciesielska-Kopacz. 2002. The relationship between the daily profile of chosen biochemical markers of bone metabolism and melatonin and other hormone secretion in rats under physiological conditions. Neuroendocrinol. Letters 23: 417–425.

Renden, J. A., S. F. Bilgili, R. J. Lien, and S. A. Kincaid. 1991. Live performance and yields of broilers provided various lighting schedules. Poult. Sci. 70: 2055–2062.

Renden, J. A., S. F. Bilgili, and S. A. Kincaid. 1992. Effects of photoschedule and strain cross on broiler performance and carcass yield. Poult. Sci. 71: 1417–1426.

Renden, J. A., E. T. Moran, Jr., and S. A. Kincaid. 1996. Lighting programs for broilers that reduce leg problems without loss of performance or yield. Poult. Sci. 75: 1345–1350.

Robson, H., T. Siebler, S. M. Shalet, and G. R. Williams. 2002. Interactions between GH, IGF-I, Glucocorticoids, and thyroid hormones during skeletal growth. Pediatr. Res. 52: 137–147.

Roth, J. A., B. Kim, W. Lin, and M. Cho. 1999. Melatonin promotes osteoblast differentiation and bone formation. J. Biol. Chem. 31: 22041–22047.

Sanotra, G. S., J. D. Lund, A. K. Ersboll, J. S. Petersen, and K. S. Vestergaard.

2001. Monitoring leg problems in broilers: a survey of commercial broiler production in Denmark. Worlds Poult. Sci. J. 57: 55–69.

Sanotra, G. S., J. Damkjer Lund, and K. S. Vestergaard. 2002. Influence of light-dark schedules and stocking density on behaviour, risk of leg problems and occurrence of chronic fear in broilers. Br. Poult. Sci. 43: 344–354.

SAS Institute Inc. 2004. SAS/STAT User's Guide. Version 9.1. SAS Inst. Inc. Cary, NC.

Sherlock, L., T. G. M. Demmers, A. E. Goodship, I. D. McCarthy, and C. M. Wathes. 2010. The relationship between physical activity and leg health in the broiler chicken. Br. Poult. Sci. 51: 22–30.

Torres, C. A. 2013. Influence of maternal flock age, maternal trace mineral nutrition and incubation temperature on bone development of embryos and chicks. Doctoral dissertation, University of Alberta.

Van der Eerden, B. C. J., M. Karperien, and J. M. Wit. 2003. Systemic and local regulation of the growth plate. Endocr. Rev. 24: 782–801.

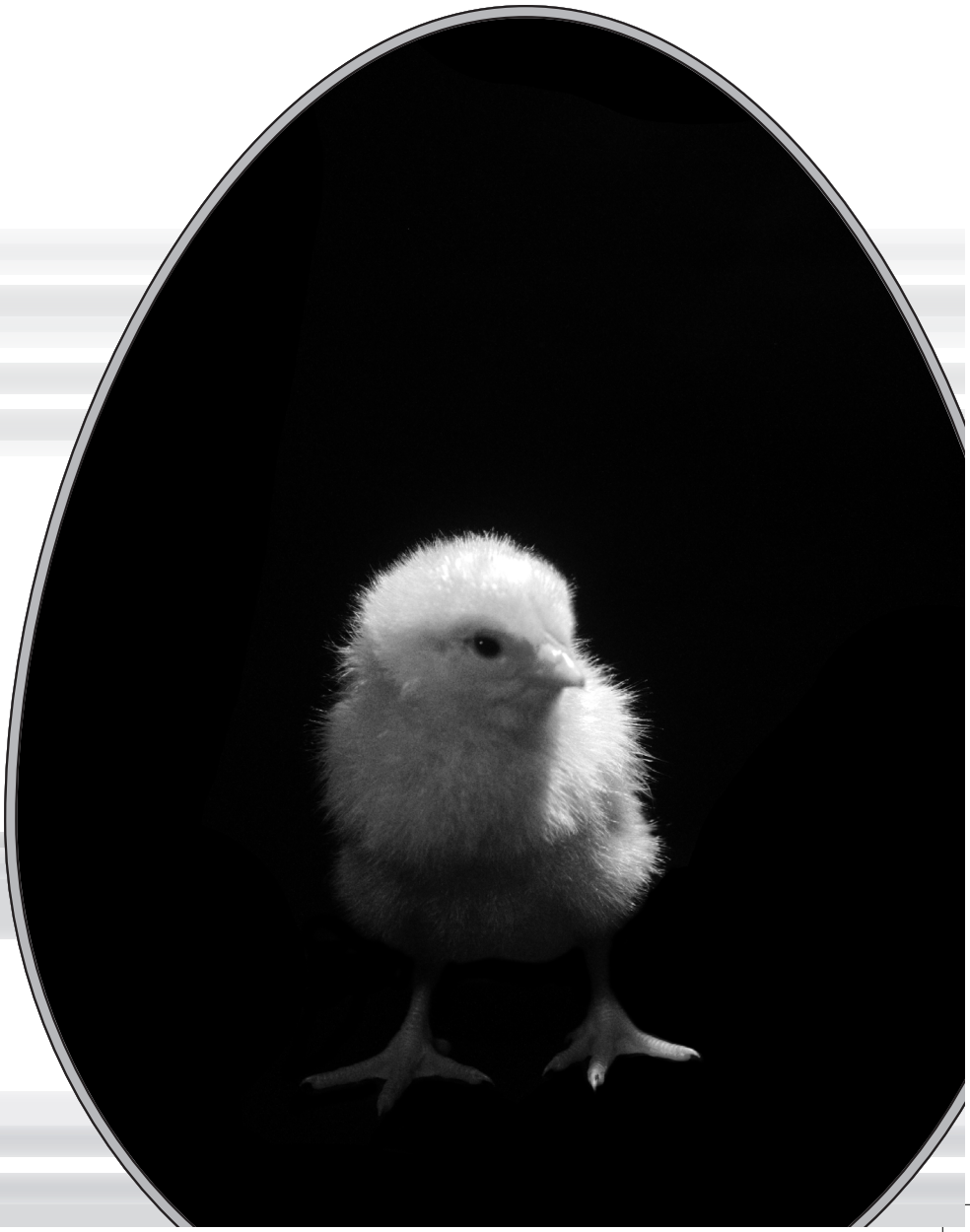
Van der Pol, C. W., I. A. M. van Roovert-Reijrink, C. M. Maatjens, H. van den Brand, and R. Molenaar. 2013. Effect of relative humidity during incubation at a set eggshell temperature and brooding temperature posthatch on embryonic mortality and chick quality. Poult. Sci. 92: 2145–2155.

Wilson, J. L., W. D. Weaver, Jr., W. L. Beane, and J. A. Cherry. 1984. Effects of light and feeding space on leg abnormalities in broilers. Poult. Sci. 63: 565–567.

Yeates, N. T. M. 1963. The activity pattern in poultry in relation to photoperiod. Anim. Behav. 11: 287–289.

Chapter 8

General discussion



1 | Introduction

Leg pathologies in broiler chickens constitute both a production and a welfare problem. The prevalence is not completely clear, but approximately 30% of the 51,000 broiler chickens observed in the field by Knowles *et al.* (2008) in the UK and the 2,800 broiler chickens observed by Sanotra *et al.* (2001) in Denmark were found to have leg scores of >2, which indicates that these broilers were mildly to severely lame (Kestin *et al.*, 1992). Leg pathologies are painful for broiler chickens (McGeown *et al.*, 1999), and they limit the broiler's ability to express its natural behaviours (Weeks *et al.*, 2000). The aetiology of several of the most common leg pathologies in broiler chickens seems to lie in suboptimal early life development of the bone (Bradshaw *et al.*, 2002). Tibial dyschondroplasia, varus/valgus deformities, rotated tibia, and growth plate abnormalities are commonly considered to be leg problems with a developmental origin (Bradshaw *et al.*, 2002; Bessei, 2006). Bacterial chondronecrosis with osteomyelitis (BCO) and femoral head separation (epiphyseolysis) may also have an aetiology that lies at least partly in suboptimal early life bone development (Wideman and Prisby, 2013; Packialakshmi *et al.*, 2015) (see Chapter 1).

During incubation, the skeleton is first formed as a cartilage model (Kürtül *et al.*, 2009). Later, a collar of bone material is formed around the cartilage model at the mid-diaphysis, in the primary ossification centres (Pechak *et al.*, 1986a,b). At the epiphyseal plate, length growth is realized by cartilage formation, which then becomes ossified; this process continues post hatch (Applegate and Lilburn, 2002; Kürtül *et al.*, 2009). Affecting cartilage formation and ossification might affect the incidence of developmental leg bone pathologies in later life. Exposing a broiler embryo to light during incubation may be a tool to improve this early life bone development. Applying a circadian lighting schedule during incubation has previously been shown to result in rhythmic melatonin release from the pineal gland, which was maximized during the dark period (Zeman *et al.*, 1999, 2004). Archer and Mench (2014a) showed that incubating broiler chickens under 12 hours of light, followed by 12 hours of darkness (12L:12D) not only led to plasma melatonin peaks in the dark periods, but also to higher average melatonin levels over a 24 hour period compared to 24D on embryonic day (E)19. Melatonin is known to stimulate embryonic bone development, as evidenced by retarded bone development in pinealectomised chicken embryos (Machida *et al.*, 1995). It could therefore be hypothesized that incubating broiler chickens under a circadian lighting schedule may result in maximized and rhythmic melatonin release (Archer and Mench, 2014a), resulting in increased bone development and improved leg health at slaughter age. It was speculated in Chapter 1 that a similar phenomenon might also be at play when chickens are exposed to a lighting schedule in the first few days post hatch compared to continuous light, with improved leg bone development for chickens exposed to a lighting schedule.

Aim of this thesis was to investigate effects of light and lighting schedules during incuba-

tion and in the early post hatch phase of broiler embryos and chickens on leg health at slaughter age. Bone morphology was studied during embryonic development, at hatch, and during the grow out period to reveal how these treatments affected bone development directly and long term. Possible underlying mechanisms were furthermore investigated, with special emphasis on the involvement of the endocrine pathways through melatonin, growth hormone (GH), and IGF-I.

2 | Leg pathologies

2.1 | Effects of light during incubation

Chapter 3 and 6 describe how lighting schedules during incubation affect leg health of broiler chickens at slaughter age (Table 1). In Chapter 3, broiler eggs were incubated under continuous cool white LED light (24L); 12L:12D of cool white LED light; or continuous darkness from set until hatch of the eggs. In Chapter 6, a similar setup was used, but a circadian lighting schedule of 16 hours of light, followed by 8 hours of darkness (16L:8D) was applied instead of 12L:12D. It was found that 12L:12D resulted in a 7.1% lower incidence of tibial dyschondroplasia compared to 24L, with 24D intermediate. When leg pathologies were scored as 1 = absent to 4 = severe, it was found that 16L:8D and 24D resulted in a lower average score for epiphyseal plate abnormalities, and 16L:8D resulted in a lower average score for BCO than 24D, with 24L intermediate. 16L:8D furthermore tended to have a lower average score for tibial dyschondroplasia than 24D, with 24L intermediate. Numerically, 16L:8D also resulted in lowest incidence of rotated tibia and valgus/varus deformities, and the highest percentage of chickens without any leg problems.

When chickens were scored as having 1, 2, 3, or ≥ 4 leg bone pathologies (in which each pathology was counted per leg; for example, a chicken with tibial dyschondroplasia in both legs was scored as having 2 pathologies), 24L was shown to result in the highest number of chickens with 3 or ≥ 4 leg bone pathologies. This shows that incubation under 24L not only resulted in fewer chickens without leg bone pathologies, but also in a higher percentage of chickens that suffered from various types of leg problems, and in more than one leg, compared to 16L:8D and 24D.

The fact that incidence and severity of BCO, tibial dyschondroplasia, and epiphyseal plate abnormalities were affected on D35 post hatch by lighting schedules that were applied only during the incubation period suggests that differences in bone morphology or physiology had developed already during embryogenesis. To better understand how embryonic bone development may have resulted in differences in leg bone pathologies at slaughter age, the aetiology of BCO, tibial dyschondroplasia, and epiphyseal plate

abnormalities is discussed in more detail.

Table 1. Overview of leg health variables (pathologies and gait scores) in broiler chickens incubated under continuous light (24L), 12 hours of light, followed by 12 hours of darkness (12L:12D; Chapter 3), 16 hours of light, followed by 8 hours of darkness (16L:8D; Chapter 6), or continuous darkness (24D) from set until hatch on D34 or D35 post hatch.

Leg problem variable				
Pathologies (% incidence D35)	**24L**	**12L:12D**	**24D**	**P < 0.05**
Tibial dyschondroplasia	8	1	2	Yes
	24L	**16L:8D**	**24D**	
BCO	3	1	6	Yes
Epiphyseal plate abnormalities	18	9	7	Yes
Epiphyseolysis	37	30	27	
Rotated tibia	18	16	17	
Tibial dyschondroplasia	13	7	15	
Valgus/varus deformity	25	25	28	
Incidence of pathologies within a chicken (% of chickens)[1]	**24L**	**16L:8D**	**24D**	**P < 0.05**
0 leg bone pathologies	23	29	24	
1 leg bone pathology	9	17	13	
2 leg bone pathologies	20	21	26	
3 leg bone pathologies	20	9	15	
≥4 leg bone pathologies	28	24	22	
Gait scores	**24L**	**16L:8D**	**24D**	**P < 0.05**
D21	1.5	1.4	1.5	
D28	2.0	1.9	1.9	
D34	2.9	2.8	2.8	

[1] Each pathology observed in 1 leg is considered 1 incidence; for example, tibial dyschondroplasia in both legs of 1 chicken counts as 2 leg bone pathologies.
Within a row, dark grey denotes the worst value in terms of leg health (highest incidence of the leg pathology or highest gait score), light grey denotes the intermediate value, and white denotes the best value in terms of leg health.

