BORN TO MOVE

Development of form and function of the locomotor system in the early juvenile phase of precocial animals

Ben Gorissen

Hirado horses

The horses on the cover of this thesis are so-called Hirado horses and are part of a private collection of Chinese and Japanese art. Hirado horses were made in the Mikawachi kilns, which were situated in Hirado, on the Kyushu island, which is the most southern island of Japan. These kilns were active from the second half of the 18th century, until early 20th century and were famous for their refined products. The horses on the cover of this thesis were made in the second half of the 19th century, probably around 1870. They are on the cover because they mimic the delicate beauty of the horse and their anatomy perfectly, as mentioned in the book "Hirado the prince of porcelains" from Louis Lawrence. He stated that these models of a horse show that nothing was beyond the capability or imagination of the Hirado potters as not only the technical achievement is beyond imagination, but also the anatomy of the horse, making it a great piece of art.

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Development of form and function of the locomotor system in the early juvenile phase of precocial animals

Geboren om te bewegen
Vroege ontwikkeling van vorm en functie van het bewegingsapparaat van nestvlieders

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Chapter I **9**
General introduction and outline of the thesis.

Chapter II **25**
The development of locomotor kinetics in the foal and the effect of osteochondrosis.

Chapter III **49**
Footing of the foal: the development of hoof balance and landing preference in the postnatal period.

Chapter IV **71**
Trabecular bone of precocials at birth; are they prepared to run for the Wolf(f)?

Chapter V **91**
Longitudinal bone development in foals.

Chapter VI **109**
Effects of longterm use of the preferential cox-2 inhibitor meloxicam on growing pigs.

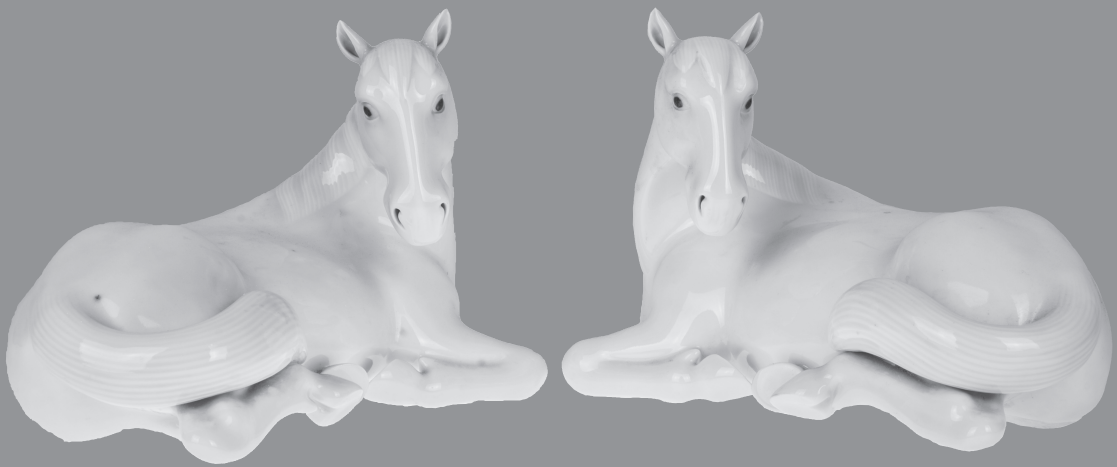
Chapter VII **129**
Hypoxia negatively affects senescence in osteoclasts and delays osteoclastogenesis.

Chapter VIII **155**
General discussion

Addendum
Summary 169
Dutch summary / Nederlandse samenvatting 173
Dankwoord 177
About the author 181

CHAPTER I:

General introduction



Introduction

In nature there is a huge diversity in maturity of the locomotor system of mammals at birth. Extremely precocial species such as the horse are able to follow the herd within hours after birth, while at the other end it may take years to develop a fully functional locomotor system, as is the case in humans. Functionality of the locomotor system is dependent on two key issues: (a) sufficient strength of the system components to generate the necessary force and withstand the loading provoked by locomotion and (b) appropriate development of neuromotor control to produce gait. The heavy loads generated by locomotion are mainly counteracted by the skeleton, parts of which are still undergoing the process of endochondral ossification in recently born individuals. Gait should be sufficiently regular and consistent to be effective.

This thesis focuses at this fascinating period of early development of locomotor functionality in precocial species from both the perspective of bone development and of gait maturation. The work seeks to identify areas in which young animals of precocial species differ from either their altricial counterparts or their mature homologues and thus where and how they do not comply with the standard paradigms, such as Wolff's law on the adaptation of bone, or well-established gait patterns. Furthermore, there is special attention for osteochondrosis, an aberration of endochondral ossification, which is the most common developmental disorder in the horse, with a huge impact on the equine industry and on equine welfare, and for the possible implications of current management practices, such as the use of NSAIDs in fattening pigs. The work goes in depth on the true nature and oxygen-dependent functionality of osteoclasts, the special multi-nucleated cells that are indispensable for the process of bone remodeling, which is extremely active in early life.

Development of gait

Gait analysis in the horse

The usage of the horse, which is in present-day society mostly for leisure and sports, requires an optimally functioning locomotor system. Although equine gait can well be assessed by the trained eye (Dyson, 2014), as is currently still common practice at for instance studbook admission tests or pre-purchase examinations, the human eye is limited in its abilities to collect detailed information about gait. With the advent of sophisticated measuring devices, able to objectively quantify gait with a much better spatial and temporal resolution than the human eye, research into (equine) gait entered

a new era. Gait can now objectively and accurately be assessed by studying kinetic and kinematic gait parameters. Kinetics covers the forces exerted by the subject that is studied and kinematics describes the motion of the segments the subject is composed of.

Force plates, which are able to measure all three components of the ground reaction force, were among the first instruments used to study the kinetic aspects of gait and lameness in the horse (Merkens and Schamhardt, 1988a; 1988b; 1988c). As they are very precise and accurate in doing so, they are still considered the gold standard for studying the kinetics of gait (Merkens et al., 1993). However, they have the drawback that many of them are not portable. Further, data collection is often laborious and tedious, as only one limb can be measured at a time and the horse has to hit the plate in the right spot.

Pressure measuring plates on the other hand are mobile and larger in size, enabling the registration of more limbs in one run (van Heel et al., 2004). Their disadvantage is that they are not as accurate in determining absolute forces as force plates (Oosterlinck et al., 2010a). Furthermore, they cannot decompose the ground reaction force in the three constituting elements in an orthogonal coordinate system, as they only report vertical forces. Nevertheless, they have been used widely and successfully for kinetic studies in several species in recent years (van der Tol et al., 2003; Oosterlinck et al., 2010a; 2010b; Meijer et al., 2014). An alternative to force or pressure plates is the use of an instrumented horseshoe (Kai et al., 2000; Chateau et al., 2009), which allows measuring the ground reaction forces during locomotion and on different surfaces. However, the weight of the shoe and the availability are limiting factors.

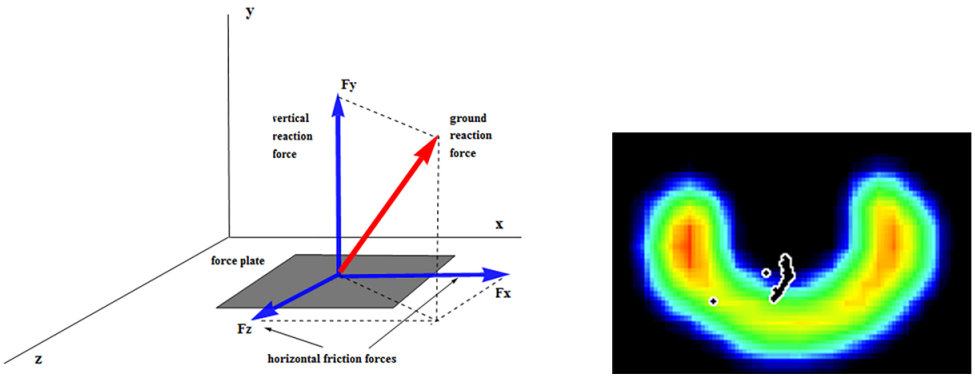


Figure 1: *Schematic representation of a force plate measuring all three components of the ground reaction force (left) and a typical example of a hoof print obtained with a pressure plate system (right). The different colors represent differences in pressure.*

Kinematic gait analysis aims at describing the movement of body segments during locomotion. In the past this was done with help of photography and film. A famous example is the series of photographs taken by Eadweard Muybridge in 1878 of the trotter "Occident", owned by the railroad magnate Leland Stanford, that solved the age-old question whether in trot there was a suspension phase with all 4 feet off the ground or not (van Weeren, 2013). The advance of opto-electronic techniques and development of systems working with skin markers made it possible to study gait kinematics in much more detail and at higher resolutions. The CODA-3 system for example was able to collect data in 3D at recording speeds of up to 300 Hz (van Weeren et al., 1990). Nowadays, three dimensional infrared camera systems are available, able to track and record multiple reflective markers simultaneously. As they are highly accurate and precise, these so-called motion capture systems are considered the gold standard in kinematic gait analysis. The other option to measure kinematic gait parameters is the use of sensors that can measure acceleration (Kastner, 1989). Nowadays, these sensors have evolved into small, but highly sophisticated inertial measurement units (IMUs) (Pfau et al., 2005). These IMUs are able to collect acceleration data and determine position by double integration (Pfau et al., 2005). Modern IMUs are very accurate in measuring acceleration (Clayton and Schamhardt, 2013), are user friendly and are very versatile, as they can be used on location. Presently, they are validated with the gold standard motion capture systems.

Development of gait in the horse

The horse is the only species that was domesticated for the athletic capacity of its locomotor system. It may hence not be surprising that the history of research into equine gait spans centuries (van Weeren, 2013) and has led to hundreds of scientific publications. However, investigations into the ontogeny of equine gait are rare. Whereas it is been stated by some, based on the precocious character of the species, that foals are born with adult-like postural abilities and coordination (Fox, 1964; Lelard et al., 2006), scientific proof to back this statement is lacking. On the contrary, recent work has indicated that young foals show relatively poor postural control after birth, which will slowly but gradually improve during the first few months of life (Nauwelaerts et al., 2013). This picture of immature locomotor activity after birth also emerges from one of the few studies that have focused on the development of equine gait. Denham et al. (2012) showed that foals have not yet achieved an even, four beat rhythm at walk when 21 weeks old, thus providing evidence for the existence of a period of gait and balance maturation, as has been demonstrated in other animals (Westerga and Gramsbergen, 1990; Muir et al., 1996).

The few studies focusing on the development of gait in foals were all kinematic in nature (Drevemo et al., 1987; Back et al., 1994; Denham at al., 2012). The development of kinetic aspects, so relevant to the mechanical loading of the limb, has never been studied. Furthermore, the youngest animals to be included in a study regarding the (kinematic) development of gait were aged three weeks, leaving the period immediately after birth completely unexplored. This is the period in which, based on what is known about the development of static balance (Nauwelaerts et al., 2013), most prominent changes can be expected.

Skeletal development

The process of endochondral ossification.

Long bone development starts with the formation of a cartilaginous anlage that will serve as a template for the bony skeleton. During the process of endochondral ossification, all but the articular cartilage is replaced by bone, starting with the formation of a bony collar in the mid-region of the bone. Subsequently, vessels invade the cartilage of the shaft, making it possible for bone progenitor cells to enter the cartilage and differentiate into osteoblasts that will form the primary ossification centre. Slightly later, the secondary ossification centres develop in the central regions of the epiphyses. They are separated from the shaft of the bone by the still cartilaginous growth plate, which enables longitudinal bone growth. The epiphysis itself also increases in size, thanks to the presence of growth cartilage that is located between the centre of ossification and the articular cartilage, the articular-epiphyseal growth cartilage (Mackie et al., 2008).

On a cellular level, the process of endochondral ossification commences as resting chondrocytes, located in the region farthest away from the ossification front, start to proliferate. After dividing and subsequent development, the chondrocytes move a bit closer to the ossification front and form multicellular columns. After this proliferating phase, chondrocytes undergo hypertrophic differentiation and secrete extracellular matrix that becomes mineralized later. After mineralization of their matrix, chondrocytes undergo apoptosis and the cartilaginous matrix is remodelled. Hereby, cells of the ossification front can invade the cartilage and use the remnant matrix spicules as a scaffold for the deposition of bone matrix (Mackie et al., 2008). In parallel to chondrocyte apoptosis, at the ossification front chondrocytes may also transdifferentiate into osteoblasts (Zhou et al., 2014).

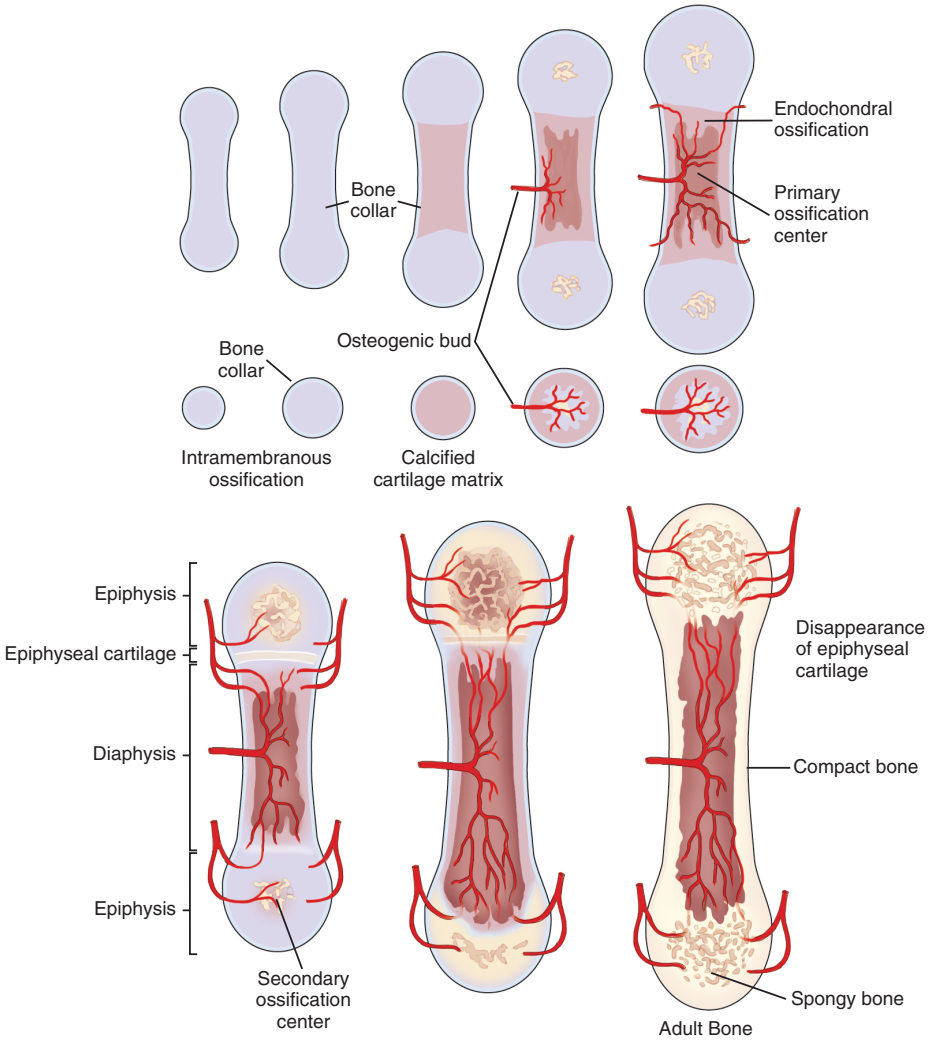


Figure 2: *Schematic representation of the endochondral ossification process of a long bone, illustrating the formation of a primary ossification centre in the diaphysis of the bone (upper part) and secondary ossification centres (lower part). Reprinted with permission from "Joint Disease In The Horse", second edition, by McIlwraith C.W., Frisbie D.D., Kawcak C.E. and van Weeren P.R. St. Louis: Elsevier.*

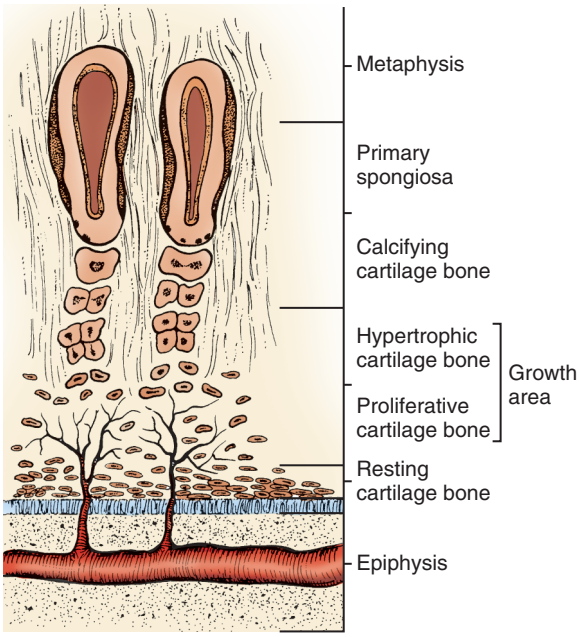


Figure 3: *Schematic representation of the growth plate, showing the consecutive phases of chondrocyte development. Reprinted with permission from "Joint Disease In The Horse", second edition, by McIlwraith C.W., Frisbie D.D., Kawcak C.E. and van Weeren P.R. St. Louis: Elsevier.*

Bone can be compared with reinforced concrete as it consists of mineral hydroxyapatite (the concrete) and a collagen type I network (the steel reinforcement), which comprises about 90% of the non-mineralised matrix of bone (Buckwalter et al., 1995). The composite character of bone is evident in its formation process. Bone is produced in two steps; the collagen network is synthesised first by the osteoblasts, followed by the deposition of the hydroxyapatite crystals within and between the newly formed collagen fibres (Pearson and Lieberman, 2004).

When endochondral ossification derails: osteochondrosis

Bone development is orchestrated by a complex system of endocrine and auto/paracrine regulators, controlling chondrocyte proliferation and differentiation. In the horse, aberrations of this development affecting joints or joint-related structures of the musculoskeletal system, indicated as juvenile osteochondral conditions (Denoix et al., 2013), are common. Of these, osteochondrosis (OC) is most common and also relevant in other species like the pig (Ytrehus et al., 2007). Osteochondrosis is defined as a disturbance of the process of endochondral ossification, either in the growth plate and/or in the articular-epiphyseal complex (Olsson and Reiland, 1978; Ytrehus et al., 2007).

Osteochondrosis in the horse is considered a multifactorial disease with several underlying causes, including high growth rate, intake of a high-energy or unbalanced diet, biomechanical influences that may be related to a poor conformation or trauma and it has also an heritable component (Laverty and Girard, 2013; Semevolos, 2017). Currently, there is reasonable consensus that damage to cartilage canals, which contain the blood vessels irrigating the growth cartilage, leading to focal areas of chondronecrosis is the principal early pathogenetic mechanism of OC (Olstad et al., 2015). The underlying causes that lead to this damage and that determine why such damage occurs in some animals and in others not are more contentious.
Theories include, among others, aberrations in collagen type II constitution or metabolism (Laverty and Gerard, 2013) and carbohydrate metabolism-related changes in gene expression patterns that affect several signalling pathways related to cartilage formation and repair, such as the Wnt/ß catenin, Indian hedgehog and TGF-ß pathways (Serteyn et al., 2010).

Wolff's law on the adaptation of bone
Already in the 19th century scientists studied bone adaptation to loading (Roux, 1881) and explained trabecular bone architecture by mathematic rules (Wolff, 1892). Their findings were slightly refined in the Mechanostat theory a century later (Frost, 2001). This work on the relationship between bone remodeling and loading, known as "Wolff's law of bone adaptation" is still very relevant today. The essence of this law is that bone adapts to loading by decreasing bone mass when loaded below a certain threshold and increasing it in reaction to loading above this threshold, achieving an optimal balance between bone weight and strength (Huiskes et al., 1987; Frost 2001; MacLatchy and Müller 2002; Christen et al., 2014). Subsequently, trabeculae will line up along the main direction of loading, as trabeculae that are loaded under a certain threshold will disappear, which will increase the degree of anisotropy of the trabecular bone (Tanck et al., 2001; Wolschrijn and Weijs, 2004).

How does Wolff cope with the lack of loading in precocials?
The process of bone adaptation is beautifully illustrated by several studies on trabecular bone ontogeny of altricial species. In both human babies and puppies, trabecular bone volume fraction follows a U-shaped curve after birth (Wolschrijn and Weijs, 2004; Ryan and Krovitz, 2006; Gosman and Ketcham, 2009), neatly following the principle of "Wolff's law". After birth, both babies and puppies are relative immobile and the mechanical stimulus in the fetal period, *i.e.* the intra-uterine movements and "kicking" against the uterine wall, is lost. This leads to the loss of bone, as mechanical strain is below the threshold. When the children or young dogs become mobile later in life, loading and associated bone strain will increase, leading to the observed increase in bone volume.

In comparison to altricial species, in precocial animals like foals and calves, the mechanical environment changes dramatically after birth. During gestation, their limbs are folded and not bearing weight, limiting loading to forces generated by intrauterine muscle contractions. After birth the limbs are extended and within hours weight-bearing and subjected to the considerable forces generated by locomotion. In this special situation the classic adaptation mechanism based on the Mechanostat theory cannot be sufficient and hence some form of anticipative bone development must exist to prepare the precocial skeleton for its future function. Indeed, in a number of species and anatomical locations evidence has been found for some form of anticipative trabecular bone development. Significant trabecular alignment, fitting postnatal loading, was shown at birth in the mule deer and ovine calcaneus (Skedros et al., 2004; 2007). These observations are not likely to be the result of strain-related adaptation, suggesting that at least part of the skeleton is anticipating future loading, possibly based on a genetic blueprint (Cunningham and Black, 2009). Given the foal's precocial behavior, it is plausible that after birth there is less bone loss and most likely an immediate gradual increase of trabecular bone volume fraction, comparable to altricials after they start movement.

The potential effect of NSAIDs on skeletal development

In the pig industry, painful procedures, like castration and tail docking, are routinely performed (Llamas Moya et al., 2008; Sutherland, 2015). Also, lameness, often associated with pain, is a common problem in both fattening pigs and sows used for breeding (Main et al., 2000; Kilbride et al., 2009). Due to the increased awareness of welfare in food producing animals, and possibly as a substitute for the use of antibiotics as a means to enhance growth, standard treatment with non-steroidal anti-inflammatory drugs (NSAIDs) is gaining ground and may increase in the future in this industry. NSAIDs provide effective pain management by the reduction of the production of prostaglandins through the inhibition of cyclooxygenase (COX)-activity. Activity of the iso-enzyme COX-1 is mostly associated with tissue homeostasis, while COX-2 activity is inducible and the driving force of an inflammatory reaction. NSAIDs have several well-known negative side effects on the gastro-intestinal tract, renal papillae and on primary hemostasis, both in humans (Conaghan, 2011; Bozimowski, 2015) and animals (Clark, 2006; Goodrich and Nixon, 2006), which are mainly caused by the suppression of COX-1 activity.

For this reason, selective COX-2 inhibitors have been developed that have to a large extent replaced the older generation of general COX-inhibitors. Prostaglandins are induced by pathologic processes, especially inflammation, and have a role in pain perception, but they are also known to be important for bone metabolism (Raisz, 1995). They influence both bone resorption by osteoclasts (Okada et al., 2000; Take et al., 2005) and formation of

bone (Weinreb et al., 1997; Suponitzky and Weinreb, 1998) and osteoblast differentiation (Arikawa et al., 2004). Besides their roles in bone metabolism, prostaglandins have also recently been shown to play an important role in the hypertrophic differentiation in endochondral ossification (Welting et al., 2011). This has led to debate about prolonged use of NSAIDs, as long-term COX-2 inhibition can lead to delayed fracture healing (Inal et al., 2014). Given the similarities between the processes of fracture healing and endochondral ossification, it can be speculated that COX-2 inhibition might affect skeletal development negatively, especially in a fast growing animal such as the pig. However, specific information regarding the effect of COX-2 inhibition on bone development in growing piglets is lacking and there is a need to generate such knowledge, given the developments in the pig industry pointed out above.

The osteoclast: a crucial but insufficiently understood player in skeletal remodeling

Within the context of bone development, osteoclasts, the bone resorbing cells, play an essential role. Osteoclasts are multinucleated cells, formed by fusion from monocyte precursor cells (Marks and Walker, 1981). They resorb bone by the production of several enzymes like cathepsin K, matrix metalloproteinase 9 and gelatinase (Odgren et al., 2016). There are numerous signaling pathways and substances known to play a role in the recruitment, development and function of osteoclasts.

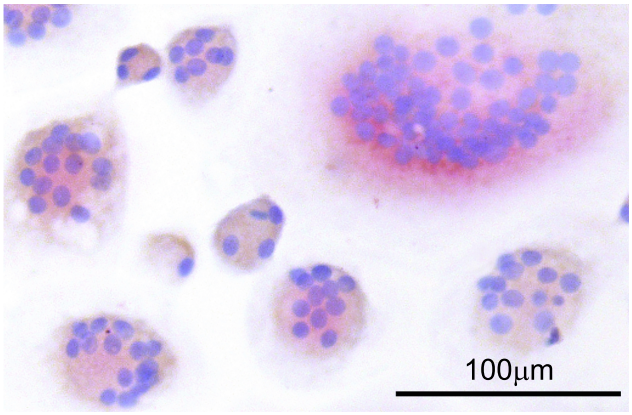


Figure 4: *Representative examples of multinucleated, tartrate-resistant acid phosphatase positive, osteoclasts. Nuclei are stained with haematoxylin.*

One essential question that remains unanswered is in what context we have to place the multinucleated nature of osteoclasts. In literature they are mostly described as "quiescent cells" (Takahashi et al., 2010) but it is not unlikely that, as in other cells with increased DNA content, the term "senescent" would be more appropriate. Senescence was first observed in cells after extensive passaging and thought to be the underlying cause of

aging (Hayflick and Moorhead, 1961). The current definition of cellular senescence entails withdrawal from the cell cycle combined with the acquirement of the senescence associated secretory phenotype. Senescence plays important roles in the development of polyploid/multinucleated cells, including megakaryocytes, formed by endomitosis without cytokinesis (Besancenot et al., 2010) and placental syncytiotrophoblast, formed by fusion of cytotrophoblasts (Chuprin et al., 2013). In a cell-specific and context dependent manner, senescence regulates their function while limiting oncogenic transformation. Furthermore, senescence has been described to be essential in tissue remodeling during embryonic development (Munoz-Espin et al., 2013). Although there are some indications that osteoclasts may also become senescent, the role of the senescence in bone resorption remains as yet underexplored.

Outline of the thesis

The main aim of this thesis was to investigate how the skeleton of precocial animals anticipates on and copes with the postnatal loading environment. With respect to gait, the longitudinal development of kinetic gait parameters is studied by means of pressure plate analysis in healthy foals and during the course of osteochondrosis development (chapter II). Furthermore, the development of dynamic balance is addressed in the same cohort of foals (chapter III). At the tissue level, the strategy used by precocials to cope with the extreme change in loading after birth is investigated by the study of structurally important bone parameters. These included trabecular bone parameters of the distal tibia and talus of warmblood and Shetland pony foals and Holstein-Friesian calves (Chapter IV). Postnatal development of these parameters in the warmblood foal is also investigated, combined with analysis of the collagen type I network (Chapter V). Potential side-effects of prolonged use of NSAIDs in the pig industry are addressed in Chapter VI, where long-term treatment with meloxicam on bone and cartilage development in growing pigs is studied. Finally, Chapter VII goes into depth on the potential senescent character of osteoclasts and explores the relationship between senescence and bone resorption by modulating oxygen tension.

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CHAPTER II:

The development of locomotor kinetics in the foal and the effect of osteochondrosis

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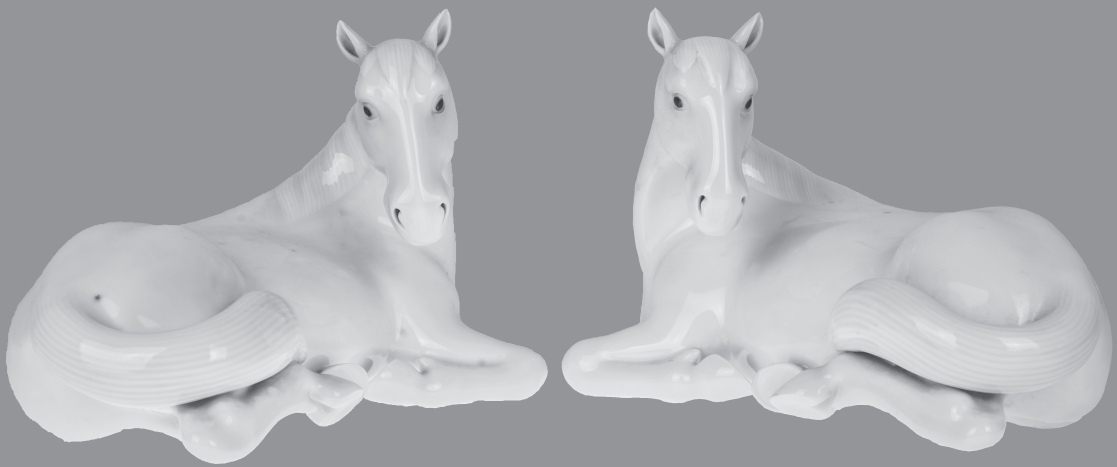

Summary

Background: Foals stand and walk immediately after birth, but insight into the subsequent longitudinal development of their gait kinetics in the early juvenile phase and the possible influence of osteochondrosis (OC) thereon is lacking.

Objectives: To quantify gait kinetics in foals during the first half year of life, taking into account their OC status.

Study design: Prospective, cohort study performed at a single stud farm.

Methods: Pressure plate measurements at walk and trot from eleven Dutch warmblood foals during the first 24 weeks of life were used to determine body mass normalised peak vertical force (nPVF), vertical impulse (nVI) and stance duration (StD). Coefficients of variation (CV) of PVF and StD were used as measures for gait maturity. Radiographs of tarsocrural and femoropatellar joints were taken at four to six weeks and after six months to check for OC. A linear mixed model was used to determine the effects of age, limb, presence of OC and speed on gait parameters.

Results: Mean walking and trotting velocity increased over time as did StD and nVI, nPVF values however remained relatively constant. During the first weeks of their life only the CV of StD decreased significantly, while the CV of PVF did not. None of the foals was visibly lame, but the presence of OC resulted in a temporarily but significantly reduced nPVF.

Main limitations: This study is limited by the relatively small sample size only containing one breed from a single stud farm, use of a stand-alone pressure plate and estimation rather than measurement of body mass.

Conclusions: Despite being precocious, foals need time to mature their gait. During growth, velocity at walk and trot increases, but nPVF remains relatively constant. Although not visibly lame, a temporary reduction in nPVF was detected in OC positive foals using a pressure plate.

Introduction

"Born to move" could be the motto of the horse, a species that has always been bred for its locomotor qualities. Research into equine gait spans centuries (van Weeren, 2013), but investigations into the development of equine gait are rare. It is stated by some that foals are born with adult-like postural abilities and coordination (Fox, 1964; Lelard et al., 2006). However, recent work has indicated that foals show relatively poor postural control after birth, which gradually improves during early life (Nauwelaerts et al., 2013). Denham et al. (2012) showed that foals have not yet achieved an even, four beat rhythm at walk when 21 weeks old, providing evidence for the existence of a period of gait and balance maturation, as has been demonstrated in other animals (Lelard et al., 2006; Muir et al., 1996; Westerga and Gramsbergen, 1990).

The few studies focusing on the development of gait in foals were all kinematic in nature (Back et al., 1994; Denham et al., 2012; Drevemo et al., 1987) and, to the authors' knowledge, kinetic aspects of the development of equine gait have never been studied. Stabilographic analysis of young foals has already shown that suboptimal postural balance is characterized by larger swaying amplitudes and velocities (Nauwelaerts et al., 2013). A similar incoordination can be expected during locomotion and would lead to inconsistent weight-bearing per limb, hence to increased variation of vertical forces between different steps.

The prevalence of osteochondrosis (OC) in young foals can be very high (van Grevenhof et al., 2009), although the majority of the lesions present at an early age will heal (Dik et al., 1999). Still, presence of OC lesions might influence gait and hence locomotor parameters, as has been reported in pigs (de Koning et al., 2012; Meijer et al., 2014). It is therefore imperative that any study looking at the longitudinal development of equine gait simultaneously monitors OC status in the most commonly affected joints (*i.e.* the tarsocrural and femoropatellar joints) to exclude any influence from OC lesions on conclusions drawn about normal gait development.

We hypothesized that foals initially exhibit an immature gait, characterized by a larger coefficient of variation (CV) of peak vertical forces (PVF), which would improve over time, in line with the development of their postural balance (Nauwelaerts et al., 2013). Moreover, it was hypothesized that the presence of OC lesions would significantly affect measured kinetic locomotor variables of the foal.

Materials and Methods

Foals

Eleven privately owned Royal Dutch Sport Horse foals (five mares, six stallions), bred for show jumping were used in this study. They were all born and housed at the same stud farm and raised following usual standards in the Dutch horse breeding industry. In this case, foals were kept together with their dams in a stable bedded with straw and had daily access to a pasture. After weaning between week 20 and 24 the foals were housed in a large, half-open group stable with straw bedding.

Before each measurement session, foals were clinically examined and only included if they were considered clinically sound. Limbs and joints were inspected and palpated for the presence of swelling and/or joint effusion and soundness was visually checked at walk and trot on a straight line on a hard surface. Height at the withers was regularly measured and body mass was estimated according to the method of Staniar et al. (2004).

Data collection

A pressure plate with a measuring surface of 1.95 x 0.32 m (Footscan 3D, 2 m system[a]), connected to a laptop computer with appropriate software (Gait Scientific[a], version 7.99 – 27.05.2014) was used. These pressure plates are equipped with 16,384 sensors (sensitivity 0.27 – 127 N/cm^2; 2.06 sensors/cm^2), measuring at 126 Hz. Before every session, the system was calibrated according to the manufacturer's instructions and offset was manually adjusted to avoid saturation of the sensors. The pressure plate was embedded in a custom made, wooden frame to create a 1.5 m wide and 2.3 m long level measuring area with a small ramp in front and behind to prevent stumbling. To protect the pressure mat, the runway including the plate was covered with a 10 m long, 1.5 m wide and 5 mm thick rubber mat[b] (natural rubber/styrene-butadiene rubber, shore hardness 65 +/- 5).

All measurements took place in an empty stable building at the stud farm. After a five minute warm-up period, foals were consistently led over the pressure plate at their preferred speed by an experienced handler. Before weaning the foals followed their dams, led by another person next to the pressure plate; after weaning foals were handled alone. A trial was considered valid if the foal moved over the pressure plate in a consistent way, looking straight ahead and with the hooves making full ground contact within the measuring area. When the foals were small, all limbs could be measured during one run, but with increasing size left and right limb data needed to be recorded in separate trials. For each limb, five valid measurements were collected.

On day two or three and on day seven after birth, data were recorded at walk. From two until 12 weeks of age, foals were measured every two weeks at both walk and trot and subsequently at 16, 20 and 24 weeks. Velocity of the foals was estimated by measuring the "limb velocity" of the first fore limb contacting the pressure plate (distance covered by two successive hoof strikes divided by the time between them). Relative velocity (Froude number) was calculated using the formula v^2/gh (v: velocity, h: height at withers and g: gravitational acceleration). All trials were recorded with a small digital video camera (Philips full HD 1080p camcorder[c]) for retrospective visual control.

Data analysis

Collected footprints were manually assigned to left fore (LF), right fore (RF), left hind (LH) or right hind (RH) based on the video images. Peak Vertical Force (PVF) and Vertical Impulse (VI) were normalized to body mass. The results of each set of five measurements per limb were averaged and considered representative for that limb at that measuring moment. Intra-individual variability of PVF and stance duration (StD) for each limb was assessed by calculating "within session" coefficients of variation (CV = SD/Mean x 100%) (Petrie and Watson, 2013). Symmetry of hind limb loading at trot was assessed by calculating asymmetry indexes (ASIs) of the PVF (Contralateral hind limbs (CHL): CHL = ((LH-RH))/(0.5*(LH+RH))*100) (Meijer et al., 2014). The resulting value is a dimensionless figure between -200 and 200, with 0 meaning perfect symmetry. Ground reaction force curves of the five OC negative foals at week two, 12 and 24 were made with a custom-made script (Matlab r2015b[d]) after extracting the raw force data. Vertical force data were normalized to body mass and stance time using linear interpolation.

Radiographic examinations

For logistic reasons radiographic examinations of the foals were clustered and took place on location using a Gierth 400 X-ray machine[d] and FDR D-EVO plus C24i panels[e]. The first examination took place at the age of four to six weeks; the second examination at an age of at least 6 months (age range 6 to 9 months). Foals were sedated with detomidine (Domosedan[f]).

Three radiographs (dorso-plantar, latero-medial and dorsomedial-palmarolateral oblique) were taken from the tarsocrural joints, and two (latero-medial and craniolateral-caudomedial oblique) from the femoropatellar and femorotibial joints. The radiographs were evaluated by two board-certified veterinary radiologists.

