THE EFFECTS OF SILICON ON SKELETAL INTEGRITY IN POULTRY

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Abstract

Intensive genetic selection for growth and breast meat yield mean modern broilers are susceptible to skeletal disorders. Issues relating to leg weakness are extremely concerning in terms of welfare and cost. Silicon (Si) has been linked to the calcification of growing bone, and deficiency in poultry diets has been shown to lead to several health and welfare issues, such as various skeletal weaknesses. Beyond avoidance of deficiency *per se*, to date, Si has not been considered as a route to addressing the skeletal issues faced by the poultry sector. The aim of this project was to assess the impact of a newly developed, highly bio-available Si supplement on the skeletal integrity of broiler chickens.

A series of *in vitro* studies and bird trials were conducted to investigate the Si supplement. Firstly, an investigation into the effect of altered milling parameters on particle size and suspension in carrier oil, this study showed that particles <100µm retained their bio-availability and remained suspended for longer than larger particles. The initial feeding trial compared efficacy of the NTU supplement with other Si supplements (all fed at 1000ppm) and showed that the NTU supplement was absorbed at a higher rate (6.19ppm in plasma compared to 2.64ppm for nearest competitor) and increased tibia breaking strength at d21 and 35 to 242.98N and 419.95N (closest competitor was 213.52N and 379.19N respectively), but without a corresponding increase in ash or mineral content. The second feeding trial examined rate of inclusion (RoI) (Si fed at 250, 500, 750 and 1000ppm) and assessed whether silicon from sand (fed at 1000ppm), could provide similar benefits. This trial showed that the sand was not absorbed into the blood as Si, but the bio-available Si was absorbed proportionally to RoI, although at approximately half the level seen previously and with no bone response observed; possibly due to issues in manufacturing the supplement, so the highest RoI was used in the final bird trial on the early post hatch period. This trial showed that Si increased tibia and femur strength significantly ($p<0.05$ and $p<0.001$ respectively) by d21, and this corresponded with a decrease in bone formation biomarkers, and an increase in the resting zone of the growth plate

and the presence of bone forming cells. This suggests a cellular response to the supplement, that in turn leads to a structural advantage to the bird.

To conclude, this project has demonstrated that modern broiler chickens appear not to receive adequate bio-available silicon in standard diets for optimum skeletal development and, therefore, would benefit from bio-available silicon supplementation.

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Glossary

AA- Amino acids

ANOVA-Analysis of variance

BW- Body weight

BWG-Body weight gain

Ca- Calcium

DCP- Dicalcium phosphate

DEXA- Dual energy x-ray absorptiometry

ECM- Extracellular matrix

EDTA- Ethylenediaminetetraacetic acid

DLS- Dynamic light scattering

FCR- Feed conversion ratio

FI- Feed intake

GIT- Gastrointestinal tract

ICP-OES- Inductively coupled plasma atomic emission spectroscopy

KS- Kolmogorov Smirnoff

MANOVA- Multiple analysis of variance

NRP- Nutrient requirements of poultry

NTU- Nottingham Trent University

P- Phosphorus

QCT- Quantitative computed tomography

RSM- Rapeseed meal

SBM- Soyabean meal

SEM- Scanning electron microscopy

Si- Silicon

TD- Tibial dyschondroplasia

UV- Ultra violet

Contents

Chapter 1: Review of the literature

1.1. Introduction

Modern broilers are susceptible to skeletal disorders, particularly those affecting the legs (Kestin *et al.,* 1992; Manohar *et al.,* 2015; Whitehead, 1997). This can be attributed to a number of factors including intensive genetic selection, an increase in the nutritional quality of feed and the increased demand for poultry as a food source, leading to a drive for faster production rates. These elements have resulted in birds that have a better feed conversion efficiency, reach slaughter weight at a much earlier age and are subject to far more intensive production processes than their counterparts from as little as 50 years ago (Bradshaw *et al.,* 2002; Julian, 1998). Silicon (Si) has been linked to the calcification of growing bone (Carlisle, 1970), and deficiency in poultry diets has been shown to lead to a number of health and welfare issues, such as various skeletal weaknesses (Carlisle, 1976; Carlisle, 1980a; Carlisle, 1980b). Beyond avoidance of deficiency *per se*, to date, Si has not been considered as a route to addressing the skeletal issues faced by the poultry sector. These issues include, but are not limited to, tibial dyschondroplasia (Waldenstedt, 2006), rickets (Carlisle, 1986), cage layer fatigue, keel bone fractures (Fleming *et al.,* 2006; Whitehead and Fleming, 2000), twisted legs, valgus and varus deformities (Bradshaw *et al.,* 2002) and lameness (Mench, 2004). Issues relating to leg weakness in particular are extremely concerning from a bird welfare perspective, and constitute some of the most costly losses to the poultry industry in terms of both bird mortality and losses at processing (Bennett *et al.,* 1999; Zuidhof *et al.,* 2014). Research focussing on human health has recognised the importance of Si in skeletal development and integrity (Jugdaohsingh *et al.,* 2006; Jugdaohsingh, 2007).