2.2 | Development of leg pathologies

Tibial dyschondroplasia, BCO, and epiphyseal plate abnormalities share a common origin in chondrocyte (cartilage cell) abnormalities at the epiphyseal plate. In tibial dyschondroplasia, hypertrophic chondrocytes fail to become vascularized and form a

surrounding calcified matrix. As a result, a plug of cartilage with necrotic and apoptotic chondrocytes is observed below the epiphyseal plate, possibly extending into the metaphysis (Hargest *et al.*, 1984). In BCO, damage to the poorly mineralized and vascularized columns of chondrocytes at the epiphyseal plate facilitates colonization by pathogenic bacteria, such as Staphylococcus aureus, Enterococcus cecorum, and Escherichia coli (Wideman and Prisby, 2013). Histologically, blood vessels of the epiphysis or epiphyseal plate are found to be partly or completely occluded by bacterial clumps, and the vessels are surrounded by cartilaginous matrix containing necrotic chondrocytes (McNamee and Smyth, 2000). The lesions observed in BCO may also extend into the metaphysis (McNamee *et al.*, 2000). Epiphyseal plate abnormalities, as described here, comprise more general abnormalities, such as widening or an irregular shape of the epiphyseal plate, without the symptoms of tibial dyschondroplasia or BCO. Possibly, epiphyseal plate abnormalities as observed in this study are actually early stages of BCO or tibial dyschondroplasia, and all three may share a common aetiology in suboptimal early life cartilage conversion and vascularization at the epiphyseal plate.

Valgus/varus deformity and rotated tibia are abnormalities in the bone's curvature, with valgus/varus showing angular deformity of the tibia and the hock joint (Duff and Thorp, 1985) and rotated tibia showing torsional deformity of the whole bone (Shim *et al.*, 2012). Their aetiology is not well understood, but it can be speculated that suboptimal joint angles and bone conformity, weakness of tissue supporting the joint, and poor ossification play a role (Bradshaw *et al.*, 2002). Valgus/varus deformity and rotated tibia were not significantly affected by incubation lighting schedules. It seems that leg bone pathologies originating in abnormalities at the epiphyseal plate are more strongly affected by incubation lighting schedule than leg bone pathologies related to abnormal curving, although they are not completely independent of each other: there are indications that presence of tibial dyschondroplasia in the leg increases the chance that a chicken will develop valgus/varus deformities (Randall and Mills, 1981).

Leg abnormalities were determined macroscopically in the current thesis. Microscopic and histological examination of the lesions found in the BCO and tibial dyschondroplasia affected chickens may have provided more insight into the extent and nature of the abnormalities, and it would be suggested to perform these more detailed analyses in future studies on light during incubation and leg bone pathologies. Leg bone morphology, on the other hand, has been studied in more detail in the present thesis and could provide information about how leg bones of broiler chickens exposed to different incubation lighting schedules differ in their development, embryonically, at hatch, and post hatch. These differences are described in the following paragraphs.

3 | Light during incubation and bone development

3.1 | Embryonic

Incubation lighting schedules were shown to have a long lasting effect on leg bone pathologies post hatch, with better leg health at slaughter age for broiler chickens exposed to a circadian lighting schedule during incubation compared to 24D (in the case of BCO) and 24L (in the case of tibial dyschondroplasia and epiphyseal plate abnormalities). It could therefore be expected that leg pathologies originate from differences in bone development that already arose during the embryonic phase. The leg problems that were found to be affected by incubation lighting schedules seemed to share a common aetiology in suboptimal epiphyseal plate development. In the epiphyseal plate, length growth takes place (Roach, 1997; Mackie *et al.*, 2008). It could therefore be speculated that incubation lighting schedules led to differences in leg bone development during embryogenesis and leg bone morphology at hatch.

Chapters 2 and 4 describe how incubation lighting schedules affected leg bone development during incubation. During embryonic development, bone structures are formed by laying out the leg bones in a cartilage model (Kürtül *et al.*, 2009), and increase in length takes place mainly in the bone's epiphyseal plate, where cartilage is converted into bone tissue through endochondral ossification (Roach, 1997; Mackie *et al.*, 2008). This process does not seem to be affected by incubation lighting schedule yet by E14. Femur and tibia length were not found to differ between 24L, 12L:12D, and 24D when measured between E8 and E14. Some differences in femur and tibia width were found on different days, but they were inconsistent and never lasted longer than a day.

The cartilage model is converted into bone in the middle of the bone structure by E9 (Kürtül *et al.*, 2009). This takes place in the primary ossification centre (Kürtül *et al.*, 2009), by replacing the cartilage with bone marrow, and depositing a mineralized osteoid (bone tissue) ring around the shaft of the bone (Pechak *et al.*, 1986a,b). In the embryonic femurs and tibias sampled in Chapter 3, the ossified portion was stained between E8 and E14. It was found that ossification started approximately one day earlier in some embryos of 24D than in all embryos of 12L:12D and 24L. By E13 and E14, the ossified percentage of the tibia was higher for 12L:12D than for 24L, but not for 24D. Ossification from the primary ossification centre is mostly the result of osteoblasts (bone forming cells), derived directly from mesenchymal progenitor cells, producing osteoid around the cartilage core (Pechak *et al.*, 1986a,b). These results suggest that ossification at the primary ossification centre is increased or onset of ossification is earlier in embryos incubated under 24D or 12L:12D compared to 24L, possibly through a stimulating effect on osteoclast activity. To conclude, when leg bone dimensions and ossification were measured up till E14, incubation lighting schedule was shown to affect ossification, but

not length growth of the whole bone.

3.2 | Hatch

Many variables that were studied at hatch did not differ significantly between incubation lighting schedules, but an overall pattern can be observed when data are combined in one table. Table 2 shows that in the femur, 12L:12D seemed to increase bone dimensions (length, weight, depth, and width) at hatch compared to 24L particularly, whereas16L:8D decreased them compared to 24D. Furthermore, femur microstructure measurements, revealed by CT scanning (cortical and medullary measurements and second moments of area), were all highest for 24D, and all lowest for 12L:12D. In the tibia, bone dimensions and microstructure were highest for 12L:12D in 7 out of 10 measurements, and always lowest for 24L. However, 16L:8D again resulted in lower bone dimensions for 3 out of 4 measurements at hatch than 24D. It appears that 24L and 16L:8D during incubation have a detrimental effect on embryonic bone development in chickens compared to 24D. 12L:12D is somewhat intermediate, but 24D appears to result in the most advanced leg bone development. The tibia seems to be more sensitive to these changes than the femur.

Because 12L:12D and 24D were found to increase ossification in the tibia's primary ossification centre during incubation in Chapter 3, this was studied in more detail in Chapter 5. It was investigated whether lighting schedules during incubation affected expression of genes involved in stimulation of cartilage and bone cells (Chapter 5). Collagen is a major component of cartilage, and three genes were chosen that are involved in collagen formation (*col1α2* for collagen type I, *col2α1* for collagen type II, and *col10α1* for collagen type X), and four genes coding for bone cell related proteins (*spp1* for osteopontin, *sparc* for osteonectin, *bglap* for osteocalcin, and *alpl* for alkaline phosphatase). Gene expression was measured on E13, E17, and at hatch in the tibia of broiler chickens exposed to 24L, 16L:8D, or 24D. Gene expression was not found to differ between treatments or time points. Possibly, 16L:8D did not result in an increased ossification rate or earlier onset of ossification compared to 24L, as was previously observed for 12L:12D in Chapter 3. Histological observations of embryonic ossification in the tibia and femur were not repeated in Chapter 5, so this cannot be confirmed.

It could also be that E13, E17, and hatch were not the right time points to find differences in gene expression. They were chosen as sampling time points, because ossified percentage was previously found to be higher in the tibia for 12L:12D than for 24L on E13 and E14, and differences in bone length were found at hatch. We expected that expression of genes involved in collagen formation and ossification would be upregulated for a circadian lighting schedule or 24D simultaneously with the increase in ossification. However, it might be possible that the increase in gene expression happened at an earlier stage and

Table 2. Overview of leg bone morphology at hatch in broiler chickens incubated under continuous light (24L), 12 hours of light, followed by 12 hours of darkness (12L:12D; Chapter 3), 16 hours of light, followed by 8 hours of darkness (16L:8D; Chapter 6), or continuous darkness (24D) from set until hatch.

Femur				
Measurement	**24L**	**12L:12D**	**24D**	**P < 0.05**
Weight	98	101	100	
Length	100	102	100	Yes
Width	99	100	100	
Depth	99	99	100	
Medullary area	94	91	100	
Cortical area	94	89	100	
Maximal cortical thickness[1]	98	89	100	
Mean cortical thickness[1]	97	94	100	
Major area moment	87	82	100	
Minor area moment	84	78	100	Yes
	24L	**16L:8D**	**24D**	
Weight	96	92	100	
Length	97	97	100	
Width	96	96	100	
Depth	98	94	100	

Tibia				
Measurement	**24L**	**12L:12D**	**24D**	**P < 0.05**
Weight	97	103	100	Yes
Length	99	101	100	Yes
Width	99	101	100	
Depth	98	99	100	Yes
Medullary area	94	100	100	
Cortical area	86	98	100	Yes
Maximal cortical thickness[1]	91	103	100	
Mean cortical thickness[1]	86	104	100	Yes
Major area moment	83	97	100	
Minor area moment	81	100	100	Yes
	24L	**16L:8D**	**24D**	
Weight	98	96	100	
Length	99	99	100	
Width	98	94	100	
Depth	97	93	100	

Within a row, dark grey denotes the lowest value, light grey denotes the intermediate value, and white denotes the highest value for that variable.

Values are expressed as % relative to 24D's value, which is set at 100%.
[1] Major and minor area moment = second moments of area around the major and minor axis.

histological differences were visible later than upregulated gene expression. We expected that differences could still be found at hatch, because Yalçin *et al.* (2007) demonstrated that collagen formation and ossification processes can still be affected at that time point. They investigated gene expression in the tibia through histology, and found differences in expression of genes between incubation temperatures at hatch (Yalçin *et al.*, 2007).

Possibly, gene expression is localized and quantitative analysis does not reveal these localized differences when a whole bone is measured. Embryonic ossification takes place in the primary ossification centres (Kürtül *et al.*, 2009) and at the epiphyseal plate (Roach, 1997). Indeed, in the hypertrophic or mineralizing zones of the epiphyseal plate, osteopontin (Yalçin *et al.*, 2007; McKee *et al.*, 1992), osteocalcin (McKee *et al.*, 1992), osteonectin (Pacifici *et al.*, 1990), and alkaline phosphatase (Väänänen, 1980; Yalçin *et al.*, 2007) have been found to be particularly expressed, which would suggest that specific sampling of these zones, or sampling the primary ossification centre and epiphyseal plate separately, may have revealed differences in embryonic and post hatch expression of genes involved in cartilage and bone formation.

It can be concluded that incubation lighting treatments affect leg bone development in broilers at hatch, but the effect is dependent upon which lighting schedule is used. 12L:12D was found to have a stimulatory effect on bone development compared to 24L especially, while 16L:8D resulted in less bone development than 24D and sometimes 24L. Therefore, when applying a circadian lighting schedule during incubation, dark periods should possibly not be shorter than 12 hours. 24D generally seems to have a positive effect on leg bone development parameters compared 24L especially. However, leg bone pathologies were improved by 16L:8D compared to both 24L and 24D. This would suggest that leg bone development measurements at hatch cannot always easily be translated to improved leg bone health in later life. Leg bone morphology was therefore measured post hatch, too, to investigate long term effects of incubation lighting schedule.

3.3 | Post hatch

Effects of incubation lighting schedule on bone morphology during incubation and at hatch were long term. An overview of all leg bone measurements at D35 from Chapter 3 and Chapter 6 can be seen in Table 3. These results suggest that applying 12L:12D during incubation has a long term effect on broiler tibia growth compared to 24L. Apparently, when a stimulatory effect of a circadian lighting schedule during incubation on leg bone length is found at hatch, results may last until slaughter age; but when no effect is found, it may not be seen at slaughter, either, as can be seen for 16L:8D. Although 24D seemed to show the most advanced bone development at hatching (Table 2), this effect

seemed to be mostly gone by D35.

The main objective of this thesis was to stimulate leg health through light during incubation and in the post hatch period. As shown in paragraph 2.1, 24L was found to be detrimental for leg health. Both 12L:12D and 16L:8D were found to improve leg health compared to 24L and 24D, and they can therefore both be considered to be potential lighting schedules to reduce leg pathologies in broiler chickens. However, 12L:12D was found to advance tibia development up till slaughter at D35 compared to 24L, while 16L:8D did not.

Bone microstructure, especially, gives insight into leg bone strength and resistance to bending forces (Augat and Schorlemmer, 2006). High bone cortical area, second moment of area, and bone size are associated with higher bone strength (Augat and Schorlemmer, 2006), and the risk of stress fractures may be reduced by a high moment of inertia (Milgrom *et al.*, 1989). Fractures were not examined in the current thesis, but it can be speculated that tibias with a high second moment of area, such as found for 12L:12D, may be less susceptible to fractures than tibias of chickens exposed to 24L.

The effect of incubation lighting schedule on post hatch growth is relevant in studies on broiler leg health and bone development, as high body weights are correlated with lameness in broiler chickens (Kestin *et al.*, 2001). Positive effects of a certain treatment could simply be due to lower load on the bones if the same treatment resulted in lower body weight gain. In Chapter 4, body weight did not differ between incubation lighting schedules for any of the weeks post hatch, or overall. In Chapter 6, body weight at slaughter age was found to be 2.3 to 2.7% (66.5 to 84.3 g) higher for 24L than for 16L:8D and 24D. Post hatch body weight was therefore added as a covariable in the statistical analysis of leg pathology scores. It did explain some of the variation, but effects of lighting treatments during incubation were still observed. It therefore appears that differences between incubation treatments in body weight and body weight gain cannot explain differences in leg bone development and leg health at slaughter age.

3.4 | Conclusions

It can be concluded that 24L had a detrimental effect on leg bone development, as was shown during embryogenesis, at hatch, and at slaughter age compared to 24D. 12L:12D had a stimulatory effect on tibia growth, especially, that became more pronounced as the chickens aged compared to 24L and, to a lesser degree, 24D. 16L:8D, did not greatly differ from 24D at D35. As 16L:8D resulted in better leg health at slaughter age compared to both 24L and 24D, leg bone morphology measurements cannot simply be translated to later life leg health.