Statistics

Statistical analysis was performed using SPSS Statistics 22[g] with statistical significance set at $P \leq 0.05$. Correction for multiple comparisons was done with the False Discovery Rate method of Benjamini – Hochberg (1995). Both for walk and trot, a linear mixed effect model with foal ID added as random intercept was used to evaluate the effects of week and limb (fore or hind) as fixed factors and velocity as a covariate on kinetic locomotor parameters and the coefficients of variation. The interaction between week and OC status was added to the model to identify temporal effects of OC within gait. Normality and homoscedasticity assumptions were met by log transformation of the kinetic parameters and square root transformation of the coefficients of variation.

Results

Average body mass of the group (n=11) of foals increased from 58.1 kg (± 4.4 kg) at two or three days of age to 225.4 kg (± 18.7 kg) when 24 weeks old. In the same period average height at withers increased from 1.02 m (±0.02 m) to 1.39 m (± 0.04 m) (Fig. 1). Clinical evaluation of the foals did not reveal abnormalities, except for two occasions at 24 weeks. In both cases foals were lame, showing mild effusion and painful passive flexion of a fetlock joint. A week later they were considered sound again and pressure plate measurements were taken, which were included in the dataset as being representative for week 24.

At the first radiographic screening (age four to six weeks), the tarsal joints of five foals were OC negative; three foals showed unilateral and three others bilateral signs of OC (Table 1). At six months, all but three of these nine tarsal OC lesions had resolved. Accurate evaluation of the stifle joints was not possible during the first radiographic examination due to the physiological irregular contour and granular subchondral bone opacity of the femoral trochlear ridges. At the second examination, no radiographic signs of OC in the stifles were found.

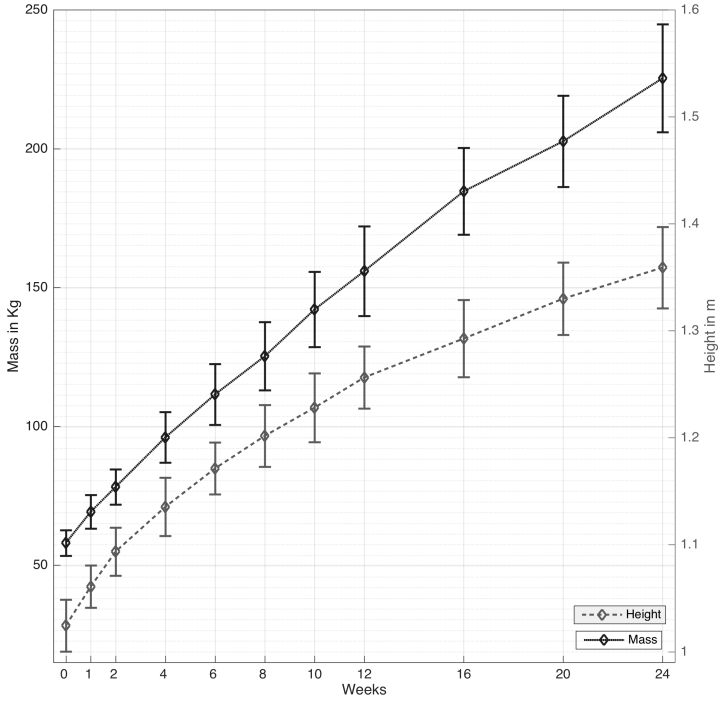

Figure 1: *Average (± SD) body mass (kg) and height at the withers (m) of all foals at the different measuring moments.*

Table 1: *Gender, and osteochondrosis (OC) status (at six and 24 weeks) of the foals. The figure in the column "OC status" corresponds with the number of OC positive limbs. All OC lesions were detected in the tarsal joints, none in the stifle joints.*

Foal number	Gender	OC status 6 weeks	OC status 24 weeks
1	stallion	2	0
2	stallion	1	0
3	stallion	0	0
4	stallion	1	0
5	mare	2	2
6	mare	0	0
7	mare	1	0
8	mare	0	0
9	stallion	2	1
10	mare	0	0
11	stallion	0	0

Walk

Gait parameters obtained at walk are presented in Table 2. The increase and variation in limb velocity at walk was limited, with average values increasing from 1.0 (±0.1) m/s to 1.2 (±0.1) m/s during the study period. Both nPVF and nVI were significantly higher in the front compared to the hind limbs. Over time nPVF values stayed relatively constant and, compared to week 24, only average nPVF of week one was significantly lower. Over the complete study period, walking speed did not have a significant effect on nPVF. At week eight average nPVF was significantly lower in the OC affected limbs, at week 10 the opposite was the case. Mean CV of nPVF was significantly higher in the hind limbs compared to the fore limbs and values fluctuated during the study; the highest variation was seen at week 24. Front limb CV of nPVF was about 10-20 %, whereas hind limbs showed about 20-30%, except for week 24 in which values of over 40% were found. Differences between OC positive and negative foals were only significant at weeks two, six and twelve; in all cases OC positive animals had lower CV values than OC negative foals. Speed had no significant effect on CV of nPVF. Compared to week 24, nVI was significantly lower in weeks zero and one. Speed had a significant effect on nVI, which decreased with increasing speed. OC affected animals had significantly lower nVI in week one whereas in week 10 nVI was significantly higher in OC positive animals. Stance duration increased significantly over time. When taking week 24 as a reference, StD was significantly lower during the first 12 weeks. Limb (fore or hind) and OC status did not have a significant effect on StD, whereas speed did; StD decreased with increasing speed. Variability of StD reduced over time, compared to week 24; CV of StD was significantly higher during the first six weeks of life. No significant effect of limb or OC status was found.

At walk the GRF curves of the OC negative foals (supplementary figures 1, 2 and 3) were quite constant. In the hind limbs, the heights of the two peaks that constitute the time-force curve were almost identical. For the front limbs, some variation in height between the peaks existed, but at week 24 a consistently higher second peak was found.

Trot

The results for the trot are presented in table 3. At trot average speed increased more compared to the walk (from 2.4 (± 0.4) m/s to 3.2 (± 0.3) m/s). Just as at walk, nPVF and nVI were significantly higher in the front limbs. Also at trot, average nPVF stayed relatively constant and only the results found in weeks two and four were significantly lower compared to week 24. At week four and six average nPVF was significantly lower in the OC positive foals. Again, when analyzed for the complete study period, also at trot, velocity did not have a significant effect on nPVF.

At trot, CV of nPVF was lower than at walk and only at week ten OC positive foals had significantly lower CV of nPVF. Speed had no significant effect on variability of PVF. At trot nVI increased over time, average values of week two to eight were significantly lower than in week 24. With increasing speed, nVI decreased significantly. OC affected animals had only significantly lower nVI values in week six. Like at walk, StD increased over time, with StD being significantly lower during the first 12 weeks, compared to week 24. Presence of OC lesions did not affect StD significantly, whereas increasing speed was associated with a significant reduction of StD. At trot CV of StD at week two was significantly higher compared to week 24 and only at week two CV of StD was significantly higher in the OC positive animals. There was no significant effect of speed on variability of StD.

At trot not much variation in the shape of the GRF curves was observed over time (Supplementary figures 1, 2 and 3). Presence of a persistent unilateral OC lesion in the left limb led to a more asymmetric limb loading pattern compared to an OC negative foal, shifting more weight to the contralateral hind limb at trot (supplementary figure 4).

Table 2: *Mean and SD of velocity and Froude numbers at walk for the different measuring moments. Per limb (front or hind) and OC status at week 6, average body mass normalised peak vertical force (nPVF), normalised vertical impulse (nVI), stance duration (StD) and coefficients of variation (CV) of nPVF and StD are presented.*

Age (weeks)	**0.5**				**1**				**2**			
	Mean		*SD*		*Mean*		*SD*		*Mean*		*SD*	
Velocity (m/s)	1.0		0.1		1.1		0.2		1.1		0.2	
Froude number	0.10		0.03		0.11		0.03		0.12		0.03	
OC status 6w	**OC -**		**OC +**		**OC -**		**OC +**		**OC -**		**OC +**	
Forelimbs	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
nPVF (N/kg)	7.7	1.4	6.8	1.6	7.1	2.0	5.9	1.0	7.6	2.0	6.6	1.5
nVI (NS/kg)	3.2	0.5	3.0	0.6	2.8	0.4	2.1	0.7	3.4	0.9	3.3	0.9
StD (ms)	619.4	89.4	642.8	75.8	588.3	64.1	698.1	113.8	661.4	35.2	714.0	92.6
CV nPVF (%)	18.6	8.0	11.3	6.6	15.7	9.8	13.9	5.1	16.3	6.2	12.2	4.8
CV StD (%)	13.7	7.0	14.9	5.7	13.4	5.9	11.2	5.3	14.1	7.8	15.3	3.8
Hindlimbs	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
nPVF (N/kg)	5.4	1.4	4.8	1.0	5.3	1.2	4.6	0.7	5.5	1.2	5.1	0.8
nVI (NS/kg)	1.9	0.3	2.0	0.4	2.2	0.7	1.8	0.7	2.5	0.5	2.4	0.4
StD (ms)	600.7	81.5	640.2	92.5	615.1	58.3	708.0	67.6	667.1	29.9	714.7	83.8
CV nPVF (%)	22.3	16.6	14.8	8.5	18.9	7.5	25.2	11.5	19.5	10.0	12.3	6.2
CV StD (%)	15.9	7.2	15.4	10.2	11.3	4.4	12.0	6.5	12.8	6.4	11.8	5.7
Age (weeks)	**4**				**6**				**8**			
	Mean		*SD*		*Mean*		*SD*		*Mean*		*SD*	
Velocity (m/s)	1.1		0.1		1.1		0.1		1.2		0.2	
Froude number	0.11		0.02		0.11		0.02		0.12		0.15	
OC status 6w	**OC -**		**OC +**		**OC -**		**OC +**		**OC -**		**OC +**	
Forelimbs	*Mean*	*SD*	*Mean*	*SD*	*Mean*	SD	*Mean*	SD	*Mean*	*SD*	*Mean*	*SD*
nPVF (N/kg)	7.9	1.3	6.5	1.3	9.0	1.6	7.2	1.1	8.6	2.2	6.9	0.6
nVI (NS/kg)	3.7	0.6	3.0	0.7	4.2	0.9	3.6	0.8	4.0	1.0	3.4	0.6
StD (ms)	701.0	77.9	689.9	58.5	707.8	49.9	749.5	48.1	705.8	63.1	725.5	95.8
CV nPVF (%)	10.9	3.5	14.9	6.9	11.4	8.3	11.4	4.7	11.9	5.2	14.2	5.7
CV StD (%)	8.2	3.7	10.8	5.4	8.4	3.0	10.4	3.4	6.5	1.7	10.2	4.3
Hindlimbs	*Mean*	*SD*	*Mean*	*SD*	*Mean*	SD	*Mean*	SD	*Mean*	*SD*	*Mean*	*SD*
nPVF (N/kg)	5.2	0.8	5.0	0.7	6.3	1.2	5.2	0.9	6.3	1.5	4.7	0.7
nVI (NS/kg)	2.5	0.5	2.4	0.4	3.1	0.6	2.8	0.6	2.9	0.9	2.4	0.4
StD (ms)	694.9	79.7	677.5	44.0	696.6	61.8	734.4	60.1	691.7	57.3	731.4	89.1
CV nPVF (%)	16.3	8.6	17.8	9.8	22.9	13.2	17.0	6.9	22.4	15.3	23.1	14.4
CV StD (%)	8.2	2.7	8.7	4.2	9.3	4.6	9.0	4.4	6.1	1.5	7.1	4.1

Age (weeks)	10				12				16			
	Mean		*SD*		*Mean*		*SD*		*Mean*		*SD*	
Velocity (m/s)	1.2		0.1		1.3		0.1		1.2		0.1	
Froude number	0.11		0.01		0.13		0.02		0.12		0.02	
OC status 6w	**OC -**		**OC +**		**OC -**		**OC +**		**OC -**		**OC +**	
Forelimbs	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
nPVF (N/kg)	7.3	1.2	7.8	1.4	8.1	1.4	7.7	1.8	9.1	1.4	8.4	1.7
nVI (NS/kg)	3.5	0.9	4.0	0.7	3.8	0.6	3.7	1.0	4.6	1.2	4.3	1.0
StD (ms)	707.1	81.1	750.5	49.3	714.5	91.5	717.0	54.6	753.1	97.5	761.6	95.3
CV nPVF (%)	15.6	7.0	13.1	7.4	15.3	6.1	12.3	5.2	16.0	7.5	12.7	5.1
CV StD (%)	6.8	3.7	7.2	2.5	7.1	3.6	6.4	2.1	5.6	2.4	6.6	3.6
Hindlimbs	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
nPVF (N/kg)	5.1	1.3	6.0	1.3	5.5	1.0	5.1	1.0	5.4	2.0	5.6	1.4
nVI (NS/kg)	2.6	0.7	3.1	0.5	2.7	0.7	2.6	0.5	2.8	0.7	3.1	0.7
StD (ms)	705.2	72.1	739.4	65.3	704.8	64.9	710.7	78.0	744.1	80.4	775.1	82.7
CV nPVF (%)	24.1	13.2	23.4	9.9	18.2	12.7	18.7	8.2	31.3	11.6	24.0	10.7
CV StD (%)	7.0	3.2	6.5	3.9	5.6	3.8	8.9	4.6	6.9	4.2	7.2	4.1

Age (weeks)	20				24			
	Mean		*SD*		*Mean*		*SD*	
Velocity (m/s)	1.3		0.2		1.2		0.2	
Froude number	0.12		0.03		0.10		0.05	
OC status 6w	**OC -**		**OC +**		**OC -**		**OC +**	
Forelimbs	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
nPVF (N/kg)	9.3	2.2	9.4	2.0	9.1	2.3	8.0	2.3
nVI (NS/kg)	4.2	0.7	4.9	1.5	4.2	1.2	4.0	1.4
StD (ms)	723.6	68.4	764.8	73.6	721.3	98.3	754.7	54.8
CV nPVF (%)	12.7	4.5	14.3	8.3	19.8	8.8	16.1	9.2
CV StD (%)	10.7	5.5	8.5	4.7	7.8	4.8	6.9	2.6
Hindlimbs	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
nPVF (N/kg)	5.3	1.5	5.8	1.6	5.2	1.1	4.9	1.5
nVI (NS/kg)	2.5	0.6	3.1	1.2	2.4	0.8	2.5	1.0
StD (ms)	717.3	98.7	760.1	71.0	720.9	72.4	758.3	47.5
CV nPVF (%)	23.2	18.3	24.9	11.9	41.7	13.0	29.4	17.3
CV StD (%)	8.1	3.6	10.1	4.9	6.6	2.6	6.3	5.1

Table 3: *Mean and SD of velocity and Froude numbers at trot for the different measuring moments. Per limb (fore or hind) and OC status at week 6, average body mass normalised peak vertical force (nPVF), normalised vertical impulse (nVI), stance duration (StD) and coefficients of variation (CV) of nPVF and StD are presented.*

Age (weeks)	**2**		**4**		**6**	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*
Velocity	2.4	0.4	2.6	0.4	2.8	0.2
Froude number	0.55	0.16	0.6	0.16	0.67	0.12

OC status 6w	**OC -**		**OC +**		**OC -**		**OC +**		**OC -**		**OC +**	
Forelimbs	*Mean*	SD	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	SD	*Mean*	SD
nPVF (N/kg)	11.6	2.5	10.0	2.8	12.9	3.8	9.6	2.4	14.4	3.3	11.1	2.4
nVI (NS/kg)	1.6	0.3	1.5	0.3	1.8	0.6	1.5	0.4	2.1	0.5	1.7	0.4
StD (ms)	253.9	27.1	267.7	30.6	253.1	17.5	274.0	18.1	269.4	13.6	268.3	24.5
CV nPVF (%)	11.2	7.8	9.3	5.9	12.6	7.0	10.1	5.7	14.9	7.2	12.1	4.7
CV StD (%)	6.6	3.6	12.3	5.7	8.8	3.6	9.7	4.8	8.6	3.5	8.4	2.8
Hindlimbs	*Mean*	SD	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	SD	*Mean*	SD
nPVF (N/kg)	9.5	2.3	8.9	1.6	10.3	1.9	8.2	1.2	11.8	1.9	9.5	2.0
nVI (NS/kg)	1.4	0.4	1.3	0.2	1.4	0.3	1.2	0.2	1.7	0.3	1.3	0.3
StD (ms)	256.3	18.8	269.9	31.6	260.0	23.9	281.1	23.3	263.5	32.6	262.9	28.5
CV nPVF (%)	15.7	12.4	15.1	9.4	12.1	4.1	14.5	6.8	16.8	8.0	14.0	7.4
CV StD (%)	6.8	4.8	13.8	11.0	11.4	7.6	14.9	9.7	8.5	4.6	11.3	8.5

Age (weeks)	**8**		**10**		**12**	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*
Velocity	3.1	0.4	3.3	0.2	3.3	0.4
Froude number	0.81	0.18	0.92	0.1	0.88	0.21

OC status 6w	**OC -**		**OC +**		**OC -**		**OC +**		**OC -**		**OC +**	
Forelimbs	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
nPVF (N/kg)	14.2	4.1	11.4	2.0	13.1	3.1	13.1	3.3	14.5	2.0	12.0	2.4
nVI (NS/kg)	1.9	0.5	1.7	0.3	1.9	0.4	1.9	0.5	2.2	0.3	1.8	0.4
StD (ms)	247.6	9.4	264.2	21.0	254.8	14.2	264.7	16.7	269.9	17.3	270.6	17.4
CV nPVF (%)	13.7	7.5	12.5	5.9	17.8	5.9	9.8	5.4	15.1	8.7	12.0	3.1
CV StD (%)	9.2	2.3	9.2	5.0	8.1	3.0	8.2	2.4	7.5	2.7	7.2	3.3
Hindlimbs	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
nPVF (N/kg)	11.5	3.7	9.2	1.4	10.1	2.4	11.0	2.5	11.3	2.4	10.1	1.9
nVI (NS/kg)	1.6	0.5	1.3	0.2	1.4	0.4	1.6	0.3	1.6	0.4	1.4	0.3
StD (ms)	248.9	8.8	263.5	32.5	263.0	13.4	266.9	23.2	264.9	21.8	267.1	17.2
CV nPVF (%)	18.9	7.3	15.9	6.0	19.3	9.9	13.3	7.2	16.2	9.2	13.0	5.7
CV StD (%)	9.2	3.4	8.1	5.2	7.7	3.3	9.4	6.0	8.3	4.7	8.4	4.2

Age (weeks)	16				20				24			
	Mean		*SD*		*Mean*		*SD*		*Mean*		*SD*	
Velocity	3.1		0.3		3.4		0.5		3.2		0.3	
Froude number	0.77		0.15		0.92		0.25		0.78		0.16	
OC status 6w	**OC -**		**OC +**		**OC -**		**OC +**		**OC -**		**OC +**	
Forelimbs	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
nPVF (N/kg)	15.1	1.7	13.4	2.3	15.5	3.4	15.1	1.5	15.1	3.7	13.5	3.5
nVI (NS/kg)	2.4	0.4	2.2	0.4	2.4	0.5	2.3	0.4	2.4	0.6	2.1	0.5
StD (ms)	284.4	12.9	285.2	18.2	276.0	18.9	276.3	32.7	287.2	14.5	289.3	12.4
CV nPVF (%)	14.9	6.0	18.1	11.3	11.7	5.4	15.3	7.4	19.2	10.3	13.8	11.8
CV StD (%)	7.1	2.9	7.6	3.0	7.3	1.6	6.7	3.6	5.7	3.3	6.9	2.6
Hindlimbs	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
nPVF (N/kg)	11.8	2.0	11.0	1.6	12.1	1.7	12.1	1.8	11.3	2.8	10.8	3.8
nVI (NS/kg)	1.8	0.3	1.7	0.3	1.8	0.3	1.8	0.5	1.7	0.4	1.6	0.5
StD (ms)	282.0	21.1	294.5	24.3	275.7	16.1	288.8	44.0	287.8	15.5	280.9	24.3
CV nPVF (%)	13.5	6.2	17.2	8.8	14.4	6.2	19.0	10.8	24.5	10.7	17.4	11.7
CV StD (%)	10.3	6.9	10.7	5.3	7.4	6.4	9.5	6.1	7.3	5.2	8.2	5.3

Discussion

The results of this study provide more insight into the effects of growth and OC on equine locomotor kinetics. Although (limb) velocity at walk and trot increased over time, nPVF stayed relatively constant during the first half year of life. Variability of StD decreased over time, suggesting the presence of a period of gait maturation in foals. Although clinically sound, OC affected foals in this study showed temporarily lower nPVF values in the affected limb, indicative of (subclinical) lameness.

For logistic reasons this study was limited to one stud farm and restricted to a cohort of 11 foals. A portable pressure plate system was used, as our force plate system is not mobile. Pressure plates are not as accurate and precise in determining absolute forces as force plates, which is a limitation of the study. Studies using the same pressure plate system simultaneously with a force plate reported that the maximal vertical forces recorded by the pressure plate are lower (Oosterlinck et al., 2010a). Nonetheless, the accuracy of the system has proven to be acceptable (Oosterlinck et al., 2010b), making it possible to use a pressure plate system to detect trends in gait kinetics over time.

Furthermore, the measuring frequency (126 Hz) and with that temporal resolution of the used pressure plate system is relatively low, especially compared to a force plate system. However, the impact of this lack of resolution is relatively low, as it will mainly affect

the smoothness of the force-time curves, but only very minimally the height of the peak (Oosterlinck et al., 2010a).

The method described by Staniar et al. (2004) is considered to be the most accurate way to estimate the mass of a foal, although this method has only been validated in Thoroughbred foals. Nonetheless, we extrapolated this method to Dutch warmblood foals as quite a considerable percentage of Thoroughbred has been bred into the Dutch warmblood. Furthermore, the birthweights of both breeds are rather similar (Barneveld and van Weeren, 1999; Hendriks et al., 2009; Whittaker et al., 2012) and the same accounts for the weight range later in life (Barneveld and van Weeren, 1999; Staniar et al., 2004) indicating a similarity in growth rate. Further, although in this particular study we were not able to validate our results by comparing the estimated mass of the foals with actual measurements, the average foal mass estimated during our study was in line with values reported in Dutch warmblood foals in studies in which mass was accurately measured (Back et al., 1994; Barneveld and van Weeren, 1999).

Five foals were OC-negative throughout the study period, representing the "normal foal"; the remaining six were OC-affected at some stage. The number of foals is limited, however, other studies in the field of equine gait and balance analysis have used comparable numbers of animals (ranging from n=5 (Oosterlinck et al., 2010b) to n=24 (Back et al., 1994). Results obtained in this study concerning body mass, growth rate and velocity during walk and trot were in line with other studies on Warmblood foals (Back et al., 1994; Barneveld and van Weeren, 1999; Denham et al., 2012). The prevalence of OC lesions at six weeks was lower than previously described (Dik et al., 1999), but foals in that study were bred from OC-positive sires and dams. At six months, beyond the age of no return for tarsal OC lesions (Dik et al., 1999), figures were similar to adult Warmblood populations (Dik et al., 1999; van Grevenhof et al., 2009; Jönsson et al., 2011), yet slightly lower than reported in yearlings (Jacquet et al., 2013; van Grevenhof et al., 2009).

Per limb and per gait, we collected five valid strides to calculate the average values for each parameter investigated. Especially when working with (very) young animals, it is a delicate balance between the number of strides collected (increasing numbers increase the accuracy of "the average stride") and the effects of the handling on the animals (inducing fatigue and thus increasing variation). A comparably study in young pigs (Meijer et al., 2014) collected only three valid strides per animal. Studies in adult horses (Oosterlinck et al., 2010a) and ponies (Oosterlinck et al., 2010b) using the same system have based their results on five strides as well.

Observed nPVF during the study period was about 9 N/kg (fore) and 6 N/kg (hind) at walk and 14 N/kg and 11 N/kg for the fore and hind limbs at trot, almost the same as in adult ponies (fore: 8.9 N/kg, hind: 7.0 N/kg at walk; fore: 12.5 N/kg, hind: 11.2 N/kg at trot (Oosterlinck et al., 2011)). Reported values in adult warmbloods are lower with 6.5 N/kg (Merkens et al., 1986) and 10.4 N/kg (Merkens et al., 1993) measured in the forelimbs at walk and trot respectively. Locomotion parameters of differently sized animals can only be compared at the same relative velocity, which is indicated by the Froude number, correcting velocity for height. Calculated Froude numbers for the foals were comparable with the values reported in ponies (Oosterlinck et al., 2011). Reported Froude numbers in adult Dutch warmbloods are similar for walk but higher for trot (Merkens et al., 1986; 1993).
The idea behind Froude numbers is that animals differing in size but comparable in proportion, display the same locomotor pattern when moving at velocities corresponding to equal values of Froude numbers (Alexander and Jayes, 1983). However, conformation and proportion of foals change during growth (Anderson and McIlwraith, 2004; Thompson, 1995), leading to variability of inertial properties and centre of mass motion (Buchner et al., 1997; 2000). Consequently, the requirements of the dynamic similarity hypothesis are not met, precluding drawing of valid conclusions on the comparison of limb loading of foals with adult horses or ponies.

Speed is known to influence most of the kinetic and kinematic gait parameters (Hoyt et al., 2002; Khumsap et al., 2001a; 2001b; 2002; Leach and Cymbaluk 1986; McLaughlin et al., 1996; Robert et al., 2002; Weishaupt et al., 2010). In adult horses, an increase in nPVF and a reduction in StD and nVI is seen when the animal moves faster (McLaughlin et al., 1996; Robert et al., 2002; Weishaupt et al., 2010). In foals however, StD and nVI increased over time, whereas nPVF values, as in young goats (Main and Biewener, 2004), remained relatively constant. This is seemingly contradictory with the observed increase in walking and trotting speed. However, in growing foals the increase in stride length due to their growing limbs is responsible for the increase in speed, whereas adult horses increase their stride frequency (Weishaupt et al., 2010) when moving at higher speeds, explaining the differences described above. Furthermore, vertical forces are influenced by limb stiffness, which changes during growth (Robilliard and Wilson, 2005).

Given the fact that stabilographic analysis of young foals has shown that suboptimal postural balance is characterized by larger swaying amplitudes and velocities (Nauwelaerts et al., 2013), comparable balance incoordination could be expected during locomotion. Elaborating on this, this imbalance would potentially lead to irregular weight-bearing over the limbs over subsequent strides and hence to increased variation

when comparing peak vertical forces between different steps, as also seen in a study on spinal ataxia in adult horses (Ishihara et al., 2009). Surprisingly, no significant reduction in variability of PVF was observed during the study period. Nevertheless, average variability of PVF, measured with the same pressure plate system in adult ponies was about 10 – 15% (Oosterlinck et al., 2011), which is somewhat lower than in our study and possibly indicating that reduction of PVF variability takes place at a later age. Variability was highest at week 24, the moment all foals were weaned. Although it is tempting to think that the increased CV was caused by more variation in velocity during the measurements, this was not the case as can be seen in table 2 and 3. It may be that weaning and consequently handling the foals alone had an increasing effect on variability. Lastly, there could also be an effect of the increased number of trials needed, as at the end of the study left and right limb data needed to be collected separately, leading possibly to more inter-trial variability.

In contrast, a reduction in variability of StD was observed in this study. This was most prominent during the first two weeks after birth. In the same period foals experience most progress in developing static balance (Nauwelaerts et al., 2013), which is indicative for maturation of the neuro-musculoskeletal system. Nauwelaerts et al. (2013) suggested that foals initially rely on so-called fast, but imprecise and ballistic motor control (open loop), later switching to the more precise, closed loop control (Collins and De Luca, 1993). More precise control leads to lower variability, therefore this parameter can be used to quantify gait maturity, as has been done in the past for toddlers (Clark et al., 1988) and warmblood foals (Back et al., 1994). Variability of StD provides information about timing, whereas CV of PVF can be seen as an indicator of how wobbly foals are. As foals need to stand and walk almost immediately after birth, the "pacemaker" in locomotor pattern development may well develop earlier in time than stability of the CoM.

Vertical limb loading at trot, measured at week four and six was significantly lower in OC affected animals and more asymmetry was seen in (unilaterally) OC-positive animals. At walk, PVF observed in the OC positive foals was also significantly lower at week eight; however, it was higher in these animals at week ten. Reduction of PVF was previously reported to be a gait adaptation mechanism in an induced lameness model (Weishaupt et al., 2006; Weishaupt, 2008). Although the foals in the present study were not visibly lame, this finding strongly suggests that around week four and six subclinical lameness was present in the OC-positive foals. The variable effect of OC on gait parameters with age may possibly be explained by the highly dynamic character of OC lesions in the early juvenile phase (Dik et al., 1999), which will most likely affect pain perception and hence manifestation of lameness. Due to ethical and economic reasons this could unfortunately not be confirmed by diagnostic anaesthesia.

Conclusions

Although foals are "born to move", it is clear that the way they move at birth is different from adult gait. Similar to the development of postural balance (Nauwelaerts et al., 2013), some time for gait maturation is needed after birth, which is reflected by a decrease in StD variability. During growth, preferred speed at walk and trot increases. Limb length and StD increase as well and increasing stride length rather than stride frequency as in the adult horse is responsible for the increase in speed. Meanwhile, nPVF and hence maximal loading of the internal structures of the distal limb remains relatively constant. Even when subjectively classified as sound, OC lesions may temporally influence PVF, as shown in this study. The (sub)clinical manifestation will most likely be related to the actual status of the lesion, which is known to change rapidly in early life. Therefore, pressure plate systems might have the potential to serve as an early identification tool for detection of foals that might need further examination.

Ethical note

This study was reviewed and approved by the ethical committee of Utrecht University (DEC no.2014.III.04.038) and all effort was taken to minimize discomfort of the foals and their dams. For all procedures carried out, written consent was obtained from the owner of the animals.

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Manufacturers' details

[a]RsScan International N.V., Paal, Belgium
[b]De Mulder Rubber and Plastics, Gent, Belgium
[c]Koninklijke Philips N.V., Eindhoven, The Netherlands
[d]MathWorks, Natick, USA
[e]Gierth X-Ray International GmbH, Riesa, Germany
[f]Fujifilm Holdings Corporation, Tokyo, Japan
[g]Orion Pharma Animal Health, Espoo, Finland
[h]IBM Corporation, Armonk, New York, USA

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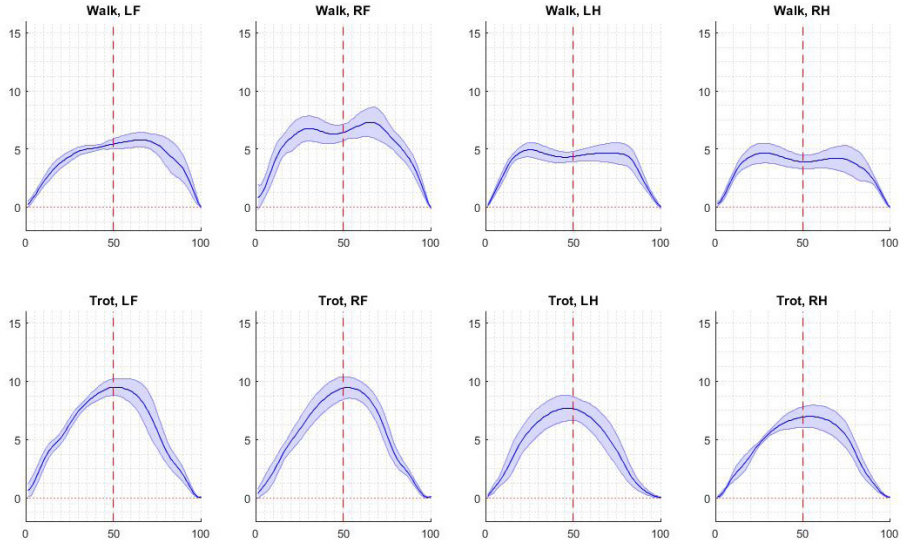
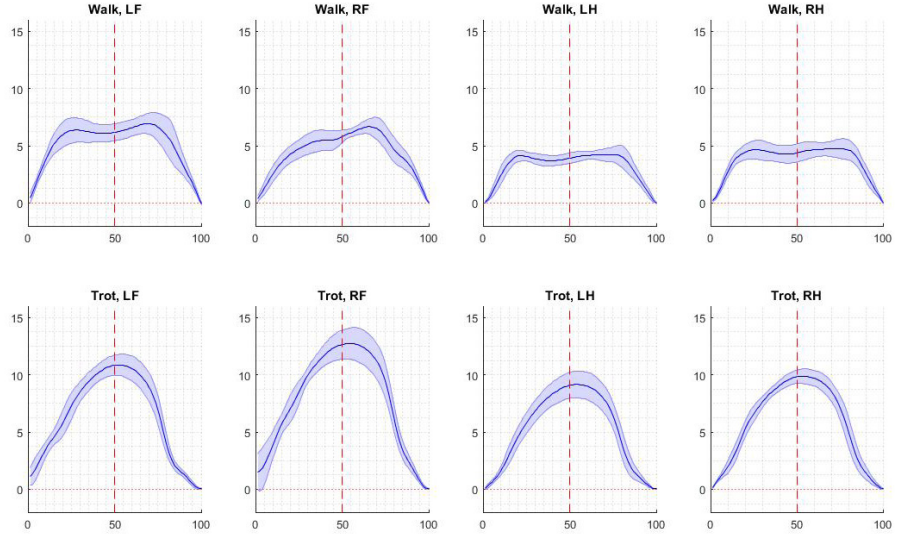
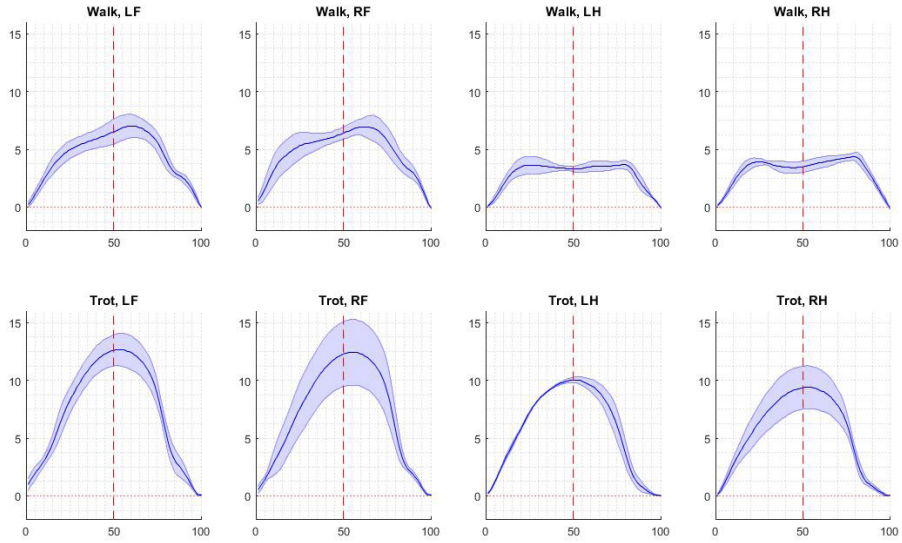


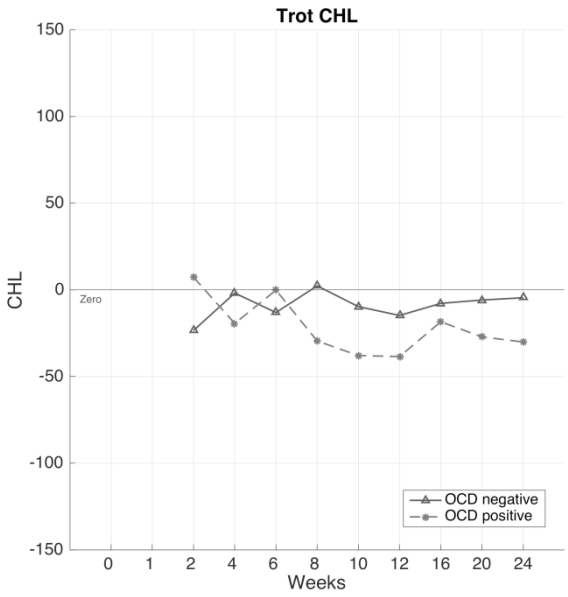
Supplementary figure 1: *Mean force (body mass normalised) time (normalized by linear interpolation) curves at week two of the OC-negative foals for each limb (LF: left fore, RF: right fore, LH: left hind, RH: right hind) at walk and trot. The central line of the force time curves represents the mean and the shaded area represents the median absolute deviation (MAD).*



Supplementary figure 2: *Mean force (body mass normalised) time (normalized by linear interpolation) curves at week 12 of the OC-negative foals for each limb (LF: left fore, RF: right fore, LH: left hind, RH: right hind) at walk and trot. The central line of the force time curves represents the mean and the shaded area represents the median absolute deviation (MAD).*



Supplementary figure 3: *Mean force (body mass normalised) time (normalized by linear interpolation) curves at week 24 of the OC-negative foals for each limb (LF: left fore, RF: right fore, LH: left hind, RH: right hind) at walk and trot. The central line of the force time curves represents the mean and the shaded area represents the median absolute deviation (MAD).*



Supplementary figure 4: *Asymmetry indexes (ASI) of the contra-lateral hind limbs (CHL) at trot of a representative OC-negative and unilateral OC-positive foal at trot.*

CHAPTER III:

Footing of the foal: the development of hoof balance and landing preference in the postnatal period

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Submitted

Summary

Background: Foals are able to follow the herd within hours from birth, but it has been shown that kinetic gait parameters and static balance still have to mature. However, development of dynamic balance has not been investigated.