1.2. Poultry production

1.2.1. The poultry industry

Between 1950 and 2017 the global population rose by 5 billion people, from 2.5 billion to 7.5 billion, and is expected to continue this exponential growth with some predications estimating the global population to exceed 9.8 billion by 2050 (Population Reference Bureau, 2017). This increase in population creates a drive for sustainable food sources. It is becoming increasingly recognized that the population increase, extended life expectancy and associated food insecurity is a major global concern (FAO, 2015). In recent years, there has been a rise in awareness surrounding food security and the need to focus on sustainable food production. This has led to an understanding that sustainability can only be achieved via a holistic approach (Burton *et al*., 2016). In addition to rising population growth, there has been a rapid movement towards public health awareness, improving current health status and healthy eating education (Burton *et al*., 2016). Government campaigns in the USA and UK have cited eating white meat as beneficial for general health and maintenance of a healthy weight. Due to a combination of the global population increase, nutritional health awareness, the need for sustainable protein production and the popularity of poultry as a meat source, pressure has been placed on the poultry industry to increase production without compromising on price or environmental impact. In response to this pressure, the poultry industry is currently the fastest growing animal production industry in the world. Between 1995 and 2005, global poultry production increased by 53% (Scanes, 2007). This has led to the UK to producing over 875 million broiler chickens annually (ADHB, 2017). The collective poultry industry is also reported to be one of the cleanest industries, producing only 0.1% of total global greenhouse gas emissions (Williams *et al*., 2006). Poultry meat currently accounts for 35% of the world's meat consumption (FAO, 2018), as a low fat, high protein meat, poultry offers low cost, rapid turnover, palatability, versatility and social

and cultural acceptability. An estimated 225,000 million head of poultry are produced annually around the globe (FAO, 2018).

As of April 2018 (in comparison to April 2017) in the UK, monthly broiler chick placings were up 2.4% at 101.9 million chicks, monthly broiler slaughtering's were 3.5% higher at 103.9 million birds and total monthly UK poultry meat production was 182.8 thousand tonnes, up 3.7%. Figures for 2017 show a rise of 6% from 2016 in the value of the poultry meat sector, giving it a contribution of £2.42 billion to the UK economy. Total UK annual poultry production increased 1% to 1.81 million tonnes, with 86% of this increase being attributable to broilers and table chickens (DEFRA, 2018). The poultry industry accounts for 5.5% of the UKs total agricultural output and chicken makes up 46% of the total meat consumed in the UK (35.4 kg/capita), nearly equalling the combined consumption of pork, beef and lamb (DEFRA, 2016). Broiler chicken is now the most produced meat in the UK, contributing to 88% of total poultry eaten. Unfortunately, the UK has seen a dramatic increase in animal feed costs, with the total cost of animal feed in 2017 rising by £577 million since 2016 (DEFRA, 2018), alongside this the increase in demand for meat has led to an increase in poultry meat prices.