Table 3. Overview of leg bone morphology at D35 post hatch in broilersincubated under continuous light (24L), 12 hours of light, followed by 12 hours of darkness (12L:12D; Chapter 3), 16 hours of light, followed by 8 hours of darkness (16L:8D; Chapter 6), or continuous darkness (24D) from set until hatch.

		Femur		
Measurement	**24L**	**12L:12D**	**24D**	**P < 0.05**
Length	100	100	100	
Weight	99	105	100	Yes
Width	99	100	100	
Depth	97	97	100	Yes
Medullary area	92	95	100	
Cortical area	95	88	100	
Maximal cortical thickness[1]	105	103	100	
Mean cortical thickness[1]	114	114	100	
Major area moment	87	79	100	
Minor area moment	89	83	100	
	24L	**16L:8D**	**24D**	
Length	100	100	100	
Weight	99	100	100	
Width	102	104	100	
Bone mineral content	110	111	100	
Bone mineral density	114	107	100	Yes
		Tibia		
Measurement	**24L**	**12L:12D**	**24D**	**P < 0.05**
Weight	97	101	100	
Length	99	101	100	Yes
Width	100	101	100	
Depth	102	102	100	
Medullary area	88	96	100	
Cortical area	88	107	100	Yes
Maximal cortical thickness[1]	117	113	100	Yes
Mean cortical thickness[1]	113	107	100	
Major area moment	79	110	100	Yes
Minor area moment	75	104	100	
	24L	**16L:8D**	**24D**	
Weight	100	102	100	
Length	99	99	100	
Width	102	104	100	
Bone mineral content	96	100	100	
Bone mineral density	99	103	100	

Within a row, dark grey lowest value, light grey denotes the intermediate value, and white denotes the highest value for that variable.
Values are expressed as % relative to 24D's value, which is set at 100%.
[1] Major and minor area moment = second moments of area around the major and minor axis

Variations in light during incubation

The experiments described in the present thesis with light during incubation used cool white LED light with a colour temperature of 6,050K and an intensity of 500 lux at the eggshell level, from set of the eggs until pull time of the chickens. The present thesis focuses on lighting schedules, but many more variations with light during incubation can be made, and it is not known how this would affect broiler leg bone development and leg bone pathologies. However, literature suggests that differences in lighting colour, intensity, and the phase during which it is applied may affect other variables of broiler embryonic development.

Colour

White LED light is not monochromatic, but rather consists of a range of wavelengths that together form light that is visually white to humans. White LED light can vary in its composition by having the peaks at slightly different wavelengths, or having different peak sizes, which together determine the colour temperature. Archer (2016) compared 24D to 12L:12D throughout incubation, with light provided by either cool (7,500 K) or warm (3,250 K) white LED light. To compare, in the present thesis, cool white LED with a colour temperature of 6,050 K was used. Archer (2016) found no effect of incubation lighting schedule on percentage of chickens with leg abnormalities at hatch, but at D14 post hatch, more composite asymmetry (indicating developmental instability) of the leg bones was found in the 24D incubated broilers than in the cool or warm white LED incubated broilers. Possibly, effects of white LED light on leg health at slaughter age do not depend on colour temperature, either.

Green light seems to have a particular stimulatory effect on embryonic development and muscle growth. Zhang *et al.* (2012) incubated Abor Acres eggs from E0 until hatch under continuous monochromatic green or blue cold LED light or darkness. Green incubated chickens had higher body weight gain, higher feed intake, and lower (better) feed conversion until D42, as well as higher relative and absolute breast muscle weight at D42 than blue and dark incubated chickens.

No difference was found between blue and dark incubated chickens. It would be interesting to investigate whether incubating broiler chickens under a circadian lighting schedule with various monochromatic light colours also leads to differences in embryonic leg bone development.

Intensity

In the present thesis, a light intensity of approximately 500 lux at the egg's level was used. Given that we found differences in several variables at hatch, it appears that this was sufficient to create differences in embryonic development. Archer (2016) observed differences in variables, such as hatchability, navel score, and post hatch behaviour for broilers incubated under 12L:12D of cool white LED light of 250 lux at the egg's level compared to 24D, suggesting that a lower intensity may also be sufficient to affect embryonic development. It is likely that many effects of light during incubation depend on the intensity of light, but evidence from literature to support this is not very strong, and often may have been confounded with embryo temperature (for example, Isakson *et al.*, 1970; Shafey *et al.*, 2005). The effect of light intensity during incubation on broiler leg bone development, specifically, has not been investigated yet.

Phase of light exposure

Özkan *et al.* (2009; 2012a) found that the final week of incubation may be the most relevant phase to affect embryonic breast muscle development through incubation lighting schedule. It is not known if bone development has a similar sensitive phase. Different developmental processes in the leg bones take place in different phases of incubation, which would suggest that lighting schedules should perhaps be applied during those phases to stimulate specific processes. For example, ossification starts around E9 and continues post hatch (Kürtül *et al.*, 2009), and melatonin release patterns become rhythmic by E16 to E18 (Csernus *et al.*, 2007). It can be speculated that applying a lighting schedule during the first week of incubation has less impact on leg bone development than applying it during the second and/or last week.

4 | Pathways

Light during incubation affected bone development and leg health in chickens as described above. Several pathways are discussed that may have influenced embryonic and post hatch bone development. The endocrine system, embryonic activity, and embryo temperature were investigated specifically in the current thesis. Brain lateralization is furthermore speculated to have played a role in differences found in later life leg health.

4.1 | Endocrine system

Providing a circadian rhythm during incubation seemed to have a more stimulatory effect on leg bone health and, depending on the rhythm, leg bone development than 24L, which is why particular focus lies on how circadian rhythmicity could have affected development. In this thesis, it was hypothesized that developing a circadian rhythm would be the most important mode of action of light during incubation on embryonic and early post hatch bone development. Circadian rhythms created by an 'endogenous clock' are present in all kinds of levels of physiology: from whole organ systems to individual cells. When embryos are exposed to 24L or 24D, they will still show a circadian rhythm in physiology of for example, heart rate, hormone release, and metabolism, that oscillates around roughly 24 hours (reviewed by Takahashi and Zatz, 1982). These rhythms are free running; each embryo can be in another phase than its neighbour, and the amplitude of circadian effects may be smaller than when a circadian lighting schedule is provided (Lamošová *et al.*, 1995).

An important endocrine factor involved in the creation of circadian rhythms is melatonin, which has been shown to stimulate embryonic chicken bone development (Machida *et al.*, 1995). Melatonin synthesis and release is known to be increased in darkness (Özkan *et al.*, 2012a). Melatonin levels were expected to be higher for 24D than for 24L or in the light periods of incubation lighting schedules. Melatonin was furthermore expected to peak highest for embryos in the dark periods of 12L:12D and 16L:8D compared to 24L or 24D, because this was observed previously (Zeman *et al.*, 1999; Özkan *et al.*, 2012a), both in plasma and in the pineal gland (Zeman *et al.*, 1999; Zawilska *et al.*, 2007). Probably most importantly for bone development, overall melatonin levels were found to be higher for broilers incubated under 12L:12D compared to broilers incubated under 24D by Archer and Mench (2014a). A total higher melatonin level could be speculated to be most beneficial for bone development. However, this was not found in the current thesis. Plasma melatonin in Chapter 3 and pineal melatonin in Chapter 5 were taken in 4 to 6 hour intervals between E18.8 and E19.5, and they were not found to differ between treatments. Literature shows that chicken pineal glands show a lighting schedule dependent release pattern in melatonin synthesis and release, both in vivo in broiler chickens (Zeman *et al.*, 1999; Zeman *et al.*, 2004) and in turkeys (Zawilska *et*

al., 2007), and in vitro in chickens (Lamošová *et al.*, 1995), using cool white fluorescent tubes, and in vitro in chickens using incandescent light bulbs (Csernus *et al.*, 2007), but this could not be confirmed in the experiments presented in this thesis.

Not all studies on incubation lighting schedules find a very clear circadian rhythm in melatonin. At internal pipping, Özkan *et al.* (2012a) did not find a difference in plasma melatonin levels between broilers incubated under 16L:8D or 24D, and no significant interaction between incubation lighting schedule and light or dark sampling time. At hatch, again no interaction between incubation lighting schedule and sampling time or main effect of incubation lighting schedule on plasma melatonin levels was found. However, a main effect of sampling time was found both at internal pipping and at hatch, showing higher plasma melatonin levels for the sampling time that corresponded with 16L:8D's dark period than for the sampling time that corresponded with its light period (Özkan *et al.*, 2012a). In Chapter 5, blood was drawn from the jugular vein in the embryos used for pineal melatonin analysis before the pineal glands were removed. The whole procedure of collecting the embryo from the incubation envrionment, sampling the blood, decapitating the embryo and collecting and storing the pineal gland took on average 5.5 minutes per embryo. It is possible that embryos were exposed to light during blood sampling for too long, which may have affected their melatonin rhythm. It is not known how quickly melatonin degrades in vivo in the pineal gland after embryos are exposed to light.

Two other factors involved in bone development, GH and IGF-I, were expected to peak along with melatonin in dark periods, as melatonin is said to have a regulatory effect on GH and IGF-I expression (Ostrowska *et al.*, 2002). In Chapter 3, plasma GH at hatch was found to be 14.5 to 21.8% higher for 24D than for 12L:12D and 24L. According to Monsonego *et al.* (1995), GH inhibited differentiation of chondrocytes at the epiphyseal plate when cultured in vitro. This allowed chondrocyte to remain in their proliferative state longer, and when combined with epidermal growth factor, chondrocyte proliferation was upregulated by GH (Monsonego *et al.*, 1995). In the epiphyseal plate, length growth of the long bones is realized by producing cartilage that then becomes ossified. Zones of resting, proliferating, hypertrophying, and calcified cartilage cells can be distinguished. Once the cartilage matrix becomes ossified, the cartilage cells become apoptotic or become osteoblast-like cells (Roach, 1997; Mackie *et al.*, 2008). If GH (combined with epidermal growth factor) increases chondrocyte proliferation, it may indirectly increase length growth of the bone. In Chapter 3, length of the tibia at hatch was found to be longer for 12L:12D and 24D than for 24L, but length of the femur was longer for 12L:12D than for both 24L and 24D. Overall, leg bone dimensions seemed to be highest for 24D compared to 24L and, to a lesser degree, light-dark rhythms during incubation (Table 2). However, plasma GH, when measured between E18.75 and E19.5, did not differ between treatments in Chapter 5, and it did not show a release pattern similar to melatonin. A clear relation between GH and leg bone dimensions at hatch was therefore

not found in the present thesis. IGF-I at hatch did not differ between treatments. In Chapter 6, no effect of incubation lighting schedule on IGF-I levels was found, and levels did not follow the same rhythm as pineal melatonin embryonically. The potential role of GH and IGF-I in bone development was therefore not clarified by the current thesis.

Although literature suggests that circadian lighting schedules during incubation affect rhythmicity of the endocrine system through the involvement of melatonin and associated factors, results from this thesis do not support this. It is therefore not confirmed by this thesis that the stimulatory effect of circadian lighting schedule during incubation on embryonic bone development takes place through the involvement of melatonin, GH, and IGF-I. The thyroid hormone T3 and its precursor T4 were also speculated to be involved in embryonic bone development in chickens (Van der Eerden *et al.*, 2003), but they were not measured in the current thesis.

4.2 | Embryonic activity

Movement is known to be an important stimulator of bone development, and post hatch lack of activity is noted to be one of the main reasons why leg health is poor in broiler chickens (Bradshaw *et al.*, 2002; Bessei, 2006). Chicken embryos are also active, with contractions starting halfway through E4 and movements peaking after the first week of incubation (Bekoff, 1981). Embryonic movements already influence bone development during chicken incubation. In paralyzed chicken embryos, cartilage and joints were retarded in their development, fused, or even completely absent (Murray and Drachman, 1969), and limb bones were shorter (Bertram *et al.*, 1996) compared to embryos that were not paralyzed, and able to move. Effects of light during incubation on embryo activity are, however, not completely revealed yet.

In Chapter 5, embryonic heart rate was measured as a reflection of embryonic activity twice a day, during 16L:8D's light and dark period. When heart rate was averaged per day, a pattern may be observed (Figure 1). From E14 till E16, embryonic heart rate was higher in 24D than in 24L, with 16L:8D mostly intermediate. On E18, it tended to be highest for 16L:8D, and on E19, it was higher for 16L:8D than for 24L and 24D. When heart rate was averaged over days, 16L:8D had higher total heart rate than 24L. In human foetuses, heart rate increases linearly with foetal movement (DiPietro *et al.*, 2001). In chicken embryos, on the other hand, Pollard *et al.* (2016) found that higher heart rate correlated with fewer embryonic movements. However, these findings may have been a result of overheating. Pollard *et al.* (2016) incubated their eggs at a constant air temperature of 37.0°C. Air temperature can differ greatly from temperature at the embryo's level, because of differences in embryonic heat production or heat transfer from the egg (Lourens *et al.*, 2011). Especially during the last week of incubation, when heat production rises (Molenaar *et al.*, 2010), embryos are at risk of overheating when air temperature

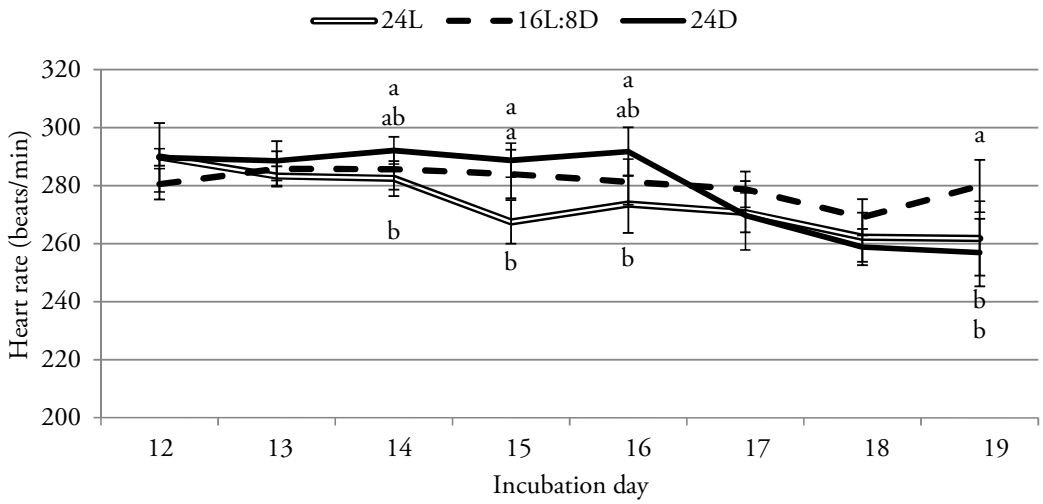


Figure 1. Heart rate between E12 and E19 of broiler chickens incubated under continuous light (24), 16h of light, followed by 8h of darkness (16L:8D), or continuous darkness (24D) from set until hatch (Chapter 5). a,b data labels with differing letters within a day differ significantly at $P \leq 0.05$.

is kept at a constant value. Noiva *et al.* (2014) demonstrated that at high air temperatures (38.9°C), broiler embryos increased their heart rate, and decreased their movements compared to broiler embryos incubated at an air temperature of 37.8°C. Both can be speculated to be mechanisms to reduce body temperature: a higher heart rate can be used to increase blood flow to the chorioallantoic membrane and thus increase heat loss, and lower activity can reduce heat production.