Objectives: To quantify the way of landing and pressure pattern dynamics under the hoof during the first half year of life.

Study design: Prospective, cohort study performed at a single stud farm.

Methods: Pressure plate measurements at walk and trot from ten Dutch warmblood foals during the first 24 weeks of life were used to quantify toe – heel and lateral – medial hoof balance asymmetry indexes and to determine preferred landing strategy. Concurrently, radiographs of the tarsocrural and femoropatellar joints were taken at four to six weeks and after six months to check for osteochondrosis. A linear mixed model was used to determine the effects of time point, limb pair (front / hind), side (left / right) and osteochondrosis status of every foal.

Results: Toe-heel balance gradually shifted to the heel and medio-lateral balance to lateral. This was accompanied by a gradual shift of landing preference in the same directions. Variability in pressure distribution decreased over time. (Subclinical) osteochondrosis did not influence any of the measured parameters.

Main limitations: This study is limited by the relatively small sample size only containing one breed from a single stud farm.

Conclusions: Dynamic hoof balance and preferred landing gradually change in the early juvenile phase in foals. Knowledge of this gradual stabilisation of hoof balance and preferred landing may be useful for the clinician when taking decisions about possible interventions such as trimming in early life.

Introduction

As precocious fright-and-flight animals, foals are able to follow the herd within hours from birth. While being able to keep up this early, their gait has to mature (Denham et al., 2012; Gorissen et al., 2017). The same applies to their static balance, as stabilographic measurements showed that both the amplitude and velocity of the body's centre of pressure movements decrease rapidly after birth (Nauwelaerts et al., 2013). At the same time, the variability of stance duration at walk and trot is reduced, suggesting a maturation of the musculoskeletal system and gait control (Gorissen et al., 2017). It is not unlikely that dynamic balance, *i.e.* the way of landing and the pressure pattern under the hoof during the stance phase, also goes through rapid development during this period, but this has never been investigated.

Pressure plate systems can objectively quantify the pressure distribution underneath the hoof during the stance phase (van Heel et al., 2004). Previous studies have successfully used this technique to quantify toe – heel and medial – lateral hoof balance using asymmetry indices (ASI) in the fore feet of adult warmblood horses and ponies (Oosterlinck et al., 2013; 2014). These results suggested that adult horses present a distinct loading pattern at walk, generally loading the lateral side of the hooves more (Oosterlinck et al., 2013). Although van Heel et al. (2004) reported lateral landing as preferred way in both front and hind limbs at trot, Oosterlinck et al. (2013) reported contralateral differences in the lateral –medial loading pattern between the front hooves at impact, whereas at the end of stance, the lateral parts of both front feet were loaded more. In ponies these contralateral differences were not observed (Oosterlinck et al., 2014). Using high speed video recordings Wilson et al. (2016) reported a large variation, both within horses and between them, in front hoof placement. Nevertheless, the authors reported that for the studied population of adult horses (age range 4-23 years), the most common hoof placement pattern at walk was the lateral heel and at trot the lateral hoof wall.

For this study, we hypothesised that foals initially show a relatively high degree of inconsistency in their toe-heel and medial-lateral loading patterns in the stance phase at walk and trot due to immature coordination. This would translate as relatively high variability in the lateral - medial and toe-heel ASIs, which would then gradually decrease to stabilise in the patterns observed in adult horses. For hoof placement at landing, we similarly hypothesised that young foals show more variation in their preferred landing than older foals.

Materials and methods

Foals

The data for this study were collected during the experimental sessions that have also yielded data for another study on the longitudinal development of kinetic gait parameters and the possible influence of osteochondrosis (OC) thereon in warmblood foals (Gorissen et al., 2017). In short, eleven privately owned Royal Dutch Sport Horse foals (five mares, six stallions) bred for show jumping, were used. All foals were born and housed at the same stud farm and kept together with their dams in a stable bedded with straw and daily access to a pasture. After weaning between weeks 20 and 24 the foals were housed in a large, half-open group stable with straw bedding.

One colt was excluded from this study due to uneven front feet. Before each measurement session, foals were visually checked at walk and trot on a straight line and hard surface to ensure that they were clinically sound. The OC status of the femoropatellar, femorotibial and tarsocrural joints was assessed by radiographic evaluation at the age of four to six weeks and at an age of at least 6 months (age range 6 to 9 months). During the study period, no hoof trimming was performed.

Data collection

Data were collected using a pressure plate with a measuring surface of 1.95 x 0.32 m, (Footscan 3D, 2 m system[a]), with a sampling rate of 126 Hz, connected to a laptop computer with the dedicated software (Gait Scientific[a], version 7.99 – 27.05.2014). The pressure plate was embedded in a wooden frame, with a small ramp in front of and behind the plate to avoid stumbling. The runway, including the pressure plate, was covered with a 10 m long, 1.5 m wide and 5 mm thick rubber mat[b] (natural rubber/ styrene-butadiene rubber, shore hardness 65 +/- 5) to protect the plate. Before each measuring session, the pressure plate was calibrated according to the manufacturer's instructions and offset was manually adjusted to avoid sensor saturation.

All measurements were performed at the stud farm in an empty stable building. After a short warm-up period, foals were consistently led over the pressure plate at their preferred speed by an experienced handler. Before weaning the foals followed their dams, led by another person next to the pressure plate; after weaning foals were handled alone. A trial was considered valid if the foal looked ahead, moved over the pressure plate in a consistent way and the hooves made full ground contact within the measuring area. When the foals were small, all limbs could be measured during one run, but with increasing size left and right side data needed to be recorded in separate trials. At each

measuring session, at least five valid measurements were collected from each hoof at walk and trot. During the first two weeks after birth, data were recorded solely at walk. From week two until week 12, foals were measured every fortnight at both walk and trot and subsequently at 16, 20 and 24 weeks. All trials were recorded with a small digital video camera (Philips full HD 1080p camcorder[c]) for retrospective visual control.

Data analysis

Collected footprints were manually assigned to left fore (LF), right fore (RF), left hind (LH) or right hind (RH) based on the video images. Hoof prints were divided in a toe and heel region by a line through the maximal hoof width and in a medial and lateral zone by a line through the middle of the hoof as described earlier by Oosterlinck et al. (2013). Any footprints that were not aligned with the pressure plate coordinate system were excluded from the analysis to ensure correctness of the calculations that followed. After exporting the raw data from the pressure plate system, data was processed using custom written matlab[d] scripts. Toe – heel and medio – lateral hoof balance ASI were calculated using the vertical forces obtained for the four hoof zones by the following formulas (Oosterlinck et al., 2013):

$$\text{ASI (toe-heel)} = \frac{VF(toe) - VF(heel)}{0.5 * (VF(toe) + VF(heel))} * 100$$

$$\text{ASI (medial-lateral)} = \frac{VF(medial) - VF(lateral)}{0.5 * (VF(medial) + VF(lateral))} * 100$$

For the toe-heel ASI, a positive value indicates higher loading towards the toe zone and a negative value higher loading towards the heel zone. For the lateral-medial ASI, a positive value indicates higher loading towards the medial zone and a negative value higher loading towards the lateral zone.

To compare the data obtained from the different measurements, all ASI data was normalized to percentage of stance. This was performed by interpolating each measurement to 200 samples using a shape-preserving, piecewise cubic interpolation method. For each hoof-impact, the preferred hoof placement zone was determined based on the force distribution over the four zones at impact with the plate (first recorded data frame). For this we have analysed the four zones in two pairs, medial-lateral and toe-heel. Based on the zone pair which had the highest force value, the measurement was later classified as toe or heel first and as lateral or medial first.

Statistics

Open software (R version 3.3.1[e]) was used for statistical analysis using the package 'nlme' (version 3.1–121) for linear mixed effects model, package 'lme4' (version 1.1-12) for logistic regression analysis and the package ggplot2 (version 2.2.1) for generating plots. To evaluate the preferred landing site a logistic regression analysis in a reparametrized binomial model was used and odds ratios were calculated by exponentiation of the model estimates.

Both for walk and trot, a linear mixed effect model, with foal ID used as a random effect and time point, limb pair (front / hind), side (left / right) and OC status as fixed effects, were used for statistical evaluation of the data. Correction for multiple comparisons was done for both models with the False Discovery Rate method of Benjamini – Hochberg (1995).

For all models, the first measurement moment (i.e. week 0.5 for walk and week 2 for trot) was used as a reference for the comparison between time points.

Results

Foals

All foals were considered clinically sound before each measurement session, except for two foals at 24 weeks. As these animals were sound again one week later, pressure plate measurements were taken then and included in the dataset as being representative for week 24. At the first radiographic screening, five foals were negative for OC, whereas in the other five at least one lesion was found. At the second screening moment, two out of the five foals were still positive for OC.

Figure 1 presents a front, hind and lateral view of a representative foal used in the study, illustrating the changes in conformation observed between week one and 12. At birth, this foal showed a subtle valgus deviation and relatively wide base of support, whereas later in life, the limbs were straight and their base of support relatively smaller.

Figure 1: *Front (a&d), hind (b&e and lateral (c&f) views of the same foal at an age of 1 week (a, b and c) and 12 weeks (d, e and f) illustrating conformational changes over time. Note the subtle valgus deviation at week 1 in all 4 limbs.*

Walk ASI

Median toe – heel ASI values during the whole stance phase are presented in figure 2. At one percent of the stance phase, only the front limbs showed significantly more loading of the heel region at week 12 in comparison with the first measurement at week 0.5. At 25% of the stance duration, front limbs showed significantly more loading of the heel region in week six until 20, whereas in the hind limbs the difference in this phase of stance with the first measurement was only significant at week 24, which was the only difference found in the hind limbs. In the front limbs, there were also differences at 50% from week 6-24 and at 75% from week 16-24. Statistical analysis at the end of stance (i.e. 99%), was not possible due to skewedness of the data, even after transformation. No significant effects of side (left / right) or OC status were found on the toe – heel balance.

Medial – lateral ASI at walk is presented in figure 3. At one percent of the stance duration, both the front and hind limbs showed significantly more loading of the lateral side of the hooves from week six until 24. At 25% of stance, the same trend was visible with significantly more loading of the lateral side of the front hoofs at week eight – 12 and from week six until 24 for the hind limbs. At half and 75% of stance, no significant changes of the medial – lateral loading distribution were found. A significant difference between the left and right limb, with more lateral loading of the right side was observed at 1, 25 and 50% of the stance duration, but not at 75%. No significant effect of OC status was found for the medial - lateral balance.

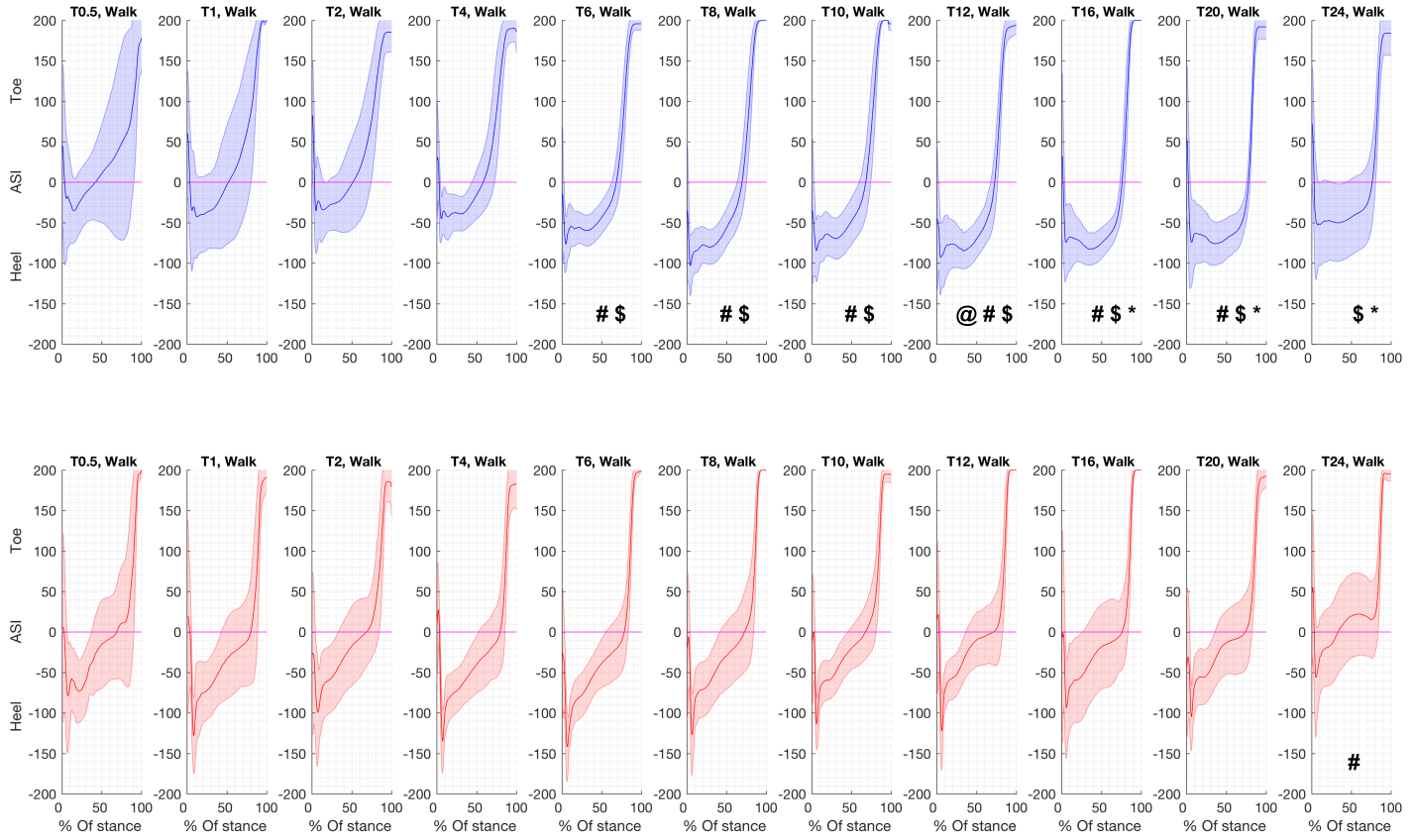

Figure 2: *Front (upper row, blue) and hind (lower row, red) Toe – Heel ASIs calculated for the complete stance duration at walk for the different ages. The line represents the median ASI of all foals and the shaded area the median absolute deviation of all foals. Statistically significant differences compared to the first measurement at 1% of stance are indicated with the symbol @, at 25% of stance with #, at 50% with $ and at 75% with *.*

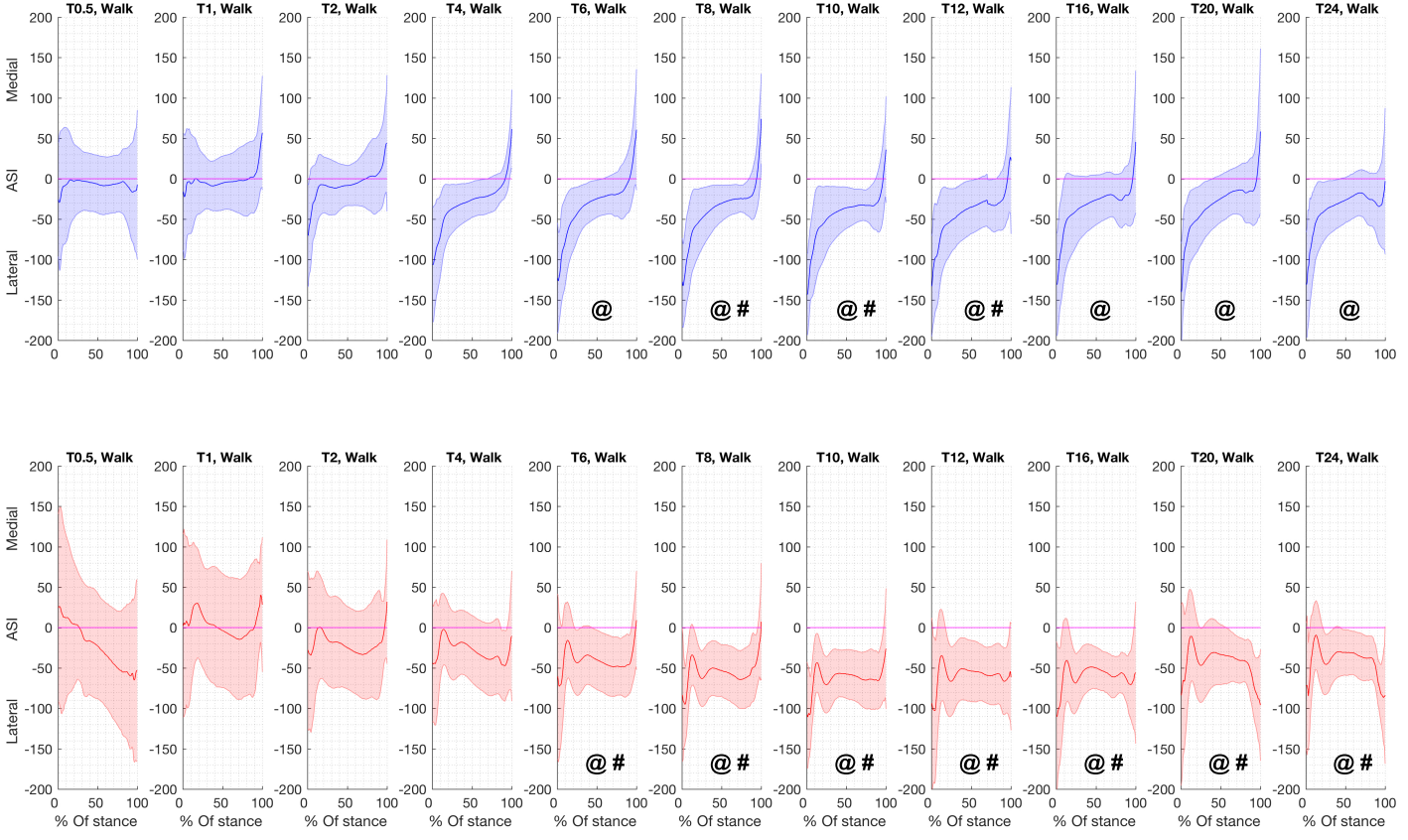

Figure 3: *Average front (upper row, blue) and hind (lower row, red) Medial - Lateral ASIs calculated for the complete stance duration at walk for the different ages. The line represents the mean ASI of all foals and the shaded area the median absolute deviation of all foals. Statistically significant differences compared to the first measurement at 1% of stance are indicated with the symbol @, at 25% of stance with # , at 50% with $ and at 75% with *.*

Trot ASI

For the toe-heel ASI at trot, only at week 16 for the front and week 10 in the hind limbs, statistically significant differences, indicative of more loading of the heel region, were found at the beginning of the stance phase (1%)(Fig. 4). At 25% of stance, the front limbs showed significantly more loading of the heel at week 12 – 20. During mid-stance, significantly more loading towards the heel was observed at weeks four and six in the hind limbs. At 75% of stance, significantly more loading of the heel region in the front limbs was observed in weeks 16 and 20, for the hind limbs this was only the case at week four. No significant effects of side (left/right) or OC status were found on the toe – heel balance.

Medial – Lateral hoof balance at trot is presented in figure 5. At one percent of stance, significant more loading of the lateral part of the front hooves was observed at week eight until 12 and in week 24. For the hind limbs this was only the case at week 10 and 24. At all points during stance, the right hooves showed significantly more loading of the lateral side. There were no effects of OC status on the medial – lateral hoof loading pattern.

Intra-trial variability

A general trend of decreasing intra-trial variability over time was observed, which was more prominent in the hind limbs (table 1). At walk, front limb intra-trial variability of the toe – heel ASI did only change significantly at 25% (week 16) and 50% (week eight, 16 and 20) of stance. In all cases, variability was lower. Hind limb variability of the toe – heel balance showed besides significantly lower variability at week 10 and 16 (25% of stance) and week 16 (50% of stance) also significantly lower variability at 75% of stance at weeks six, eight, 16 and 24. At trot, only one difference was significant, in the hind limbs at week 10 at 75% percent of stance.

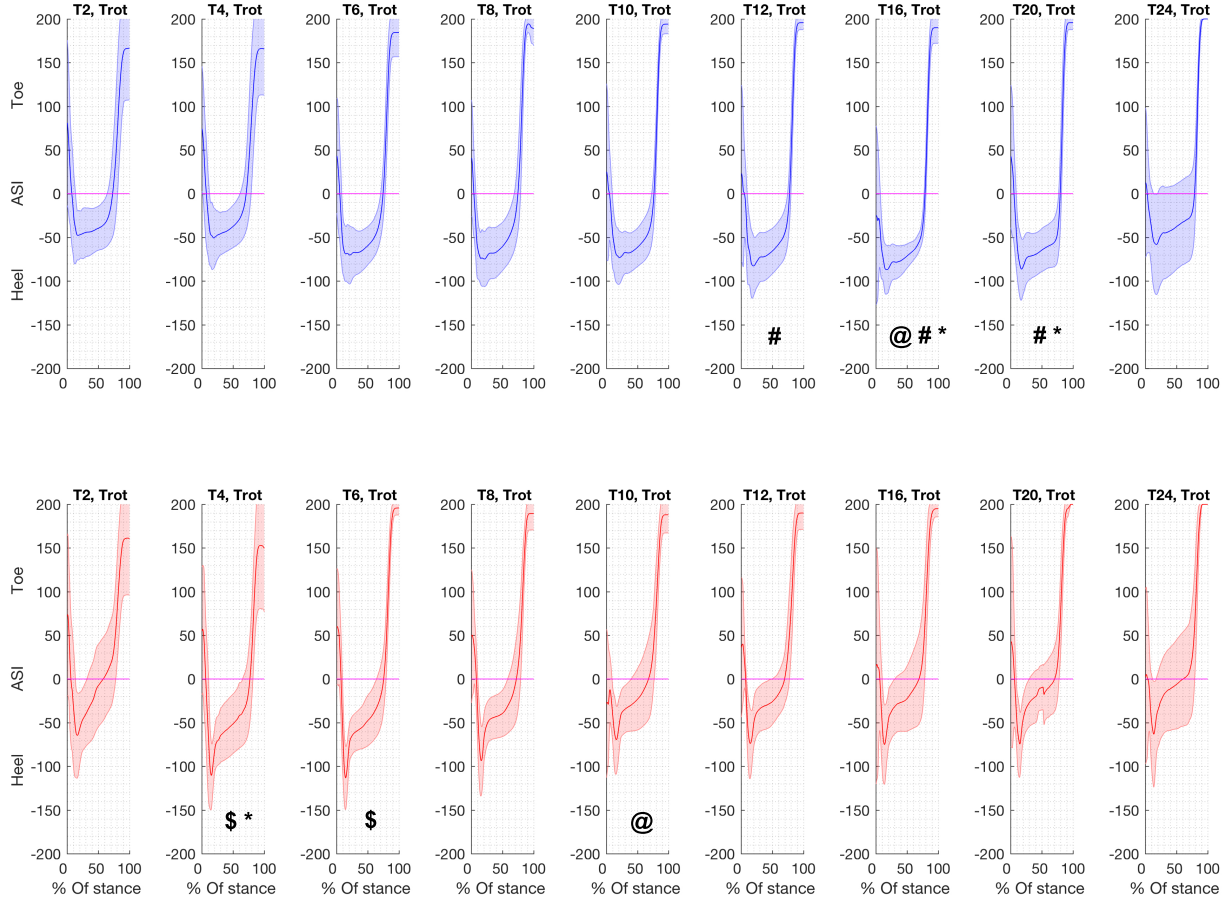

Figure 4: *Average front (upper row, blue) and hind (lower row, red) Toe – Heel ASIs calculated for the complete stance duration at trot for the different ages. The line represents the mean ASI of all foals and the shaded area the median absolute deviation of all foals. Statistically significant differences compared to the first measurement (P>0.05) at 1% of stance are indicated with the symbol @, at 25% of stance with #, at 50% with $ and at 75% with *.*

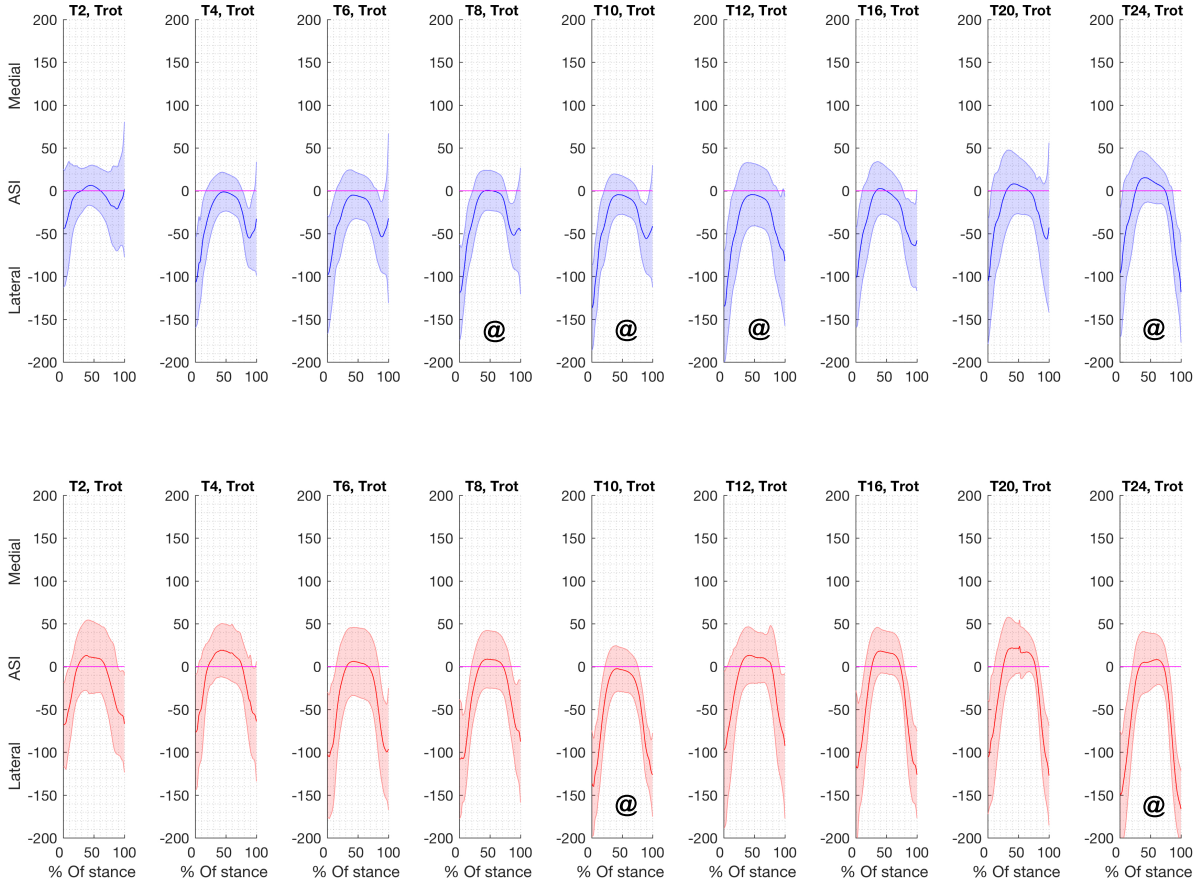

Figure 5: *Average front (upper row, blue) and hind (lower row, red) Medial - Lateral ASIs calculated for the complete stance duration at trot for the different ages. The line represents the mean ASI of all foals and the shaded area the median absolute deviation of all foals. Statistically significant differences compared to the first measurement (P>0.05) at 1% of stance are indicated with the symbol @, at 25% of stance with #, at 50% with $ and at 75% with *.*

Table 1: *Average intra-trial variability (median absolute variation) of the Toe - Heel ASI (T-H) and Medial - Lateral ASI (M-L) of the front and hind limbs at trot at 1, 25, 50 and 75%. An asterisk indicates a significant difference when compared with the first measurement.*

Time (weeks)	Limb	Gait	T-H 1%	T-H 25%	T-H 50%	T-H 75%	M-L 1%	M-L 25%	M-L 50%	M-L 75%
0.5			49.18	23.86	23.41	18.04	54.14	20.41	18.19	20.25
1			48.28	18.30	18.11	19.31	64.85	24.47	26.14	37.28 *
2			33.16	30.57	22.69	21.56	51.19	23.97	22.96	23.49
4			49.97	19.17	16.92	23.85	36.11	27.68	21.59	22.04
6			61.99	14.66	15.47	17.96	32.69	20.97	21.70	23.36
8	Front	Walk	49.82	15.01	13.25 *	23.41	36.17	16.11	20.23	21.25
10			33.63	14.22	14.70	19.66	22.09	18.72	15.57	16.97
12			41.13	18.23	16.56	22.47	29.11	26.24	19.85	19.15
16			34.02	12.85 *	13.07 *	17.43	27.87	18.68	17.63	18.91
20			50.81	18.76	13.89 *	15.35	18.00 *	15.91	15.93	16.50
24			48.67	15.07	14.75	15.15	33.51	26.37	17.65	20.06
0.5			54.47	24.81	24.15	27.05	44.39	24.15	31.13	33.76
1			43.80	26.60	27.37	25.16	41.51	32.41	32.05	32.09
2			34.70	17.33	18.25	21.31	50.43	25.65	20.86 *	23.45 *
4			60.59	19.55	23.05	24.59	46.24	16.52	26.65	26.36 *
6			37.51	19.70	19.47	16.91 *	35.27	21.34	14.94 *	16.92 *
8	Hind	Walk	53.61	18.40	17.34	15.85 *	41.24	17.84	15.32 *	17.21 *
10			45.51	16.06 *	20.47	21.64	27.68	19.62	17.29 *	14.91 *
12			52.32	20.39	23.02	17.88	33.80	24.12	23.42	15.78 *
16			21.55	14.06 *	14.25 *	16.06 *	23.62	15.01	13.37 *	17.70 *
20			52.98	16.20	21.32	16.50	38.72	14.48	16.56 *	16.66 *
24			55.40	18.92	19.04	15.11 *	42.20	25.76	20.99 *	21.62 *
2			39.23	23.60	20.58	22.96	49.32	19.54	18.07	19.89
4			46.32	17.30	17.77	19.80	32.75	25.84	19.91	20.85
6			63.08	14.84	14.99	18.38	55.45	20.20	13.53	15.65
8			68.24	18.20	17.15	22.69	25.78	22.93	18.23	18.45
10	Front	Trot	56.27	14.05	11.61	17.16	23.43	18.24	15.09	16.54
12			43.34	18.10	15.91	14.72	24.52	23.34	18.56	22.27
16			46.27	15.64	10.73	16.27	28.52	17.15	13.18	18.68
20			45.61	14.28	12.82	13.95	42.52	17.21	11.74	14.84
24			64.54	16.71	15.13	16.77	29.45	17.08	12.96	12.88
2			54.23	24.98	21.08	34.85	51.64	32.21	27.67	41.86
4			48.00	16.37	22.58	22.84	37.65	19.42	17.56	20.16 *
6			40.65	17.56	16.36	22.83	40.72	17.06 *	14.67 *	18.26 *
8			55.86	15.49	14.05	22.36	33.52	18.31 *	16.28	18.00 *
10	Hind	Trot	60.22	14.68	19.18	18.81 *	28.07	18.13 *	14.78 *	16.52 *
12			42.12	17.17	17.68	29.99	29.75	19.75 *	17.58	23.53 *
16			32.76	19.44	20.13	35.11	23.68 *	20.62	16.55	19.63 *
20			27.27	19.65	16.10	25.96	33.68	23.20	15.75	15.33 *
24			38.28	14.40	15.60	24.08	27.56	21.89	16.83 *	18.41 *

Front limb medial – lateral balance did not show much change over time with only week 20 at walk having significantly lower variability. Also at 75% of stance a significant difference was found for the front limbs at week one, however in this case, variability was more compared to the first measurement a few days earlier. In contrast, hind limb variability showed many significant differences for the medial – lateral ASI, all decreasing over time with respect to baseline. At trot there was no change in the front limbs, but many changes over time, again all to values less than baseline, in the hind limbs (table 1).

Hoof placement

During the first measurements at walk, more toe landing was observed (OR 1.5), which changed to more heel landing from week six onwards, although no significant changes compared to the first measurement were found (table 2). At trot, the same trend was observed with young foals showing relatively more toe landing compared to later in life. This difference was significant from week eight on. No effects of OC or differences between front and hind limbs were noticed and although not present at walk, at trot a significant difference was observed when comparing left and right, with more pronounced heel landing at the right side.

After two weeks, foals landed significantly more on the medial side of their hooves when walking. Also, a significant difference between front and hind and left and right was found, whereas OC status did not have a significant effect. Hind limbs showed relatively more medial landing and the right side showed more lateral landing. At trot there was from the beginning more lateral landing compared to medial, which difference increased over time and became significantly different from baseline in weeks 8-12 to get back to the original level afterwards. At trot no significant differences were found when comparing front and hind and again, significantly more lateral loading was observed at the right side.

Table 2: *Odds ratios (OR) and 95% confidence intervals for toe or heel (T-H) and medial or lateral (M-L) landing at walk and trot at the different time points. A value > 1 is indicative of preferential toe or medial landing, < 1 is indicative of preferential heel or lateral landing. An asterisk indicates a significant difference when compared with the first measurement. (T=0.5 for walk and T=2 for trot). Significance between left and right and front and hind is also indicated with an asterisk.*

Time (weeks)	Limb/ Side	Gait	T-H OR			M-L OR			Gait	T-H OR			M-L OR		
			Estimate	95% Lower	95% Upper	Estimate	95% Lower	95% Upper		Estimate	95% Lower	95% Upper	Estimate	95% Lower	95% Upper
0.5			1.5	0.9	2.6	1.1	0.7	2.0							
1			1.1	0.6	2.1	0.7	0.4	1.2							
2			1.1	0.6	2.0	0.4 *	0.2	0.8		4.0	2.3	7.1	0.7	0.4	1.2
4			1.3	0.7	2.2	0.3 *	0.2	0.5		0.8	0.4	1.4	0.5	0.3	0.9
6			0.7	0.4	1.3	0.2 *	0.1	0.3		0.7	0.4	1.2	0.5	0.3	0.9
8	Front limbs		0.6	0.4	1.1	0.2 *	0.1	0.3		0.5	0.3	1.0	0.4 *	0.2	0.8
10		Walk	0.6	0.4	1.1	0.1 *	0.1	0,3	Trot	0.3 *	0.2	0.6	0.3 *	0.1	0.5
12			0.6	0.4	1.1	0.2 *	0.1	0,3		0.5	0.3	0.9	0.4 *	0.2	0.7
16			1.1	0.6	1.8	0.2 *	0.1	0.3		0.3 *	0.2	0.5	0.5	0.3	0.9
20			0.9	0.5	1.5	0.2 *	0.1	0.3		0.5	0.3	0.9	0.6	0.3	1.1
24			1.6	0.9	2.9	0.2 *	0.1	0.4		0.4 *	0.2	0.7	0.6	0.3	1.0
	Hind limbs		0.9	0.7	1.1	2.2 *	1.7	2.9		1.1	0.9	1.4	1.0	0.7	1.3
	Right Side		0.9	0.7	1.1	0.5 *	0.4	0.7		0.7 *	0.5	0.9	0.4 *	0.3	0.5

Discussion

In line with our first hypothesis, dynamic hoof balance changes significantly during the first weeks of life. Toe-heel balance shifts towards increased loading of the heel as the foals grow, with a relative increase of the odds ratio of landing heel-first. The medial-lateral ASI shifts gradually towards increased loading of the lateral side, accompanied by an increase of the odds of landing with the lateral part of the hoof first. In general, intra-trial variability decreases over time, although this is not the case for all parameters investigated. The most prominent reduction in intra-trial variability is observed for the medial-lateral hoof balance of the hind limbs.

The hoof balance in mature horses is influenced by conformation, their locomotor pattern, the quality and frequency of trimming and the equestrian discipline the horse is bred for or competing in (Johnston and Back, 2006; Oosterlinck et al., 2013; Trotter, 2004). In the foal, subtle limb deviations are a common finding with carpal and fetlock valgus deviations being most prevalent (Robert et al., 2013). New-born foals also tend to splay their limbs, hereby increasing the width of their base of support (Acworth, 2003; Adams and Mayhem, 1984), which is thought to be a compensation for poor balance and muscle tone early in life (Nauwelaerts et al., 2013). In the current study population, such mild deviations in conformation were also observed (Figure 1) during the first few weeks of life. Where these were mild and resolved spontaneously without treatment and/or farrier intervention, they might help to explain the relatively higher loading of the medial part of the hooves observed during the first weeks of life. Their gradual resolution can be related to the decrease in percentage and odds ratios for medial landing over time, as observed especially in the hind limbs at walk. For the toe-heel balance the ASI curves are surprisingly constant, however, over time. This loading pattern closely resembles the previously described situation in adult warmblood horses (Oosterlinck et al., 2013). Further, during the first weeks of the study period, the hind limbs showed relatively more loading of the heel region of the hooves compared to the front limbs. This could be related to a mild degree of digital hyperextension ("weak tendon"), as is commonly seen in new-born foals (Korosue et al., 2015) and which is more prominent in the hind limbs. Although we did not measure joint angles, retrospective evaluation of the pictures made before each measurement session confirmed that this was also the case in the majority of the foals included in this study.