1.2.2. Meeting demand for poultry meat

The popularity of poultry can be attributed to a number of factors. These include its versatility as a food ingredient, lack of religious or cultural restrictions, low fat and high protein content compared to other meats, and relatively low cost to the consumer (Magdalaine *et al*., 2008). In order to meet the seemingly ever-increasing demand, the poultry industry has implemented a number of strategies. Perhaps the largest factor in improving growth rate in broilers has been intense genetic selection. A combination of selective breeding and, more recently, genetic

manipulation, has produced the modern day broiler chicken. The heavy selective pressure applied to the modern broiler has led to significant changes in the conformation and skeletal architecture of the bird. However, these changes have led to a range of problems resulting from rapid growth and increased muscle to bone ratio. Duggan *et al*. (2015) found that the bone architecture of the modern broiler displayed significant curvature compared to its lighter con-specifics. As such, the gait and mobility of these birds was likely to be adversely affected. Modern strains of broiler exhibit much more efficiency when converting feed into body mass. Comparisons between genetically preserved strains from the 1950s and modern strains have shown an increase in carcass yield and overall bird size. Modern strains grow to nearly five times the size of their historic counterparts by day 42, and show a 100% increase in breast meat to body weight ratio (Havenstein *et al.,* 2003). Unfortunately, selection for large pectoral mass did not occur alongside selection for a stronger musculoskeletal support system, with an investigation by Robinson *et al.* (2007) revealing that high breast-yielding strains had reduced carcass frames. Part of this increased feed conversion efficiency is also thought to be due to an increase in digestive efficiency. This is indicated by earlier maturation of the liver thought to improve nutrient utilisation in increasingly carbohydrate heavy feeds, and a 20% increase in jejunum and ileum length, which would allow greater surface area for nutrient absorption (Schmidt *et al*., 2009).

Aside from genetic selection, other methods for increasing production efficiency include the development of highly efficient rearing and processing systems designed to handle the high throughput required to meet demand. As of 2013, there were 1,982 broiler farms across the UK, housing a total of 142.5 million birds at any one time (DEFRA and AHVLA, 2013). While these large-scale intensive operations enable the demand for meat to be met, they do place more physiological strain on the birds. Within rearing systems, space is at a premium and access to food and water, while freely available, is not regulated among the flock meaning that weaker or

smaller individuals may struggle for access. Methods of transportation and slaughter are also geared toward this high throughput, and are often highly mechanised, meaning that broiler carcasses also undergo substantial stresses during processing.

Arguably, the most important tool at the farmer's disposal to increase meat production is nutrition. This often goes hand in hand with welfare, and there has been increasing recognition of the importance of good nutrition in producing birds that are able to handle demanding rearing processes. The importance of positive welfare amendments and feed provision is being increasingly highlighted due to the health limitations seen in animals bred for enhanced developmental characteristics. Nutritional advances are of particular interest, as a relatively simplistic, and often cost effective, measure by which developmental disorders can be mitigated (Klasing, 2007).

Genetic awareness is also increasing in the practical aspects of sustainability, in order to promote genetic diversity on the basis that future adaptations will be required to meet production demand. Change in demand must concentrate on a long-term approach, with the consideration for future economic, environmental, cultural and disease challenges at the forefront. Genetic parameters are already often controlled by computer software, such as best linear unbiased prediction, to assess genetic trends and polygenic health, meaning there is potential for the accidental elimination of certain traits if they are not currently desirable (Hill, 2016).

1.3. Poultry musculo-skeletal system

1.3.1. Type and structure of bone

There are four main types of cell associated with the formation, growth, remodelling and maintenance of bone; osteoblasts, bone lining cells, osteocytes and osteoclasts (Sommerfeldt and Rubin 2001).

The osteoblast, derived from the stromal marrow cells known as osteoprogenitor cells (Downey and Siegel, 2006), is the cell that is responsible for the active formation of bones, and the mineralisation of the bone matrix. The primary function of the osteoblast is to lay down the osteoid (Owen, 1963), and they are also the synthesisers of collagen and many of the protein carbohydrate complexes which make up the matrix (Vaughan 1981). Osteoblasts have also been implicated in the deposition of calcium, and the exchange of its ions (Talmage and Grubb, 1977). They play a major role in the mineralisation of the matrix, and once their function as mineralisers has been fulfilled, they revert to bone lining cells (Florencio-Silva *et al.,* 2015).

Bone lining cells cover the surfaces of bone when they are inert and no formation, growth or remodelling is taking place (Miller *et al.,* 1989). They possess processes which extend into the bones canaliculi and communicate via gap junctions (Florencio-Silva *et al.,* 2015). They have the ability to reacquire their osteoblastic function if the status of the bone changes (Donahue *et al.,* 1995). While their precise functions are not fully understood, they have been shown to participate in both bone resorption, via osteoclast differentiation, and in bone remodelling by preparation of the resorbed area for the laydown of new matrix (Everts *et al.,* 2002).