In the current thesis, embryos were incubated at a constant eggshell temperature (EST) of 37.8°C and were therefore not overheated (paragraph 4.3). It can therefore be speculated that heart rate indeed reflected higher activity in Chapter 5. This would suggest that 24D embryos were more active than 24L embryos between E14 and E16, but 16L:8D embryos were more active than 24L embryos on E19 and overall. As overall leg bone development seemed to be most advanced in 24D at hatch (see paragraph 3.2), it can be speculated that the time period between E14 and E16 is particularly important when it comes to the stimulation of embryonic activity on leg bone development. However, it does not explain why 16L:8D incubated broilers showed the lowest leg bone dimensions at hatch. It seems likely that embryonic activity, when measured through heart rate, can explain only part of the differences in leg bone development found at hatch under different incubation lighting schedules.

4.3 | Embryo temperature

An important regulator of chicken embryonic metabolism and developmental rate is embryo temperature, and it may differ from air temperature, because of differences in heat transfer from the egg and heat production of the embryo (Lourens *et al.*, 2011). Because of the large impact of EST on embryonic development, it is important to consider EST when studying light during incubation. Applying light during incubation may additionally heat up the eggs if no preventative measures are taken. To examine how temperature during incubation affected embryonic leg bone development in broiler chickens, broiler eggs were exposed to 4 different EST from set until hatch of the eggs in Chapter 2: low (36.9°C), normal (37.8°C), high (38.6°C), and very high (39.4°C). Largest differences in absolute bone sizes at hatch were found between the very high and normal or high temperature treatments, with lower tibia (-6.7 to -8.4%) and metatarsus (-6.0 to -6.7%) lengths for very high than for normal and high EST, irrespective of body weight (which was added as a covariable in the model during statistical analysis). These results emphasize that EST affects leg bone development. All incubation experiments in the current thesis were therefore performed at an EST of 37.8°C, to eliminate a confounding temperature effect. EST was maintained at this level by constantly and automatically adjusting air temperature to meet the set point.

4.4 | Brain lateralization and fear

Potential effects of light during incubation on embryonic activity are discussed in paragraph 4.2. Incubation conditions can also have effects on post hatch behaviour and activity, and differences in activity post hatch may play an additional role in long term development of leg bones and leg bone pathologies. A lot of research concerning light during incubation has focused on the development of the embryonic brain, lateralization of brain functions, and post hatch behaviour (e.g. Rogers, 1990, 2000, 2008). This could provide an explanation for the fact that effects of lighted incubation on bone development were found up until slaughter age, and why the stimulating effect of 24D on bone dimensions at hatch are mostly gone by D35 post hatch. Brain lateralization means that cognitive processes or neural functions are specialized in one hemisphere of the brain. The right hemisphere (linked to the left eye) is in control of fear and escape responses, while the left hemisphere can inhibit attention to distractions as it is involved in focusing on details (reviewed by Rogers, 2008). Lateralization has the advantage that post hatch chickens are able to process multiple tasks at the same time (Rogers, 2000). Providing embryos with light during incubation is thought to stimulate brain lateralization compared to dark incubation, because of a chicken embryo's position in the egg. The sensitive period is thought to be the last three days of incubation (Deng and Rogers, 2002), when the left eye is occluded by the embryo's body, while the right eye is turned towards the air chamber and can be exposed to light (Rogers, 1990).

Adam and Dimond (1971) exposed chicken embryos to 5 minute light pulses every hour for 12 hours on various days during incubation. Chickens that had their last exposure to this treatment on E19, when the visual system is thought to be complete and the left eye is occluded, showed a shorter time to approach a novel object at 48 hours post hatch than chickens last exposed to this treatment on E15 or E17. A shortened approach time can be viewed as a measurement of reduced fear behaviour (Adam and Dimond, 1971). On the other hand, Archer and Mench (2014b) exposed broiler eggs to 24D or 16L:8D throughout incubation, 16L:8D from E7 onward, or 16L:8D from E14 onward. At 3 weeks post hatch, chickens exposed to 16L:8D from E0 or E7 onward showed less fear behaviour than chickens exposed to 24D or 16L:8D from E14 onward. This would suggest that light exposure during the first two weeks of incubation already results in differences in brain development, even though the visual system is not complete yet and the embryo's left eye is not yet occluded by its body. Chickens exposed to 16L:8D from E0 onward, furthermore, had lower corticosterone levels after crating at 3 weeks post hatch than 24D incubated chickens (Archer and Mench, 2014b).

Reduced fear might impact on bone development through lower corticosterone levels. In adult laying hens, corticosterone release was found to increase in response to stressors, such as handling, followed by social isolation (Fraisse and Cockrem, 2006) or low environmental temperature (2°C for 6 hours) (Etches, 1976). High levels of corticosterone are a negative regulator of chondrocyte proliferation and differentiation (Robson *et al.*, 2002; Van der Eerden *et al.*, 2003). However, no evidence was found for the corticosterone pathway in the current thesis, as corticosterone levels did not differ between incubation lighting treatments between E18.8 and E19.5 (Chapter 5), nor before slaughter (Chapter 6). Chickens were not exposed to specific stressful situations, such as tonic immobility, in the current thesis; it can only be said that basal corticosterone levels did not differ.

5 | Post hatch lighting schedules

5.1 | Brooding period

So far, effects of incubation lighting schedule on leg health and bone development have been described. Another important phase in bone development is the early post hatch period, when a high rate of leg bone ossification and length growth takes place (Applegate and Lilburn, 2002; Kürtül *et al.*, 2009). It was expected that light-dark rhythms in the first 4 days post hatch (the brooding phase) would be beneficial for leg bone development. In two experiments, described in Chapter 7, broiler chickens were incubated under 24D and then brooded in a HatchBrood system (Van der Pol *et al.*, 2013) under 24L, 2h of light, followed by 1h of darkness (2L:1D), or 2h of light, followed by 6h of darkness

(2L:6D). 2L:1D was chosen because practical experience showed that this lighting schedule stimulated activity and feed intake in chickens housed in HatchBrood compared to 24L. 24L was chosen as the most contrasting schedule, without dark periods, and 2L:6D was chosen as a lighting schedule with prolonged dark periods to possibly stimulate bone development. Leg bone dimensions were measured at D4 post hatch. Higher femur and tibia diameter was found for 24L than for the light-dark schedules in the first experiment. Within the light-dark schedules, longer femurs and tibias were found for 2L:1D than for 2L:6D. In the second experiment, relative asymmetry of femur and tibia length and femur diameter was higher for 24L than for applying a light-dark schedule. Relative asymmetry is a measure of developmental instability (Møller *et al.*, 1999), and 24L as a post hatch lighting schedule has previously been found to result in higher developmental instability than applying a lighting schedule (Møller *et al.*, 1999; Campo *et al.*, 2007). It can be speculated that asymmetry of leg bones creates uneven weight load on the legs, which may predispose a chicken to leg pathologies in later life. Unfortunately, leg pathologies at slaughter age were not measured in the experiments described in Chapter 7, so this speculation cannot be supported with later life data.

Chickens in the brooding phase, such as in Chapter 7, are beyond the window of opportunity to affect brain lateralization through light. According to Rogers (2008), lateralization of the brain occurs during late incubation, because then one eye is exposed to light, while the other is covered. Post hatch, both eyes are exposed to light. An effect of lighting schedule in the brooding phase on behaviour can still be observed. Bayram and Özkan (2010) housed 2-day-old broiler chickens under 24L or 16L:8D. They found more activity, more comfort behaviours, and less stress behaviour in the light period in 16L:8D housed broiler chickens than in 24L housed broiler chickens. Behaviour was not observed in this thesis, but it can be speculated that activity is stimulated in 2L:1D brooded chickens compared to 24L brooded chickens.

Lighting schedules in the brooding phase may also affect early life bone development through differences in metabolism, but the mechanism is different post hatch than in ovo. While in ovo, the chicken embryo is limited in its gas exchange through the eggshell, and fat oxidation may be limited because of low O2 availability (Maatjens *et al.*, 2014). Post hatch, low O2 levels are rarely an issue, and chickens can use nutrients from exogenous feed. In Chapter 7, it was found that femur and tibia diameter were higher for chickens brooded under 24L compared to a light-dark schedule, and within the light-dark schedules, 2L:1D led to longer femurs and tibias than 2L:6D. This may simply be a result of the bones growing in proportion to body weight, as yolk free body mass was higher for 24L than for the lighting schedules, and higher for 2L:1D than for 2L:6D.

Based on literature and the fact that some differences in leg bone development and leg bone pathologies are not well understood from the pathways studied in the current thesis, post hatch activity might be involved, and it would be interesting to investigate this

into more detail.

5.2 | Interaction incubation and post hatch lighting schedule

In Chapter 6, the interaction between incubation lighting schedule (Inc24L, Inc16L:8D, and Inc24D) and a matching or mismatching post hatch lighting schedule (PH24L or PH16L:8D) from D0 till slaughter at D35 was investigated. No effects of the incubation x post hatch lighting schedule interaction on bone development at slaughter age, gait scores on D21, D28, or D34, or leg bone pathologies were found. PH16L:8D has the same total number of light and dark hours per day as PH2L:1D, which was tested in Chapter 7 in the brooding phase, but no interaction with incubation lighting schedule was tested in that experiment (see paragraph 4.1). Post hatch lighting schedule did have a main effect on leg health in Chapter 6: at slaughter, PH16L:8D had poorer gait scores and a higher score (indicating a higher and/or more severe incidence) of tibial dyschondroplasia and epiphyseal plate abnormalities, but also higher femur and tibia mineral content and mineral density, than PH24L. PH16L:8D did not lead to worse leg health in the whole grow out period, as gait scores on D21 and D28 post hatch were better for PH16L:8D than for PH24L. Other leg health variables or bone dimensions were not measured before slaughter age, so it cannot be said when differences in bone development arose, and how the brooding phase contributed to bone development in this experiment.

Even though no clear circadian rhythm in melatonin release was found for embryos incubated under a lighting schedule in the current thesis, chickens may still have been entrained to a certain post hatching environment. Özkan *et al.* (2012b) incubated broiler chickens under 24D or 16L:8D during the whole incubation period or in the last week of incubation, and hatched chickens were housed under 16L:8D or 24L. Plasma was collected at D6 post hatch. They propose that housing chickens under lighting conditions that matched their incubation conditions reduced stress, because they found lower corticosterone levels at D6 post hatch in chickens incubated under 16L:8D and then housed under the same schedule. Very high corticosterone levels reduce differentiation and proliferation of the chondrocytes (Robson *et al.*, 2002; Van der Eerden *et al.*, 2003), thereby possibly impairing length growth of the leg bones. With these results in mind, leg bone development was hypothesized to be increased, and leg bone health hypothesized to be better, for chickens that were housed under the same lighting schedule during incubation and post hatch (Chapter 6). Eggs were incubated under 24L, 16L:8D, or 24D, and housed under 24L or 16L:8D post hatch. No differences were found in plasma corticosterone at D35 post hatch, but this may have been too late to still observe stress from a mismatch between incubation and post hatch lighting schedule. Furthermore, no differences were found for leg pathology scores, gait scores, or leg bone morphology at slaughter age. Entraining embryos to a post hatch lighting environment did not prove to be effective in improving leg health in this thesis. Possibly, newly hatched chickens are

Light during incubation and production performance

Ideally, treatments aiming to improve leg health in broiler chickens do not simultaneously reduce broiler production. Hatchability, chick quality at hatch, and post hatch body weight gain, feed intake, and feed conversion were considered in this thesis.

Hatchability and chick quality

No differences were found between incubation lighting treatment in hatchability (Chapter 4 and 5) and the chick quality parameters; culls at hatch, chick length, or navel score (Chapter 4). However, in Chapter 4, yolk free body mass at hatch was higher for 12L:12D than for 24L, with 24D intermediate; and in Chapter 5, body weight at hatch was higher for 24D than for 24L and 16L:8D. This seems to be in line with the conclusion that a circadian lighting rhythm during incubation may have a positive effect on bone development, but a minimum length of darkness is needed for optimal development. Based on yolk free body mass and body weight at hatch, 24L during incubation may result in suboptimal chick quality.

Post hatch production parameters

In Chapter 4, no differences of incubation lighting schedule on body weight gain, feed intake, and feed conversion were found. In Chapter 6, 24L applied during incubation was found to result in highest body weights at slaughter age, because of higher feed intake in week 4, with no differences between 16L:8D and 24D. No difference in mortality was found. This means that providing a circadian lighting schedule during incubation will in any case not have a negative impact on production performance in the post hatch phase.