Especially at trot there is a marked preference to start loading at the lateral side of the hoof; after that loading becomes more even with the hoof balance centring around zero and at the end of the stance phase in general the lateral side is again loaded more. This

pattern resembles the loading patterns described previously in adult warmblood horses (Oosterlinck et al., 2013; van Heel et al., 2004). We observed significant side influences, with right limbs being loaded more laterally than left limbs. This observation is in line with the findings of Oosterlinck et al. (2013) in adult Warmbloods, but the phenomenon was not observed in the work of van Heel et al. (2004) or in adult ponies (Oosterlinck et al., 2014). Possible explanations are the effect of the handler (Oosterlinck et al., 2013), laterality (Kroekenstoel et al., 2006; McGreevy and Rogers, 2005; van Heel et al., 2006) and functional differences between the limbs (Oosterlinck et al., 2013), which is a normal finding in humans (Sadeghi et al., 2000). In our study, the pressure plate was positioned next to the wall (at the right side of the foal). Most likely, due to the effect of the handler, which has been shown to play a role in very young and untrained foals (Lucidi et al., 2013) and the mare, the foals leant slightly to the right side, probably responsible for the significantly more loading of the lateral side of their right hooves.

Confirming our hypothesis, a substantial decrease in variability of (part of) the hoof loading pattern over time was observed. The hypothesis was based on the observation that static balance development in young foals, as studied by means of stabilography, is characterized by relatively large swaying amplitudes and velocities immediately after birth that decrease later in life (Nauwelaerts et al., 2013). Comparable suboptimal balance during locomotion could lead to inconsistent pressure patterns in the different zones of the hooves during subsequent strides and hence to increased variation when calculating the intra-trial variability of an individual foal. Most prominent changes were observed in the medial – lateral hoof balance of the hind limbs in the second part of the stance phase. Already during the first weeks of life a significant reduction in variability was observed, which is in line with the fast initial improvement of static balance (Nauwelaerts et al., 2013) and the significant reduction in variability of the stance duration (Gorissen et al., 2017) observed in young foals and is likely due to better control thanks to maturation of the neuromuscular system. In this study we observed more prominent changes in medial –lateral balance during the first weeks of life, whereas it were the cranio-caudal sway amplitudes that were larger shortly after birth when studying static balance development (Nauwelaerts et al., 2013). This difference may be explained by the stabilising action of the forward momentum, which is present during locomotion, but obviously not when standing still. Similar observations were made in horses suffering from spinal ataxia, a situation that is to some extent comparable with an immature neurological system. These horses also showed increased lateral forces, but cranio-caudal forces were not different (Ishihara et al., 2009). The fact that the age-related decrease in variability was much more outspoken in the hind limbs than in the front limbs may have several explanations. The stabilising effect of the handler that mainly acts on the fore quarter of the horse may

be a factor here. Another difference is that the placement of the front limbs can benefit from visual input, whereas hind limbs solely have to rely on proprioception. Postnatal development of the proprioceptive system may therefore be responsible for the gradual decrease in variability observed in the hind limbs.

Some between-foal variability was seen at weeks 20 and 24, as can be deduced from the larger shaded area at these time points in Figs. 2-5. This may have been related to the fact that at week 20 about half of the foals and at week 24, all foals were weaned and consequently handled without their dam. Whereas handling of the foals without the presence of the mare was uneventful and all animals were accustomed to the location and procedure of the pressure plate data collection, the absence of the dam may have led to more variability in the trials. In the earlier study an increase in variability of kinetic gait parameters was observed in the period directly after weaning (Gorissen et al., 2017).

Osteochondrosis was not a significant factor in our models, while there was a small, yet significant effect of OC on kinetic parameters in our previous study (Gorissen et al., 2017). This leads to the conclusion that the small changes observed in kinetic parameters due to subclinical lameness (resulting in a reduction of nPVF in the affected limb) are not reflected in the hoof-balance ASI. The observation, suggests that the primary component of compensation to pain (i.e. lameness) is the even unloading of the lame limb and that ground reaction forces are more accurate in detecting low degree lameness than pressure patterns (Weishaupt et al., 2004).

Through the study period, hoof development and conformation was normal and no hoof trimming was performed, which is considered normal practise in horses with acceptable limb conformation (O'Gray, 2017). This was a deliberate choice. Whereas uneven hoof growth could have induced some variability in this study due to changes in hoof-balance (van Heel et al., 2004) hoof trimming would most likely have induced more variation unrelated to development, ultimately inducing a bias in the data.

There were several limitations to this study. The number of animals included in the study was limited and all animals were bred for show jumping, making it impossible to draw firm conclusions regarding the whole warmblood population. Furthermore, only the hoof prints that were aligned with the pressure plate coordinate system could be used, which made it necessary to collect more hoof prints. For the understanding of the development of hoof asymmetries as often observed in adult horses and their relation to lameness, further studies monitoring hoof pressure patterns for longer periods than the first weeks of life are needed.

Conclusions

Dynamic hoof balance changes in foals and these alterations seem to follow the conformational changes reported and observed in foals. The toe-heel balance gradually shifts to the heel and the medio-lateral balance to lateral. This is accompanied by a gradual shift of landing preference in the same directions. Variability in pressure distribution, especially the medio-lateral balance in the hind limbs decreased, which is similar to the decreasing trend shown in a number of kinematic parameters in the same phase of life. Knowledge of this gradual stabilisation of hoof balance and preferred landing during the first weeks of life may be useful for the clinician when taking decisions about possible interventions such as trimming in early life.

Manufacturers' addresses

[a] RsScan International N.V., Paal, Belgium
[b] De Mulder Rubber and Plastics, Gent, Belgium
[c] Koninklijke Philips N.V., Eindhoven, The Netherlands
[d] MathWorks, 3 Apple Hill Drive, 01760, Natick, USA
[e] R-Studio, 250 Northern Ave, MA 02210, Boston, USA

Ethical note

This study was reviewed and approved by the ethical committee of Utrecht University (DEC no.2014.III.04.038) and all effort was taken to minimize discomfort of the foals and their dams. For all procedures carried out, written consent was obtained from the owner of the animals.

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CHAPTER IV:

Trabecular bone of precocials at birth; are they prepared to run for the Wolf(f)?

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Abstract

Bone is a dynamic tissue adapting to loading according to "Wolff's law of bone adaptation". During very early life, however, such a mechanism may not be adequate enough to adapt to the dramatic change in environmental challenges in precocial species. Their neonates are required to stand and walk within hours after birth, in contrast to altricial animals that have much more time to adapt from the intra-uterine environment to the outside world. In this study, trabecular bone parameters of the talus and sagittal ridge of the tibia from stillborn but full-term precocials (calves and foals) were analysed by micro-CT imaging in order to identify possible anticipatory mechanisms to loading.

Calculated average bone volume fraction in the Shetland pony (49–74%) was significantly higher compared to Warmblood foals (28–51%). Bovine trabecular bone was characterised by a low average bone volume fraction (22–28%), however, more directional anisotropy was found. It is concluded that anticipatory strategies in skeletal development exist in precocial species, which differ per species and are most likely related to anatomical differences in joint geometry and related loading patterns. The underlying regulatory mechanisms are still unknown, but they may be based on a genetic blueprint for the development of bone. More knowledge, both about a possible blueprint and its regulation, will be helpful in understanding developmental bone and joint diseases.

Keywords neonate; development; micro-CT; Wolff's law.

Introduction

Loading of bone starts already during intrauterine skeletal development (Pitsillides, 2006; Rot et al., 2014) and continues during growth after birth and in all phases of life thereafter (Yokota et al., 2011; Dowthwaite et al., 2014; Torcasio et al., 2014). According to "Wolff's law of bone adaptation" (Wolff, 1892), which forms the basis for the mechanostat theory, explaining the relationship between loading and bone remodelling (Frost, 2001), bone mass will decrease when loaded below a certain threshold and increase in reaction to loading above this threshold, achieving an optimal form to withstand prevailing forces (Huiskes et al., 1987; Frost, 2001; MacLatchy, 2002; Christen et al., 2014).

Eutherian mammals differ in the degree of development at birth. Precocials, such as foals and calves, are born relatively mature and are able to stand and walk within hours after birth. On the other hand, altricials, like dogs, produce offspring born in a relatively poor state of development. No sharp division can be made between altricial and precocial, as it is a gradient characteristic. Being precocial has major consequences for the bones of the axial and appendicular skeleton, as skeletal loading changes dramatically after birth. During gestation, limbs are folded and not weight bearing, limiting loading to forces generated by intrauterine muscle contractions. After birth limbs are extended, carry weight and in precocials are subjected to considerable forces generated by locomotion, only hours after leaving the uterine environment.

To withstand these forces, the ossification process has progressed much further in the precocial neonate, compared to altricial species. In new-born puppies the ossification process of short bones starts around birth and most epiphyses are still completely cartilaginous (Evans, 1993). Ossification has progressed much further in neonatal foals and calves, with only a small band of growth and articular cartilage still present at their epiphyses and short bones (Küpfer and Schinz, 1923; Nickel et al., 2005; Fontaine et al., 2013).

Most research on trabecular bone structure of neonates has been performed in long bones of altricial species, and it has been reported that bone volume fraction (BV/TV; BV= bone volume, TV= total volume) followed a U-shaped curve after birth. For example, in the human femur and tibia, average BV/TV at birth is about 50% and decreases to values around and sometimes even under 20% between 6 and 12 months of age. After this period, BV/TV increases again, to reach final values, similar to adult, at an age of 6 years (Ryan and Krovitz, 2006; Gosman and Ketcham, 2009). In dogs BV/TV follows a comparable U-shaped curve, with lowest BV/TV values found at about 8 weeks after

birth (Wolschrijn and Weijs, 2004). As both in humans and in dogs lowest BV/TV values are reported around the onset of walking, the drop in BV/TV could be explained by mini-modeling, associated with the development of a more preferential orientation of trabeculae, due to the increasing demands of locomotion. During this process, trabeculae that are loaded under a certain threshold disappear and as a result of this, directional anisotropy (DA) increases (Tanck et al., 2001; Wolschrijn and Weijs, 2004).

There is evidence that at least in a number of species, this process begins during gestation. Significant trabecular bone DA was shown at birth in the mule deer and ovine calcaneus (Skedros et al., 2004, 2007). Furthermore, a trabecular architecture fitting with loading during bipedal locomotion has been reported in human neonatal ilia (Cunningham and Black, 2009). The observed DA of the pelvis is not likely to be the result of intra-uterine strain-related adaptation, suggesting that at least parts of the skeleton are anticipating future loading, possibly based on a genetic blueprint (Cunningham and Black, 2009).
Bone strength is highly dependent on bone volume (Kabel et al., 1999; Borah et al., 2000; Ryan et al., 2010) and architecture (Ulrich et al., 1997; Mittra et al., 2005). Besides having a further advanced ossification process and hence a higher bone volume, we hypothesized that in neonatal calves and foals trabecular bone microarchitecture already anticipates postnatal loading requirements and thus reflects future rather than past function. Since the Warmblood horse has increased considerably in height at withers over the past 50 years due to selective breeding (Ducro et al., 2009), we also investigated Shetland ponies which have not been subject to strong selection pressure, to exclude possible artificial influences. To verify our hypothesis, microarchitecture of the distal tibia and the talus of stillborn but full-term calves, Shetland pony and Warmblood foals was analysed and compared by means of micro-CT imaging. More insight into prenatal bone development and its underlying regulatory mechanisms may lead to a better understanding of bone growth and development. It might also provide clues for unravelling pathogenic mechanisms and even for the development of new therapeutic approaches for both developmental (joint) diseases and problems associated with aberrant endochondral ossification later in life, such as osteoarthritis (Staines et al., 2013).

Materials and Methods

Animals

Because of ethical considerations no animals were euthanized for this study. Tarsal joints of full-term Holstein-Friesian (HF) calves (*Bos Taurus*, *n*=5), Dutch Warmblood foals (*Equus ferus caballus*, *n*=5) and Shetland ponies (*Equus ferus caballus*, *n*=3) were obtained

with owners' consent from (para)clinical departments of the Faculty of Veterinary Medicine, Utrecht University, and from private veterinary practices. To exclude effects of postnatal loading, we collected only material from animals that were either stillborn or euthanized within 12 hours after birth. Gestational time and cause of death were recorded, and animals were only included if the cause of death could be considered to have no effect on development. Except for two Dutch Warmblood foals, all animals died because of dystocia, caused by either an abnormal intrauterine position or a foetus that was too large. One Dutch Warmblood foal was euthanized because of a combination of a ruptured inguinal hernia and bladder rupture, the other because of an acute equine herpes virus, type 1 infection.

Sample preparation

Tarsal joints were collected within 2 h after death and stored at 4 °C, or –18 °C, the latter when further processing would occur more than 12 h after collection. If necessary, the joints were thawed in running tap water before removing all soft tissue. Articular surfaces were macroscopically inspected, photographed, and fixated in 4% buffered formaldehyde for at least a week. In order to fit in the micro-CT sample holder (diameter 7.85 cm), the distal tibias were cut transversely through the distal metaphysis about 1 cm proximal to the distal physis. The Warmblood foals' tali were cut in longitudinal direction through the middle of the talar trochlea and the two parts were scanned separately.

Micro-CT

Micro-CT imaging was performed with a μCT 80 scanner (Scanco Medical AG). Bone samples were placed in the sample holder in a consistent manner and secured with synthetic foam. Scanning was performed in air at an isotropic spatial resolution of 37 μm, with a peak voltage of 70 kV and intensity (current) of 114 μA. To reduce beam hardening effects, the scanner was equipped with an aluminium filter.

Trabecular microarchitecture was quantitatively determined in volumes of interest (VOIs) that were manually drawn and included only trabecular bone. Blood vessels present in the bone structure were not included. The VOIs comprised areas where high postnatal loading was expected. For the distal tibiae these areas were similar for foals and calves. The tibial VOI contained the trabecular bone of the dorsal 25% of the sagittal ridge (Fig. 1A, B). The average volume of this VOI was about 1500 mm^3 (range ~800–2100 mm^3) for the calves, 1250 mm^3 (~900–2000 mm^3) for the Warmblood foals, and 175 mm^3 (~100–300 mm^3) for the Shetland foals.

Species specific differences in anatomy led to the selection of different anatomical areas in the talus as "high postnatal loading" regions. As bovine tali exhibit a rather upright position, with little developed ridges, bovine talar VOIs comprised the distal part of the talus (caput tali), an area that is not supported by the os malleolare (Nickel et al., 2005). The caput tali was further divided into a lateral part (average volume 1700 mm^3, ~1000–2500 mm^3) and medial part (average volume 2200 mm^3, ~1000–3500 mm^3, Fig. 1C), by drawing a vertical line through the middle of it.

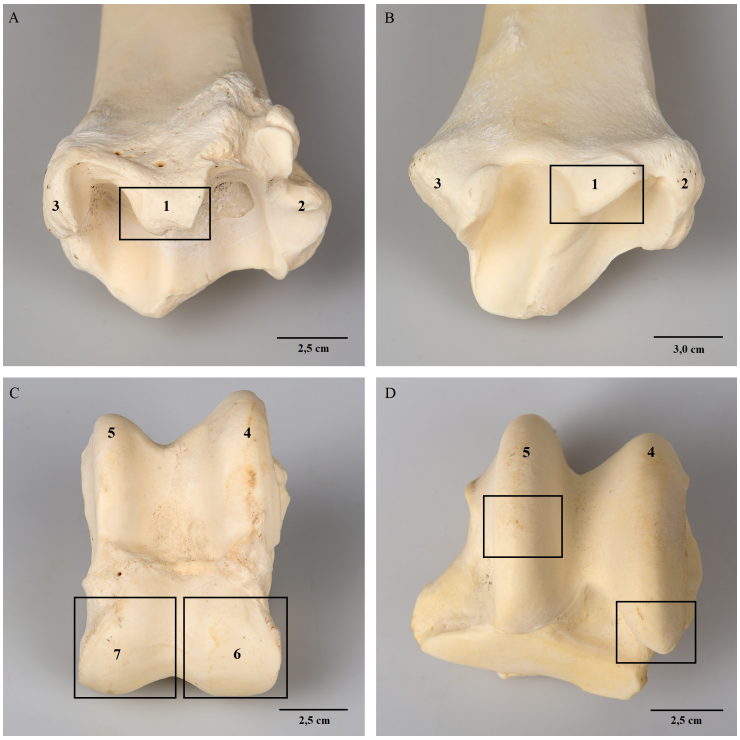

Figure 1. *Volumes of interest (VOIs) indicated with a box in samples of an (adult) distal tibia and talus. The VOI in the bovine (A) and equine (B) distal tibia consisted of the dorsal part of the sagittal ridge of the tibia. The VOI in the bovine talus (C) contained the medial and lateral part of the caput tali. The VOI in the equine talus (D) contained the distal part of the lateral and middle region of the medial talar ridge. 1: Dorsal part of the sagittal ridge of the tibia, 2: Lateral malleolus of the tibia, 3: Medial malleolus of the tibia, 4: Lateral trochlear ridge of the talus, 5: Medial trochlear ridge of the talus, 6: Lateral part of the caput tali, 7: Medial part of the caput tali.*

In the horse, the trochlear ridges are prominent and slanting and the caput tali is very small (Nickel et al., 2005). Consequently, the load transmitted from the tibia has both a shear component and a component perpendicular to the ridges (Badoux, 1987), justifying the selection of the trochlear ridges as areas where high postnatal loading is expected.

The lateral talar VOI consisted of the distal part of the lateral ridge (Warmblood foals: average volume 1100 mm^3, ~800–1500 mm^3; Shetland foals: 200 mm^3, ~150–250 mm^3; Fig. 1D). The medial talar VOI contained the middle region of the medial talar ridge (Warmblood foals: average volume 1900 mm^3, ~1000–3000 mm^3; Shetland foals: 300 mm^3, ~200–500 mm^3; Fig. 1D). Both VOIs had a length of approximately 25% of the total length of the ridge and were limited by a virtual line following the deepest part of the talar trochlea.

Thresholding (i.e., distinguishing between bone and non-bone) was performed visually by comparing segmented images at different threshold levels with the original scans (representative examples of each can be seen in supplementary figures 1 and 2), choosing the best fit (Wolschrijn and Weijs, 2004).
This procedure led to a threshold of 145 per mille of the maximum possible voxel value, corresponding to 1222 Hounsfield units and appeared to be identical in all animals studied. This threshold is less than commonly used for trabecular bone in full-grown animals, because the bone tissue in neonatal animals is not fully mineralized and may contain cartilaginous fragments. Quantitative trabecular parameters were calculated from these segmented images with the manufacturer's software. Bone volume was calculated as the number of bone voxels divided by the total number of voxels in the VOI after segmentation. Structural parameters included trabecular number (Tb.N.), trabecular thickness (Tb.Th.), and trabecular separation (Tb.Sp.) and were calculated using a distance transformation method. The DA was based on the Mean Intercept Length fabric tensor and defined as the largest principal fabric value over the smallest one.

Statistical analyses

Statistical analyses were performed using SPSS Statistics 22 (IBM Corporation). A paired Student's *t*-test showed no significant differences between left and right limb results for all parameters, justifying the calculation of one average value for each parameter and region studied in each animal. Trabecular parameters were log-transformed to meet normality and homoscedasticity assumptions. A linear mixed effect model, with 'animal ID' added as random intercept to account for correlation between observations in the same animal, was used to check for significant differences in homologous bone regions between animal species and compare trabecular bone parameters of the lateral talar VOI with the medial talar VOI within the foals and calves. Statistical significance was set at $P \leq 0.05$ and correction for multiple comparisons was done with the False Discovery Rate method of Benjamini – Hochberg (1995).

Results

Trabecular bone parameters for the VOIs are given in figure 2. An average bone volume fraction (BV/TV) of up to 74% was found in the dorsal part of the sagittal ridge of the tibia in Shetland pony foals, whereas in Warmblood foals this was 51% and in calves 28% (Fig. 2A). The BV/TV in the lateral VOI of the talus was in the same range as in the tibia, whereas the medial VOI showed much lower values: 49% and 28% for Shetland pony and Warmblood foals, respectively, and 22% for calves. All differences in BV/TV were statistically significant. Significantly lower trabecular numbers were found in the tibial and lateral talar VOI in calves compared to foals, whereas in the medial VOI of the talus no significant differences between the species were found (Fig. 2B). In the Shetland pony foals, trabecular thickness was between 0.20 and 0.24 mm, which was significantly larger than in Warmblood foals (0.13–0.20 mm) and calves (0.13–0.14 mm) for all VOIs. Between Warmbloods and calves, differences were only significant in the tibia (Fig. 2C).

The highest trabecular separation values were found in the calves and differences were significantly higher compared to the foals in both the tibial and lateral talar VOIs (Fig. 2D). In the medial talar VOI, no significant differences were found. Only in the tibial VOI differences found between Warmblood and Shetland foals were significant. The highest DA values were found in the lateral VOI of the talus of calves and were significantly different from the foals (Fig. 2E). In the tibia and medial talar VOIs, lower and not significantly different DA values were found. When comparing trabecular architecture found in the medial talar VOI with the lateral talar VOI of the same species, BV/TV was significantly higher in the lateral talar VOI of both foals and calves. In foals, Tb.N. and Tb.Th were significantly higher, whereas Tb.Sp. was significantly lower in the lateral VOI. No difference in DA was found in the foals, whereas calves showed significantly higher DA in the lateral compared to the medial VOI of the talus.

Supplementary figure 1 shows two-dimensional, unsegmented micro-CT transections of the left distal tibia and talus of a calf (A-C), Shetland pony foal (D-F) and Warmblood foal (G-I) showing the differences in anatomy between the animals studied. Supplementary figure 2 contains transections of the segmented 3D reconstructions of representative examples of all studied VOIs, visualizing and illustrating the described differences in trabecular bone architecture.

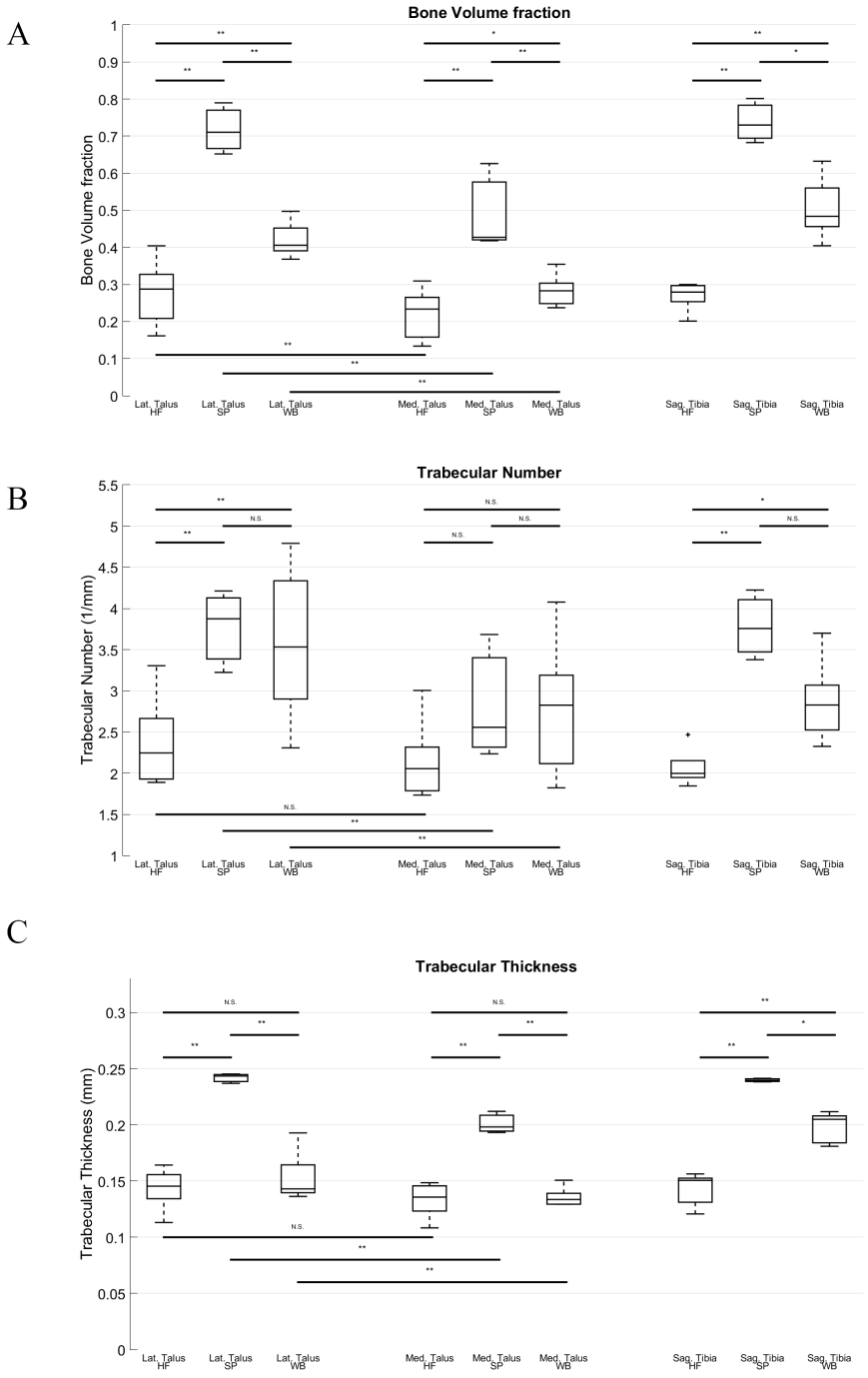
A
Bone Volume fraction
Bone Volume fraction
Lat. Talus HF
Lat. Talus SP
Lat. Talus WB
Med. Talus HF
Med. Talus SP
Med. Talus WB
Sag. Tibia HF
Sag. Tibia SP
Sag. Tibia WB
B
Trabecular Number
Trabecular Number (1/mm)
N.S.
C
Trabecular Thickness
Trabecular Thickness (mm)

Figure 2A-C.

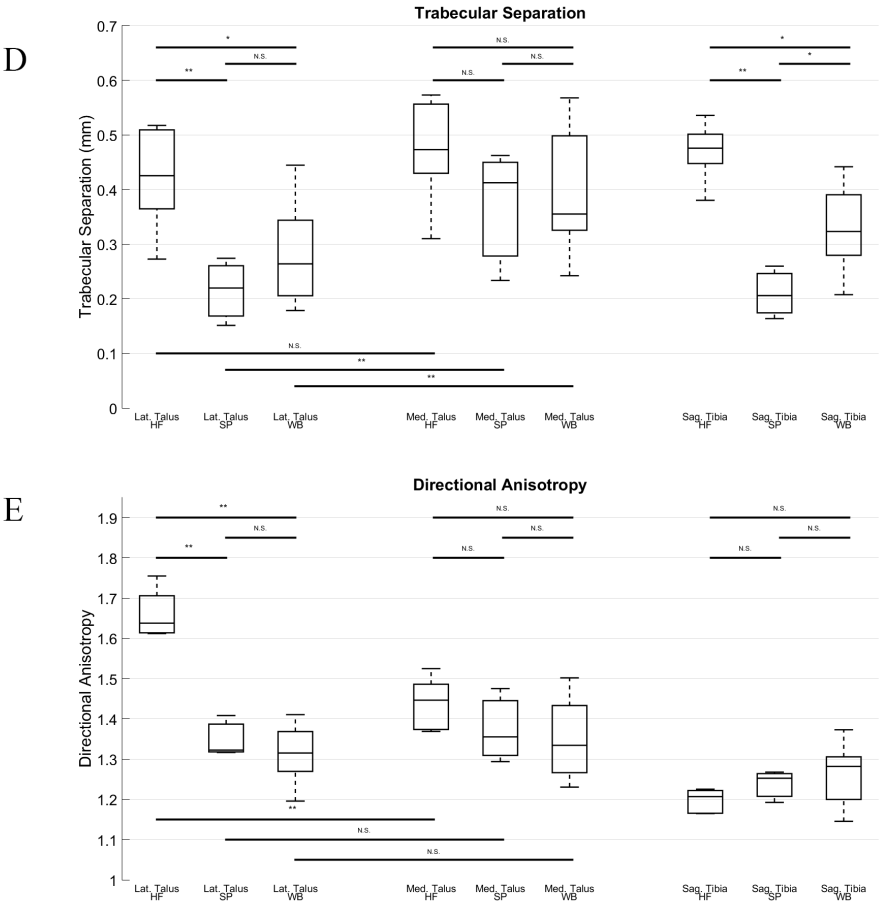

Figure 2A-E. *Boxplots of A: Bone Volume fraction (BV/TV), B: Trabecular number (Tb.N.), C: Trabecular Thickness (Tb.Th.), D: Trabecular Separation (Tb.Sp) and E: Directional Anisotropy (DA) calculated for the different VOIs (Lat. Talus: lateral part of the talus; Med. Talus: medial part of the talus; Sag. Tibia: sagittal ridge of the tibia) and species (HF: Holstein-Friesian calves; SP: Shetland pony foals; WB: Warmblood foals). N.S., not significant; * 0.01 < P < 0.05; ** P < 0.01.*

Discussion

In order to identify possible anticipatory strategies in bone development, this study compared trabecular bone architecture at birth in the distal tibia (epiphysis of a long bone) and talus (short bone) of precocials. As it is difficult to obtain still born animals meeting the inclusion criteria, the number of animals in this study was relatively small. Nonetheless, we were able to show that foals and calves appear to have different strategies for reinforcement in the talus. In foals BV / TV is higher, whereas in calves a less

dense but more anisotropic architecture is present. The DA in the calf is along a proximo-distal line through the talus, matching postnatal loading during standing and walking. As the limbs of foals and calves cannot be extended during the last part of gestation, it is tempting to hypothesize that the observed DA in the calf is not a result of the common mechanostat principle of bone development and loading (Frost and Jee, 1994; Tanck et al., 2001), but presents evidence for the existence of an anticipatory mechanism in trabecular bone growth, as has been suggested previously (Skedros et al., 2007; Cunningham and Black, 2009).

If trabecular bone in precocial neonates is indeed anticipating loading, its architecture must reflect future (postnatal) instead of past (prenatal) loading. To identify these anticipatory strategies, it is important to study areas in which significant postnatal loading is expected. In the talus noticeable anatomical differences are present, affecting the loading of this bone. In ruminants the talus is more rectangular, the trochlear ridges are aligned in the sagittal plane but are less developed; in the horse the trochlear ridges are prominent and slanting. In addition, the bovine talus is stabilized on the lateral side by the os malleolare and lateral parts of the calcaneus, whereas the equine talus is stabilized by the tight interlocking of the talar ridges in the tibial cochlea (Nickel et al., 2005). These anatomical differences have biomechanical consequences with the ruminant talus mainly perpendicularly loaded and hence experiencing compression, whereas the equine talus experiences both shear and compressive forces (Badoux, 1987).

The "volumes of interest" used in this study were chosen based on the expected loading condition immediately after birth. Defining and comparing bone regions in different species is difficult due to the species specific differences in anatomy and associated loading, which could be considered a limitation of this study. We studied the caput tali of the bovine samples as we expected to find most prominent anticipatory behaviour in this region. We divided the caput tali into a lateral and medial part as it is reported that maximum pressure is found under the dorsolateral part of the hind claws in adult cattle (van der Tol et al., 2002). Loading of the equine tibia leads to tensile strain on the lateral part and compression on the medial part of the talus (Schneider et al., 1982).
Strain is reported to be highest on the dorsal and lateral parts of the equine tarsal joint (Schamhardt et al., 1989; Murray et al, 2004). Accordingly, subchondral bone thickness of the os tarsi centale, positioned under the talus, was reported to be greatest on the dorsal and lateral aspects. This suggests that compressive loading is transferred from medial to lateral through the talus (Branch et al., 2005), justifying our choice for the mid region of the medial and distal part of the lateral trochlear ridges as VOI.

Average birthweight of HF calves is about 45 kg, but can easily be in excess of 60 kg (Linden et al., 2009). The calves included in this study were all above average in size and bodyweight. Warmblood foals weigh about 55-60 kg at birth (van Weeren et al., 1999; Hendriks et al., 2009). Scientific information regarding the birthweight of Shetland pony foals is difficult to obtain and limited to a paper from the 1930s, mentioning birthweights of about 20 kg (Walton and Hammond, 1938). The bodyweight of the dams from this study are still representative for the standard sized Shetland pony nowadays, making this estimation still useful. As Shetland pony foals are much smaller at birth, VOI sizes were proportionally scaled to the size of the foal to prevent oversampling or analysing areas not homologous in function with the Warmblood horse (Fajardo and Müller, 2001; Lazenby et al., 2011).

Bone volume fraction is a good indicator of bone strength (Kabel et al., 1999; Borah et al., 2000; Ryan et al., 2010) and it has been shown in inter-species comparisons (mainly from adult animals), that this parameter is independent of body weight (Doube et al., 2011; Barak et al., 2013; Christen et al., 2015) enabling comparison of differently sized animals. Bone volume fractions found in (Warmblood) foals were the highest, while BV/TV in the calves was much lower, although both species are comparable in birth weight and postnatal behaviour. However, in the foal's talus, loading is multi-directional, as explained above, which may explain lower DA and higher BV/TV compared to the calves. Both in the calves and foals, highest BV/TV and/or DA was observed in the lateral part, which is in line with the load distribution in the postnatal animal, providing further evidence for the anticipatory development of bone.

The BV/TV fraction in the Shetland pony was higher than in the Warmbloods. A similar result has been reported for the carpal bones, in which relative bone density was higher in ponies compared to thoroughbreds (Abdunnabi et al., 2001). This observation may be somewhat unexpected at first site, given the fact that, at least in adults, BV/TV is independent of body mass (Barak et al., 2013), but can most likely be attributed to differences in growth rate. High rates of bone growth are associated with less dense bone (Martin and Burr, 1989; Leterrier and Nys, 1992; Williams et al, 2004; Prisby et al., 2014) and growth rate in pony breeds is much lower than in horse breeds.

Other variables measured and statistically compared between groups in this study (Tb.N., Tb.Th. and Tb.Sp.) scale with negative allometry with body mass (Doube et al., 2011; Ryan and Shaw, 2013; Christen et al., 2015). Besides differences in loading described above, the lower Tb.N found in calves compared to Warmblood foals could in part be explained by their lower birth weight. However, Shetland pony foals showed the highest

Tb.N. whereas their birth weight is the lowest. Just as for the BV/TV this could be caused by differences in growth rate. Furthermore, it must be emphasized that scaling factors are based on studies performed in (more) mature subjects and information about neonatal bone is lacking in literature.

In the physes of long bones, very young, newly formed bone, is reported to be anisotropic as its structure reflects the parallel columnar organization of the endochondral ossification process (Lai and Mitchell, 2005; Gosman and Ketcham, 2009). In foals ossification of the talus starts at 8 months of gestation and progresses distally and radially away from the center (Fontaine et al., 2013); for the calf this moment has not been determined yet, but it seems reasonable to assume that in this species ossification of the talus also starts in the last trimester of gestation. Therefore, it is plausible to assume that the DA observed in this study, which does not resemble the original organisation, is not a primary feature of newly formed bone, but is the result of mini-modeling.

Our results, combined with previous work (Cunningham and Black, 2009; Skedros et al., 2004, 2007) show that bone (mini-)modeling can occur unrelated to loading during 'special occasions', like prenatal development. Hibernation is another of such occasion as bears keep bone formation and resorption well-balanced during the hibernation period, whereas disuse would normally lead to bone resorption and osteoporosis. Through this mechanism bears preserve their bone strength to prevent problems when waking up and becoming mobile again (McGee-Lawrence et al., 2009; 2015). This situation is comparable with that of precocial animals after birth, as in both cases the skeleton must be prepared for a sudden increase in loading and this strategy saves valuable energy.

Although our data confirm anticipatory development of trabecular bone architecture to postnatal loading, which could be the result of a genetic blueprint as proposed by others (Cunningham et al., 2009), convincing explanations for the observed differences between foals and calves are still lacking. Besides species-specific anatomy and related loading patterns, the availability of calcium could also play a role. From the literature it is known that at equal levels of intake, horses absorb more dietary calcium from their intestine than ruminants (Schryver et al., 1983), which may help in increasing bone volume. Furthermore, gestational length in horses (11 months) is longer than in cows (9 months), giving them more time to increase their bone volume.

Conclusion

The results of this study illustrate that bone is a highly efficient tissue that is able to anticipate postnatal loading on a very local scale. On the level of trabecular bone, different strategies in anticipatory bone development seem to be present in the talus of calves and foals, which is likely related to anatomical differences in joint geometry and related loading patterns.