Osteocytes are formed when matrix secretion has nearly been concluded. Around 10% of osteoblasts, which have become surrounded by matrix during the process, modulate to become osteocytes (Eurell and Frappier, 2013). The osteocyte is the most common cell found in mature

bone, and they reside inside a lacuna, surrounded by mineralised matrix (Feng *et al.,* 2006; Tate *et al.,* 2004). Osteocytes are dendritic, connected to one another via processes which extend from the cell body and run via canaliculi within the matrix, with gap junctions being present at the contact point that enables communication between cells (Aarden *et al.,* 1994). They are thought to play a role in mineral homeostasis and initiate bone remodelling (Cullinane, 2002). This is due to their ability to act as mechanosensors, as their interconnected network of processes can detect the mechanical pressures and loads placed on bone, sending signals that allow the bone to adapt (Rochefort *et al.,* 2010).

Osteoclasts are derived from bone marrow stem cells, and are primarily responsible for bone resorption, playing a vital role in bone homeostasis. They are also thought to be an important part of bone marrow production, thus making them an important factor in the production of blood cells (Miyamoto and Suda, 2003). During resorption they attach to the matrix via a sealing zone, a ruffle border is formed through which projections infiltrate the matrix, hydrochloric acid is secreted to dissolve the hydroxyapatite crystals, then proteolytic enzymes degrade the collagen matrix that remains and the products of these processes are removed (Vaananen *et al.,* 2000).

In the mature skeleton there are two types of bone that can be easily identified, spongey (also known as trabecular or cancellous) bone and compact cortical (also known as lamellar) bone (Vaughan, 1981). Long bones, such as the tibia or femur, contain both types of bones with the inner spongey bone being protected and given strength by the outer layer of compact bone (Clarke, 2008).

Cancellous bone is made up of a three dimensional network of interlacing trabeculae, or spicules, which enclose numerous medullary cavities that contain either red or fatty marrow, loose connective tissue and blood vessels (Sommerfeldt and Rubin, 2001). This type of bone is typically

found in the vertebrae, the majority of flat bones (such as the skull and craniofacial bones and the pelvis) and in the ends of the long bones (Hall, 2015). In flat bones, trabecular bone is generally found sandwiched between two layers of cortical bone (Bacha and Bacha, 2012). It is also found inside the diaphysis of long bones, containing the bone marrow and surrounded by a layer of compact bone (Mescher and Junqueira, 2016).

Compact cortical bone is generally composed of osteoid matrix that has been laid down in layers. The layers that surround the inner and outer circumference of the bone are known as circumferential lamellae with other internal layers being referred to as concentric lamellae (Burr and Akkus, 2014). These concentric lamellae form layers of rings, arranged into columns, known as Haversian systems or osteons. The osteons run parallel to the axis of the long bones where the majority of compact cortical bone is found. Down the centre of each Haversian system there is a Haversian canal, also known as a central canal, which allows blood vessels and nerves to run through the bone tissue (Datta *et al.,* 2008). Running between the Haversian canals and the inner medullary cavity, or the external periosteum, are Volkmann's canals, also known as perforating canals, which allow blood vessels and nerves to connect to the external environment and the inner cavities of the bone (Boskey, 2006).

There is also a third type of bone, known as woven, or coarse bundled, bone. This is the first type of bone to appear during embryonic development, or in the repair of damage to mature bone (Vaughan, 1981). Its structure is very similar to that of cancellous bone, but with the significant difference that any collagen fibrils within the matrix are irregularly arranged (Su *et al.,* 2003).

The matrix of bone is composed of osteoid, produced by the osteoblasts. The major organic component of the matrix is collagen (predominantly type I, but types III, V and X are also present) which it is thought acts as a scaffold for other, non-collagenous proteins to attach to (Gehron Robey, 2008). These proteins then go on to act as nucleators that allow the deposition of the hydroxyapatite crystals that constitute the inorganic component (Gehron Robey, 2008).