Because incubation lighting schedules have been found to result in fewer leg bone pathologies, applying them in practical hatcheries may be considered as a tool to reduce leg problems in later life, even if production performance in terms of body weight gain is not improved.

able to adapt quickly when they are moved from a free running rhythm to a fixed rhythm (like in Inc24L or Inc24D to PH16L:8D); in nature, too, chickens are moved to a true circadian rhythm only after hatching as a broody hen will not leave her nest daily (Archer and Mench, 2014b). Moving newly hatched chickens from a fixed rhythm to a free

running rhythm (Inc16L:8D to PH24L) may not cause too much trouble, either, because chickens can continue their internal physiological rhythms and are not disturbed by external cues. It can be speculated that moving newly hatched chickens from one rhythm to another, asynchronous rhythm (where the dark period is longer, shorter, or starts at a different time) is more problematic and stressful than the situations provided in the current thesis. This is something to keep in mind when considering practical application of incubation lighting schedules, because the exact lighting schedule and timing of that schedule may differ per broiler farmer.

To conclude, the interaction between incubation and post hatch lighting schedule is probably something to consider and investigate into more detail, but transitions to and from free running or fixed schedules were not found to differ in the current thesis. Post hatch lighting schedule is known to affect leg bone health and development, and differences can already arise in the brooding phase: lighting schedules applied only during brooding did result in differences in bone development and asymmetry, emphasizing that leg bone development can be affected by lighting schedules during early life development.

6 | Conclusions

Continuous light during incubation was found to be detrimental for leg bone development and leg bone health. Applying a circadian incubation lighting schedule during incubation may reduce leg pathologies in broiler chickens at slaughter age, but a dark period exceeding 8 hours is possibly required for additional optimal bone development. The underlying mechanisms were not well understood, and the involvement of an endocrine pathway was not evidenced in this thesis. Because effects of applying a circadian lighting schedule during incubation did not show a negative effect on chick quality or post hatch production parameters, lighting schedules may be considered as a viable tool for hatcheries to improve leg health, and thereby welfare, in broiler chickens.

7 | References

Adam, J., and S.J. Dimond. 1971. The effect of visual stimulation at different stages of embryonic development on approach beahviour. Anim. Behav. 19: 51-54.

Applegate, T. J., and M. S. Lilburn. 2002. Growth of the femur and tibia of a commercial broiler line. Poult. Sci. 81: 1289-1294.

Archer, G. S., and J. A. Mench. 2014a. The effects of the duration and onset of light stimulation during incubation on the behavior, plasma melatonin levels, and productivity of broiler chickens. J. Anim. Sci. 92: 1753-1758.

Archer, G. S., and J. A. Mench. 2014b. Natural incubation patterns and the effects of exposing eggs to light at various times during incubation on post-hatch fear and stress responses in broiler meat chickens. Appl. Anim. Behav. Sci. 152: 44-51.

Archer, G. S. 2016. Spectrum of white light during incubation: warm vs cool white LED lighting. Int. J. Poult. Sci. 15: 343-348.

Augat, P., and S. Schorlemmer. 2006. The role of cortical bone and its microstructure in bone strength. Age Ageing 35(suppl 2): ii27-ii31.

Bayram, A., and S. Özkan. 2010. Effects of a 16-hour light, 8-hour dark lighting schedule on behavioral traits and performance in male broiler chickens. J. Appl. Poult. Res. 19: 263-273.

Bekoff, A. 1981 Embryonic development of chick motor behavior. Trends NeuroSci. 4: 181-184.

Bertram, J. E., L. S. Greenberg, T. Miyake, and B. K. Hall. 1996. Paralysis and long bone growth in the chick: growth shape trajectories of the pelvic limb. Growth Dev. Aging 1997;61: 51-60.

Bessei, W. 2006. Welfare of broilers: a review. World Poultry Sci J. 62: 455-466.

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 Biol. Res. 13: 45–103.

Campo, J. L., M. G. Gil, S. G. D´avila, and I. Muñoz. 2007. Effect of lighting stress on fluctuating asymmetry, heterophil-tolymphocyte ratio, and tonic immobility duration in eleven breeds of chickens. Poult. Sci. 86: 37–45.

Csernus, V. J., A. D. Nagy, and N. Faluhelyi. 2007. Development of the rhythmic melatonin secretion in the embryonic chicken pineal gland. Gen. Comp. Endocrinol. 152: 148-153.

Deng, C. and L. J. Rogers. 2002. Prehatching visual experience and lateralization in the visual Wulst of the chick. Behav. Brain Res. 134: 375-385.

DiPietro, J. A., R. A. Irizarry, M. Hawkins, K. A. Costigan, and E. K. Pressman. 2001. Cross-correlation of fetal cardiac and somatic activity as an indicator of antenatal neural development. Am. J. Obstet. Gynecol. 185: 1421-1428.

Duff, S.R.I., and B.H. Thorp. 1985. Patterns of physiological bone torsion in the pelvic appendicular skeletons of domestic fowl. Res. Vet. Sci. 40: 333-

338.

Etches, R. J. 1976. A radioimmunoassay for corticosterone and its application to the measurement of stress in poultry. Steroids 28: 763-773.

Fraisse, F. and J. F. Cockrem. 2006. Corticosterone and fear behaviour in white and brown caged laying hens. Br. Poult. Sci. 47: 110-119.

Hargest, T. E., R. M. Leach, and C. V. Gay. 1984. Avian tibial dyschodroplasia. 1. Ultrastructure. Paper 6916 in the Journal Series of the Pennsylvania Agricultural Experiment Station, USA.

Hill, W. L., K. L. Bassi, L. Bonaventura, and J. E. Sacus. 2004. Prehatch entrainment of circadian rhythms in the domestic chick using different light regimes. Dev. Psychobiol. 45: 174-186.

Isakson, S. T., B. J. Huffman, and P. B. Siegel. 1970. Intensities of incandescent light and the development of chick embryos in ovo and in vitro. Comp. Biochem. Physiol. 35: 229-235.

Kestin, S. C., T. G. Knowles, A. E. Tinch, N. G. Gregory. 1992. Prevalance of leg weakness in broiler chickens and its relationship with genotype. Vet. Rec. 131: 190-194.

Kestin, S. C., S. Gordon, G. Su, and P. Sørensen. 2001. Relationships in broiler chickens between lameness, liveweight, growth rate and age. Vet. Rec. 148: 195-197.

Knowles, T. G., S. C. Kestin, S. M. Haslam, S. N. Brown, L. E. Green, A. Butterworth, S. J. Pope, D. Pfeiffer, and C.J. Nicol. 2008. Leg disorders in broiler chickens: prevalence, risk factors and prevention. PlosOne 3(2): e1545.

Kürtül I., S. H. Atalgin, K. Aslan, and E. U. Bozkurt. 2009. Ossification and growth of the bones of the wings and legs in prehatching period of the Hubbert strain broiler. Kafkas Univ. Vet. Fak. 15: 869-874.

Lamošová, D., M. Zeman, M. Macková, and E. Gwinner. 1995. Development of rhythmic melatonin synthesis in cultured pineal glands and pineal cells isolated from chick embryo. Experientia 51: 970-975.

Lourens, A., R. Meijerhof, B. Kemp, and H. van den Brand. 2011. Energy partitioning and consequences for embryo temperature: A theoretical approach. Poult. Sci. 90: 516–523.

Maatjens, C. M., I. A. M. Reijrink, I. van den Anker, R. Molenaar, C. W. van der Pol, B. Kemp, and H. van den Brand. 2014. Temperature and CO2 during the hatching phase. II. Effects on chicken embryo physiology. Poult. Sci. 93: 655-663.

Machida, M., J. Dubousset, Y. Imamura, T. Iwaya, T. Yamada, and J. Kimura. 1995. Role of melatonin deficiency in the development of scoliosis in pinealectomised chickens. J. Bone Joint Sur. 77: 134-138.

Mackie, E. J., Y. A. Ahmed, L. Tatarczuch, K.-S. Chen, and M. Mirams. 2008. Review. Endochondal ossification: How cartilage is converted into bone in the developing skeleton. Int. J. Biochem. Cell Biol. 40: 46-62.

McGeown, D., T.C. Danbury, A.E. Waterman-Pearson, and S.C. Kestin.

1999. Effect of carprofen on lameness in broiler chickens. Vet. Rec. 144: 668-671.

McKee, M. D., M. J. Glimcher, and A. Nanci. 1992. High-resolution immunolocalization of osteopontin and osteocalcin in bone and cartilage during endochondral ossification in the chicken tibia. Anat. Rec. 234: 479-492.

McNamee, P. T., and J.A. Smyth. 2000. Bacterial chondronecrosis with osteomyelitis ('femoral head necrosis') of broiler chickens: a review. Avian Pathol. 29: 477-495.

Milgrom, C., M. Gildadi, A. Simkin, N. Rand, R. Kedem, H. Kashtan, and M. Gomori. 1989. The second moment of area of the tibia: a risk factor for stress fractures. J. Biomech. 22: 1243-1248.

Molenaar, R., R. Meijerhof, I. van den Anker, M. J. W. Heetkamp, J. J. G. C. van den Borne, B. Kemp, and H. van den Brand. 2010. Effect of eggshell temperature and oxygen concentration on survival rate and nutrient utilization in chicken embryos. Poult. Sci. 89: 2010–2021.

Møller, A. P., G. S. Sanotra, and K. S. Vetergaard. 1999. Developmental instability and light regime in chickens (Gallus gallus). Appl. Anim. Behav. Sci. 62: 57–71.

Monsonego, E., O. Halevy, A. Gertler, S. Hurwitz, and M. Pines. 1995. Growth hormone inhibits differentiation of avian epiphyseal growth-plate chondrocytes. Mol. Cell. Endocrinol. 114: 35-42.

Murray, P. D. F., and B. Drachman. 1969. The role of movement in the development of joints and related structures: the head and neck in the chick embryo. J. Embryol. Exp. Morph. 22: 349-371.

Noiva, R. M., A. C. Menezes, M. C. Peleteiro. 2014. Influence of temperature and humidity manipulation on chicken embryonic development. BMC Vet. Res. 10: 1.

Ostrowska, Z., B. Kos-Kudla, B. Marek, D. Kajdaniuk, and N. Ciesielska-Kopacz. 2002. The relationship between the daily profile of chosen biochemical markers of bone metabolism and melatonin and other hormone secretion in rats under physiological conditions. Neuro Endocrinol. Lett. 23: 417-425.

Özkan, S., S. Yalçin, E. Babacanoğlu, and H. Kozanoğlu. 2009. Effect of photoperiod on prenatal and early post hatch growth of broiler chickens. In Proceedings of the 2nd Mediterranean Summit of WPSA, 4-7 October 2009: Antalya, Turkey, pp. 263-268.

Özkan, S., S. Yalçin, E. Babacanoğlu, H. Kozanoğlu, F. Karadaş, and S. Uysal. 2012a. Photoperiodic lighting (16 hours of light: 8 hours of dark) programs during incubation: 1. Effects on growth and circadian physiological traits of embryos and early stress response of broiler chickens. Poult. Sci. 91: 2912-2921.

Özkan, S., S. Yalçin, E. Babacanoğlu, H. Kozanoğlu, F. Karadaş, and S. Uysal. 2012b. Photoperiodic lighting (16 hours of light: 8 hours of dark) programs during incubation: 2. Effects on early posthatching growth, blood

physiology, and production performance in broiler chickens in relation to posthatching lighting programs. Poult. Sci. 91: 2922-2930.

Pacifici, M., O. Oshima, L. W. Fisher, M. F. Young, I. M. Shapiro, and P. S. Leboy. 1990. Changes in osteonectin distribution and levels are associated with mineralization of the chicken tibial growth cartilage. Calcif. Tissue Int. 47: 51-61.

Packialakshmi, B., N. C. Rath, W. E. Huff, and G. R. Huff. 2015. Poultry femoral head separation and necrosis: a review. Avian Dis. 59: 349-354.

Pechak, D. G., M. J. Kujawa, A. I. Caplan. 1986a. Morphological and histochemical events during first bone formation in embryonic chick limbs. Bone 7: 441-458.

Pechak, D. G., M. J. Kujawa, and A. I. Caplan. 1986b. Morphology of bone development and bone remodelling in embryonic chick embryos. Bone 7: 459-472.

Pollard, A. S., A. A. Pitsillides, and S. J. Portugal. 2016. Validating a Non-ninvasive Technique for Monitoring Embryo Movement In ovo. Physiol. Biochem. Zool. 89: 000-000.

Randall, C.J., and C.P.J. Mills. 1981. Observations on leg deformity in broilers with particular reference to the intertarsal joint. Avian Pathology 10: 407-431.

Roach, H. I. 1997. New aspects of endochondral ossification in the chick: chondrocyte apoptosis, bone formation by former chondrocytes, and acid phosphatase activity in the endochondral bone matrix. J. Bone Miner. Res. 12: 795-805.

Robson, H., T. Siebler, S. M. Shalet, and G. R. Williams. 2002. Interactions between GH, IGF-I, Glucocorticoids, and thyroid hormones during skeletal growth. Pediatr. Res. 52: 137-147.

Rogers, L. J. 1990. Light input and the reversal of functional lateralization in the chicken brain. Behav. Brain Res. 38: 211-221.

Rogers, L. J. 2000. Evolution of hemispheric specialization: advantages and disadvantages. Brain Lang. 73: 236-253.

Rogers, L. J. 2008. Development and function of lateralization in the avian brain. Brain Res. Bull. 76: 235-244.

Shafey, T. M., H. A. Al-Batshan, M. M. Ghannam, and M. S. Al-Ayed. 2005. Effect of intensity of eggshell pigment and illuminated incubation on hatchability of brown eggs. Br. Poult. Sci. 46: 190-198.

Shim, M. Y., A. B. Karnuah, N. B. Anthony, G. M. Pesti, and S. E. Aggrey. 2012. The effects of broiler chicken growth rate on valgus, varus, and tibial dyschondroplasia. Poult. Sci. 91: 62-65.

Takahashi, J. S., and M. Zatz. 1982. Regulation of circadian rhythmicity. Science 217: 1104-1111.

Väänänen, H. K. 1980. Immunohistochemical localization of alkaline phosphatase in the chicken epiphyseal growth cartilage. Histochemistry 65: 143-148.

Van der Eerden, B. C. J., M. Karperien, and J. M. Wit. 2003. Systemic and local regulation of the growth plate.

Endocr. Rev. 24: 782-801.