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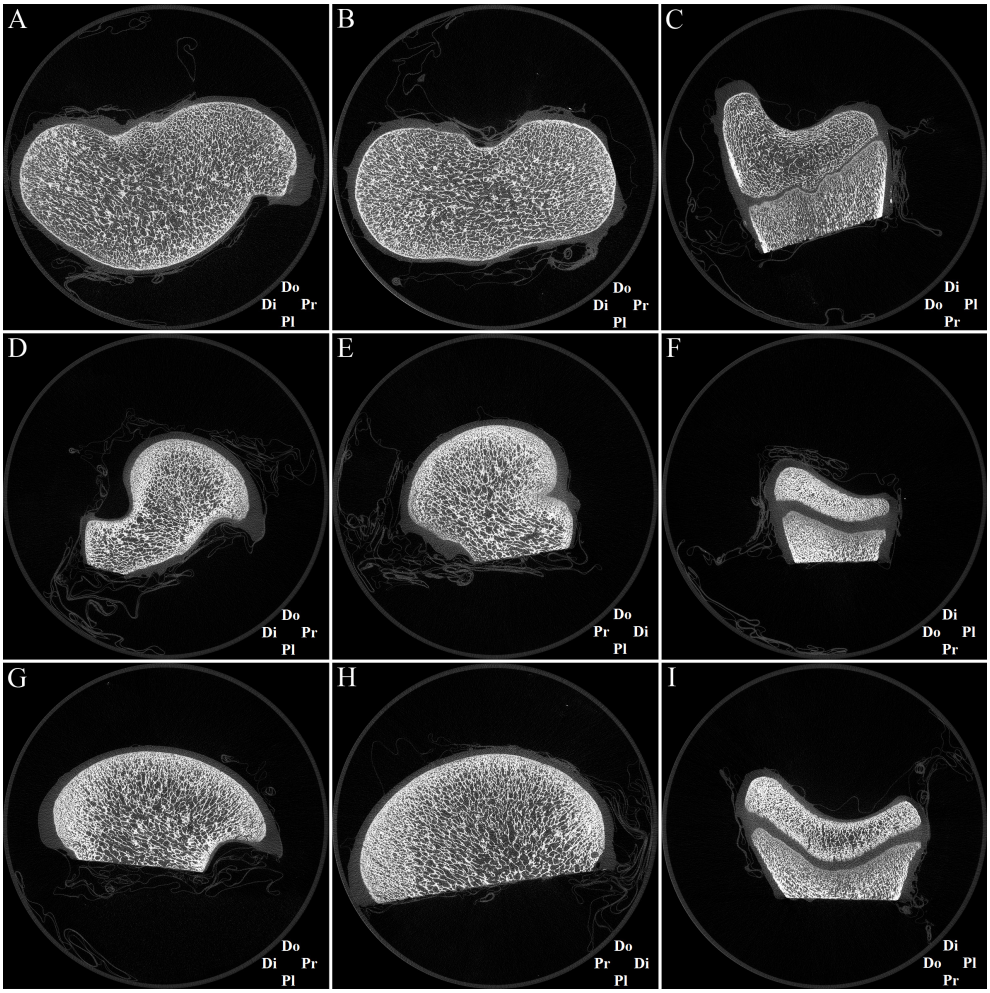
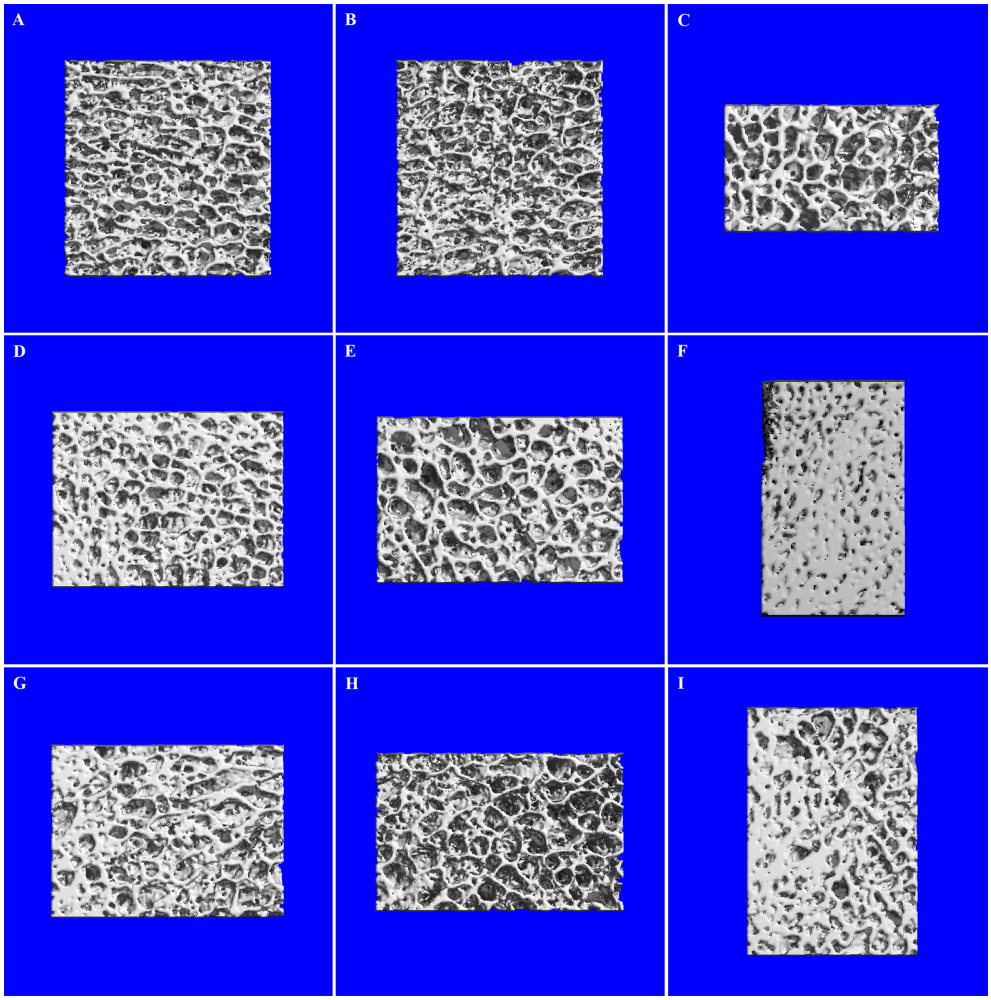


Supplementary Figure 1. *Two-dimensional, unsegmented microCT transections the lateral part of the talus of a Holstein-Friesian calf (A), medial part of the talus of a Holstein-Friesian calf (B), distal part of the tibia of a Holstein-Friesian calf (C), lateral part of the talus of a Shetland pony foal (D), medial part of the talus of a Shetland pony foal (E), distal part of the tibia of a Shetland pony foal (F), lateral part of the talus of a Warmblood foal (G), medial part of the talus of a Warmblood foal (H) and distal part of the tibia of a Warmblood foal. (Do: Dorsal; Pl: Plantar; Pr: Proximal; Di: Distal).*



Supplementary Figure 2. *Transections of the segmented 3D reconstructions of representative parts of the lateral part of the caput tali of the talus of a Holstein-Friesian calf (A), medial part of the caput tali of the talus of a Holstein-Friesian calf (B), dorsal part of the sagittal ridge of the distal tibia of a Holstein-Friesian calf (C), distal part of the lateral trochlear ridge of the talus of a Shetland pony foal (D), middle region of the medial trochlear ridge of the talus of a Shetland pony foal (E), dorsal part of the sagittal ridge of the distal tibia of a Shetland pony foal (F), distal part of the lateral trochlear ridge of the talus of a Warmblood foal (G), middle region of the medial trochlear ridge of the talus of a Warmblood foal (H) and dorsal part of the sagittal ridge of the distal tibia of a Warmblood foal (I).*

CHAPTER V:

Longitudinal bone development in foals

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Submitted

Abstract

Horses are precocial animals and able to stand and walk within hours after birth. In order to cope with associated loading, intra-uterine bone development has shown to be anticipative. This study provides further insight in the postnatal development of structurally important features of trabecular and subchondral bone of the talus and sagittal ridge of the tibia of warmblood horses.

In all areas studied, the average bone volume fraction showed a gradual increase over time, which was the result of a significant increase of trabecular thickness, without significant changes in the degree of anisotropy. Similar to the mineralised part of the bone, collagen content, measured as average retardation using polarised light microscopy, increased significantly, but the degree of anisotropy of the collagen type I network did not. At birth, the subchondral bone layer had a more trabecular aspect, gradually changing to an even surface with only few openings at an age of two months.

Presented results indicate the necessity for a stronger structure, but not for a different structural design after birth, providing further evidence for anticipatory bone development in the horse. More knowledge about the strategies used to cope with mechanical loading after birth might be helpful in understanding developmental bone and joint diseases.

Keywords: bone development; collagen type I; polarised light microscopy; micro-CT; Wolff's law.

Introduction

According to Wolff's law on bone adaptation (Wolff, 1892) and the Mechanostat theory (Frost, 2001), bone adapts reactively to loading. However, precocial animals need to stand and outrun predators within hours after birth. To withstand the forces associated with this, foetal bone development has shown to be anticipative, reflecting future rather than past function, both in calves and foals (Gorissen et al., 2016). This phenomenon, which is possibly based on a genetic blueprint, seems general as it has been described in several other precocial species (Skedros et al., 2004; 2007) and even in altricial man (Cunningham and Black, 2009).

After birth, the biomechanical situation changes dramatically and trabecular bone development is strongly influenced by loading, as has been shown in infants (Ryan and Krovitz, 2006; Gosman and Ketcham, 2009; Acquaah et al., 2015) and puppies (Wolschrijn and Weijs, 2004). Their altricial behaviour after birth is responsible for the initial reduction of trabecular bone volume fraction (BV/TV). As the reduction in the bone volume fraction (BV/TV), at least in babies, coincides with relatively high levels of parathyroid hormone (David and Anast, 1974; Rubin et al., 1991; Land and Schoenau, 2008), it has been suggested that the relatively high bone volume at birth might serve as a reservoir for calcium during bone growth (Acquaah et al., 2015). After the onset of locomotion, BV/TV in these species increases again in reaction to the higher loading, making the curve describing BV/TV after birth U shaped. Additionally, a certain degree of anisotropy develops, because trabeculae aligned with loading will grow in thickness, whereas trabeculae which are not will shrink and ultimately disappear (Tanck et al., 2001; Wolschrijn and Weijs, 2004). Given the extreme precocial behaviour of many of the larger ungulates, including equidae, one might expect a different BV/TV curve with less bone loss and most likely an immediate gradual increase of trabecular BV/TV after birth, in line with what is seen in altricials after the onset of walking, but this has not been reported yet. In the same line of thinking, it can be hypothesised that, unlike in altricial species, directional anisotropy (DA) will not change significantly after birth in the foal.

Other features of epiphyseal bone that might behave differently in altricial and precocial species include the density of the subchondral bone layer and characteristics of the collagen I network. The subchondral bone layer supports the overlying cartilage (Radin and Rose, 1986; Brama et al., 2001) and transmits forces to the underlying trabecular bone (Holopainen et al., 2008). Its development is mechanically driven (Wolschrijn and Weijs, 2005; Tanck et al., 2006; Skedros et al., 2007), as solid patches of subchondral bone were shown to appear first on the locations where bone loading is highest (Wolschrijn and

Weijs, 2005). Given the extreme precocial behaviour and associated high loading of the skeleton in equids, it is plausible to expect these patches to develop early in foals. Due to the fact that a solid subchondral bone layer limits further growth by endochondral ossification (Carter, 1987; Radin et al., 1995) a delicate balance between loading resistance and growth requirements will have to be maintained.

The collagen type I network comprises about 90% of the non-mineralised matrix of bone (Buckwalter et al., 1995) and is of major influence on the strength of bone. This network is laid down before calcification takes place and it has been postulated that it plays a leading role in regulating mineralisation (Wassen et al., 2000; Wang et al., 2012). This would mean that the changes in trabecular bone orientation during the early juvenile phase would occur simultaneously with or be preceded by changes in collagen orientation.

Indirectly, this assumption is supported by the observations that both in the altricial rabbit (Turunen et al., 2012) and in the precocial foal (Holopainen et al., 2008) the collagen network of the cortical and subchondral bone reaches a mature organisation far before the mineralised part of the bone does.

The aim of this study is to elucidate the postnatal development of structurally important parameters in the subchondral bone of the distal tibia and talus of Dutch warmblood foals using micro-computed tomography (micro-CT) imaging and polarised light microscopy (PLM). We hypothesise that: 1) the BV/TV curve of the trabecular bone will not show a U-shape, but a gradual increase; 2) trabecular DA will show only minor changes during growth; 3) a patched subchondral bone layer, with solid patches on the locations where most bone loading can be expected, is present in young foals; 4) there are no or only minor differences in the orientation and degree of anisotropy of the collagen type I network after birth.

Materials and Methods

Animals

Tarsal joints of Dutch Warmblood foals and adult horses (*Equus ferus caballus*), that were still-born or euthanized due to reasons unrelated to the tarsocrural joint or general orthopaedic disorders were obtained with owners' consent from private veterinary practices and the (para)clinical departments of the Faculty of Veterinary Medicine, Utrecht University. Five neonatal foals and four older foals (63, 65, 81 and 145 days old) were included. For comparison, three adult (age range 12 - 20 years old) warmbloods

were also included. The CT data of the 5 neonatal foals have been used as well in a previous study on early skeletal development (Gorissen et al., 2016).

Sample preparation

Sample preparation and micro-CT imaging were carried out as described previously (Gorissen et al., 2016). In short, tarsal joints were collected within two hours after death and stored cooled (4 °C) if processing could be done within 12 hours, or frozen (–18 °C) when this took longer. After the removal of all soft tissue, the articular surfaces of the distal tibia and talus were macroscopically inspected and photographed. Samples were subsequently fixated in 4% buffered formaldehyde for at least a week. To fit in the micro-CT sample holder (diameter 7.85 cm), distal tibias were cut transversely through the distal metaphysis about 1 cm proximal to the distal physis and tali were cut in longitudinal direction through the middle of the talar trochlea. If necessary, also a small piece of the proximal part of the trochlear ridges of the talus was removed.

Micro-CT

Micro-CT imaging was performed with a μCT 80 scanner (Scanco Medical AG, Brüttisellen, Switzerland), fitted with an aluminium filter to reduce beam hardening effects. Bone samples were consistently positioned in the sample holder and secured using synthetic foam. Scanning was performed in air at an isotropic spatial resolution of 37 μm, a peak voltage of 70 kV and intensity (current) of 114 μA.

Trabecular bone was quantitatively determined in manually drawn volumes of interest (VOIs) only including trabecular bone. Selected VOIs were in line with previous work (Gorissen et al., 2016). The tibial VOI contained the trabecular bone of the dorsal 25% of the sagittal ridge. The lateral talar VOI consisted of the distal part of the lateral talar ridge and the medial talar VOI contained the middle region of the medial talar ridge. The subchondral bone layer was analysed in these same areas.
As there was no clear subchondral bone layer visible in the neonatal foals, a layer of 40 voxels (40 x 37 μm = 1480 μm) thick, just under the cartilage was selected to represent this layer.

Based on the histograms and visual comparison of segmented images at different threshold levels with the original scans (Wolschrijn and Weijs, 2004), a threshold of 145/1000 of the maximum possible voxel value (246 mgHA/cm^3) was chosen as best fit to analyse the juvenile bone samples. The samples of the adult horses were analysed at 170/1000, corresponding to 327 mgHA/cm^3.

Quantitative trabecular and subchondral bone parameters were calculated from the segmented images using the Scanco Medical software. Bone volume was calculated by dividing the number of bone voxels by the total number of voxels in the VOI. All structural parameters, including trabecular number (Tb.N.), trabecular thickness (Tb. Th.), and trabecular separation (Tb.Sp.) were obtained using a distance transformation method. The DA was based on the Mean Intercept Length fabric tensor and defined as the largest principal fabric value over the smallest one. Subchondral bone density was calculated by defining the subchondral bone layer as mentioned above first. After thresholding, BV/TV and density of the tissue above the threshold was measured.

Histology

From each region of interest analysed by MicroCT imaging, a 0.5-cm-thick sample (one cm apart), was cut with a handsaw and subsequently decalcified in 10% ethylenediaminetetraacetic acid (EDTA, Amresco LLC Solon, USA). After embedding in paraffin, three five-µm thick sections were cut, which were left unstained and covered using Eukitt (Sigma Aldrich, Darmstadt, Germany). Polarised light microscopy was performed with an Abrio PLM system (CRi, Inc., Woburn, USA), consisting of a green bandpass filter, a circular polarizer, and a computer-controlled analyser with two liquid crystal polarizers and a CCD camera mounted on a conventional light microscope (Nikon Diaphot TMD, Nikon, Inc., Shinagawa, Tokyo, Japan).

Four (talus) or three pictures (size of one image: 2.59 x 3.52 mm) per slide covered the total width of trabecular bone between the overlying cartilage and the middle of the talus, or the distal growth plate of the tibia. Two images were produced, one for the calculation of optical retardance and the other one for measuring the angular orientation of birefringent structures (e.g. collagen). As optical retardance depends on the amount of birefringent material and its anisotropy, it can provide an estimate of the collagen content in the tissue (Rieppo et al., 2007). Retardance and orientation images were analysed using custom Matlab (Matlab r2015b, Natick, USA) scripts. First, the retardance image was used to remove the empty space due to pores from the analysis. Then, the mean orientation angles were calculated for each image. The randomness of the orientation angles was quantified by calculating the entropy of the orientation values. The entropy was defined as:

$$E = -\sum_i P_i \log_2(P_i)$$

where *Pi* contains the count of each orientation angle within the window. The anisotropy was calculated as:

$$A = \frac{1}{E+1}$$

As the minimum of entropy is 0, the range of this anisotropy parameter is between 0 and 1 (with one representing only one orientation angle within the analysis window, and 0 representing a situation where all orientation angles within the analysis windows are represented evenly).

Statistical analyses

All statistical analyses were performed using SPSS Statistics 22 (IBM Corporation). As there were no statistically significant differences between the left and right limb, one average value for each parameter and region studied in each animal was calculated. To analyse the microCT and PLM data two linear mixed effect models with foal ID added as random intercept were made using a backward approach based on the Akaike's Information Criterion (AIC). To meet the normality and homoscedasticity assumptions of the model, several parameters needed transformation (Tb.N, density trabecular and subchondral bone, average and SD of the retardation data were square rooted; Tb.Sp and BV/TV subchondral bone layer were logarithmically transformed). Unfortunately, it was not possible to make the SD of the orientation and the anisotropy data of the PLM analysis fit to the model assumptions and therefore, the non-parametric Mann-Whitney U test was used for this data. Statistical significance was set at $P \leq 0.05$ and outcomes were corrected for multiple comparisons using the False Discovery Rate method of Benjamini – Hochberg (1995).

Results

Trabecular bone

Trabecular bone parameters for the analysed VOIs are presented in figure 1. After birth, average BV/TV increased significantly in all three analysed VOIs. Differences observed between the older foals and adult horses were not significant. (Fig. 1a). Only the difference in Tb.N. between the neonatal foals and adult horses in the medial part of the talus was significant. (Fig. 1b). Compared to the neonatal foals, Tb.Th was significantly higher in all three regions of both the older group of foals and adult horses. In both the tibia and the lateral part of the talus, trabeculae were significantly thicker in adult horses than in the group of older foals, whereas in the medial part of the talus this was not the case. (Fig. 1c). In all VOIs and age groups studied, no significant differences in Tb.S. and DA were observed (Figs. 1d and e). Bone density increased gradually with increasing age, except for the lateral talus where new-born and older foals had the same density (Fig. 1f).

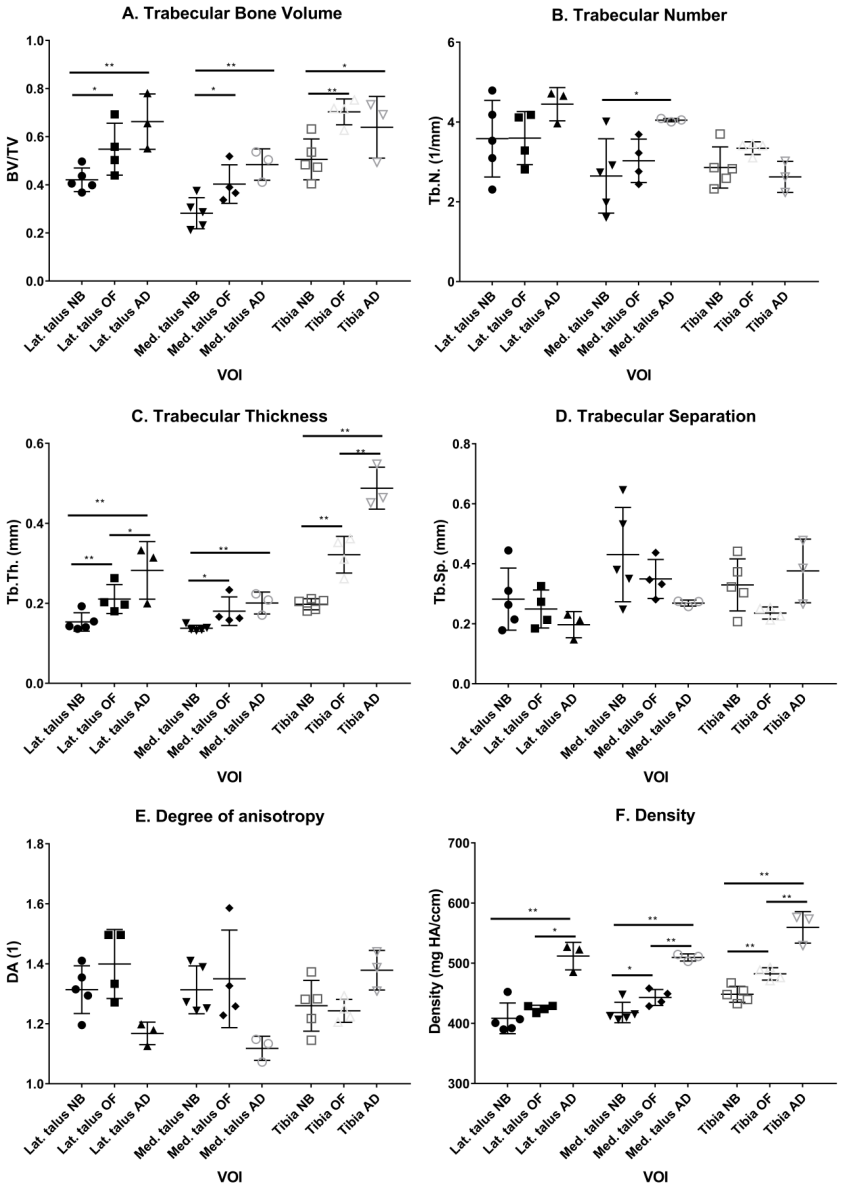

Figure 1: *Mean (+/- SD) trabecular bone parameters calculated for the different Volumes of Interests (VOIs, Lat. talus: lateral part of the talus; Med. Talus: medial part of the talus; Tibia: sagittal ridge of the tibia) and age groups (NB: new born foals; OF: older foals; AD: Adult horses). * 0.01 < P < 0.05; ** P < 0.01.*

a) Bone volume fraction (BV/TV).
b) Trabecular number (Tb.N.).
c) Trabecular thickness (Tb.Th.).
d) Trabecular separation (Tb.Sp.).
e) Directional anisotropy (DA).
f) Density

Subchondral bone

The results of the subchondral bone analysis are presented in figure 2. At birth, no solid subchondral bone layer was present in any of the foals and the average percentage of bone was about 70% for all studied VOIs. In the older group of foals, this volume increased to values of over 80%, which increase was significant in the lateral talus (Fig. 2a). A strong and significant increase in the degree of mineralisation of all regions was observed from new-borns to older individuals (Fig. 2b). See for illustration supplementary figure 1, which shows representative examples of the visual aspects of the subchondral bone at birth and later in life.

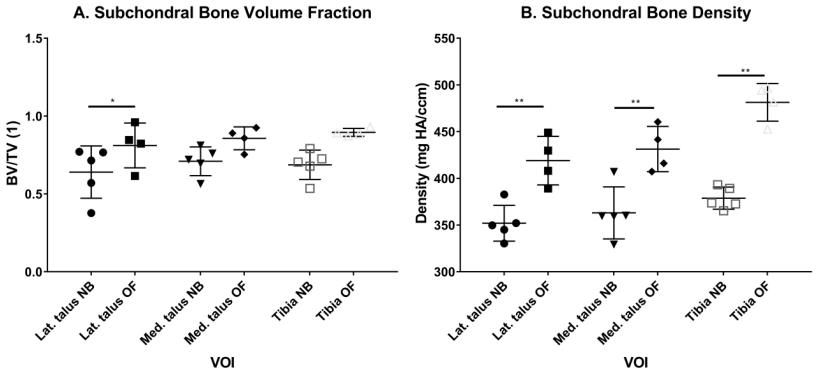

Figure 2: *Mean (+/- SD) subchondral bone parameters calculated for the different Volumes Of Interest (VOIs, Lat. talus: lateral part of the talus; Med. Talus: medial part of the talus; Tibia: sagittal ridge of the tibia) and age groups (NB: new born foals; OF: older foals). * 0.01 < P < 0.05; ** P < 0.01.*

a) Bone volume fraction (BV/TV)

b) Density

Collagen network

In all VOIs and depths studied a significant increase in average retardation was observed (table 1). Also the variation of the retardation signal significantly increased in the older group of foals. The average orientation of the collagen type I network only changed in the deeper parts of the medial trochlear ridge of the talus and stayed similar in all other locations. In all locations and depths, both the variation of the orientation angles and degree of anisotropy of the collagen type I network did not change over time. Supplementary figure 2 shows representative examples of PLM images of the collagen network.

Table 1: *Mean (+/- SD) Polarised Light Microscopy (PLM) parameters calculated for the different Volumes of Interests (VOIs), age groups and depths (1=superficial, 3 and 4 =deepest). * 0.01 < P < 0.05; ** P < 0.01.*

		Average Retardation					Standard Deviation Retardation			
		Newborn foals		Older foals			Newborn foals		Older foals	
	Depth	Mean	SD	Mean	SD	Depth	Mean	SD	Mean	SD
Lateral part of the talus	1 **	4,0584	2,1600	11,0463	1,6888	1 **	2,1876	1,2943	5,7574	0,9316
	2 **	3,8181	1,9820	11,4547	2,0228	2 **	2,1153	1,1483	5,7964	0,9280
	3 **	3,9024	2,0439	11,6886	2,1248	3 **	2,1218	1,2185	5,8980	1,0067
	4 **	4,0254	2,0506	11,2537	2,6871	4 **	2,1954	1,1145	5,6164	1,1952
Medial part of the talus	1 **	6,0745	3,1144	11,2477	2,1300	1 *	3,7726	1,5588	5,8482	1,2020
	2 **	7,6087	2,4240	12,8514	3,0764	2 **	4,2346	1,2339	7,2081	3,3001
	3 **	7,0928	1,2646	12,0382	2,1419	3 **	3,5084	0,9299	6,2624	1,2997
	4 *	6,2402	1,3375	10,5530	2,4651	4 **	2,9334	1,1243	5,0454	1,4340
Tibia	1 **	5,6093	3,0609	12,9550	2,5087	1 **	3,1800	1,5663	6,9114	2,4877
	2 **	6,6327	1,7825	12,5324	1,6876	2 **	4,0080	0,9164	6,6573	1,9330
	3 **	7,1468	1,6784	12,1337	2,6464	3 **	3,9779	1,0098	6,0049	1,6362
		Average Orientation					**Standard Deviation Orientation**			
		Newborn foals		Older foals			Newborn foals		Older foals	
	Depth	Mean	SD	Mean	SD	Depth	Mean	SD	Mean	SD
Lateral part of the talus	1	49,4749	11,0144	42,7368	2,5858	1	23,4554	2,9415	25,5006	0,3553
	2	45,7456	7,8133	41,0646	2,0658	2	24,9643	1,3633	25,4046	0,3633
	3	45,4571	3,6816	42,5746	2,1579	3	25,9176	0,3069	25,6996	0,2997
	4	47,1897	2,7361	45,6648	1,6902	4	25,7958	0,4835	25,6693	0,2007
Medial part of the talus	1	38,4557	4,4079	41,1603	4,8405	1	25,3196	0,9501	25,3270	0,5915
	2 **	38,6081	7,7788	49,3621	4,1727	2	24,4817	1,8609	24,7602	0,8419
	3 **	40,6235	7,8532	51,6843	3,7798	3	24,8268	1,2102	24,6841	0,3551
	4 **	42,1042	9,1937	52,1770	4,6046	4	24,8416	0,9979	25,1162	0,7649
Tibia	1	40,3431	3,6003	44,3497	4,0831	1	24,9698	1,1057	25,7129	0,6809
	2	42,6789	3,8052	38,5930	4,0845	2	25,7780	0,3845	25,2556	0,8264
	3	45,1640	2,3641	43,8060	4,0680	3	25,8318	0,2905	25,2954	0,9328

		Degree of Anisotropy			
		Newborn foals		Older foals	
	Depth	Mean	SD	Mean	SD
Lateral part of the talus	1	0,1369	0,0043	0,1336	0,0002
	2	0,1347	0,0021	0,1339	0,0003
	3	0,1337	0,0004	0,1336	0,0003
	4	0,1337	0,0005	0,1335	0,0002
Medial part of the talus	1	0,1347	0,0016	0,1343	0,0009
	2	0,1355	0,0031	0,1344	0,0011
	3	0,1351	0,0025	0,1349	0,0008
	4	0,1352	0,0020	0,1356	0,0014
Tibia	1	0,1344	0,0009	0,1339	0,0008
	2	0,1337	0,0004	0,1346	0,0012
	3	0,1336	0,0002	0,1344	0,0011

Discussion

This study provides further insight in the postnatal bone development of the precocial foal, which can be seen as a reflection of the strategies how this precocial animal copes with the mechanical loading associated with locomotion. The equine tarsal joint is loaded in multiple directions, as weight bearing of the hind limb leads to tensile strain of the lateral part and compression of the medial part of the talus (Schneider et al., 1982). Additionally, simultaneous shear and compressive forces are exerted on the talus (Badoux, 1987), as the slanting talar trochlear ridges tightly interlock in the tibial cochlea to stabilize the tarsus (Nickel et al., 2005). Whereas absolute forces obviously increase with growth, relative loading remains stable in the early juvenile phase, as bodyweight normalised peak vertical forces at walk and trot stay relatively constant during the first half year of the foal's life (Gorissen et al., 2017).

The absence of a reduction in trabecular BV/TV after birth in foals as seen in this study, and of the increase in BV/TV in the following months, mirrors the gradual increase in BV/TV after the onset of walking in altricials (Wolschrijn and Weijs, 2004; Ryan and Krovitz, 2006; Gosman and Ketcham, 2009). The increase of trabecular BV/TV is caused by the significant increase of Tb.Th, which is a common hallmark of trabecular bone growth, and reported in many other species and skeletal locations (Wolschrijn and Weijs, 2004; Mulder et al., 2005; Ryan and Krovitz, 2006; Gosman and Ketcham, 2009). A possible explanation for the lower BV/TV values observed in babies and puppies at the onset of walking compared to the foal may be that in the latter, the process of learning to walk is much more gradual, whereas foals need to be able to outrun predators from day one. The increase in trabecular BV/TV is also reflected by the increase in collagen type I. Optical retardance is dependent on the amount of birefringent material and its anisotropy (Rieppo et al., 2007). As the anisotropy of the trabecular bone did not change significantly over time, the increase in retardation is due to an increase of collagen.

In foals, Tb.N. stays relatively constant after birth, which is in contrast to reports in altricial (Wolschrijn and Weijs, 2004) and semi-precocial (Tanck et al., 2001) animals and in humans (Ryan and Krovitz, 2006; Gosman and Ketcham, 2009). According to the Mechanostat theory (Frost, 2001) and Wolff's law of bone adaptation (1892) this would indicate that in the foal after birth no trabeculae are loaded under their threshold, which would lead to resorption. This observation supports the idea of prenatal anticipatory bone development, as proposed earlier (Gorissen et al., 2016). Further support for this concept can be found in the relative constant orientation of both trabeculae and collagen network. These orientations are highly correlated with the main direction of loading

(Ulrich et al., 1997; Wang et al., 2001; Mittra et al., 2005; Silva et al., 2006) and hence the lack of changes means that both are already in line with postnatal loading with form preceding function, in contrast to Wolff's paradigm. A comparable situation has been shown to exist in equine articular cartilage where an anisotropic collagen network was already evident prior to birth (Cluzel et al., 2013). Also in the subchondral bone layer, the basic collagen framework seems to have attained the mature configuration at an age of five months (Holopainen et al., 2008). This may seem extremely early given the fact that horses reach skeletal maturity (as defined by growth plate closure) at an age of 4 years (Kainer, 1987), but it is questionable whether growth plate closure is the event to relate to. A relation with the maturation process of the collagen network in the articular cartilage may be more logical, as this structure is functionally much more closely related to the subchondral bone than the growth plates. In foals, the functional adaptation of articular cartilage indeed essentially takes place as early as during the first five months of life (Brama et al., 2002).

In line with previous studies (Wolschrijn and Weijs, 2004), the subchondral bone layer in the foal was shown to develop in the form of patches, which first appeared in those locations with highest loading. At birth, quite some variation was found in the composition and structure of this layer, as reflected by the differences in BV/TV. It can be hypothesised that there might be a relationship with the susceptibility to osteochondrosis. Osteochondrosis (OC) is a disorder of the process of endochondral ossification as this takes place at the articular side of the epiphyseal ossification centre (Ytrehus et al., 2007). During this process the cartilaginous anlagen, called the growth cartilage, gradually ossifies until only the layer of articular cartilage remains. Whereas the ultimate articular cartilage is avascular, the growth cartilage is richly vascularised (Lecocq et al., 2008; Olstad et al., 2008a; Olstad et al., 2008b; Olstad et al., 2008c; Olstad et al., 2009) These vessels, which are located in so-called cartilage canals originally receive their blood from perichondrial arteries. With the advancement of the ossification front, this supply is cut off and partially replaced by newly formed anastomoses between the vessels in the cartilage canals and epiphyseal subchondral arteries, which hence cross the ossification front (Olstad et al., 2008b; Olstad et al., 2008c). It is hypothesized that the vessels are vulnerable at this site before entering the cartilage canals as there is a sharp transition in biomechanical characteristics of these different environments (Ytrehus et al., 2004). Although foals are able to follow their dam from day one, the development of static balance (Nauwelaerts et al., 2013) and gait (Gorissen et al., 2017) takes time, which increases the risk of stumbling and consequently the generation of focal peak loads in joints. It can be conjectured that foals featuring biomechanically less favourable subchondral and trabecular bone parameters at birth would be more susceptible than

others to sustaining vascular damage and consequently develop OC due to hypoxia of the chondrocytes.

Due to the difficulty obtaining material from foals meeting the inclusion criteria, the number of animals included in this study was limited, which is considered a limitation. Nevertheless, all animals had developed normally until death or euthanasia and can be seen as representative for the Dutch Warmblood breed. All also showed similar developmental patterns with regard to the variables investigated in this study.

Conclusion

We confirmed in this study that, in contrast to more altricial species, foals have an anticipating way of bone development, both with respect to composition (BV/TV) and architecture (trabecular DA and collagen orientation). These observations support and expand earlier observations pointing at the existence of such a prenatal anticipatory bone development (Gorissen et al., 2016) and demonstrate the strategy followed by the horse to cope with the extreme changes in mechanical environment the locomotor system experiences around birth. Furthermore, they can be helpful in better understanding the aetiology of development orthopaedic diseases.

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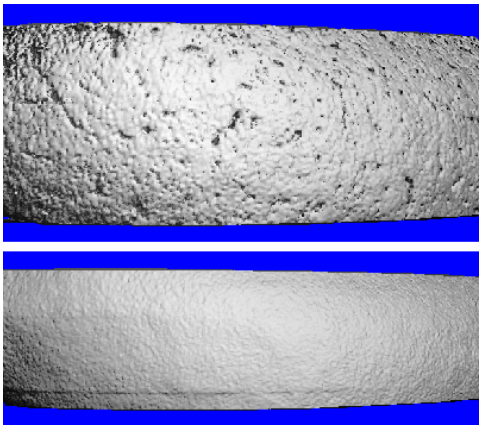
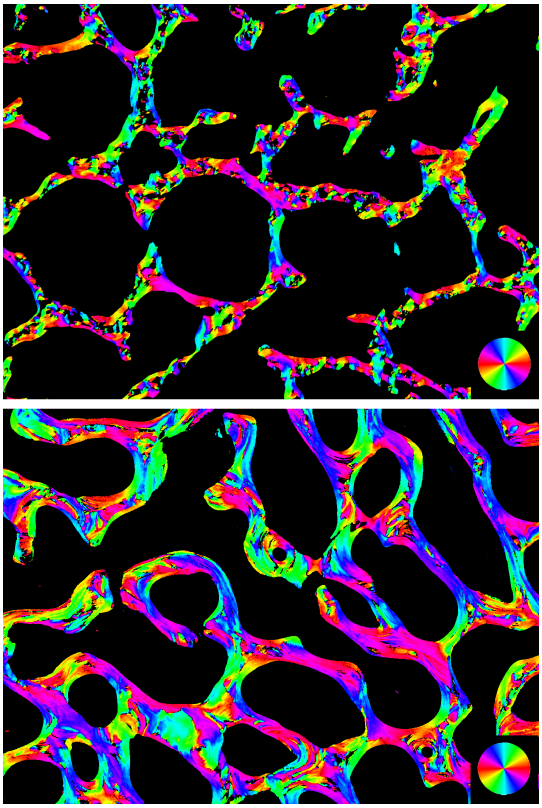


Supplementary figure 1: *Part of the reconstructed subchondral bone layer of the right lateral trochlear ridge of the talus of a new-born (upper part) and 145 days old foal (lower part).*



Supplementary figure 2: *Representative example of a Polarised Light Microscopy (PLM) image from the trabecular bone of the distal part of the lateral trochlear ridge of a neonatal (upper part) and older (145 days old; lower part) foal. The different colours represent the orientation of the collagen type I network as indicated in the legend in the right hand corner.*

CHAPTER VI:

Effects of longterm use of the preferential COX-2 inhibitor meloxicam on growing pigs

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Submitted

Abstract

Meloxicam, a preferential COX-2 inhibitor, is a commonly used non-steroidal anti-inflammatory drug in pigs. Besides having potential side effects on the gastro-intestinal tract, this type of drug might potentially affect osteogenesis and chondrogenesis, processes relevant to growing pigs. Therefore, the effects of long-term meloxicam treatment on growing pigs were studied. Twelve piglets (n=6 receiving daily meloxicam 0.4 mg/kg orally from 48 until 110 days of age; n=6 receiving only applesauce (vehicle control) were subjected to visual and objective gait analysis by pressure plate measurements at several time points. Following euthanasia a complete necropsy was performed and samples of the talus and distal tibia, including the distal physis, were collected. Trabecular bone microarchitecture was analysed by microCT scanning, bone stiffness by compression testing and growth plate morphology using light microscopy. Animals were not lame and gait patterns did not differ between the groups. Pathological examination revealed no lesions compatible with known side effects of non-steroidal anti-inflammatory drugs. Trabecular bone microarchitecture and growth plate morphology did not differ between the two groups. The findings of this in vivo study reduce concerns regarding the long-term use of meloxicam in young, growing piglets.