The organic matrix of bone is similar in construction to dense fibrous tissues such as tendons and ligaments (Buckwalter *et al.,* 1995). It accounts for around 22% of the matrix, and is comprised of roughly 90% collagen, with the remaining 10% being non-collagenous structural proteins such as proteoglycans, sialoproteins, gla-containing proteins and 2HS-glycoproteins (Kini and Nandeesh, 2012). Osteoblasts are responsible for synthesis and lay down of collagen precursors, and produce the most common non-collagenous protein found in bone (osteocalcin) as well as proteoglycans (Brodsky and Persikov, 2005). The collagen produced by the osteoblasts is packaged into fibrils and fibres, then laid down in concentric layers that form lamellae, and it is these lamellae that form the structural unit of compact (lamellar) bone (Frandson *et al.,* 2009). The organic matrix is responsible for giving the bone its form and ability to withstand tension, while the inorganic matrix is responsible for bones ability to withstand compressive forces (Buckwalter *et al.,* 1995).

Once the organic matrix has been laid down by osteoblasts, it is ready for the addition of the inorganic matrix, in a process known as mineralisation. The inorganic matrix makes up about 69% of the total structure of bone, of which 99% is thought to consist of crystalline hydroxyapatite. Hydroxyapatite [$(Ca_{10}(PO_4)_6(OH)_2$] crystals are deposited along the collagen fibrils in the organic matrix (Kini and Nandeesh, 2012). It is believed that the ordered deposition of these crystals is regulated by several non-collagenous proteins (including osteocalcin,

osteopontin and bone sialoprotein) which control the size and volume of crystals that are formed (Clarke, 2008). The hydroxyapatite crystals are plate shaped (Weiner and Traub, 1992), and fit into discrete spaces (known as hole zones) between the collagen fibrils. Their placement between collagen fibrils limits their growth, causing them to be discontinuous, and to have a specific orientation – their c axis runs parallel to the long axis of the fibrils (Rho *et al.,* 1998). At this point there are still un-mineralised collagen fibrils separating the mineralised hole zones but, as mineralisation continues and the remaining hole zones become full, the deposition and growth of the crystals continues until it includes the fibrillary zones between the hole zones and mineral deposits occupy all available space (Buckwalter *et al.,* 1995).

Once the bone is fully formed, its organic and inorganic phases create a composite material that possess properties that differ from the two components individually but make it well suited to its various roles within the body. These roles include, but are not limited to, protection of vulnerable organs and structures, locomotion and movement via their roles as levers to which tendons and ligaments are attached, formation of blood cells, storage of minerals and maintenance of mineral homeostasis (Frandson *et al.,* 2009).

The epiphyseal growth plates are present at either end of long bones, and their main purpose is for growth in the length of bone. Once this has been achieved, they will disappear due to the completion of bone development in adulthood. The epiphysial growth plate displays distinct regions, the resting zone, the proliferative zone and the hypertrophic zone, each with its own purpose and range of cellular activity (Mescher *et al.*, 2016). They are composed of a layer of hyaline cartilage where ossification occurs in immature bones. On the epiphyseal side of the epiphyseal plate, cartilage is formed. On the diaphyseal side, cartilage is ossified, and the diaphysis grows in length. There are also areas referred to as the zone of calcified matrix, and

the ossification zone, although these are not typically considered part of the growth plate, as the majority of chondrocytes within them are typically dead and are more like a part of the diaphysis.

The resting zone (sometimes referred to as the reserve zone) appears at the outermost region of the growth plate, proximal to the articular cartilage layer at the epiphyseal end of the plate and is composed of quiescent chondrocytes. These chondrocytes do not participate actively in bone growth but are responsible for securing the epiphyseal plate to the osseous tissue of the epiphysis to maintain structural integrity during growth. They are irregularly scattered in a bed of cartilage matrix and are thought to act in a similar manner to stem cells. The resting zone produces a growth plate organisational factor called morphogen that directs the alignment of cells in the proliferative zone into columns that run parallel to the long axis of the bone. It is thought that the resting zone may also produce another morphogen that inhibits the terminal differentiation of proliferative zone chondrocytes which may be responsible for the organisation of the growth plate into the distinct zones of proliferation and hypertrophy (Abad *et al*., 2002). The resting zone is also thought to maintain the growth plate by expressing parathyroid hormone-related protein (PTHrP) and provides a source of chondrocytes. Skeletal stem cells have been shown to be formed among PTHrP-positive chondrocytes within the resting zone of the postnatal growth plate in mice and recent cell-lineage analysis conducted by Mizuhashi *et al*. (2018) has revealed that PTHrP-positive chondrocytes in the resting zone continued to form columnar chondrocytes in the long term, with these chondrocytes then going on to hypertrophy and become osteoblasts and marrow stromal cells beneath the growth plate. This suggests that the potential for the future growth of bones begins in the resting zone, making it an area of key importance for bone elongation, even though its cells do not actively participate in the actual deposition and mineralisation of bone tissue on the cartilage anlage.