Van der Pol, C. W., I. A. M. van Roovert-Reijrink, C. M. Maatjens, H. van den Brand, and R. Molenaar. 2013. Effect of relative humidity during incubation at a set eggshell temperature and brooding temperature posthatch on embryonic mortality and chick quality. Poult. Sci. 92: 2145–2155.

Weeks, C. A., T. D. Danbury, H. C. Davies, P. Hunt, and S. C. Kestin. 2000. The behaviour of broiler chickens and its modifications by lameness. Appl. Anim. Behav. Sci. 67: 111-125.

Wideman, R. F., and R. D. Prisby. 2013. Bone circulatory disturbances in the development of spontaneous bacterial chondronecrosis with osteomyelitis: a translational model for the pathogenesis of femoral head necrosis. Front. Endocrinol. 3: 1-14.

Yalçin, S., H. B. Molayoglu, M. Baka, O. Genin, and M. Pines. 2007. Effect of temperature during the incubation period on tibial growth plate chondrocyte differentiation and the incidence of tibial dyschondroplasia. Poult. Sci. 86: 1772-1783.

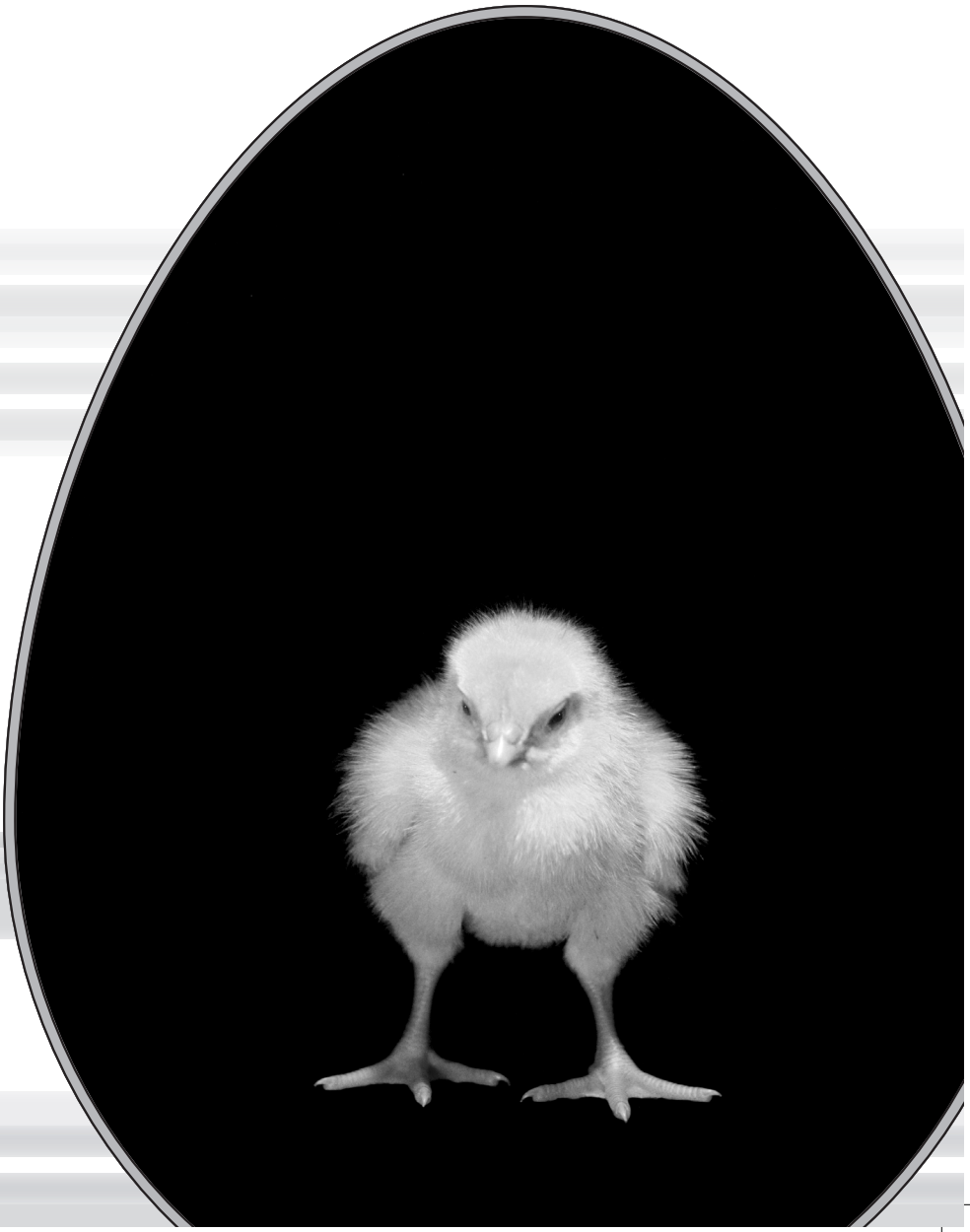
Zawilska, J. B., A. Lorenc, M. Berezińska, B. Vivien-Roels, P. Pevet, and D. J. Skene. 2007. Photoperiod-dependent changes in melatonin synthesis in the turkey pineal gland and retina. Poult. Sci. 86: 1397-1405.

Zeman, M., E. Gwinner, I. Herichová, D. Lamošová, and L. Koštál. 1999. Perinatal development of circadian melatonin production in domestic chicks. J. Pineal Res. 26: 28-34.

Zeman, M., P. Pavlik, D. Lamošová, I. Herichová, and E. Gwinner. 2004. Entrainment of rhythmic melatonin production by light and temperature in the chick embryo. Avian Poulty Biol Rev. 2004;15: 197-204.

Zhang, L., H. J. Zhang, X. Qiao, H. Y. Yue, S. G. Wu, J. H. Yao, and G. H. Qi. 2012. Effect of monochromatic light stimuli during embryogenesis on muscular growth, chemical composition, and meat quality of breast muscle in male broilers. Poult. Sci. 91: 1026-1031.

Summary



Summary

Leg problems constitute a major welfare problem in broilers. Broilers are selected for high muscle gain, which results in an imbalance between body weight load on the leg bones and leg strength. This increases the risk of leg bone fractures, rotational deformities, and developmental pathologies. In this thesis, it is hypothesized that providing optimal circumstances for bone growth and development during the incubation and in the early post hatch period may improve the leg bones' capacity of carrying high body weight loads, thus improving leg health. During embryonic development, the chicken limb bones are laid out in a cartilage model, and the limbs are then ossified. This process continues post-hatch, with highest ossification and length growth of the bones occurring in the first few days post-hatch. It is therefore speculated that large differences in leg bone development can be made during incubation and early post-hatch brooding (day 0 to 4 post hatch).

The main focus of the current thesis lies on stimulating leg bone development and health through light during incubation and in the brooding phase of broilers. Effects of light cannot be studied without taking temperature into account. Depending on the type of lighting, light may transfer heat to chickens or embryos through convection (as the light source heats up itself, and warm air is blown to the eggs) or radiation (as light waves reach a mass). Previous studies may not have looked at how light was confounded with temperature. In all experiments described in this thesis, cool white LED light was used as a source of illumination during incubation or brooding. However, even in modern LED systems, where heat transfer from the lights is minimized, some radiation may heat up the eggs or chickens and it is important to correct for this by maintaining embryo temperatures at a constant level. Eggshell temperature (EST) is used in this thesis as a reliable reflection of embryo temperature. The magnitude of effects of temperature on embryonic bone development was addressed in Chapter 2. Four different EST were applied throughout incubation of Ross 308 eggs: Low (36.9°C), Normal (37.8°C), High (38.6°C), and Very high (39.4°C). At hatch, tibia, femur, and metatarsus weight, length, width, and depth, and ash content were measured. Relative asymmetry was considered to be a sign of developmental instability and was calculated from the relative dimensions of the left and right sided bones. The Normal and High EST never differed from each other in bone variables. A Very high EST resulted in the lowest metatarsus weight and metatarsus, tibia, and femur length compared to High EST and, to a lesser degree, Normal EST, with Low EST intermediate. Yolk free body mass (body weight minus residual yolk weight) was added to the statistical model as a covariable to correct for differences in bone dimensions that were due simply to the size of the chicken itself. Asymmetry and ash content (expressed as a percentage of the bone) did not differ between treatments. From these results, it can be concluded that overheating during incubation (>39.4°C EST) has a negative effect on embryonic bone growth compared to an EST between 37.8 and 38.6°C. It was therefore decided that incubation experiments on bone development

would best be performed at a constant EST of 37.8°C, as this is also an EST that leads to good hatchability and chick quality.

Chapter 3 describes the first experiment on lighting schedules applied during incubation. It was speculated that applying a circadian lighting schedule during incubation would have a stimulating effect on embryonic leg bone development. Circadian lighting schedules are known to stimulate high levels of synchronized rhythmicity in melatonin release from the pineal gland of chickens. Melatonin, in its turn, may stimulate bone development. Ross 308 eggs were incubated at a constant EST of 37.8°C and exposed to 1 of 3 treatments: continuous light (24L), 12 h of light, followed by 12 h of darkness (12L:12D), or continuous darkness (24D) from set until hatch of the eggs. Total bone length and ossified length of the femur and tibia were measured between E8 and E14. Plasma melatonin was measured every 6 h between embryonic day (E)18.8 and E19.5, and growth hormone (GH) and insulin-like growth factor I (IGF-I) were measured at hatch. Femur and tibia weight, length, depth, and width, as well as bone microstructure were measured through CT scanning, on D0 (at hatch), D21, and D35 post hatch. It was found that 12L:12D resulted in higher ossified length of the tibia at E13 and E14 compared to 24L. At hatch, 12L:12D furthermore resulted in higher femur length compared to both other treatments. Tibia weight, length, cortical area, cortical thickness, and second moment of area of the minor axis were higher for both 12L:12D and 24D than for 24L. Effects of incubation lighting schedule was still found on D35 post hatch, with higher femur weight and tibia length, cortical area, and second moment of area for 12L:12D compared to 24L. In terms of leg health, incidence of tibial dyschondroplasia (a defect in ossification in the growth plate of the tibia) was higher in 24L than in 12L:12D. It can be concluded that a lighting schedule of 12L:12D during incubation had a long term stimulatory effect on leg bone development and health compared to 24L during incubation especially, with 24D mostly intermediate. This suggests that a degree of darkness is necessary for optimal broiler embryonic bone development. However, the experiment did not reveal the underlying mechanisms, as embryonic plasma melatonin rhythm did not differ between treatments, despite the fact that samples were taken throughout the lighting schedule.

Improvements in leg bone development and health should ideally not come at the expense of chick quality at hatch, or post hatch production parameters. In Chapter 4, production results of the experiment in Chapter 3 are described. Chick quality was measured through yolk free body mass, chick length, and navel scores at hatch. Organ weights were measured at hatch and D21, and production parameters (body weight, feed intake, and feed conversion) were measured weekly post hatch. At hatch, yolk free body mass and liver weights were higher for 12L:12D than for 24L, and intestine weight was higher for 12L:12D than for 24L and 24D. Body weight and feed intake during the grow out period did not differ between treatments. These results suggested that 12L:12D stimulated chick quality and development at hatch compared to 24L in particular, but incubati-

on lighting schedule did not have an effect on post hatch performance.

In Chapter 5, Ross 308 eggs were again incubated at 1 of 3 light treatments: 24L, 16 h of light, followed by 8 h of darkness (16L:8D), or 24D. Aim was to explore endocrine mechanisms affecting embryonic bone development, in more detail. Pineal melatonin was therefore measured every 4 h between E18.8 and E19.5, and simultaneously blood samples were taken for GH and corticosterone analysis. An alternative pathway that may have caused differences in embryonic bone development previously could have been movement of the embryo. Heart rate was therefore measured from E12 till hatch as a reflection of embryonic activity. Another aim was to reveal the mechanisms behind embryonic ossification by measuring gene expression. Genes involved in collagen synthesis and synthesis of proteins involved in ossification were measured in the tibias of embryos or chickens on E13, E17, and moment of hatch. Femur and tibia weight, length, width, and depth were again measured at hatch. On E14, E15, and E16, heart rate was lower for 24L than for 24D, with 16L:8D mostly intermediate. On E19, heart rate was higher for 16L:8D than for 24L and 24D. Gene expression did not differ between treatments and time points. Pineal melatonin showed an erratic pattern, with peaks for 16L:8D right before and at the beginning of its dark period, but also in the middle of the light period. 24L and 24D also showed random peaks, and no clear difference in circadian rhythm was visible. Unlike in Chapter 3, the circadian lighting schedule (in this case: 16L:8D) did not result in increased bone dimensions at hatch. The lighting schedule 16L:8D had lower femur and tibia weight, femur length, and femur and tibia depth and width than 24D, with 24L mostly intermediate or similar to 24D. These results were unexpected, as they did not show the clear positive effect at hatch of the circadian lighting schedule 12L:12D that was found in the previous experiment. Possibly, a longer dark period than 8h is required for optimal embryonic leg bone development. The involved mechanisms were furthermore still unclear. The involvement of a circadian rhythm in melatonin release was not proven.

In Chapter 6, hatched chickens from the same experiment as Chapter 5 were used in a grow out trial until D35. Incubation lighting conditions may not only affect bone development at hatch, but they may also program a chicken for its post hatch lighting environment. Chickens were therefore housed post hatch either at 24L (PH24L) or 16L:8D (PH16L:8D) from D0 till D35 and the interaction with incubation light treatment (Inc24L, Inc16L:8D, and Inc24D) was tested in a 3 x 2 factorial arrangement. Performance data were measured weekly. Gait was scored on D21, D28, and D34. On D35, incidence and severity of several leg problems, femur and tibia weight, length, width, mineral density, and mineral content, and plasma corticosterone and GH were assessed. Only a few effects of the incubation x post hatch lighting schedule interaction were found, with no clear biological meaning. This suggests that providing a chicken with a post hatch lighting schedule that matches the one experienced during incubation was not very effective to stimulate leg bone development. However, a main effect of incubation lighting

schedule was found, with a positive effect of Inc16L:8D on leg health. Inc16L:8D and Inc24D resulted in a lower score for growth plate abnormalities, indicating better leg health, than Inc24L. Inc16L:8D also resulted in lower scores for femoral head necrosis than Inc24D, with Inc24L intermediate. Inc24L showed the highest total growth, with higher body weight on D35 than for Inc16L:8D and Inc24D. Femur mineral density was furthermore higher for Inc24L than for Inc24D.