Introduction

In the pig industry, procedures generating pain, like castration and tail docking, are routinely performed (Llamas Moya et al., 2008; Sutherland, 2015; Nordquist et al., 2017). Additionally, pain and inflammation are frequently related to lameness, which is a common clinical observation in rearing piglets and sows (Main et al., 2000; Kilbride et al., 2009). However, despite increased awareness and attention for welfare in food producing animals, (knowledge of) pain management significantly lags behind compared to companion animals and horses (Thomsen et al., 2012). Therefore, the beneficial effects of pain relief on clinical presentation and animal welfare, as well as on hidden financial costs such as decreased production (Bonde et al., 2004; Jensen et al., 2012) and premature culling (Engblom et al., 2007; Anil et al., 2009; Jensen et al., 2010; Jensen et al., 2012) may not be appreciated appropriately. Further factors likely contributing to the underuse of anti-inflammatory pain medication in the pig rearing industry are both the labor intense burden of selective treatment and concerns about associated side-effects.

In medical (Mathews, 2014) and veterinary pain ladders, treatment with non-steroidal anti-inflammatory drugs (NSAIDs) is a base step in relieving pain. A recent meta-analysis of effective pain treatment in piglets following surgical procedures early in life concluded that high heterogeneity in study designs precluded definitive recommendations, yet treatment with NSAIDs was the only intervention with proven efficacy (O'Connor et al., 2014). The most commonly prescribed NSAID in pigs is meloxicam (Ison and Rutherford, 2014; Wilson et al., 2014), which is also marketed for use in several other domestic species as well as humans. Previous studies of meloxicam administration in pigs have reported both COX-1 and more potent COX-2 inhibition (Fosse et al., 2008). In pigs, meloxicam is licensed for single use in a dose of 0.4 mg/kg given intramuscularly or orally with an option to repeat 24 hours later (Friton et al., 2003). However, as in other species, longer usage is necessary to treat more chronic conditions associated with pain and inflammation (e.g. joint disease).

Side effects of NSAID treatment on the gastro-intestinal tract, renal papillae and on primary hemostasis are well known and have been reported in humans (Conaghan, 2011; Bozimowski, 2015) and animals (Clark, 2006; Goodrich and Nixon, 2006), especially after prolonged use. In pigs, the information on the use of meloxicam and possible side-effects is limited (Rauser et al., 2010; Fosse et al., 2011; Friess et al., 2012). Currently, COX-2 inhibition during bone fracture healing is under debate in human medicine, as it could possibly lead to delayed fracture healing by reducing prostaglandin concentrations (Goodman et al., 2002; Simon et al., 2002; Gerstenfeld et al., 2003; Herbenick et al., 2008;

Boursinos et al., 2009; Inal et al., 2014). Given the similarities between the processes of fracture healing and endochondral ossification, COX-2 inhibition could also possibly negatively affect skeletal development, especially in a fast growing animal such as the pig. Studies on the effect of NSAID-mediated COX-2 inhibition on cartilage and bone formation are thus far conflicting, reporting both negative (Jakob et al., 2004; Retamoso et al., 2010) and neutral to positive effects (Rainsford et al., 1997; Su et al., 2014). Only one *in vitro* study used porcine cartilage explants and reported that meloxicam did not interfere with cartilage repair (Rainsford et al., 1997). However, Welting et al. (2011) found that in growing rabbits the COX-2 inhibitor celecoxib negatively affected the hypertrophic zone of the growth plate. Specific information regarding the effect of COX-2 inhibition on bone development in growing piglets is lacking, but piglets have been used as a model to study the effects of prenatal, neonatal and perinatal glucocorticoid administration. Birthweight and growth rate were not affected, but glucocorticoid treatment in perinatal piglets negatively affected structural bone development and associated mechanical properties (Sliwa et al., 2010).
The lack of scientific information regarding the effects on bone and cartilage formation of meloxicam in growing pigs urges the in vivo assessment of the use of meloxicam in these animals.

In this study, which is part of a larger study assessing efficacy and possible side-effects of prolonged daily administration of meloxicam to rearing pigs with experimentally induced mono-arthritis (J. J. Uilenreef, F. J. van der Staay, E. Meijer, unpublished observations), we focused on identifying possible clinically relevant effects of meloxicam administration on the locomotor system. Objective evaluation of the locomotion by pressure plate analysis, combined with postmortem MicroCT and bone compression testing were used as outcome parameters. Additionally, the gastro-intestinal and renal systems were assessed by (histo-) pathological examination. We hypothesized that long-term, daily treatment of piglets with meloxicam at the registered dose would result in an increased incidence of gastro-intestinal side-effects compared to the negative control group. Further, we anticipated adverse effects on chondro- and osteogenesis, more in particular a decreased hypertrophic chondrocyte differentiation in the growth plate and inferior trabecular bone parameters such as lower bone volume fractions (BV/TV). The study aims at contributing to clinical decision making in (growing) pigs with regard to the administration of meloxicam for anti-inflammatory and pain management under conditions requiring prolonged treatment.

Materials and Methods

Ethical statement

The study was reviewed and approved by the ethical committee of Utrecht University (no. 2014.I.11.085, date of approval December 17, 2014), The Netherlands, and was conducted in accordance with the recommendations of EU directive 86/609/EEC. All efforts were taken to minimize the number of animals used and their suffering.

Animals and housing

The 12 pigs used for this study were a subset of a larger group of 40 Topigs 20 x Piétrain piglets from the breeding herd of the Utrecht University teaching farm used to study the efficacy of meloxicam for treatment of experimentally induced osteoarthritis by injection with mono-iodoacetate (J. J. Uilenreef, F. J. van der Staay, E. Meijer, unpublished observations), in which this subset served as controls (injected with saline as placebo). Piglets were group-housed and provided with a covered nest area and environmental enrichment (metal chains, balls, chewing sticks). The nest area had a roof that could be pulled up. Each nest area had two heating lamps and the floor was covered with a rubber mat and thick layer of straw. Transparent rubber flaps hung down from the front side of the roof to provide extra shelter during the first weeks. Piglets were housed according to litter (8 piglets per litter) to minimize aggression and fighting. Animals had *ad libitum* access to water from a drinking nipple, straw and commercial standard food (supplier: De Heus Voeders B.V., Ede, The Netherlands) for growing pigs. Starting at one week of age, all piglets were fed with "Romelko nurse", followed by "Romelko prevent 3" in the week before weaning. Subsequently, pigs were fed as recommended by the feed supplier using "Prevent 5", Stimulans 6 and "Vital Plus". Information regarding ingredients relevant to bone development (Calcium, Phosphorus and Vitamin D) and gastric ulceration (crude fibre) can be found in supplementary table 1. During the 20-day acclimatization period and in the first two weeks of the experiment, pigs were weighed twice a week, thereafter once a week.

Experimental design

After the acclimatization period, the animals in this study received an intra-articular injection with 0.25 ml sterile 0.9% saline solution (B. Braun Melsungen AG, Germany) as a placebo treatment against the arthritis-induced animals (not included in this study), as pointed out above. For this, animals were lightly anesthetized in a two-step procedure consisting of an intramuscular (i.m.) injection with dexmedetomidine (15 μg/kg, Dexdomitor® 0.5 mg/ml, Orion Pharma, Finland; 10 ml) followed 15 minutes later by an i.m. injection with ketamine (10 mg/kg, Narketan® 100 mg/ml, Vétoquinol S.A.,

France; 10 ml) in combination with midazolam (0.5 mg/kg, Midazolam Actavis 5 mg/ml, Actavis Group PTC ehf., Iceland; 10 ml). After five minutes the animal was transported to a dedicated area for surgery. After aseptic preparation the left intercarpal joint was injected. Recovery of anesthesia was accelerated by administration of atipamezole (0.5 mg/kg Atipam®, 5 mg/kg, Eurovet Animal Health BV, Bladel, Netherlands).

From day one (48 days of age) until the end of the study (110 days of age), half (n=6) of the animals received applesauce freshly spiked with meloxicam (0.4 mg/kg, Metacam® 15 mg/ml oral suspension, Boehringer Ingelheim Vetmedica GmbH, Germany), the other half (n=6) only received untreated applesauce.

Gait analysis

Before each pressure plate measurement session, animals were visually checked to make sure all animals were sound. Video recordings, obtained one day before and one, three and 28 days after left carpal intervention, were assessed by two experienced porcine veterinarians, blinded for intervention and treatment. If present, lameness was scored according to the protocol of Main et al. (2000). Quantitative gait parameters were obtained by pressure plate measurements (Footscan®, RSscan, Belgium) at one day before and one, three, seven, 14, 28 and 56 days after intra articular injection using the same setup as used before in piglets (Meijer et al., 2014a, b). During the habituation period preceding gait analyses, piglets were trained to trot over the runway at a steady pace without stopping. Runs were considered valid if the pig moved in a straight line and looked straight ahead. Measurements were repeated until at least 4 valid runs were collected.

Footfalls were manually assigned to the corresponding limb using the manufacturer's software. Peak Vertical Force (PVF) and Vertical Impulse (VI) were extracted from the data for each limb and normalised for bodyweight. Asymmetry indices (ASI) comparing contralateral limbs within one run were calculated for both PVF and VI using the following formulas:

Contralateral front limbs (CLF): $CLF = \frac{(LF-RF)}{0.5*(LF+RF)} * 100$

Contralateral hind limbs (CLH): $CLH = \frac{(LH-RH)}{0.5*(LH+RH)} * 100$

This yielded a dimensionless number between -200 (indicating that no weight was put on the left limb) and 200 (indicating that no weight was put on the right limb). An ASI of 0 meant that weight bearing was perfectly symmetrical (Oomen et al., 2012).

Euthanasia

Animals were sedated and general anesthesia was induced in the same way as described for the intra-articular injections. When the animals had reached a sufficient anesthetic depth, they were euthanized by IV injection of 50 ml of Pentobarbital (Euthanimal, Alfasan, Woerden, The Netherlands, 400 mg/ml).

Gross pathology, tissue sampling and histopathology

Following euthanasia a complete necropsy was performed, including opening of the carpal, tarsal, shoulder, knee and elbow joints. Samples of the stomach, duodenum, jejunum, ileum, cecum and colon and both kidneys were taken and fixated in 10% neutral buffered formalin. All samples were paraffin embedded and 3 μm thick sections were cut using a microtome. After haematoxylin and eosin (HE) staining, samples were evaluated under light microscopy (Olympus BX-45, Zoeterwoude, The Netherlands).

Four mm thick samples of the left talus and distal tibia were taken using a K430 band saw (Kolbe, Germany; blades Munkfors, Sweden). After fixation in paraformaldehyde (4%), bone samples were decalcified in 10% ethylene-diamine-tetra-acetic acid (EDTA), which took between two and six weeks. Bone samples were paraffin embedded and three μm thick samples were obtained and HE stained. Photographs of the distal growth plate of the tibia were taken and the thickness of the hypertrophic and proliferative zones was independently measured by two observers with Fiji for ImageJ (version 2.0.0-rc-43/1.50e) using the protocol of Welting et al. (2011).

MicroCT imaging and tissue mechanics

Right tali were stored at -18 °C before microCT imaging and subsequent tissue testing. After thawing, cylindrical trabecular bone samples (diameter 7.5 mm) were obtained from the lateral and medial part of the caput tali with a hollow drill. With a diamond blade saw the distal ends of the samples were cut just above the cartilage; proximally the samples were cut to a length of 10 mm, ensuring plane parallel ends. Micro-CT imaging was performed using a μCT 80 scanner (Scanco Medical AG), equipped with an aluminium filter to reduce beam hardening effects. Scanning was performed in air at a spatial resolution of 37 μm [voltage of 70 kV; intensity (current) 114 µA]. Based on the histograms and visual comparison of differently thresholded images with the original scans (Wolschrijn and Weijs, 2004), a global threshold of 212 per mille of the maximum grey value was chosen. From the segmented images, quantitative trabecular bone parameters were calculated using the Scanco Medical software. Bone volume fraction (BV/TV) was calculated as the number of bone voxels divided by the total number of voxels in the sample. Structural parameters (trabecular number Tb.N.; trabecular thickness Tb.Th.

and trabecular separation (Tb.Sp.) were calculated by a distance transformation method. The degree of anisotropy (DA) was based on the Mean Intercept Length fabric tensor and defined as the largest principal fabric value over the smallest one.

After scanning, the stiffness of the bone samples was determined by non-destructive compression. Before testing, metal endcaps were glued at both sides of the cylindrical bone samples to reduce end artifact effects (Keaveny et al., 1993, Linde, 1994). Then, bone samples were pre-loaded four times with 20N, followed by a gradual compression with a force of 200 N at a speed of 0.1 mm/min. As mechanical behavior of all bone samples tested was still in the elastic range, experimental stiffness of the samples was determined by calculating the slope of the force-displacement curve in the 100-200 N region with Matlab r2015 (MathWorks, Natick, USA).

Statistical analysis

Normality of the data distribution was checked both visually and using the Shapiro-Wilks test. Since the data were not normally distributed, differences between the meloxicam treated and vehicle control group (no meloxicam) were assessed using the Mann-Whitney U test. Data were analyzed using SPSS statistics 22 (IBM) and R Statistical software version 3.1.2 (R Development Core Team 2008).

Meloxicam effects were tested with P set at <0.05 and a correction for multiple comparisons was performed according to the False Discovery Rate method of Benjamini and Hochberg (1995). Unless indicated otherwise, results are presented as mean ± standard deviation (SD).

Effect sizes (ES) were retrieved as Cliff's delta (Cliff 1993). The interpretation for the present work is the following: <0.11, very small or no effect; 0.11 - 0.28, small effect; 0.29 - 0.43, medium effect; and >0.43, large effect. Differences were considered relevant if a P value < 0.05 was found and the effect size was medium or large.

Results

Average weight of the pigs at the end of the study was 61 (± 4.1) kg and did not differ between the two groups.

Gait analysis

No animals were considered lame before the pressure plate measurements and no gait abnormalities were observed on the video recordings. During the study period, average nPVF values fluctuated between seven and ten N/kg, but lower values were found in both groups on day 28 (Figure 1A). The same trend can be seen in the nVI, with average values between 0.7 and 1.0 Ns/kg and about 0.6 Ns/kg at week 28 (Figure 1B). No differences in bodyweight normalized, kinetic gait parameters were found between the meloxicam treated and vehicle control animals.

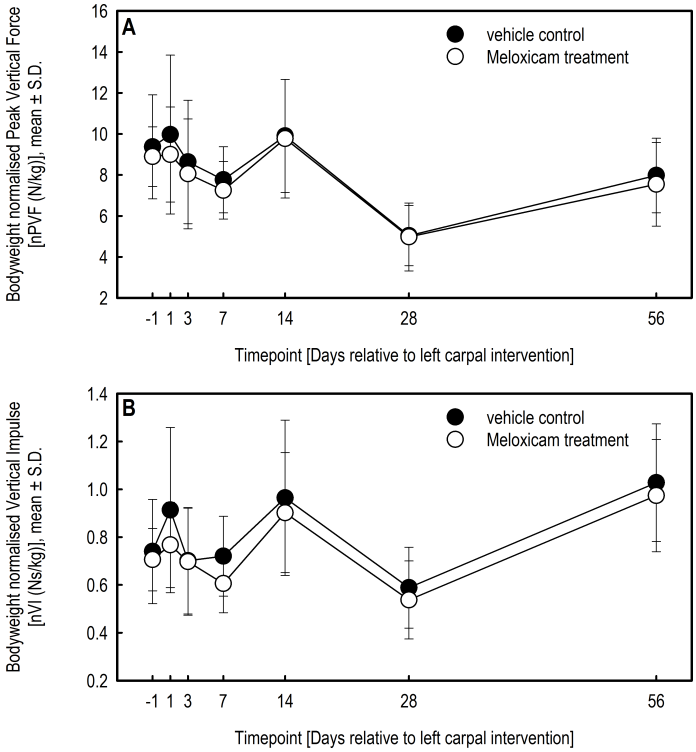

Figure 1: *Mean (± standard deviation) body weight normalised Peak Vertical Force (nPVF N/kg, A) and Vertical Impulse (nVI Ns/kg, B) at the different time points for the vehicle control and meloxicam treated animals.*

Over time, ASI values fluctuated around 0, with a slight dip in both contra-lateral front limb PVF and VI on day 1 (Figure 2). No effects of NSAID treatment were found.

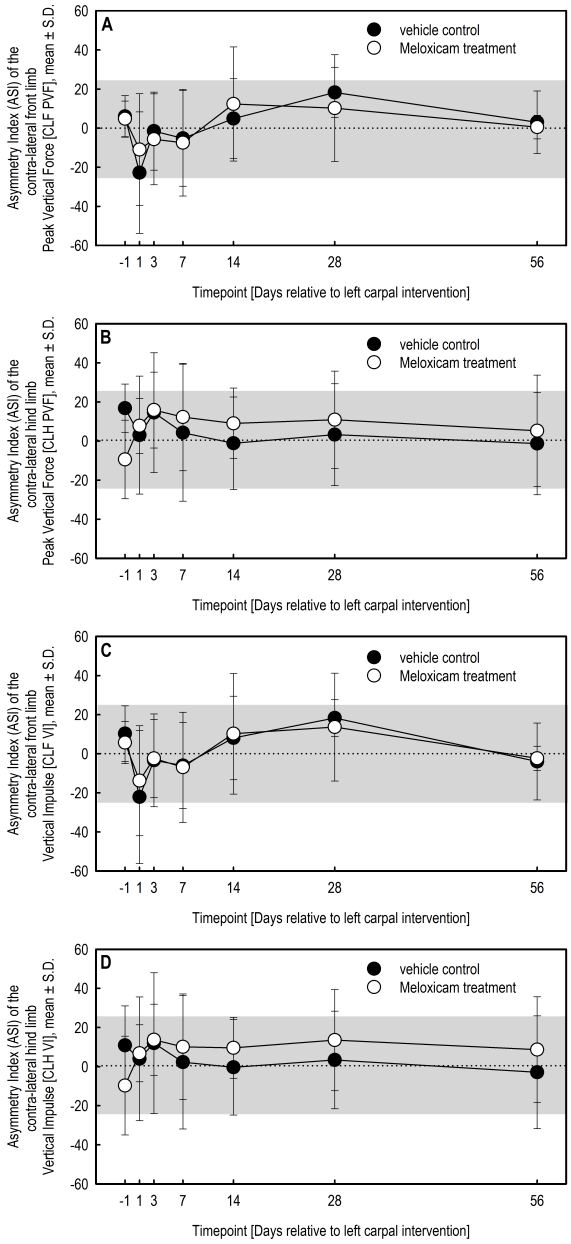

Figure 2: *Asymmetry indices (ASI) comparing Peak Vertical Force of the left front limb and the right front limb (A). Asymmetry indices (ASI) comparing Peak Vertical Force of the left hind limb and the right hind limb (B). Asymmetry indices (ASI) comparing Vertical Impulse of the left front limb and the right front limb (C). Asymmetry indices (ASI) comparing Vertical Impulse of the left hind limb and the right hind limb (D). Mean values over time of the group that received NSAIDs and the group that did not receive NSAIDS are shown. PVF= Peak Vertical Force. VI= Vertical Impulse. CLF= Asymmetry index of the contralateral front limbs. CLH= asymmetry index of the contralateral hind limbs.*

Histo(patho)logy

At necropsy, all pigs were normally developed and in good condition. Signs of mild enteritis were found in all animals, but in none of the animals, macro- or microscopic signs of gastric or enteric ulceration were encountered. Evaluation of the kidneys did not reveal renal papillary necrosis. In three pigs (all from the vehicle control group) 0.1-0.2 cm sized (osteo-)chondral lesions were found at macroscopic evaluation of the tarsal joints. Microscopically, OC associated lesions were found in five animals (two from the vehicle control group and three from the meloxicam treated group). Two of these animals (meloxicam treated) showed two lesions on different locations within the tarsal joint.

Growth plate morphology

In both groups, the hypertrophic zone of the growth plate was thicker compared to the proliferative zone (ratio about 60:40). Meloxicam treatment did not affect the relative thickness of these zones (Figure 3).

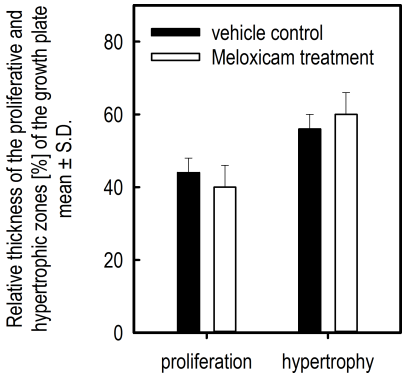

Figure 3: *Relative thickness of the proliferative and hypertrophic zones respectively in the distal tibial growth plate for the meloxicam treated and vehicle control animals.*

Trabecular bone parameters are presented in table 1. The differences observed between the meloxicam treated and vehicle control animals were not statistically significant.

Table 1: *Trabecular bone parameters of the lateral and medial part of the talus (BV/TV: bone volume fraction; Tb.N.: trabecular number; Tb.Th.: trabecular thickness; Tb.Sp. trabecular separation; DA: degree of anisotropy).*

Parameter	**BV/TV [1]**		**Tb.N. [1/mm]**		**Tb.Th. [mm]**		**Tb.Sp. [mm]**	
Metacam?	yes	no	yes	no	yes	no	yes	no
Average lateral part	0,39	0,37	2,53	2,58	0,16	0,15	0,35	0,34
SD lateral part	0,04	0,04	0,24	0,31	0,01	0,01	0,05	0,04
Average medial part	0,34	0,31	2,41	2,31	0,14	0,14	0,37	0,40
SD medial part	0,05	0,05	0,20	0,27	0,01	0,01	0,04	0,07
Parameter	**DA [1]**		**Density [mg HA/cm^3]**		**Stiffness [N/mm]**			
Metacam?	yes	no	yes	no	yes	no		
Average lateral part	1,67	1,73	844.72	844.02	2165,2	1462,5		
SD lateral part	0,09	0,08	13.87	12.85	405,5	247,1		
Average medial part	1,91	1,92	849.89	849.59	1302,4	1377,4		
SD medial part	0,21	0,20	12.44	11.28	468,1	564,0		

Discussion

This study did not show adverse side effects of long-term meloxicam usage in growing pigs on weight gain, gait, trabecular bone parameters, growth plate morphology, gastro-intestinal integrity and kidney histology. These findings indicate that prolonged daily treatment with meloxicam at the licensed dose does not lead to detrimental side-effects in growing pigs with regard to these body systems and their function. Based on the results of this study, meloxicam is a good candidate to consider for prolonged treatment of inflammation and pain, ultimately contributing to improvement of welfare in the pig industry.

None of the animals in this study was lame on subjective gait analysis, however subtle changes may be missed when gait is only visually assessed (Keegan, 2007). Therefore, gait was objectively evaluated using a pressure mat system. No differences in bodyweight normalised kinetic gait parameters were found, indicating that limb loading was comparable between the meloxicam treated and vehicle control animals.
Mean ASI's fluctuated around 0 and were comparable to values previously found in sound piglets (Meijer et al., 2014a, b). In theory, healthy animals are expected to have perfect symmetry and thus ASI's of 0. In practice, perfect symmetry is almost never observed, neither in humans nor in animals (Herzog et al., 1989; Colborne et al., 2008;

Jelén et al., 2008; Oosterlinck et al., 2011). This normally occuring deviation from perfect symmetry is considered to be related to limb dominance. Functional differentiation of limbs and brain hemispheres may be responsible for this finding, resulting in small asymmetries in limb loading and other kinetic and spatiotemporal characteristics of gait (Sadeghi et al., 2000).

The "dip" in front limb ASI's that was observed on day one was indicative of reduced loading of the left front limb. This may have been due to the intra-articular injection with saline. Although saline does not induce changes in cartilage, the increase in volume in the joint space may have stretched the articular capsule and may have caused some pain. This effect has been observed in humans (Jayson et al., 1970) and in horses (Thomsen et al., 2010), although in horses the effect was only observable for 2 hours. Also in the hind leg ASI, a small but progressive change from decreased to increased weight bearing of the left hind leg was observed, which can also be explained by initial subtle weight shifting from the left to the right side in response to the slightly stretched joint capsule. Limb loading and mean ASI's did not differ between pigs that received meloxicam and pigs that did not. We therefore concluded that long-term administration of meloxicam to healthy pigs did not result in functional changes in locomotion and thus the locomotor apparatus.

In both groups, some small OC lesions were found, but incidence and severity of the lesions did not differ. Locomotion of pigs can be affected by the presence of OC lesions (De Koning et al., 2012). In growing foals, presence of radiographically visible OC lesions led to a temporary subclinical lameness, identified by a significant reduction of peak vertical force (Gorissen et al., 2017). Nonetheless, in the present study no significant effects of the presence of OC lesions on gait kinetics were observed, possible due to the relatively small (microscopic) size of the lesions.

To our knowledge, no in vivo studies about possible NSAID-associated side-effects on bone and cartilage development in pigs have yet been published. Welting et al. (2011) reported negative effects of celecoxib treatment on the hypertrophic chondrocytes of the growth plate in rabbits. In contrast, we did not find any effects on bone and cartilage morphology. Although COX selectivity for celecoxib in rabbits is not established, in humans celecoxib is much more selective for COX-2 than meloxicam (Hawkey, 1999). Additionally, growth plate morphology of the pig is different compared to that of rabbits. In the pig, the hypertrophic zone of the growth plate is thickest, whereas the proliferative zone is the thickest in the rabbit. This might be explained by differences in (relative) growth rate, as has been shown in dogs (Tryfonidou et al., 2010).

In the gastro-intestinal system and kidneys no lesions consistent with NSAID side-effects were found. We did not expect to find renal changes, as meloxicam is considered relatively safe for kidneys. Short term usages in pigs did not show adverse effects (Friton et al., 2003) and in older cats, even when suffering from chronic kidney disease, long-term maintenance doses of meloxicam were considered safe (Gowan et al., 2011). The total absence of gastro-intestinal ulcerations is somewhat surprising. In intensive farming litters are mixed and housing conditions may not completely satisfy normal (rooting) behavior. This may give rise to increased stress levels, associated with the development of gastro-intestinal ulcerations (Amory et al., 2006). Possibly, the fact that we used a very pig-friendly system in which we kept littermates together in an environment that was substantially more enriched compared to commercial housing may have either prevented formation of ulcers and/or the exacerbation of those by meloxicam. Furthermore, pigs had permanent access to straw, which has a protective effect on the gastric mucosa (Amory et al., 2006; Di Martino et al., 2013; Herskin et al., 2016). In the group of animals with induced arthritis, gastric and duodenal ulcerations were found, but the incidence and severity of the lesions were comparable between the meloxicam treated and vehicle control group (J. J. Uilenreef, F. J. van der Staay, E. Meijer, unpublished observations). The effect of housing conditions on particularly the gastro-intestinal side-effects needs to be followed up in further research using commercial housing conditions.

There are several limitations to this study. The small sample size prohibits drawing firm conclusions. Furthermore, the current study did not aim at evaluating dose-response effects of meloxicam. Only prolonged oral administration at the licensed dose of 0.4 mg/ml once daily was investigated, representing the most likely conditions in practice under which meloxicam would be used. In piglets, the licensed dose has been reported to result in inadequate tissue levels to inhibit COX-2 to an extent sufficient for a good anti-inflammatory effect (Fosse et al., 2011). However, in that study the piglets were only two weeks old. Increasing the dose is likely to result in a more potent anti-inflammatory effect, but may also produce more or stronger unwanted side-effects.

Conclusion

The results of this in vivo study indicate that prolonged daily use of oral meloxicam at the licensed dose of 0.4 mg/kg did not lead to any of the thus far known NSAID-associated side effects in growing pigs. Given the high incidence of painful interventions and conditions in the modern pig-farming industry, this information may help veterinarians and farmers to decide to treat pigs that are in pain with a NSAID and may

thus improve the welfare of pigs. Clinical decision making with regards to administering or withholding NSAIDs because of possible side-effects should not be made on *in vitro* data only, but should be backed up by subsequent *in vivo* validation in the target species.

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Supplementary table 1: *Food ingredients relevant to bone development.*

	Ca (g/kg)	P (g/kg)	Vit D (IE/kg)	Crude fibre (%)
Nurse pellet	4.5	4.4	2000	0.5
Prevent 3	4.7	5.1	2000	5.7
Prevent 5	7.0	4.4	2000	5.8
Stimulans 6	6.6	4.4	2000	4.0
Vital Plus	6.0	4.5	2000	9.3

CHAPTER VII:

Hypoxia negatively affects senescence in osteoclasts and delays osteoclastogenesis

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Abstract

Cellular senescence, *i.e.* the withdrawal from the cell cycle, combined with the acquirement of the senescence associated secretory phenotype has important roles during health and disease and is essential for tissue remodelling during embryonic development. Osteoclasts are multinucleated cells, responsible for bone resorption and cell cycle arrest during osteoclastogenesis is well recognized. Therefore, the aim of this study was to investigate whether these cells should be considered senescent and to assess the influence of hypoxia on their potential senescence status. Osteoclastogenesis and bone resorption capacity of osteoclasts, cultured from CD14+ monocytes, were evaluated in two oxygen concentrations, normoxia (21% O_2) and hypoxia (5% O_2). Osteoclasts were profiled by using specific staining for proliferation and senescence markers, qPCR of a number of osteoclast and senescence-related genes and a bone resorption assay. Results show that during *in vitro* osteoclastogenesis, osteoclasts heterogeneously obtain a senescent phenotype. Furthermore, osteoclastogenesis was delayed at hypoxic compared to normoxic conditions, without negatively affecting the bone resorption capacity. It is concluded that osteoclasts can be considered senescent, although senescence is not uniformly present in the osteoclasts population. Hypoxia negatively affects the expression of some senescence markers. Based on the direct relationship between senescence and osteoclastogenesis, it is tempting to hypothesize that contents of the SASP not only play a functional role in matrix resorption, but also may regulate osteoclastogenesis.

Introduction

The term cellular senescence was introduced in the early 1960's (Hayflick and Moorhead, 1961), based on the observation that normal human fibroblasts stopped proliferating over time, which was speculated to be the underlying cause of aging. Currently, the process of cellular senescence is recognized as having important roles in both health and disease. It is often linked to tumor suppression, as cell cycle exiting after malignant transformation prevents tumor growth (Collado and Serrano, 2010), but more recently senescent cells have also been identified during embryological development in mammals and birds (Nacher et al., 2006; Munoz-Espin et al., 2013; Storer et al., 2013; Storer and Keyes, 2014). Developmental senescence, which is independent of DNA damage and dependent on the cyclin dependent kinase inhibitor p21, leads to the recruitment of macrophages to clear the embryo from the senescent cells (Munoz-Espin et al., 2013). Hence, it is essential in tissue remodeling during embryonic development.

Cyclin dependent kinase inhibitors, like p16 and p21 are well-known markers of senescent cells; they indicate cell cycle exit (Munoz-Espin and Serrano, 2014). Their presence often coincides with the absence of Ki67, which is present in actively proliferating cells (during G1, S, G2 and M phases of the cell cycle), but absent in resting (G0 phase) cells (Gerdes et al., 1984; Scholzen and Gerdes, 2000). Exit from the cell cycle is also a feature of quiescent cells (Terzi et al., 2016). In contrast to the quiescence state, senescent cells acquire the so-called senescence associated secretory phenotype (SASP) (Terzi et al., 2016), characterized by the production and secretion of soluble signaling factors (Coppé et al., 2010). The most widely used histological method to differentiate between quiescent and senescent cells is positivity for the senescence associated beta-galactosidase staining (Dimri et al., 1995; Itahana et al., 2007).

Cellular senescence is also observed in healthy adult individuals and considered to be physiological. Both megakaryocytes, formed by endomitosis without cytokinesis (Besancenot et al., 2010) and placental syncytiotrophoblasts, formed by fusion of cytotrophoblasts (Chuprin et al., 2013) become senescent during their development. Senescence of these cells is speculated to play an essential, yet largely unexplored, role in their specific function while it limits oncogenic transformation.

Osteoclasts contain multiple nuclei as well. They are formed by fusion from monocyte precursor cells under the influence of M-CSF (Dobbins et al., 2002; van Wesenbeeck et al., 2002) and RANK-L (Wong et al., 1997; Lacey et al., 1998; Yasuda et al., 1998). A fairly recent concept of osteoclasts is that they can renew themselves by fusion of new

mononuclear precursor cells, by splitting off multinucleated daughter cells (fission) or even by fusion of existing multinucleated cells (Jansen et al., 2012). They show several characteristics of senescence, such as being beta galactosidase positive at pH 7.0-8.0 (Kopp et al., 2007) but also at pH 6.0 (Chen et al., 2007), which is a hallmark of senescent cells (Dimri et al., 1995). The necessity of cycle arrest during osteoclastogenesis is well recognized (Sankar et al., 2004; Kwak et al., 2005; Zauli et al., 2007; Mizoguchi et al., 2009; Takahashi et al., 2010; Kwon et al., 2016) and indeed several different cyclin dependent kinase (CDK) inhibitors, among others p21, p27 and p38 are reported to be expressed during osteoclast differentiation (Okahashi et al., 2001; Chen et al., 2007; Cong et al., 2017). Further, they secrete hydrochloric acid and proteases, among others cathepsin K and matrix metallopeptidase 9 (MMP-9) (Odgren et al., 2016) that can degrade bone, which are also found in the SASP (Coppé et al., 2010). However, only Chen et al. (2007) regarded osteoclasts as senescent cells, whereas others described them as being quiescent (Sankar et al., 2004; Kwak et al., 2005; Zauli et al., 2007; Mizoguchi et al., 2009; Takahashi et al., 2010; Kwon et al., 2016), leaving the exact classification of these cells and the role of senescence in their functioning open to debate.

Osteoclasts are closely associated with vessels and play an important role during embryonic and post-natal skeletal development, as well as in pathologic conditions of the skeleton such as periodontitis (Hienz et al., 2015) and rheumatoid arthritis (Harre and Schett, 2017). In all activities hypoxia plays an important role: the hypoxia inducible transcription factor (HIF) stimulates angiogenesis and new bone formation (Shomento et al., 2010; Wang et al., 2015). Furthermore, hypoxia and more importantly, subsequent reoxygenation have a stimulating effect on the differentiation and bone resorbing capacity of osteoclasts (Arnett et al., 2003; Fukuoka et al., 2005; Knowles, 2015), possibly mediated by HIF-1α. Mechanistically, hypoxia and reoxygenation activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Rupec and Baeuerle, 1995) and the production of reactive oxygen species (Granger and Kvietys, 2015), which are both linked to the induction of senescence and the SASP (Nelson et al., 2012; Hubackova et al., 2012; Acosta et al., 2013). Reoxygenation is essential, as culturing osteoclasts under constant hypoxia led to extensive cell death and dramatically reduced numbers of osteoclasts (Knowles and Athanasou, 2009). However, it remains undetermined how oxygen tension and potential senescence are related and how these factors may affect osteoclast function.

The aim of this study was to investigate the profile of osteoclasts to answer the question whether these cells should be considered senescent or not and to assess the influence of hypoxia on osteoclast function and senescence status. We hypothesized that functional osteoclasts have a senescent phenotype that is stimulated by hypoxia. To verify these

hypotheses, we studied osteoclastogenesis and bone resorption capacity of osteoclasts, cultured from CD14+ monocytes under the influence of M-CSF, RANK-L in two oxygen concentrations, i.e. normoxia (21%) and hypoxia (5%). Osteoclasts were profiled by using specific staining for proliferation and senescence markers, qPCR of a number of osteoclast and senescence-related genes and bone resorption assay.

Materials and Methods

Monocyte isolation

Buffy coats from healthy donors were obtained with donor's consent from Sanquin Blood supply (Amsterdam, the Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coats using Ficoll-Paque density centrifugation (Ficoll-Paque PLUS, GE Healthcare). Monocytes were positively selected by magnetic-activated cell sorting (MACS) with anti-CD14 labelled microbeads (Miltenyi Biotec, Cat# 130-050-201, RRID:AB_2665482) according to the manufacturer's instructions, using an autoMACS (Miltenyi Biotec). Purity of the isolated monocyte population was confirmed using flow cytometry on a FACSCanto II cytometer (Becton Dickinson) after incubation with a monoclonal mouse anti-human CD45-FITC/CD14-PE dual-tag antibody (Beckman Coulter, Cat# 6603909, RRID:AB_2665483). Purity was on average >90%.

Osteoclastogenesis

Isolated CD14+ monocytes were either seeded on glass in Nunc® Lab-Tek® II 8 wells Chamber Slides (Sigma-Aldrig) or in plastic 96-wells tissue culture plates (Cellstar, Greiner Bio-One) at a cell density of $3.0x10^6$ cells per cm^2, corresponding with a cell concentration of 2.2×10^6 cells per chamber and 1.0×10^6 cells per well (for quantitative PCR and Western blotting). For the bone resorption assay, $1 x10^5$ monocytes per well were seeded on top of bovine cortical bone chips in 96-well plates according to Limonard et al. (2016). The culture medium consisted of αMEM (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (16000-044; Gibco), 1% Penicillin/Streptomycin (P11-010; GE Healthcare Life Sciences), 1% Fungizone (15290; Invitrogen), 10 ng/ml of macrophage colony stimulating factor (M-CSF, R&D Systems) and recombinant human nuclear factor kappa B ligand (RANK-L, Peprotech) dissolved in 0.1% human serum albumins in PBS (HSA/PBS). Cell cultures were maintained at 37°C, 5% CO_2 and 5% or 21% of O_2 and media were changed twice weekly. In total three experiments were performed, each making use of three different donors and cells cultured at 5% or 21% of O_2. Cells were harvested after one day (n=3), one week (n=6), two weeks (n=6) and three weeks (n=3) of culturing (supplementary table 1).

Immunocytochemistry

One, seven, 14 and 21 days cultured cells were fixated at room temperature for 10 minutes in 4% paraformaldehyde in PBS. After washing with PBS, cells were incubated with PBS containing 0.2% Triton X-100 (Sigma-Aldrich) followed by washing in PBS. Thereafter, an incubation with 10% normal goat serum in PBS was performed to reduce background staining followed by incubation with the different primary antibodies: Monoclonal rabbit anti Ki-67 (13.3 μg/ml; Thermo Fisher Scientific Cat# RM-9106-S0 RRID:AB_2341197), anti p21 (2.5 μg/ml; Santa Cruz Biotechnology Cat# sc-471 RRID:AB_632123) and anti p16 (6.7 μg/ml; Santa Cruz Biotechnology Cat# sc-1207 RRID:AB_632106). Goat anti rabbit\biotin (4 μg/ml; Vector Laboratories Cat# BA-1000 RRID:AB_2313606) in PBS containing 5% normal goat serum was employed as a secondary antibody. Antibody binding was made visible using 3,3'-diaminobenzidine (DAB; Dako) and nuclei were counter-stained with haematoxylin (H3404, Vector). Subsequently, TRAP staining was performed according to the manufacturer's instruction using a commercially available kit (Leukocyte Acid Phosphatase Staining Kit, Sigma-Aldrich). Staining for senescence was performed at 7 and 14 days by incubating the cells overnight in freshly prepared senescence associated beta galactosidase staining solution at 37°C, according to the protocol of Dimri et al. (1995). Digital images were obtained using an Olympus BX-60 microscope, equipped with a Leica DFC450C camera and LAS 4.7 software. For each time point, oxygen concentration, donor and staining, two chamber slides were analysed by counting and categorizing all cells present in 4 standardised sites of the chamber.

Quantitative PCR (qPCR)

After one, seven, 14 and 21 days of culture, cells were harvested for RT-qPCR analysis. RNA was extracted using a commercial spin-column kit (RNeasy Micro Kit, Qiagen) according to the instructions of the manufacturer. RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). cDNA was made using the iScript cDNA synthesis kit with similar RNA input for all the samples. RT-qPCR reactions were performed using Sybr Green Master mix (Thermo Fisher Scientific) for a total reaction volume of 10 μl. The primers for genes related to the osteoclast phenotype and function and senescence related genes are listed in supplementary table 2. These primers were validated by a gradient PCR (for retrieving the melting temperature), followed by sequencing of the amplicon (supplementary table 2). Ct values were normalised with four reference genes (HMBS, B2M, GAPDH and HPRT), corrected for the RT-qPCR efficiency and further relativized with the *norm-first* method. The stability of the reference genes was assessed by the use of NormFinder (Andersen et al., 2004).

Bone resorption

Cells were cultured for three weeks on bovine cortical bone chips in the presence of M-CSF and RANKL. Thereafter, cells attached to the bone were fixated at room temperature for 10 minutes in 4% paraformaldehyde in PBS. Non-specific background staining was blocked with 20% normal goat serum (Vector Laboratories) for 60 min.

After washing with PBS, bone chips were incubated with Alexa Fluor 647-labeled bisphosphonates (Thompson et al., 2006; Coxon et al., 2008) for one hour at room temperature, followed by washing. Nuclei were visualized with propidium iodide (Sigma). Bone slices were stored at 4 °C in PBS until they were analysed by confocal laser scanning microscopy (Leica SPE-II DMI-4000, Leica Microsystems), using a 10x ACS APO (NA 0.3) objective at a pixel size of 733 nm (zoom 1.5, 1024x1024 image size) and a quadruple dichroic filter that does not reflect the excitation lines in the detector path. 3D datasets were compiled from 11 slices, spaced in Z by 5 μm. Bisphosphonate fluorescence was recorded using the 635 nm laser line and emission was detected over the 646-706 nm range using the spectral detector. Buffer control staining was performed to determine background. Propidium Iodide signal was recorded using the 561 nm laser over an emission range of 571-635 nm. Quantification of the Bisphosphonate fluorescence was performed using the surface object wizard of Imaris (version 8.2.0 RRID:SCR_007370). Representative images of the different groups are shown with minor linear intensity adjustments (Red 20/190, Blue 15/230).

Semi-quantitative HIF-1α analysis by Western blot

In order to investigate whether the cells experienced hypoxia, HIF-1α protein expression was quantified. After washing with cold PBS, cell contents were harvested by scraping the 96 wells plates with RIPA buffer containing 0.06 nM phenylmethylsulphonyl fluoride, 17 μg/mL aprotin and 1 mM sodium orthovanadate (Sigma Aldrich). Cells were lysed on ice for 20 minutes, to prevent HIF-1α degradation followed by centrifugation for 10 minutes at 12,000 g. The supernatant was stored at -20 °C until analysis. Proteins were fractionated by electrophoresis using a 7.5% acrylamide gel (Bio-Rad Laboratories) and electro-blotted on a Nitrocellulose Membrane (9004-70-0, Bio-Rad Laboratories). After blocking with milk powder dissolved in PBS containing 1% Tween (TBS-T 1%) for one hour, the blots were incubated overnight at 4°C with the primary antibody against HIF-1α (1.0 μg/ml; BD Biosciences Cat# 610959 RRID:AB_398272) in TBS-T 1%. After washing with TBS-T 1%, the blots were incubated with an HRP conjugated secondary anti-rabbit antibody (0.33 μg/ml; Cell Signaling Technology Cat# 7074 RRID:AB_2099233) for an hour at room temperature. After obtaining the results for HIF-1α, the blots were stripped (strip buffer containing Glycin and SDS, pH 2.0) and incubated following the same

protocol as described above with a primary antibody against γ tubuline (1.0 μg/ml; Sigma-Aldrich Cat# T6557 RRID:AB_477584) to serve as a loading reference. The protein expression was visualised using ECL (GE Healthcare) in a ChemiDoc XRS System (Bio-Rad Laboratories). Images were obtained and densities were quantified by using Image Lab software (Bio-Rad Laboratories RRID:SCR_014210).

Statistical analysis

Data were analyzed using R Studio Statistical software version 3.1.2 (RRID:SCR_001905). The *P* value threshold was set at 0.05 and a correction for multiple comparisons was done with the False Discovery Rate method of Benjamini and Hochberg (1995). For the RT-qPCR and cell counting analysis, normality of the data distribution was checked graphically and with a non-parametric bootstrapped Shapiro-Wilks test. Since the RT-qPCR data were not normally distributed and the sample size was low (n=3), differences between the groups were assessed by a non-parametric bootstrapped permutation test without replacement. The number of permutations for each experimental group and gene was set at 1000 and the differences between the mean values of bootstrapped ΔCt were assessed.

Since the cell counting data were not normally distributed, differences between the groups were assessed by a Cox proportional hazard model (coxph), considering donor and the different experiments as random effects. Unless indicated, results are presented as mean ± standard deviation (SD). Confidence Intervals (C.I.) were set at 95%. Effect sizes (ES) were retrieved in all cases as the non-parametric Cliff's delta (Cliff, 1993), after bootstrapping 1000 times by a Monte Carlo simulation.
The interpretation for the present work is the following: <0.11, very small or no effect; 0.11-0.28, small effect size; 0.29-0.43, medium effect size; and >0.43, large effect size. Differences were considered as (biologically) relevant if a p value < 0.05 was found and the effect size was medium or large.

Results

Hypoxia delays osteoclastogenesis

Osteoclastogenesis was studied in CD14+ monocytes cultured in the presence of M-CSF, RANK-L and two oxygen concentrations, i.e. normoxia (21%) and hypoxia (5%) by determining the number of multinucleated TRAP positive cells representative of an osteoclast, gene expression profiling of OCL phenotypic markers and the respective bone resorbing capacity. To confirm that cells experienced hypoxia, gene and protein

expression of markers of hypoxia were determined. There were no detectable differences in the relative mRNA expression of the HIF target gene *NIX* over time between the culture conditions. Although at protein level no significant difference in amount of HIF-1α was present at week 2 in 5% O_2 compared to 21% O_2 (Fig. 1), there seems to be a tendency towards more HIF-1α in 5% O2 compared to 21% O2 given the (corrected) *P* value of 0.090 and medium effect size (Cliff's delta 0.361).

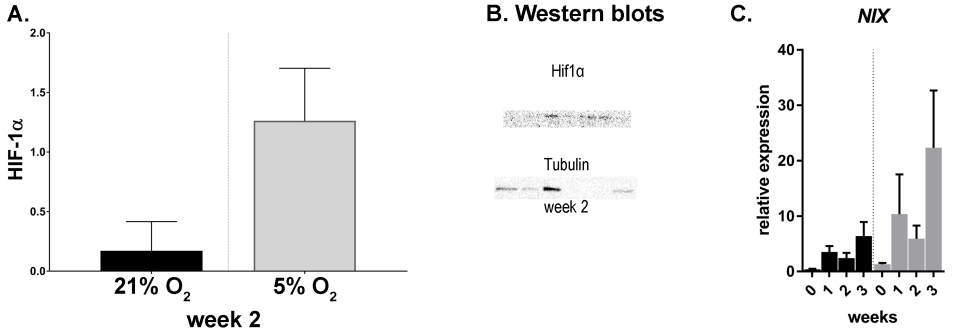

Figure 1: Osteoclasts seem to experience hypoxia *as there is a tendency towards increased average quantity (+/- SD) of HIF-1α present at week 2 in 5% versus 21% 0_2 (A; n=3 donors per time point) and corresponding blots of HIF-1a (upper) and tubulin (lower) (B). qRT-PCR results of the HIF target gene NIX (BCL2 Interacting Protein 3 Like) over time (C; n=3 donors per time point). There were no significant differences between culture conditions.*

After one week, significantly less osteoclasts were formed at 5% O_2 compared to 21% O_2. After two weeks, significantly more small osteoclasts (3-5 nuclei) were present at 5% O_2, whilst significantly larger osteoclasts (> 11 nuclei) were present at 21% O_2. No significant differences were observed in the osteoclast group with 6-10 nuclei. After three weeks, significantly more small osteoclasts were present at 5% O_2 (Fig. 2A). TRAP staining was less intense in the osteoclasts cultured at 5% O_2 compared to 21% O_2 (Fig. 2B). RT-qPCR analysis revealed no significant differences in the relative expression of *Carbonic Anhydrase II*, *Cathepsin K* and *Integrin β3* between culture conditions. The relative expression of *TRAP* was significantly lower at week 2 in the presence of 5% O_2 compared to 21%. *DCSTAMP* was lower expressed at 5% O_2 compared to 21% O_2 regardless of time (Fig. 2C).

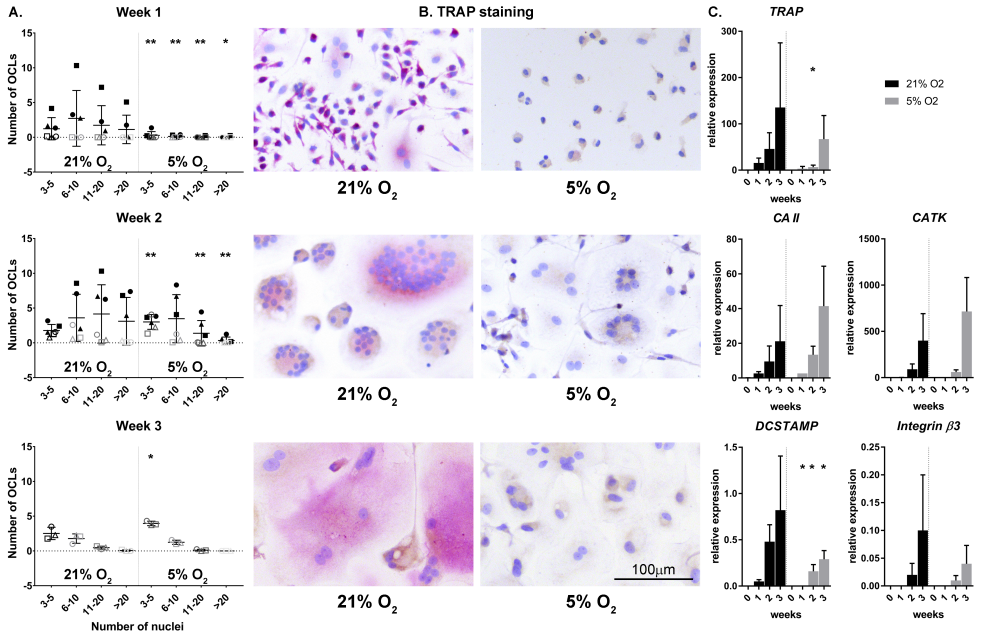

Figure 2: *Hypoxia delays osteoclastogenesis as indicated by the number of multinucleated, TRAP positive osteoclasts (OCL) over time (A) with representative examples (B), and corresponding results of the RT-qPCR analysis of genes associated with osteoclast differentiation and function (C).*
TRAP: Tartrate-resistant acid phosphatase, CA II: Carbonic Anhydrase 2, CATK: Cathepsin K, DCSTAMP: Dendritic Cells (DC)-Specific Transmembrane Protein, Integrin β3: Integrin subunit β3. Each symbol represents a single donor. (0.01 < P < 0.05; ** P < 0.01 hypoxia vs normoxia at the same time point).*

The delayed osteoclastogenesis in the presence of hypoxia may be related either to decreased survival/adhesion of the cells at the initiation of the culture or indeed to disturbed fusion of the mononucleated cells. Therefore, the single and double nucleated cells were counted as well. No significant difference in number of single nucleated cells was observed after one day of culturing under normoxia or hypoxia. This indicates that at the initiation of the experiment similar numbers of mononucleated cells were present in both oxygen culture conditions.

Nonetheless, while significantly less single nucleated cells were present after one week of culturing under hypoxia, after two and three weeks culturing the numbers of single nucleated cells were significantly higher at hypoxia compared to normoxia (Fig. 3, left panel). In line with this observation, after two and three weeks of culturing under hypoxic conditions, significantly more double nucleated cells were also present (Fig. 3, right panel). Altogether, these results support further the notion that osteoclastogenesis was delayed in the presence of hypoxia.

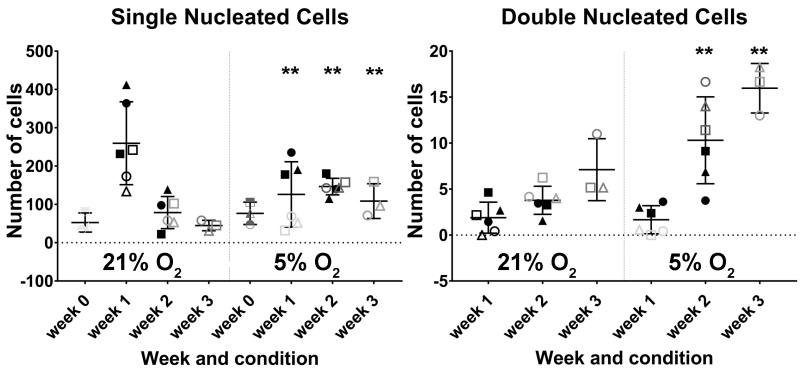

Figure 3: *Number of single (left) and double nucleated cells after over time in normoxic (21% O2) and hypoxic (5% O_2) culture conditions 21%. (* 0.01 < P < 0.05; ** P < 0.01 hypoxia vs normoxia at the same time point).*

Despite the differences in OCL numbers and gene expression profile between 21% and 5% O_2, there were no significant differences observed in bone resorption after three weeks of culturing, as quantified by the integrated intensity of bisfosfonate (Fig. 4, left panel).

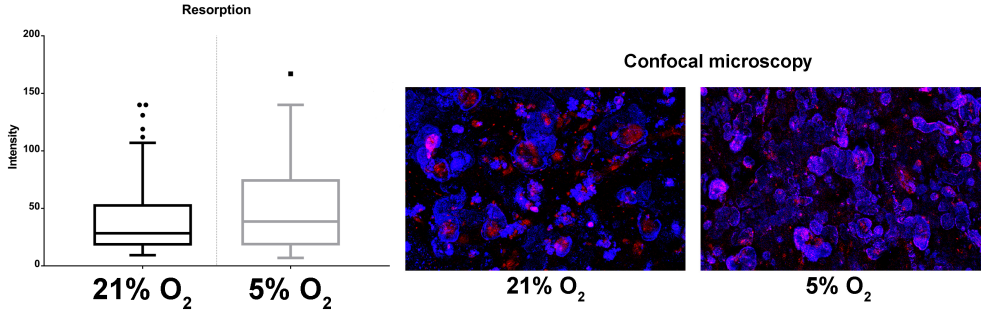

Figure 4: *Oxygen concentration during culture does not seem to affect the bone resorption capacity of osteoclasts as indicated by the integrated intensity of biphosphonate staining after three weeks of culturing osteoclasts on bovine bone chips in 21% and 5% of O_2 (A). Representative examples with resorption lacunae stained blue and nuclei stained red (B). There were no significant differences between culture conditions.*

Osteoclasts heterogeneously express both markers of proliferation and senescence, while hypoxia seems to negatively affect senescence

During osteoclastogenesis in two oxygen concentrations, i.e. normoxia (21%) and hypoxia (5%), osteoclasts were profiled by using specific staining for proliferation and senescence markers and senescence-related genes expression analysis. Surprisingly, osteoclasts expressed Ki67; much heterogeneity existed in the proportion of positive nuclei within osteoclasts (Fig. 5). In some, all nuclei stained positive, whereas in others only one or two

did so. There were no significant differences between osteoclasts cultured at hypoxia or normoxia (Fig. 5).

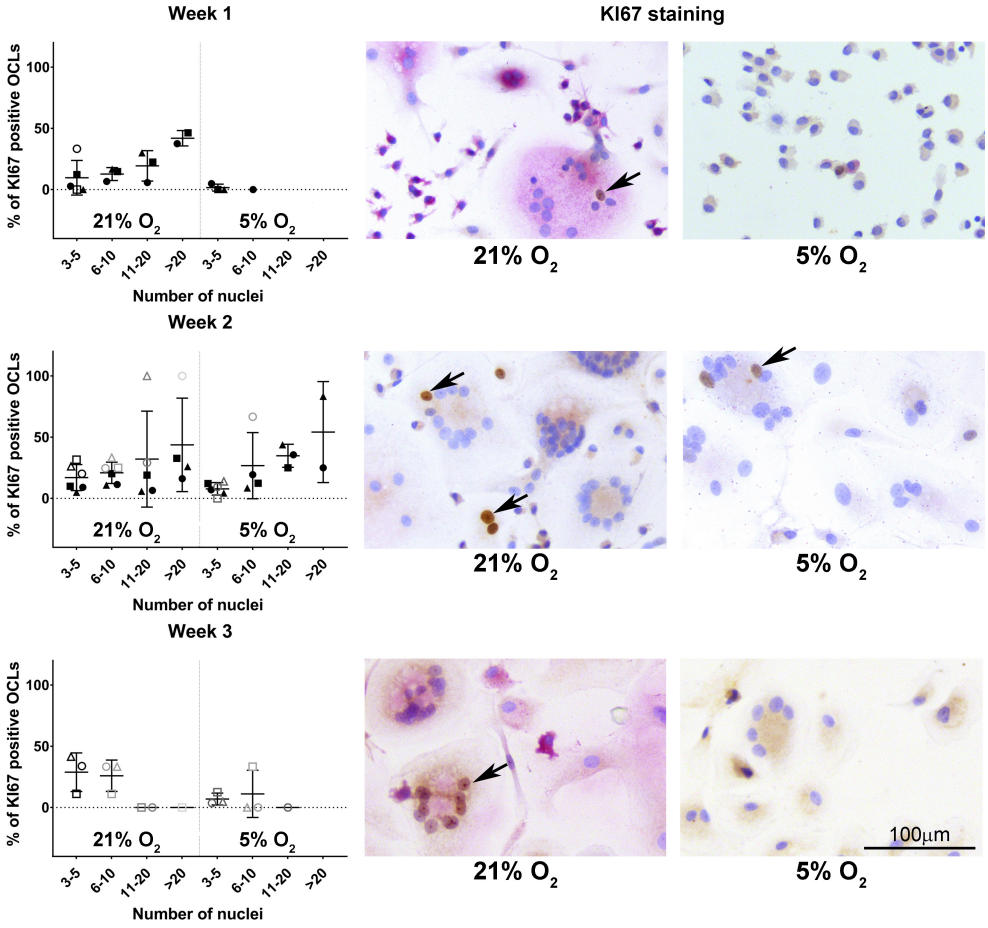

Figure 5: *The proliferative marker Ki67 is expressed heterogeneously in the nuclei during osteoclastogenesis and seems not to be affected by oxygen tension. Percentage of Ki67 positive, multinucleated, TRAP positive osteoclasts (OCL) over time (left) with representative examples (right) in normoxic (21% O_2) and hypoxic (5% O_2) culture conditions. The arrows indicate Ki67 positive nuclei in multinucleated cells. There were no significant differences between culture conditions.*

While mononuclear cells stained positive for both nuclear and cytoplasmic p16, only a small fraction of the osteoclasts showed a positive nuclear staining for p16; no significant differences were observed between cells cultured under normoxia and hypoxia (Fig. 6). The cytoplasm seemed to stain positive for p16 for both oxygen conditions after one and two weeks of culture. Only at week three, in osteoclasts cultured at 5% O_2 cytoplasmic

staining was consistently less intense compared to 21% O2 (Fig. 6, third row). If an osteoclast stained positive, the majority of its nuclei were positive for p16.

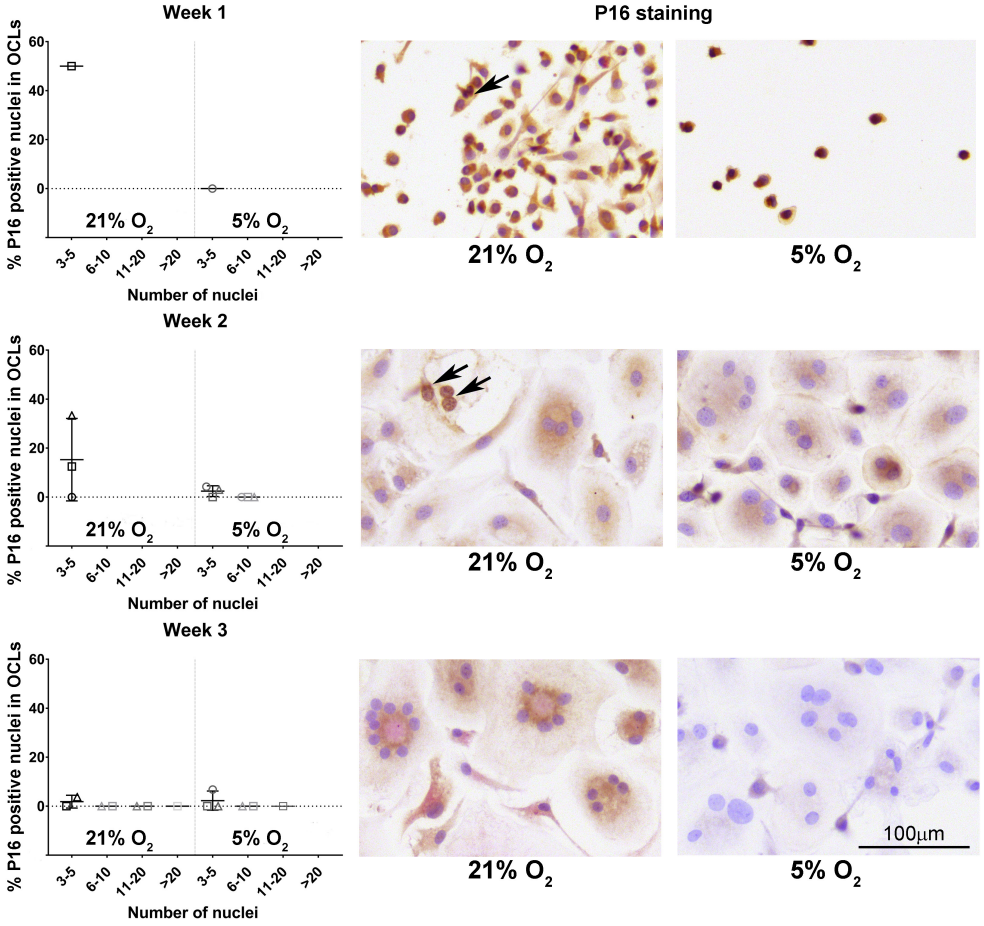

Figure 6: *The senescence marker p16 is limited expressed during osteoclastogenesis. Percentage of p16 positive nuclei in multinucleated, TRAP positive osteoclasts (OCL) over time (left) and representative examples (right) in normoxic (21% O_2) and hypoxic (5% O_2) culture conditions. The arrows indicate p16 positive nuclei in multinucleated cells. Note that while mononuclear cells were p16 positive, no osteoclasts were present after 1 week of culture. There were no significant differences between culture conditions.*

Furthermore, the majority of the osteoclasts showed a positive nuclear staining for p21 (Fig. 7). Significantly less osteoclasts containing 3-5 and 11-20 nuclei and cultured at 5% O_2 were positive compared to 21% O_2 at week 1. At week two, only larger osteoclasts (> 11 nuclei) showed significantly less positive nuclei, whereas at week three, significantly less small p21 positive (3-5 nuclei) osteoclasts were present in hypoxia compared to normoxia. Also here, variation was observed in the proportion of osteoclast nuclei that stained positive in all culture conditions and time points.

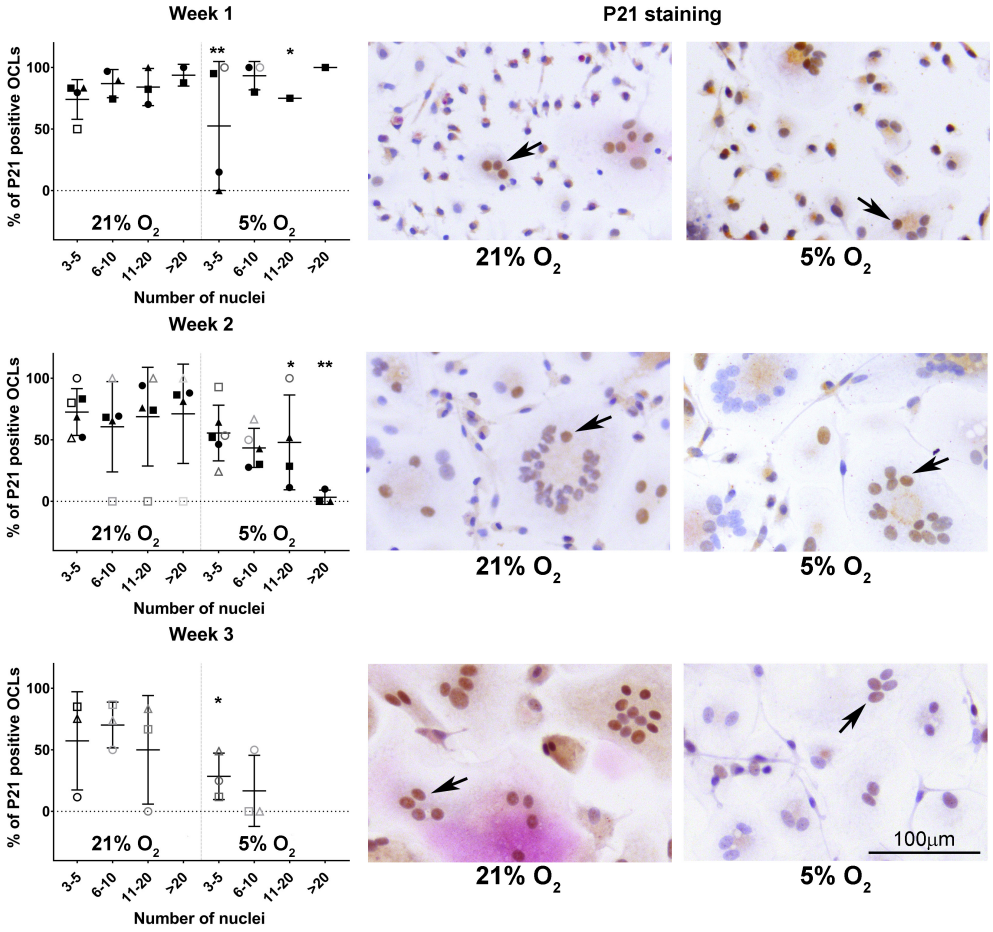

Figure 7: *The senescence marker p21 is abundantly expressed during osteoclastogenesis and negatively affected by hypoxia. Percentage of p21 positive nuclei in multinucleated, TRAP positive osteoclasts(OCL) over time and representative examples (right) in normoxic (21% O2) and hypoxic (5% O2) culture conditions. The arrows indicate p21 positive nuclei in multinucleated cells. (* 0.01 < P < 0.05; ** P < 0.01 hypoxia vs normoxia at the same time point).*

Almost all multinucleated cells stained positive for senescence associated beta galactosidase, as evidenced by the presence of blue cytoplasmic precipitate, independent of O_2 concentration during culture (Fig. 8A). Comparison of the relative expression of senescence associated genes showed significantly lower expression of *CCL2* after one week and of *p21* after two weeks under hypoxia compared to normoxia, while the opposite is true for *CCL5* expression after one week. No significant differences were observed in the relative expression of *MMP9* (Fig. 8B).

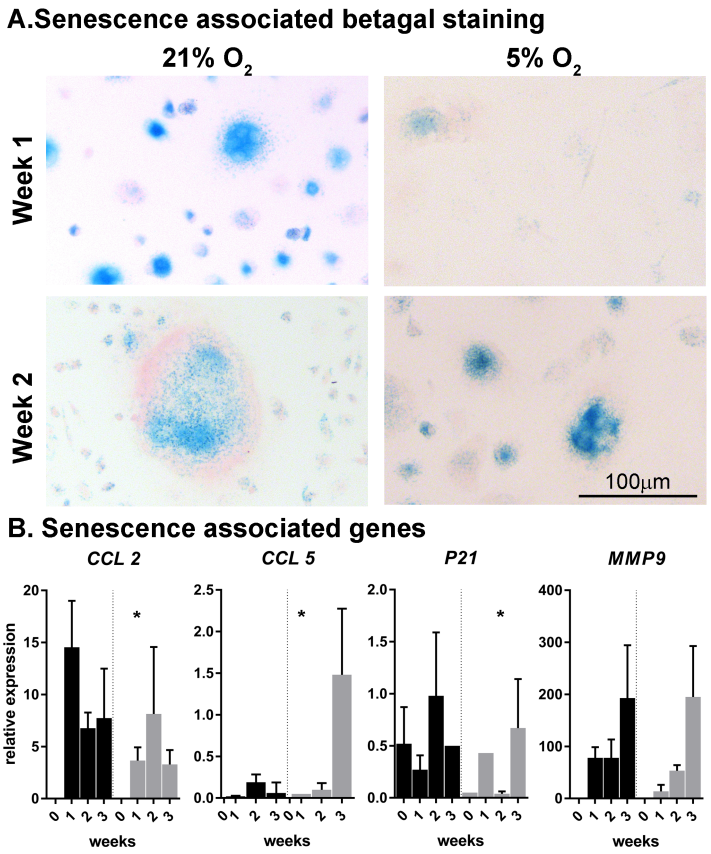

Figure 8: *Senescence associated beta galactosidase staining is present at week 1 and 2 (A) supporting the senescent phenotype of osteoclasts. RT-qPCR analysis of the senescence associated genes CCL 2, CCL5, p21 and MMP9 (B; n= 3 donors) shows differential gene expression profiles in normoxia vs hypoxia. CCL2: C-C Motif Chemokine Ligand 2, CCL 5: C-C Motif Chemokine Ligand 5, p21: Cyclin Dependent Kinase Inhibitor 1A, MMP9: Matrix Metalloproteinase. (* 0.01 < P < 0.05; ** P < 0.01 hypoxia vs normoxia at the same time point).*

Discussion

This study shows that during *in vitro* osteoclastogenesis, osteoclasts obtain a senescent phenotype and that osteoclastogenesis was delayed at hypoxic (5% O_2) compared to normoxic conditions (21% O_2) without negatively affecting the bone resorption capacity of the cells.

Osteoclasts express a senescent phenotype

Cell cycle arrest is a hallmark of osteoclastogenesis and based hereon osteoclasts have always been described as quiescent cells (Sankar et al., 2004; Kwak et al., 2005; Zauli et al., 2007; Mizoguchi et al., 2009; Takahashi et al., 2010; Kwon et al., 2016). However, the present study indicates that they should be considered senescent rather than quiescent. Senescence is not limited to the arrest of proliferation, but also includes the acquirement of the senescence associated secretory phenotype (SASP) (Terzi et al., 2016), characterized by the production and secretion of soluble signaling factors (Coppé et al., 2010). In the current study, osteoclasts indeed expressed typical markers of senescence, including p21 and senescence associated beta galactosidase, in line with a previous report (Chen et al., 2007). In fact, the expression profile of the osteoclasts derived from PBMCs stimulated with M-CSF and RANK-L overlaps with the SASP.

We observed increasing expressing of *CCL2* and *CCL5,* which are known to be part of the SASP and have chemotactic properties (Ruhland et al., 2016). Notably, these substances also stimulate osteoclastogenesis (Kim et al., 2015; Khan et al., 2016) and production of MMP-9 (Chuang et al., 2009). MMP-9 is also a part of the SASP and an important enzyme for resorbing bone (Coppé et al., 2010). The expression of *Cathepsin K,* which is an enzyme produced by mature osteoclasts to break down the non-mineralized bone matrix, increased over time in both culturing conditions. Interestingly, besides its matrix degrading characteristics, *Cathepsin K* can also induce senescence in osteoclasts, possibly to control and limit their number (Chen et al., 2007). Altogether, these findings indeed imply that osteoclasts obtain a senescence-associated secretory phenotype and strongly suggest that the SASP secretome exerts paracrine effects that possibly regulate osteoclastogenesis and bone resorption.

Oxygen tension influences osteoclast senescence, as determined by senescence and proliferation markers. Contrary to our hypothesis, low oxygen concentration correlated with decreased percentages of positively stained p21 nuclei in osteoclasts, indicating that senescence was negatively affected by hypoxia. Cyclin-dependent kinase inhibitor 2A or p16 is a senescence marker with very low expression in young and healthy tissue. However, as it is activated by cellular damage or stress, it is abundant in aged tissues (Zindy et al., 1997; Krishnamurthy et al., 2006) and considered to be a good senescence marker for *in vivo* studies (Yamakoshi et al., 2009; Baker et al., 2011; Burd et al., 2013). In the present study, only a very limited number of nuclei were positive for this marker, whereas cytoplasmic p16 was present in the majority of the cells, both in single and multinucleated ones. Cytoplasmic p16 localization has been related to malignancy (McCluggage and Jenkins 2003; Reid-Nicholson et al., 2006; Zhao et al., 2012), but its

biological meaning is still under debate. Within the context of osteoclastogenesis, the presence of p16 together with p21 has been reported in murine monocytes cultured to become osteoclasts (Cong et al., 2017). Most likely, p16 was only to a limited extent expressed in our *in vitro* model as this generated 'young and healthy" osteoclasts.

To our surprise, we observed that a considerable percentage of osteoclasts was positive for Ki67. The presence of Ki67 in the osteoclasts can obviously not be related to proliferation, as their cell cycle is arrested. However, Ki67 has also been shown to be present in quiescent cells, possibly associated with ribosomal RNA transcription (Bullwinkel et al., 2006; Rahmanzadeh et al., 2007). Another potential reason for the presence of Ki67 positive nuclei might be the possible occurrence of fission of osteoclasts (Jansen et al., 2012). We did not study this, but it has been described that multinucleated osteoclasts can split into two or more multinucleated daughter cells. This cytoplasmic separation has some resemblance with the last phases of mitosis, which might explain presence of Ki67.