In the final chapter, two experiments are described in which lighting schedules only in the early post hatch (brooding) phase were tested. Newly hatched Ross 308 chickens were taken from the hatcher at pull time and housed in a HatchBrood system for 4 days. After an initial light period of 12h for all chickens, they were kept at 1 of 5 light treatments: 24L, 2L:1D, or 2L:6D, and the treatments with a dark period experienced either an abrupt transition from light to dark ("abrupt"), or a gradual transmission ("dimming"). Dimming took 3 minutes in the first experiment, and 15 minutes in the second experiment. At D4 post hatch, femur and tibia weight, length, and width, femur and tibia relative asymmetry, yolk free body mass, organ weights, and production parameters were determined. In the first experiment, 24L resulted in higher femur and tibia diameter than applying a lighting schedule. Within the lighting schedules, 2L:1D resulted in higher tibia and femur length than 2L:6D. In the second experiment, it was found that 24L compared to applying a lighting schedule, and an abrupt transition compared to dimming, led to higher relative asymmetry of femur and tibia length, suggesting that these treatments suffered from more developmental instability. It can be concluded that leg bone development decreased with prolonged dark periods in the brooding phase. This may have been the result of lower (opportunity for) feed intake in 2L:6D, or because of lower physical activity for that treatment than for 24L and 2L:1D. However, relative asymmetry indicated that leg bone development was less stable in 24L, suggesting that applying a lighting schedule may be more beneficial for leg health in the long term. Given that treatments were applied for only 3.5 days post hatch, the results of these experiments emphasize the importance of perinatal lighting conditions on leg bone development and possibly later life leg health.

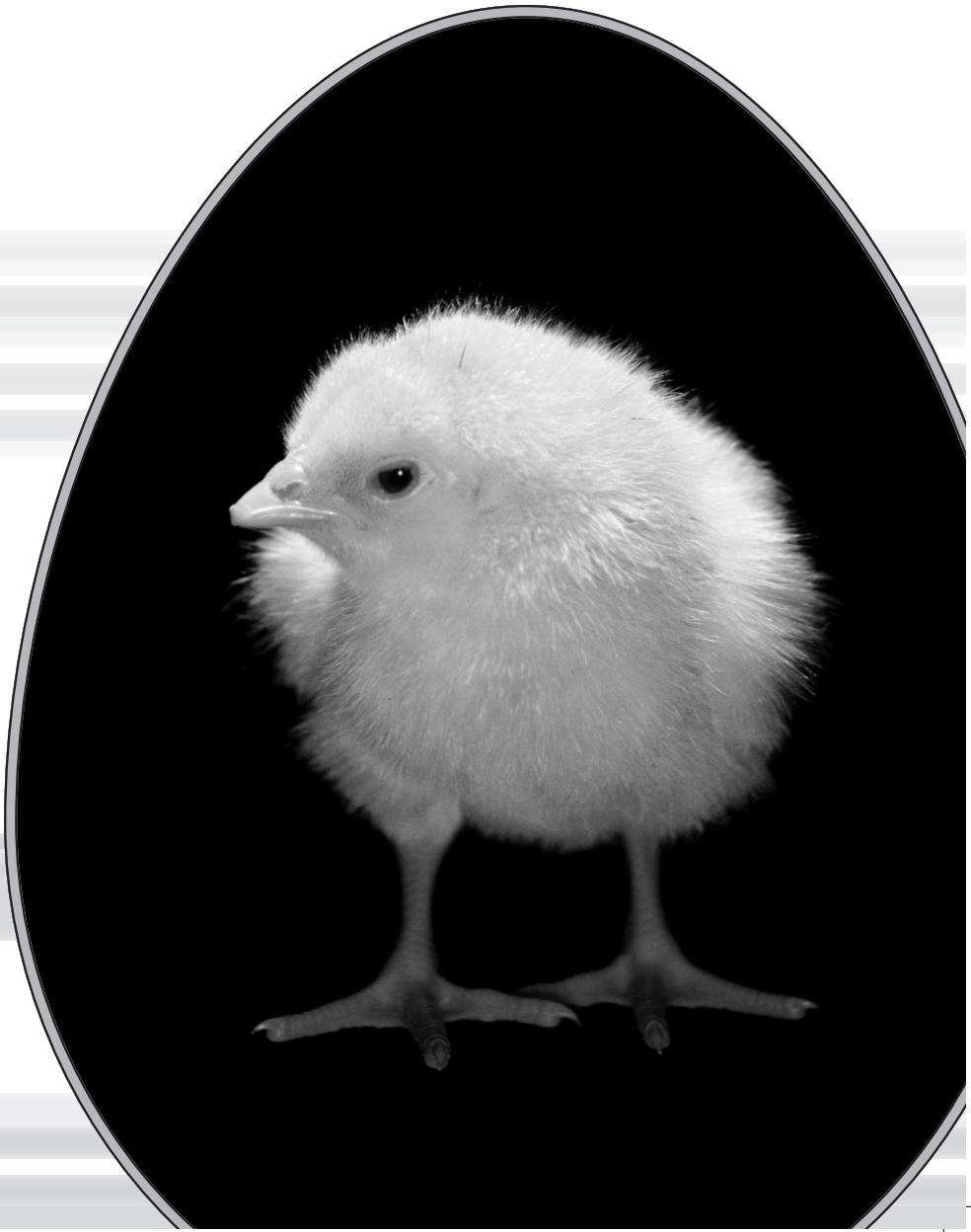
To conclude, continuous light during incubation had a negative effect on embryonic and post hatch leg bone development and health compared to continuous darkness. Applying a light-dark rhythm during incubation may improve leg bone development compared to continuous light and continuous darkness, without affecting post hatch production performance, but the dark period should perhaps last longer than 8 hours per day.

Dankwoord (Acknowledgements)

About the author

Training and supervision plan

Colophon



Dankwoord (Acknowledgements)

Het was een heerlijke tijd, ik zou zo nog een PhD willen doen!

Toen de vacature voorbij kwam voor een combinatie van toegepast onderzoek bij HatchTech en een PhD bij Adaptatiefysiologie moest ik wel even nadenken. De industrie in, was ik daar wel geschikt voor? Maar had me geen leukere baan kunnen wensen. Ik kan direct het resultaat van onze onderzoeken terugzien in de praktijk. En binnen de PhD kon ik mijn liefde voor de wetenschap en het vergroten van kennis kwijt. Tjitze, heel erg bedankt dat jij me deze kans hebt gegeven. Ik vind het gaaf om te zien hoe jouw passie voor innovatie bijdraagt aan een betere pluimveesector. Inge, jij begeleidde me vanuit HatchTech, en het onderzoek was heel anders geweest zonder jou erbij. Jouw precieze, kritische blik, en jouw drive om alles tot in de puntjes uit te zoeken waren erg waardevol. Maar daarnaast was het vooral ook heel erg gezellig en hebben we veel gelachen. Je bent een topcollega!

Vanuit Adaptatiefysiologie (ADP) werd ik begeleid door Henry van den Brand en Bas Kemp. Henry, je enthousiasme voor de pluimveewetenschap is erg aanstekelijk. Als wij een uurtje gepraat hadden zat ik weer vol inspiratie, en wist ik gelijk ook alle laatste nieuwtjes van de sector. Bas, ik vind het indrukwekkend hoe jij iedere keer weer met een paar rake opmerkingen de gedachtegang achter een onderzoek op het juiste spoor kan zetten, zowel in de eerste opzet als in de laatste discussie. Je combineert je brede kennis tot heel specifieke adviezen en daar heb ik veel aan gehad.

Binnen mijn PhD wilde ik een combinatie maken van de praktische blik van ADP op fysiologie en een wat meer fundamentele onderbouwing van wat we zoal vonden. Binnen de leerstoelgroep Experimentele Zoölogie vond ik een berg kennis en ervaring waar ik veel van geleerd heb in de afgelopen jaren. Sander Kranenbarg en Sander Gussekloo hebben dit proefschrift naar een hoger niveau getild met hun grote kennis over embryologische ontwikkeling en kritische vragen over mijn doelen. Karen Leon, Henk Schipper, en Remco Pieters hebben geholpen met de voor mij totaal nieuwe metingen, van CT-scans tot genexpressie tot histologie. Bedankt EZO'ers – zonder jullie hulp en kennis was ik op een enthousiast maar vaag idee blijven hangen.

Voor het soort experimenten dat ik deed heb je veel hulp nodig. Vaak ook 's nachts… Mijn collega's stonden gelukkig altijd voor me klaar. Uren heb ik met Ilona en Monique in het lab aan kippenpoten zitten peuteren. Het was altijd gezellig! Jan, je hebt me met van alles geholpen, van de vieste klusjes tot de ingewikkeldste techniek – dat beviel zo goed dat je me nu opvolgt als HatchTech-PhD'er. Bjorge en Fleur bleken minstens zo goed te zijn met kippen als met varkens en lieten me vaak lachen met hun gortdroge humor. Ger en Rudie, jullie zijn echte labgoeroes met engelengeduld en hebben keer op keer meegedacht over de methodes die ik in mijn hoofd had. En het secretariaat was voor mij de spil in het wiel van ADP. Lora en Nanette, jullie hebben talloze brandjes in de vorm

van visa, postpakketen, epjesstickertjes en posters geblust. Heel erg bedankt allemaal!

Veel andere collega's binnen Zodiac of HatchTech hebben natuurlijk ook meegeholpen met proeven, waren mijn reisgezelschap op congres, of waren er gewoon tijdens de pauze voor algemene gezelligheid. Cor en Christian, jullie hebben gezorgd dat er licht op mijn eieren kon schijnen – een nogal belangrijk detail, bedankt! Gerald, ik heb jouw droge opmerkingen in het algemeen en je hulp tijdens de experimenten in het bijzonder erg gewaardeerd. Britt, je had altijd tijd voor een cola en paardenverhalen! Conny en Kristina, mede-kippenfans, wat hebben we een lol gehad met de kuikentjes. In het bijzonder wil ik daarnaast de PhD'ers in de industrial room bedanken. Mariska, Maarten, Moniek, en David (met zijn prachtige grappen op promovendusniveau) als mede-industrials; Merel en Renny, met wie ik "broeden in het water" nieuw leven inblies; en Pieter, die goed was met kuikens maar nog beter bleek te zijn met IJslandse paarden.

Het leven van een onderzoeker kan een heel stuk aangenamer worden met een paar goede studenten die helpen bij het praktische werk. Sascha, Mariska, Loes, Ilse, Francisca, Anne-Lise, en Marieke, bedankt and thanks! Marieke, als ik zie hoe zorgvuldig jij aan de slag ging met de ingewikkelde materie van de genexpressie krijg ik al helemaal zin om jou nu te gaan begeleiden in jouw eigen PhD.

Inspannen gaat beter als je ook af en toe kunt ontspannen, het liefst met vrienden erbij. Dit is niet het eerste boekje waar ik aan gewerkt heb. In 2006 maakte Almanakcie 23 ("Alles op z'n tijd") al een prachtig oranje jaarboek, en we zijn nu wat meer burgerlijk maar nog altijd gezellig. Bedankt, Almanakcie, voor alle avonden, weekenden, appjes, en onzin! Ingrid, ook jij bedankt voor alle gezellige avonden en dat ik me bij jou in ieder geval nooit hoef af te vragen of ik teveel over honden praat :) Mirjam en Myrthe, wat fijn dat jullie met mij op het podium willen staan. Mirjam, je bent een zuivere Rotterdamse en toch de echtste Veeteler van ons allemaal – jij leert dit gezelschapsdierenmeisje anders naar de veehouderij te kijken. Myrthe, je bent een van de meest nuchtere mensen die ik ken, en je weet dat dat een compliment is! We delen met z'n drieën nu alweer 12,5 jaar lief, leed, en heel veel flauwe grappen, en ik ben nog lang niet uitgelachen.

Lieve families van der Pol en Bijker, schoonfamilie van den Berg, en Chela, bedankt voor jullie belangstelling, steun en gezelligheid. Jeroen en Karin, jullie zijn mijn trouwe festivalmaatjes, sportmotivators, Bourgondiërs, en bron van cynische humor – allemaal essentieel voor de geestelijke gezondheid. Mamma, jij gaf me de liefde voor dieren. Samen met Erik ben je daarnaast de beste hondenopvang voor Zorro, Dalí en Lucca die ik me kan wensen, waardoor ik mijn grootste hobby naast mijn drukke baan kon houden. (I'm the Doctor!) Pappa, jij hebt me altijd gestimuleerd om door te leren, om kritisch te denken, en om te wereld te ontdekken. Je hebt de wetenschapper in mij aangewakkerd. Je nam me al jong mee op reizen over de hele wereld en naar concerten uit allerlei genres. Samen met Atie zijn we daar nog lang niet klaar mee! Nog heel erg bedankt voor het doorlezen van de héle thesis op domme spelfouten. Sorry dat ik alle Oxford commas heb

laten staan.

Lieve Roel, jij was zo mogelijk nóg enthousiaster over mijn werk en PhD dan ikzelf. Zelfs als mijn wekker weer om 4 uur 's ochtends ging, of als ik in het weekend een dag moest werken, of opeens een week in het buitenland zat. Dat laatste was hooguit een mooie gelegenheid om ook die kant op te komen voor vakantie. *It's so rare to find someone who brings on the sunshine the way you do.*

Tot slot:

He believed, against all experience, that the world was fundamentally understandable, and that if he could only equip himself with the right mental toolbox he could take the back off and see how it worked. He was, of course, dead wrong. *Terry Pratchett, The Light Fantastic*

About the author

Carla Willemien van der Pol was born in Nieuwegein, the Netherlands, on August 10, 1986. She grew up in Houten and Geldermalsen, and started her bachelor studies in Animal Sciences at Wageningen University and Research in 2004. She obtained her MSc degree in Animal Sciences in 2010, specializing in Animal Health and Behaviour. For her major thesis for Adaptation Physiology, she studied energy partitioning and production of artificially defeathered laying hens exposed to variable ambient temperatures. For her major thesis for Ethology and Animal Welfare, she studied the influence of the handler-dog relationship on training success. Her internship was conducted at the Scottish Agricultural College in Edinburgh, and focused on maternal dietary protein, nematode infection, and lamb behaviour.