Hypoxia delays osteoclastogenesis without long term effect on bone resorption capacity

The fact that we observed in general less large osteoclasts and more single and double cells under hypoxic conditions pointed at delayed osteogenesis. Furthermore, at the gene expression level, several osteoclast-markers were markedly downregulated by hypoxia, e.g. *TRAP* and *DCSTAMP*. RANK-L in presence of M-CSF stimulates proliferation of the mononuclear osteoclast precursors. In this process, the cell density and number of cells present prior to cell fusion can influence the number of osteoclasts that eventually form (Motiur Rahman et al., 2015; Cong et al., 2017). However, these two factors were not different between the hypoxic and normoxic culture conditions in our study and similar numbers of adherent cells were observed after 24 hours of culturing in either culture. Possibly, the lower number of mononuclear cells observed after one week is the result of the inhibiting effect of hypoxia on their proliferation (Naldini and Carraro, 1999). While delayed osteoclastogenesis seems to be in contrast to several other papers reporting positive effects of hypoxia on osteoclastogenesis (Arnett et al., 2003; Fukuoka et al., 2005; Knowles, 2015), it confirms others reporting negative effects of hypoxia on osteoclast formation (Leger et al., 2010; Hulley et al., 2017). These contradictory observations may be related to differences in culture set up and to the pH sensitivity of the medium, where even different brands of fetal calve serum could have an influence. Although 2% O_2 has been reported to be the optimal concentration for bone resorption (Knowles et al., 2015), culturing osteoclasts under constant hypoxia leads to extensive cell death and dramatically reduced osteoclast numbers (Knowles and Athanasou, 2009). Therefore, we cultured at 5% O_2 and allowed reoxygenation twice a week during

culture medium change. This frequency of medium change and intermittent exposure to normoxia is comparable to the setup of Hulley et al. (2017), who also reported decreased osteoclastogenesis. Nonetheless, while osteoclastogenesis was delayed in hypoxic culture conditions, gene expression of *CAII, CATK* en *MMP9*, secreted by osteoclasts to resorb bone did not differ between conditions and resorption capacity at week 3 was comparable in the two culture conditions.

The study has several limitations

Donor background information is lacking, which could have influenced our results, as for example a clear relation with age has been shown for the presence of p16 positive staining of nuclei in healthy tissue (Zindy et al., 1997; Krishnamurthy et al., 2006). There was distinct donor variability resulting in differences in pace of osteoclastogenesis between the different experiments. For this reason, each donor was represented by a unique symbol in the figures. This limitation does not necessary affect interpretation and generalization of the results, as each donor was employed in both culture conditions (hypoxia and normoxia). Inherent to the *in vitro* model and chosen set up is the discrepancy with osteoclast formation on bone that follows a different dynamic than on plastic (De Vries et al., 2009). Sometimes, inhibitory effects on plastic can be nullified on bone (De Vries et al., 2015).

Conclusion

The present study demonstrates that osteoclasts can be considered senescent instead of quiescent. Notably, senescence is not uniformly present in the osteoclast population, which may represent different stages in the life of the osteoclast. This may also be the background of the heterogeneous expression of Ki67, which is indicative of augmented ribosomal RNA transcription rather than proliferative activity. The direct relationship between senescence and osteoclastogenesis might mean that contents of the SASP not only play a functional role in matrix resorption but also may regulate osteoclastogenesis in a paracrine manner. Hypoxia negatively affects the expression of senescence markers and osteoclastogenesis is delayed at hypoxic (5% O_2) compared to normoxic conditions (21% O_2). This, however, does not affect the resorption capacity of the osteoclasts on the longer term.

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Supplementary table 1: *Setup of all experiments and number of different donors used for each of the read out parameters.*

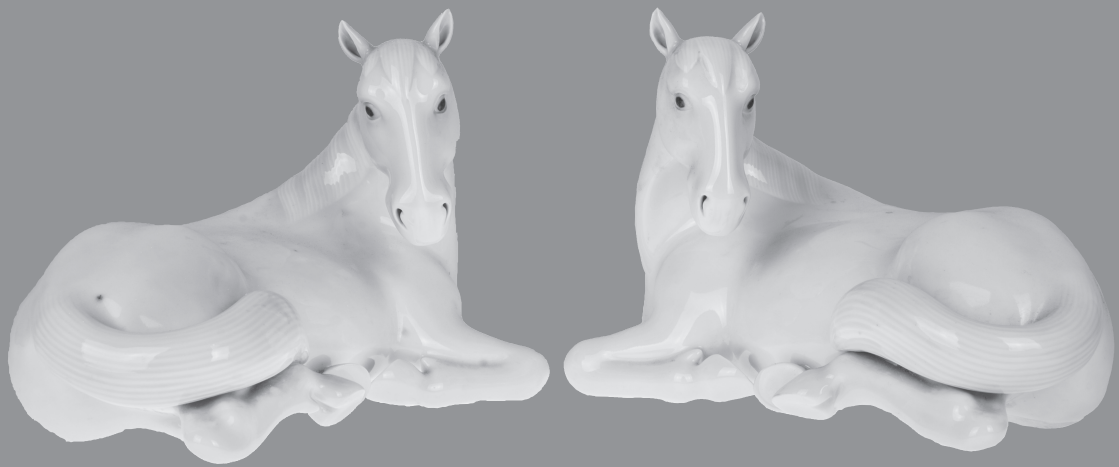
Technique		Immunocytochemistry (Nunc® Lab-Tek® II 8 wells Chamber Slides)	RT-qPCR (plastic 96-wells tissue culture plates)	Western blot (plastic 96-wells tissue culture plates)
Cells harvested after:	Medium	Number of donors used:		
1 day	plain medium	3	3	
1 week	RANK-L & M-CSF	6	3	
2 weeks	RANK-L & M-CSF	6	3	3
3 weeks	RANK-L & M-CSF	3	3	

Supplementary table 2: *Primer sequences, amplicon size, annealing temperature (°C) and Genbank accession number of the primers used for the RT-qPCR analysis. CDC73 (HRPT1): cell division cycle 73, HMBS: hydroxymethylbilane synthase, B2M: beta-2-microglobulin, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, CA2 (CAII): Carbonic Anhydrase II, CTSK (CATK): Cathepsin K, ACP5 (TRAP): Tartrate-resistant acid phosphatase, DCSTAMP: Dendritic Cells (DC)-Specific Transmembrane Protein, ITGB3: (Integrin β3): Integrin subunit beta 3, CCL2: C-C Motif Chemokine Ligand 2, CCL 5: C-C Motif Chemokine Ligand 5, MMP9: Matrix Metallopeptidase 9, CDKN1A (P21): Cyclin Dependent Kinase Inhibitor 1A,.* BNIP3L (*NIX): BCL2 Interacting Protein 3 Like.*

Genes	Forward sequensce 5′ - 3′	Reverse sequence 5′ - 3′	Amplicon size	Annealing temp. (°C)	Accession no.
Reference genes					
CDC73	TATTGTAATGACCAGTCAACAG	GGTCCTTTTCACCAGCAAG	192	60	NM_024529.4
HMBS	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC	64	56	NM_000190.3
B2M	CTTTGTCACAGCCCAAGATAG	CAATCCAAATGCGGCATCTTC	83	58	NM_004048.2
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	87	62	NM_001256799.2
Target genes osteoclasts					
CA2	TGGACTGGCCGTTCTAGGTATT	TCTTGCCCTTTGTTTTAATGGAA	100	59	NM_000067.2
CTSK	CCATATgTGGGACAGGAAGAGAGTT	TGCATCAATGGCCACAGAGA	149	66	NM_000396.3
ACP5	CACAATCTGCAGTACCTGCAAGAT	CCCATAGTGGAAGCGCAGATA	128	68	NM_001111035.2
DCSTAMP	ATTTTCTCAGTGAGCAAGCAGTTTC	AGAATCATGGATAATATCTTGAGTTCCTT	101	61,5	NM_030788.3
ITGB3	AGGCTGGCAGGCATTGTC	AGCCCCAAAGAGGGATAATCC	100	66	NM_000212.2
Target genes senescence					
CCL2	GATCTCAGTGCAGAGGCTCG	TGCTTGTCCAGGTGGTCCAT	153	60	NM_002982.3
CCL5	GCTGCTTTGCCTACATT	CATTTCTTCTCTGGGTTG	141	59	NM_002985.2
MMP9	CCTGGAGACCTGAGAACCAATC	TTCGACTCTCCACGCATCTCT	100	66	NM_004994.2
CDKN1A	CTCTAAGGTTGGGCAGGGTGACC	CAGAGGGGGGTATCAAGAGCCAG	91	68	NM_000389.4
Target genes hypoxia					
BNIP3L	AGTAGCTTATTTGAACTTGAGACCATTG	TGAGGGTTACTGGAATTGGATATGTA	83	61,5	NM_004331.2

CHAPTER VIII:

General discussion



Gait can be seen as the product of the locomotor system. Efficient and effective gait can only be generated if the musculoskeletal system, of which the bony skeleton forms the core, functions reliably and well. Optimal function of the musculoskeletal system is of especially great importance for so-called fright-and-flight animals, species that rely on their locomotor capacity to flee from predators or other threats. Interesting enough, many of these species are precocial, *i.e.* they do not have a prolonged period of postnatal development and maturation of their musculoskeletal system and are often able to follow the herd within hours after birth. This combination of instant optimal performance with apparently minimal preparation makes the development of both the musculoskeletal system itself and its product, gait in the early juvenile phase, into a highly interesting, though challenging research topic. It is this challenge that was taken up in this thesis.

Development of gait

Feasibility of gait analysis in the foal

Little work has been done on gait analysis in the foal in the immediate post-natal period. This is somewhat surprising, as this is the period in which most significant development of balance and gait can be expected. Practical considerations regarding the handling of very young foals might play a role here. However, in our study handling of the foals went surprisingly well, probably because all foals were in hands from the moment they were born and measurements took place at the stud farm. Also, the mares were all well-behaved and very cooperative, which also contributed to the uneventful collection of reliable data from these very young animals.

Wishing to relate function (*i.e.* gait) with form of the developing skeleton, we were especially interested in the forces that were generated by locomotion and that determine the loading of the structures of the musculoskeletal system. Hence, kinetic gait parameters of the foals had to be established, for which force plates are considered the gold standard (Merkens et al., 1993). However, due to logistical and practical constraints, we chose to perform all measurements at the stud farm and therefore a (mobile) pressure plate system was used. Pressure plates are less accurate in measuring forces than force plates, but there are several advantages that outweigh this limitation. Apart from being easy to transport, data of more than one limb per run can be collected. This feature drastically reduces the number of runs necessary to collect enough good data. Especially in very young foals this is beneficial, as they become tired fast. Furthermore, pressure plate systems are able to measure the distribution of forces underneath the hoof, enabling us to study landing preference and development of hoof balance as well.

The interpretation of gait analysis data in foals may suffer from several confounders. It is known that in gait analysis there may be a handler effect, as he or she will always walk on the same side of the horse (Farmer et al., 2010). This is also true for very young and untrained foals (Lucidi et al., 2013).

Until weaning between 20 and 24 weeks of age, the foals were handled together with their dam, which can be supposed to have a reassuring effect on the young foals. This was indeed illustrated indirectly by the observed increase in variability of both the vertical limb forces and distribution of forces under the hooves just after weaning, when the foals were being handled alone. Furthermore, the specific spatial set-up of the runway and pressure plate system may have influenced the results. To guide the foal the best way possible, the pressure plate was positioned against the wall to the right of the foals and handler and mare walked left to the foal.

Based on our experiences we can conclude that the uneventful collection of quantitative gait parameters is also possible in (very) young foals without too much difficulty and under field conditions. For similar studies focusing on kinematic parameters, inertial measurement unit (IMU) systems, which have recently become available (Clayton and Schamhardt, 2013) would probably be the best tool.

Development of gait in the foal

In contrast to the original notion that foals are born with adult-like postural abilities and coordination (Fox, 1964; Lelard et al., 2006), we observed marked changes in the gait of warmblood foals during the first 24 weeks of their lives. The maturation of gait is reflected by the reduction of variability in stance duration and the distribution of forces under the hooves, which is thought to be the result of better timing and coordination. Based on the known postnatal improvement of static balance, as analysed by stabilographic measurements, it was hypothesised that foals initially rely on so-called fast, but imprecise and ballistic motor control (open loop), to switch to the more precise, closed loop control later on (Collins and De Luca, 1993). Given the similarities in the development of static balance and gait, such a switch to more precise and subtle closed loop control is most likely also responsible for (part) of the reduction in variability observed in our study. This may be particularly true for the limitation of sway as an overcompensation associated with open loop balance control. Movement in itself, subtle correction of balance and the interplay between agonist and antagonist muscles require training and maturation, as illustrated in the work of Back et al. (1999). Those authors reported significant differences in gait between foals subjected to box rest during the first 5 months of their lives, compared to foals kept all-day at pasture. In their kinematic

study they reported a more retracted hind limb, more flexed stifle and tarsal joints, and a more hypermetric movement in the box rest group. All these observations are indicative of poor coordination of muscle activity during growth, and of changes in conformation and proportion of the foals (Anderson and McIlwraith, 2004; Thompson, 1995). These lead to changes in inertial properties of body segments and hence influence centre of mass motion (Buchner et al., 1997; 2000), necessitating constant adaptation. In riding horses, a similar, but less gradual event takes place when they get to be ridden and have to deal with position and mass of the rider.

The work in this thesis has shown that bodyweight normalised peak vertical forces in foals stay relatively constant during the first 24 weeks of life. This does not necessarily mean that bone strain remains constant. This has been shown in growing goats, in which an increase in *in vivo* measured bone strain was noted with, as in our study, relatively constant peak vertical forces (Main and Biewener, 2004). The hypothesis of the authors was that young goats have relatively stronger bone than adult ones, with a bigger "safety factor" to compensate for the suboptimal balance and associated higher and more variable loading patterns in the period after birth. The same may be true for foals where the marked variability of the force distribution under the hooves will also lead to relatively large variation in loading.

It can be anticipated that the variability in loading under field conditions will still be more than in our highly standardized measuring situation. This may lead to the hypothesis that under those field conditions the physiological limits of the still developing skeleton may occasionally be violated, which might cause local damage and could contribute to the development of developmental orthopaedic diseases, such as osteochondrosis.
Circumstantial evidence for this hypothesis may be the observation that an increased incidence and severity of developmental orthopaedic diseases is seen in foals with access to a large field or fields with very rough terrain at early age (Caure et al., 1998; Lepeule et al., 2009).

Skeletal development

Practical considerations when studying growing bone
Micro-CT analysis is one of the most common and best methods to evaluate bone in a quantitative way. Using this method, thresholding is one of the most important issues, as it defines what tissue is considered bone and what not. In very young bone, threshold settings must forcibly be low, as the tissue is not yet completely mineralized. In contrast,

bone of older animals is fully mineralized and should be scanned at higher thresholds. The use of different thresholds obviously poses challenges when comparing young and old bone. In the rare literature where development of bone is longitudinally monitored, thresholds are adapted, depending on the age and thus degree of mineralization (Hara et al., 2002; Wolschrijn and Weijs, 2004; Ryan and Krovitz, 2006; Gosman and Ketcham, 2009). We based the threshold values on the histograms and on visual comparison of segmented images at different threshold levels with the original scans, which is considered to be a valid procedure (Hara et al., 2002; Wolschrijn and Weijs, 2004).

Bone development

Wolff's law states that loading results in modification of the bone architecture (Wolff, 1892). It is known that in the adult skeleton the osteocytes sense the mechanical environment. Load-driven changes in fluid flow through the lacunar-canalicular system (You et al., 2008) lead to the secretion of signaling factors, of which nitric oxide is one of the most important for mechano-sensing factors (Pitsillides et al., 1995; Klein-Nulend et al., 1998; Westbroek et al., 2000). These signaling substances influence bone mass, mineral density and matrix formation to match form and function of the skeleton and thereby guide skeletal remodeling. Although the process of mechano-sensing in the developing skeleton is largely unexplored, it is known that the phenomenon exists and that loading is essential for normal joint and bone development. Individuals with congenital neuromuscular disorders are known to suffer from joint contractures and bone with thinner cortexes (Rodriguez and Palacios, 1989; Fanconi et al., 1995). Furthermore, experiments with *in ovo* or *in utero* immobilized chicken and mice have shown that muscle contraction is important for the development of bony ridges and entheses (Blitz et al., 2009; Tatara et al., 2014) and for limb proportionality (Pollard at al., 2014). The need for intra-uterine loading seems to differ with the anatomical location. In a mouse model using mice with disrupted skeletal muscle development, thus lacking mechanical stimulation from embryonic muscle contractions, bone development was affected in the scapula, humerus, ulna, femur, and elbow joint. However, the tibia and knee joints developed normally, indicative of location-specific regulation of bone and joint development (Nowlan et al., 2010). The exact underlying mechanism remains elusive, but could potentially give a clue about the mechanism of anticipatory bone development in precocial species as documented in this thesis. In that context, it would be interesting to also study other, more proximal locations in foals, known to develop abnormally in the affected mouse model, to see if bone development in these regions is comparable.

The strategy in anticipating bone development of precocial animals seems to be species-specific. Calves rely on less but more aligned trabeculae, which is different to foals. The most plausible explanation is differences in anatomy and associated postnatal loading. An interesting observation of the thesis was that in Shetland pony foals bone volume fractions were higher than in warmblood foals. In adult individuals, bone volume fraction is independent of size and body weight (Doube et al., 2011; Barak et al., 2013; Christen et al., 2014). Although it can be debated to what extent different breeds can be compared, a possible explanation for the difference between the Shetland pony and the Warmblood may be that the latter breed has been selected heavily for size and a high growth rate. High rates of bone growth are associated with less dense bone (Martin and Burr, 1989; Leterrier and Nys, 1992; Williams et al, 2004; Prisby et al., 2014), but also with reduced skeletal adaptability (Pitsillides et al., 1999) and response to mechanical loading (Rawlinson et al., 2009). Possibly, these negative effects on bone quality are the reasons behind the observation that high growth rate has also been related to the developmental orthopaedic disorders, such as osteochondrosis (Donabédian et al., 2006).

Osteochondrosis

Osteochondrosis is a very relevant problem in the equine industry, provoking huge economical losses. Apart from this, it is also an important welfare issue as yearly hundreds of thousands of horse have to be operated upon worldwide. In the current thesis we focused on possible effects on gait parameters of clinically silent lesions. It was interesting to see that sub-clinical osteochondrosis did not affect hoof placement preference or pressure patterns under the hoof during the stance phase, but could be detected by a temporary but significant reduction in normalized peak vertical force where temporal and spatial stride parameters remained unaffected. This finding stressed once more the high sensitivity of objective gait analysis for the detection of gait irregularities and provides a potential tool for early detection of subclinical lesions of osteochondrosis.

The potential effect of NSAIDs on skeletal development

Partially instigated by the much stricter regulation of the use of antibiotics in fattening pigs, administration of non-steroidal anti-inflammatory drugs (NSAIDs) is on the increase in commercial pig farming. However, usage of NSAIDs in growing pigs may be questionable based on the role of prostaglandins in normal bone and cartilage biology (Raisz, 1995) Also, data from *in vitro* research (Jakob et al., 2004; Retamoso et al., 2010) suggest that negative effects on both chondro- and osteogenesis after the prolonged use of NSAIDs in fast growing pigs can possibly be expected. When investigating this, we did not find differences in trabecular bone architecture, gait, growth plate morphology and presence of osteochondral lesions, suggesting that the long-term administration

of the selective COX-2 inhibitor meloxicam at the dose registered for the pig can be considered safe in growing pigs. However, this conclusion should be interpreted with caution. Methodologically, our study had a relatively small sample size. Apart from that, the study used the regular dose as advised by the manufacturer. In practice, such advice is not always followed and there may be a tendency to overdose. It should also be realized that the animals used were reared under well-controlled experimental conditions that were quite different to those common in commercial pig farming. Nevertheless, the observation that usage of meloxicam did not have detrimental effects on skeletal development may have a positive effect on welfare in the pig industry. Specifically in the case of (chronic) lameness, which is a common clinical observation in rearing piglets and sows (Main et al., 2000; Kilbride et al., 2009), and longer period of treatments are warranted.

The osteoclast: a crucial but insufficiently understood player in skeletal remodelling

Endochondral ossification is a crucial process in bone development and maturation. In this process, an essential cell type in the gradual replacement of the original cartilaginous matrix by bone is the chondroclast, a cell that is very similar to the bone resorbing osteoclast. Chondroclasts and osteoclasts are both multinucleated and contain many mitochondria and lysosomes (Savostin-Asling and Asling, 1975). Their gene expression profiles seem largely similar (Knowles et al., 2012), with only a few differences. For example, chondroclasts express more MMP13 than osteoclasts (Sakakura et al., 2007). It has even been shown that human osteoclasts, cultured from PBMCs, are able to resorb cartilage (Nordahl et al., 1998). This observation suggests that our study design and results from osteoclasts could potentially be translated to chondroclast function as well.

Bone/cartilage resorbing cells play an essential role in bone remodelling and as such can be expected to be involved in the repair process of osteochondrotic lesions that is known to immediately follow the formation of lesions (Olstad et al., 2008). To study phenotype and function of PBMC derived multinucleated cells we simulated *in vitro* two conditions with 21% and 5% oxygen tension, in which the latter was meant to reflect the hypoxic environment in an OC lesion caused by interruption of the local vascular supply. The hypothesis was that a low oxygen environment would negatively affect the capacity of the multinucleated cells to resorb bone and thereby delay the process of endochondral ossification; thus leading to the retention of cartilage cores in the ossifying tissue, which is a histological hallmark of OC lesions. Indeed, we demonstrated that osteoclastogenesis was delayed at 5% of O_2, but after three weeks no differences in bone resorption were observed. However, it is known that culturing osteoclasts under constant hypoxia (2% O_2), leads to extensive cell death and dramatically reduced numbers of osteoclasts,

(Knowles et al., 2009). That situation, with very low levels of O_2 may reflect the situation in an OC lesion better and it would, therefore, be very interesting to repeat this set of experiments at even lower O_2 concentrations.

Conclusion

In contrast to what has been stated in earlier reports in literature, the gait pattern of the new-born foal has to go through a period of development and stabilization. Direct postnatal loading in precocial animals is made possible by anticipatory bone development that already starts *in utero.* It is clear that, unlike in altricials, this process does not follow the classic pathway as described by Wolff's law and the Mechanostat theory. The exact underlying mechanism remains still elusive. This thesis has tried to address various aspects of the quick adaptation of the musculoskeletal system of precocials to postnatal life, from fundamental cell biology to highly practical applications in commercial pig farming, and as such has contributed to our knowledge about this crucial process. Having said this, it should be recognized with humbleness that such a contribution cannot represent but a few tiny pieces of the very complex puzzle of the many intricately interwoven processes that go on in this very fascinating period in early life that deserves further exploration.

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ADDENDUM:

Addendum

Summary

Dutch summary / Nederlandse samenvatting

Dankwoord

About the author

Summary

From a locomotor perspective precocial animals like the foal are truly extraordinary creatures, as they are able to set their first steps within an hour after birth. Despite the current wealth of techniques that are available for gait analysis, there was hardly any information regarding the early development of gait in the horse. Also the ways in which the precocial skeleton copes with the sudden changes in loading after birth were largely unexplored. Therefore, the main aims of this thesis were to investigate the early development of gait in the foal and relate this to the (development of the) trabecular and subchondral bone of the distal tibia and talus in order to investigate how the skeleton prepares for postnatal loading.

Chapters II and III of this thesis cover the longitudinal development of relevant kinetic gait parameters and hoof balance in a cohort of 11 warmblood foals during the first 24 weeks of life by means of pressure plate analysis. During growth, the preferred speed at walk and trot increases due to increased limb length and consequently larger stride length. Meanwhile, relative limb loading remains fairly constant. It is clear that being able to stand and walk immediately after birth does not mean that foals move like "mini adult horses", as also in this extremely precocial animal gait and balance need time to mature. This maturation of gait is characterised by a reduction of variability of the stance duration and of the pressure distribution under the hooves, which is indicative of better coordination and balance. Although none of the animals showed clinical signs associated with osteochondrossi (OC), like joint effusion or lameness, there was radiographic presence of OC lesions in some animals, which led to a temporary, but significant, reduction in nPVF of the affected limb.

Chapter IV uses micro-CT imaging to study trabecular bone of calves and foals (Shetland ponies and warmbloods) in order to investigate how the skeleton of these large and precocial animals anticipates the extreme changes in loading that occur after birth. It appeared that these anticipatory strategies were not universal. Foals rely on a relatively high bone volume fraction; in calves the trabecular bone volume fraction is lower, but trabeculae are more aligned. These differences are likely related to species-related anatomical peculiarities in joint geometry and hence in loading. Surprisingly, Shetland pony bone was characterised by a higher bone volume fraction than in warmblood foals. This may be related to differences in growth rate, as high growth rates are associated with relatively less dense bone.

The longitudinal development of the mineralised and non-mineralised parts of the distal tibia and talus of warmblood foals is described in chapter V. The development of trabecular and subchondral bone was quantified using micro-CT, whereas polarised light microscopy was used to investigate the collagen type I network. Due to increasing trabecular thickness, the bone volume fraction increases during growth. However, no significant changes in trabecular orientation and alignment were found. Similar to the mineralised part of the bone, collagen content increased significantly, but the degree of anisotropy of the collagen type I network did not. These results indicate the necessity for a gradual increase in strength, but not for a different architecture after birth, providing further evidence for prenatal anticipatory bone development in the horse.

The effects of the NSAID meloxicam on skeletogenesis and chondrogenesis in growing pigs are discussed in chapter VI. Prostaglandins are known to play important roles during bone growth and homeostasis, among others during the hypertrophic differentiation of chondrocytes and with regard to osteoblast and osteoclast function, suggesting that suppression of prostaglandin production by NSAIDs might have a negative effect. However, we did not observe adverse effects on skeletal development after the long-term administration of the regular dose of the COX-2 inhibitor meloxicam in growing pigs. Histology of the growth plates and micro-CT analysis of the trabecular bone did not reveal significant differences between the metacam-treated and control animals. Furthermore, bone stiffness, determined by non-destructive compressive testing, did not differ between the treated and untreated pigs. Also, prevalence of the more commonly known side effects, like gastric ulcerations and renal papilla necrosis did not differ. Altogether, these findings reduce concerns regarding the long-term use of meloxicam in young, growing piglets and suggest that the drug might be helpful for treating pigs that are in pain, thus improving welfare.

The osteoclast, a multinucleated cell responsible for the resorption of bone and therefore of key importance for bone remodelling was the subject of chapter VII. Cellular senescence, characterised by the withdrawal from the cell cycle and the acquirement of the senescence associated secretory phenotype (SASP), is essential for tissue remodelling during embryonic development. It is known that osteoclasts exit the cell cycle during their differentiation (which is commonly seen as quiescence rather than senescence). The similarities of bone remodelling with tissue remodelling during embryonic development incited us to investigate whether osteoclasts are senescent instead of quiescent. Given the important role of oxygen, both during normal and abnormal (osteochondrosis) bone development, we assessed the influence of hypoxia on osteoclastogenesis and possible senescence status of these cells. Our results demonstrated that osteoclasts

harvested during in vitro osteoclastogenesis under the influence of M-CSF and RANK-L can indeed be considered senescent rather than quiescent. Hypoxia (5% $O_{2)}$ delayed osteoclastogenesis, without negatively affecting the resorption capacity of the bone after three weeks of culturing. Based on the direct relationship between senescence and osteoclastogenesis, it is tempting to hypothesize that contents of the SASP not only play a functional role in matrix resorption but also may regulate osteoclastogenesis.

Dutch summary / Nederlandse samenvatting

Het blijft bijzonder om te zien hoe snel veulens kunnen staan en lopen na de geboorte. Ondanks dat er bij het paard veel onderzoek gedaan wordt naar beweging en kreupelheid is er vrij weinig bekend over de ontwikkeling van beweging bij deze dieren, zeker in de periode net na de geboorte. Ook de manier waarop het skelet omgaat met de plotse verandering van de mechanische belasting na de geboorte is nog grotendeels onbekend. Het verkrijgen van meer inzicht in de ontwikkeling van beweging en de manier waarop het skelet van nestvlieders (dieren zoals het veulen, die meteen na de geboorte kunnen lopen) zich voorbereidt op de periode net na de geboorte waren dan ook belangrijke doelen van het onderzoek in dit proefschrift. Ook is er gekeken naar de mogelijke effecten van de pijnstiller en onstekingsremmer meloxicam op de ontwikkeling van bot bij opgroeiende varkens. Tot slot is de rol onderzocht van een hoge of lage zuurstofconcentratie op de ontwikkeling en functie van osteoclasten, cellen die bot kunnen "opeten" en daarmee samen met de botvormende osteoblasten verantwoordelijk zijn voor de hoeveelheid en architectuur van bot.

De ontwikkeling van kinetische gangenparameters, die informatie geven over de krachten bij de beweging, worden besproken in hoofdstuk II van dit proefschrift, de ontwikkeling van hoefbalans in hoofdstuk III. Hiervoor zijn drukplaatmetingen verricht bij een groep van 11 springpaardveulens, gedurende de eerste 24 weken van hun leven. Doordat de benen tijdens de groei langer worden en de paslengte toeneemt, neemt de snelheid in stap en draf toe. Ondanks de toegenomen snelheid blijft de relatieve verticale belasting van de benen redelijk constant. Verder is er, met name in de eerste weken na de geboorte, een duidelijke afname van variatie in standtijd en de verdeling van de krachten onder de hoef, die aangeven dat de coördinatie van het veulen na de geboorte geleidelijk verbetert. Ondanks dat veulens dus ongelofelijk snel kunnen staan en lopen, bewegen ze dus niet meteen als een miniversie van volwassen paarden, maar hebben ze tijd nodig hebben om hun beweging en balans door training te ontwikkelen. Hoewel geen van de veulens op het oog kreupel was, bleek er in de veulens waarbij middels radiologisch onderzoek osteochondrose was vastgesteld, een tijdelijke maar significante reductie van de belasting van het aangedane been meetbaar met de drukplaat.

Bot is qua opbouw te vergelijken met gewapend beton en bestaat uit calciumzouten (het beton) en een collageen type I netwerk (de wapening). De stevigheid van bot wordt zowel bepaald door de hoeveelheid aanwezig botweefsel, als de opbouw van het bot. Onder invloed van de mechanische belasting door de beweging vindt er een continue finetuning van zowel de hoeveelheid, als opbouw van het bot plaats, wat dit weefsel bijzonder

dynamisch en efficiënt maakt. In dit licht is de periode rond de geboorte bij nestvlieders erg interessant gezien de extreme verandering in belasting van het skelet. Hoofdstuk IV beschrijft de resultaten van micro-CT onderzoek van het bot in het spronggewricht van kalveren, Shetland- en KWPN-veulens. Onderzocht is in hoeverre het bot voorbereid is op zijn taak na de geboorte. Botweefsel van deze dieren bereidt zich inderdaad al tijdens de dracht voor op de belasting na de geboorte, al verschillen de gebruikte strategieën per diersoort. Veulenbot wordt gekenmerkt door een relatief grote hoeveelheid botweefsel, terwijl er bij kalveren relatief gezien veel minder bot aanwezig is, maar de structuur van de beenbalkjes is meer in lijn met de hoofdrichting van de mechanische belasting. Deze verschillen zijn waarschijnlijk te verklaren door de subtiele verschillen in anatomie van het spronggewricht van runderen en paarden en daarmee in (de richtingen van) de belasting tijdens staan en lopen. Verder viel het op dat het relatieve botvolume bij Shetland-veulens nog hoger was dan bij de KWPN-veulens, wellicht veroorzaakt door de veel hogere groeisnelheid van de KWPN-veulens tijdens de dracht.

In hoofdstuk V is te lezen hoe zowel het gemineraliseerde als niet gemineraliseerde deel van het botweefsel van de talus en distale tibia, onderdelen van het spronggewricht, bij het veulen ontwikkelt na de geboorte. Hierbij is er gebruik gemaakt van zowel micro-CT, om het gemineraliseerde bot te kwantificeren, als gepolariseerd licht microscopie om het collageen type I netwerk (niet gemineraliseerde deel van bot) in beeld te brengen. Tijdens groei neemt logischerwijs de hoeveelheid bot (zowel het gemineraliseerde als niet gemineraliseerde deel) toe. De architectuur en richting van de beenbalkjes en het collageennetwerk daarentegen veranderen niet na de geboorte, wat verder illustreert dat het skelet van veulens zich al tijdens de dracht voorbereidt op de mechanische belasting tijdens staan en lopen na de geboorte.

Niet steroïde ontstekingsremmers (NSAID) zijn binnen de diergeneeskunde vaak de middelen van eerste keuze bij pijn. Naast de meer bekende bijwerkingen, namelijk het veroorzaken van ulceraties in de maag en twaalfvingerige darm en eventuele nierproblemen kan het gebruik van deze stoffen ook negatieve bijwerkingen hebben op de skelet- en kraakbeenontwikkeling. Prostaglandines, waarvan de productie door NSAIDs geremd wordt, spelen namelijk niet alleen maar een rol bij ontsteking en pijn, maar vervullen ook belangrijke signaalfuncties voor bot en kraakbeen (-ontwikkeling). Daarom is er gekeken naar de effecten van langdurige toediening van het NSAID meloxicam op de skeletontwikkeling bij opgroeiende biggen. De resultaten van deze studie zijn te lezen in hoofdstuk VI van dit proefschrift. Micro-CT, histologisch onderzoek en compressietesten van het botweefsel lieten geen significante effecten van meloxicam op het botweefsel en de groeischijven zien. Verder was de beweging van de dieren, zowel

gemeten met een drukplaat, als op het oog, niet verschillend bij de behandelde dieren ten opzichte van de controledieren. Ook bleek er geen verschil te zijn in het voorkomen van maagulceraties en nierproblemen, de meer bekende bijwerkingen van NSAIDs. Hierdoor lijkt meloxicam een geschikte keuze voor het behandelen van ontsteking en pijn bij jonge varkens.

De osteoclast is een bijzondere, meerkernige cel, die bot kan "opeten". Samen met de osteoblast, die botweefsel maakt, is hij verantwoordelijk voor de eerder genoemde balans tussen opbouw en afbraak van bot. Een verstoorde ontwikkeling of functie van de osteoclasten heeft dan ook direct effect op deze balans en daarmee het skelet. Ook tijdens de groei en ontwikkeling van het skelet zijn deze cellen essentieel. Ze ruimen dan het kraakbeen op, waaruit het skelet eerst bestaat. Daarna kan het vervangen worden door bot, een proces dat endochondrale ossificatie wordt genoemd. Osteochondrose is een belangrijke aandoening bij het paard, waarbij dit proces vertraagd of verstoord is. Aangezien zuurstofgebrek door beschadigde, voedende vaatjes een belangrijke rol speelt bij het ontstaan van osteochondrose, is er gekeken naar het effect van een hoge en lage zuurstofconcentratie op de ontwikkeling en functie van osteoclasten.

Cellulaire senescentie is een bijzondere toestand van de cel, waarbij deze niet meer deelt en een heel pallet aan (signaal)stoffen gaat produceren. Het is een belangrijk onderdeel van veroudering en speelt ook een rol in de tumorbiologie, waarmee voorkomen kan worden dat een tumorcel verder gaat met delen. Daarnaast is dit proces ook essentieel tijdens remodelleren van weefsel tijdens de embryonale ontwikkeling, bijvoorbeeld bij het laten verdwijnen van het weefsel tussen de vingers. Gezien het feit dat osteoclasten stoppen met delen tijdens hun ontwikkeling en hun rol in het remodelleren van botweefsel, is tevens gekeken of osteoclasten ook senescentie gaan vertonen en of de verschillende zuurstofconcentraties hierop van invloed zijn. Osteoclasten blijken tijdens hun ontwikkeling inderdaad cellulaire senescentie te gaan vertonen. Verder bleek dat een lage zuurstofconcentratie de ontwikkeling van osteoclasten remt, maar uiteindelijk niet van invloed is op hun capaciteit om bot te resorberen (hoofdstuk VII).

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About the author

Ben Gorissen was born in Geleen, the Netherlands, on the 28th of April, 1983. He graduated in 2001 from high school (Graaf Huyn college, Geleen) and started studying Veterinary Medicine at Utrecht University. During his research internship, he studied the effects of uneven feet on the sports performance in warmblood horses. He followed the equine track and graduated "cum laude" in 2008. After working in practise for almost two years he became a junior lecturer in veterinary anatomy and physiology and obtained his university teaching qualification. In 2012 he started as a PhD candidate under the supervision of Prof. Dr. M. E. Everts, Prof. Dr. P. R. van Weeren, Dr. C. F. Wolschrijn and Dr. M. A. Tryfonidou. His research focused on the early development of the skeleton in precocial species in relation to postnatal loading. Aside from being a PhD candidate, he combined research with teaching veterinary anatomy and physiology. He is married to Fleur and has one son, Ivan. Their second son will be born in October, just before the defence of this thesis.