In 2011, Carla started as a researcher at HatchTech B.V., Veenendaal, the Netherlands, where she conducts research into incubation equipment and procedures, and their effects on pre and post hatch poultry physiology and performance. In 2012, Carla started her PhD project at the Adaptation Physiology group of Wageningen University and Research in collaboration with HatchTech B.V. The PhD project focused on the effect of lighting schedules applied during incubation and in the brooding phase on broiler chicken leg bone development and leg health, the results of which are presented in this thesis. After obtaining her PhD degree, Carla will continue to work as a researcher at HatchTech B.V.

Publications

Pol, C. W. van der, I. A. M. van Roovert-Reijrink, C. M. Maatjens, H. van den Brand, and R. Molenaar. 2013. Effect of relative humidity during incubation at a set eggshell temperature and brooding temperature posthatch on embryonic mortality and chick quality. Poult. Sci. 92: 2145–2155.

Lamot, D. M., I. B. van der Linde, R. Molenaar, C. W. van der Pol, P. J. A. Wijtten, B. Kemp, and H. van den Brand. 2014. Effects of moment of hatch and feed access on chicken development. Poult. Sci. 93: 2604 - 2614.

Maatjens, C. M., I. A. M. Reijrink, R. Molenaar, C. W. van der Pol, B. Kemp, and H. van den Brand. 2014. Temperature and CO2 during the hatching phase. I. Effects on chick quality and organ development. Poult. Sci. 93: 645 - 654.

Maatjens, C. M., I. A. M. Reijrink, I. van den Anker, R. Molenaar, C. W. van der Pol, B. Kemp, and H. van den Brand. 2014. Temperature and CO2 during the hatching phase. II. Effects on chicken embryo physiology. Poult. Sci. 93: 655 - 663.

Pol, C. W. van der, I. A. M. van Roovert-Reijrink, C. M. Maatjens, I. van den Anker, B. Kemp, and H. van den Brand. 2014. Effect of eggshell temperature throughout incubation on broiler hatchling leg bone development. Poult. Sci. 93: 2878 - 2883.

Pol, C. W. van der, R. Molenaar, J. Buitink, I. A. M. van Roovert-Reijrink, C. M. Matjens, H. van den Brand, and B. Kemp. 2015. Lighting schedule and dimming period in early life: consequences for broiler chicken leg bone development. Poult. Sci. 94: 2980 - 2988.

Maatjens, C. M., I. A. M. van Roovert-Reijrink, B. Engel, C. W. van der Pol, B Kemp, and H. van den Brand. 2016. Temperature during the last week of incubation. I. Effects on hatching pattern and broiler chicken embryonic organ development. Poult. Sci. 95: 956-965.

Maatjens, C. M., I. A. M. van Roovert-Reijrink, I. van den Anker, B. Engel, C. W. van der Pol, B Kemp, and H. van den Brand. 2016. Temperature during the last week of incubation. II. Effects on first week broiler development and performance. Poult. Sci. 95: 2136-2144.

Maatjens, C. M., I. A. M. van Roovert-Reijrink, B. Engel, C. W. van der Pol, B. Kemp, and H. van den Brand. 2016. Temperature during the last week of incubation. III. Effects on chicken embryo physiology. Poult. Sci. pew 390.

Conference proceedings and abstracts

Maatjens, C. M., I.A.M. van Roovert-Reijrink, R. Molenaar, C. W. van der Pol, B. Kemp, and H. van den Brand. 2012. Effects of temperature and CO2 during the last 4 days of incubation on chick quality. Incubation and Fertility Research Group Meeting, Pisa, Italy.pp. 171-172.

Van der Pol, C. W., I. A. M. van Roovert-Reijrink, C. M. Maatjens, H. van den Brand, and R. Molenaar. 2012. Effect of relative humidity during incubation on embryonic mortality, chick quality, and postnatal development. Proceedings of the WPSA Incubation and Fertility Research Group Meeting, Pisa, Italy. pp. 184.

Lamot, D. M., I. B. van der Linde, R. Molenaar, C. W. van der Pol, C. M. Maatjens, P. J. A. Wijtten, B. Kemp, and H. van den Brand. 2013. The effect of hatch moment and moment of feed access after hatch on broiler development. Proceedings of the 38th Animal Nutrition Research Forum, Roeselare, Belgium. pp. 32.

Maatjens, C. M., C. W. van der Pol, and I. A. M. van Roovert-Reijrink. 2013. Effect of Diet Composition and Particle Size on the Performance of Chicks Hatched from Small Eggs. Proceedings of the 38th Animal Nutrition Research Forum, Roeselare, Belgium.

Van der Pol, C. W., C. J. Buitink, C. M. Maatjens, I. A. M. van Roovert-Reijrink, H. van den Brand, and R. Molenaar. 2013. Effect of light schedule and dimming in early life on bone development in the broiler chicken. Poster Poultry Welfare Syposium, Welfare and health, Uppsala, Sweden.

Van der Pol, C. W., C. M. Maatjens, I. A. M. van Roovert-Reijrink, B. Kemp, and H. van den Brand. 2013. Effect of incubation temperature on leg bone development in broiler hatchlings. Incubation and fertility research group (IFRG) meeting, Göttingen, Germany. pp. 8-9.

Maatjens, C. M., I. A. M. van Roovert-Reijrink, I. van den Anker, R. Molenaar, C. W. van der Pol, B. Kemp, and H. van den Brand. 2014. Effects of temperature and CO2 during the hatching phase on chicken embryo physiology. Proceedings of the XIVth European Poultry Conference. Stavanger, Norway. pp. 422.

Molenaar, R., T. Gooding. D. Lamot, P. J. A. Wijtten, C. W. van der Pol, C. M. Matjens, and I. A. M. van Roovert-Reijrink. 2014. Incubation and brooding conditions essential for the optimisation of neonatal nutrition. Aust. Poult. Sci. Symp. pp.17-20. 186

Van der Pol, C. W., R. Molenaar, C. M. Maatjens, I. A. M. van Roovert-Reijrink, and H. van den Brand. 2014. Effect of light schedule during the brooding period on leg bone development and growth at slaughter age. Proceedings of the XIVth European Poultry Conference. Stavanger, Norway. pp. 470.

Pol, C. W. van der, I. A. M. van Roovert-Reijrink, C. M. Maatjens, B. Kemp, and H. van den Brand. 2014. Effect of lighted incubation from set till hatch on hatch moment and chick development. Proceedings of the WPSA Working group 6 (reproduction); incubation and fertility research. Lunteren, the Netherlands. pp. 13.

Roovert-Reijrink, I. A. M. van, C. W. van der Pol, and C. M. Maatjens. 2014. Effect of the warming profile at the start of incubation on hatchability and chick quality in short stored eggs. Proceedings of the XIVth European Poultry Conference. Stavanger, Norway. pp. 433.

Maatjens, C. M., I. A. M. van Roovert-Reijrink, C. W. van der Pol, B. Kemp, and H. van den Brand. 2015. Effect of temperature during the last week of incubation on embryonic development and chick quality. Abstract Book of the 2015 IFRG meeting and 7th Combined Workshop on Fundamental Physiology and Perinatal Development in Poultry. Berlin, Germany. pp. 24.

Van der Pol, C. W., I. A. M. van Roovert-Reijrink, C. M. Maatjens, B. Kemp, and H. van den Brand. 2015. Effect of lighted incubation from set till hatch on broiler leg bone development. Abstract Book of the 2015 IFRG meeting and 7th Combined Workshop on Fundamental Physiology and Perinatal Development in Poultry. Berlin, Germany. pp. 32.

Van Roovert-Reijrink. I. A. M., C. W. van der Pol, C. M. Maatjens, G. Aalbers, A. J. Wade, and A. D. Nicholson. 2015. Effect of SPIDES and slow warming profile on hatchability of long stored eggs. Abstract Book of the 2015 IFRG meeting and 7th Combined Workshop on Fundamental Physiology and Perinatal Development in Poultry. Berlin, Germany. pp. 24.

Van der Pol, C. W., C. M. Maatjens, G. Aalbers, and I. A. M. van Roovert-Reijrink. 2015. Effect of feed and water access on hatchling body weight changes between hatch and pull. Proceedings of the 20th European Symposium on Poultry Nutrition (ESPN). Prague, Chech Republic. pp. 255.

Van der Pol, C. W., I. A. M. van Roovert-Reijrink, R. Pieters, S. Gussekloo, S. Kranenbarg, and H. van den Brand. 2016. Effects of applying a light-dark schedule during incubation on broiler leg bone geometry. The Proceedings of the WPC 2016. Beijing, China. pp 578.

Van Roovert-Reijrink, I. A. M., C. W. van der Pol, and G. Aalbers. 2016. Effect of feed and water access on hatchling BW changes between hatch and pull. IFRG meeting in Brugge, Belgium.

Other publications related to this thesis

Van der Pol, C. W. 2012. Incubating eggs of different sizes: it's all about incubator design. Middle East Agrifood Publishers, http://www.agrifoodpublishers.com/meap/.

Van der Pol, C. W. 2014. New, more effective breakout procedure improves hatchability. International Hatchery Practice 28: 7-8.

Van der Pol, C. W. 2015. The beautiful mystery of hatching. World Poultry magazine 2, 2015.

Van Roovert-Reijrink, I. A. M., and C. W. van der Pol. 2015. Superior chick quality is the result of optimal incubation conditions. International Hatchery Practice 30: 25-26.

Van Roovert-Reijrink, I. A. M., and C. W. van der Pol. 2016. Superior chick quality: where and how should we hatch our chickens? International Hatchery Practice 30: 11-13.

Training and Supervision Plan

Completed Training and Supervision Plan of graduate school WIAS

The Basic Package	3.0 ECTS
WIAS Introduction Course	2013
Course on philosophy of science and/or ethics	2013

International conferences	10.6 ECTS
World's Poultry Congress, Beijing, China	2016
20th European Symposium on Poultry Nutrition, Prague, Czech Republic	2015
Fundamental Physiology and Perinatal Development in Poultry, Berlin, Germany	2015
Incubation and Fertility Research Group Meeting, Berlin, Germany	2015
Incubation and Fertility Research Group Meeting, Lunteren, the Netherlands	2014
XIVth European Poultry Conference, Stavanger, Norway	2014
IX European Symposium on Poultry Welfare , Uppsala, Sweden	2013
Incubation and Fertility Research Group Meeting, Gottingen, Germany	2013
Fundamental Physiology and Perinatal Development in Poultry, Gottingen, Germany	2013
Incubation and Fertility Research Group Meeting, Pisa, Italy	2012
Fundamental Physiology and Perinatal Development in Poultry, Wageningen, the Netherlands	2011
Incubation and Fertility Research Group Meeting, Ede, the Netherlands	2011

Seminars and workshops	0.6 ECTS
WIAS Science day, Wageningen, the Netherlands	2014
WIAS Science day, Wageningen, the Netherlands	2015

Presentations	10.0 ECTS
Oral - World's Poultry Congress, Beijing, China	2016
Oral - Fundamental Physiology and Perinatal Development in Poultry, Berlin, Germany	2015
Oral - WIAS Science day, Wageningen, the Netherlands	2014
Oral - XIVth European Poultry Conference, Stavanger, Norway	2014
Oral - Incubation and Fertility Research Group Meeting 2014, Lunteren, the Netherlands	2014
Oral - Incubation and Fertility Research Group Meeting, Pisa, Italy	2012
Oral - Incubation and Fertility Research Group Meeting, Gottingen, Germany	2013
Poster - 20th European Symposium on Poultry Nutrition, Prague, Czech Republic	2015
Poster - WIAS Science day, Wageningen, the Netherlands	2015
Poster - IX European Symposium on Poultry Welfare , Uppsala, Sweden	2013

Research Skills Training	6.0 ECTS
Preparing own PhD research proposal	2012

Disciplinary and interdisciplinary courses	3.4 ECTS
Energy metabolism and body composition in nutrition and health research	2016
Incubation Biology and Management at Wageningen Business school	2014
Poultry Welfare Assessment, Ghent, Belgium	2015
Poultry Nutrition at Wageningen Business School	2015

Advanced statistics courses	3.0 ECTS
Design of Experiments	2012
Statistics for the life sciences	2016

Professional Skills Support Courses	3.2 ECTS
Techniques for writing and presenting a scientific paper	2013
Pitch perfect	2015
Philosophy and ethics in food science and technology	2016

Lecturing	0.5 ECTS
Presentation Incubation Biology and Hatchery Management	2016
Lecture course Adaptation Physiology	2013
Lecture course Adaptation Physiology	2014

Supervising practicals and excursions	1.0 ECTS
Supervising for course Adaptation Physiology	2013

Supervising theses	11.5 ECTS
MSc minor - Francisca Scholl	2016
MSc major - Marieke Priester	2016
MSc major - Anne-Lise Mary	2016
MSc major - Loes Verrijt	2014
MSc major - Mariska Niesten	2013
BSc thesis - Sascha Smits	2013
BSc thesis - Ilse Poolen	2015

Membership of boards and committees	3.0 ECTS
WAPS Council	2013-2014

Education and training total	56 ECTS

The funding of this thesis by

HatchTech BV

Providing superior chick quality

is greatly appreciated.

Cover photo	Carla van der Pol
Thesis design and layout	Roel van den Berg
Cover formatting and printing	Proefschriftmaken.nl

Propositions

1. Continuous light during incubation of broilers increases the risk of leg bone pathologies at slaughter age.

   (this thesis)

2. Providing at least 12 hours of darkness per day during incubation enhances embryonic leg bone development.

   (this thesis)

3. Melancholy music can enhance sad moods for a variety of reasons (van den Tol and Edwards, 2014), but it enhances good moods because of its aesthetics only.
4. Industry funded scientific research should focus on gaining knowledge, not products.
5. Rational thinking takes life long training.
6. The housing of top sport horses is a form of intensive animal farming.

Propositions belonging to the thesis, entitled:

"Bringing eggs and bones to light. Affecting leg bone development in broiler chickens through perinatal lighting schedules".

Carla W. van der Pol

Wageningen, 12 May 2017