The proliferative zone is the next layer of the growth plate, toward the diaphysis, and contains stacks of slightly larger chondrocytes. It makes new chondrocytes via mitosis (to replace those that die at the diaphyseal end of the plate) and these chondrocytes undergo repeated division under the influence of growth hormone. The cells enlarge and excrete type II collagen and proteoglycans and then go on to become organised into columns parallel to the long axis of the bone, guided by the morphogen excreted in the resting zone (Mescher *et al*., 2016).

The hypertrophic zone (sometimes referred to as the maturation zone) contains swollen terminally differentiated chondrocytes and is present toward the diaphyseal end of the growth plate, beneath the resting and proliferative zones. These chondrocytes are older and larger than the cells of the proliferative or resting zones and compress the matrix into aligned spicules which stiffen the structure by the secretion of type X collagen. Type X collagen limits diffusion in the matrix and, with the use of growth factors, promotes vascularisation from the neighbouring primary ossification centre (Mescher *et al.,* 2016). The longitudinal growth of bone is a result of cellular division in the proliferative zone and the maturation of cells in the zone of maturation and hypertrophy.

The next area is the zone of calcified matrix, which lies closest to the diaphysis, and is often considered a part of the bone rather than the growth plate. Most of the chondrocytes in this zone are dead because the matrix around them has calcified. Capillaries and osteoblasts from the diaphysis infiltrate this zone, and the osteoblasts secrete bone tissue onto the remaining uncalcified cartilage, connecting the epiphyseal plate to the diaphysis. This zone is also where chondrocytes about to undergo apoptosis release matrix vesicles and osteocalcin to start matrix calcification with the formation of hydroxyapatite crystals (Anderson *et al*., 2004).

Finally, the ossification zone is where true bone tissue first appears. Osteoprogenitor cells and capillaries penetrate the vacant chondrocytic lacunae and merge to form the initial marrow

cavity. Osteoblasts settle in a layer over the spicules of calcified cartilage matrix and release osteoid to become woven bone. This woven bone will then go on to be remodelled as lamellar bone (Mescher *et al*., 2016).

1.3.2. Development and Formation of Bones

Skeletogenesis begins with differentiation and condensation of mesenchymal stem cells in early embryonic development (Weaver and Fuchs, 2014). Bone formation can generally be divided into one of two types, either intramembranous or endochondral. While these two processes are similar, both involving the deposition of an organic matrix known as the osteoid which is then mineralised, there are some significant differences regarding both the environment where ossification is initiated and the cells that produce the matrix (Vaughan, 1981).

In intramembranous ossification, a collection of mesenchyme cells condensate and differentiate directly into osteoblasts, going on to become centres of ossification (Ham and Cormack, 1987). This type of ossification occurs in well-vascularised connective tissue, such as the skull and craniofacial areas (Eurell and Frappier, 2013) and is primarily associated with embryonic development, although it does sometimes occur later in life in relation to bone healing (Allen and Burr, 2014). Osteoblasts at the centres of ossification deposit bone matrix in or beneath a pre-existing membrane, beginning with secretion of collagen and other proteinaceous fibres that become the osteoid. This osteoid is laid down in projections called trabeculae that form around blood vessels and membranous pores, leading to spongey, or cancellous, bone (Bacha and Bacha, 2012).

Endochondral, also known as intracartilaginous, ossification begins with the formation of a hyaline cartilage model (also known as a cartilage anlage) of the bone (Mackie *et al.,* 2008; White

and Wallis, 2001). In the chick embryo, by 6 to 6.5 days of incubation, chondrocytes (the cells which secrete the cartilaginous matrix) in the diaphysis of the cartilage anlage of long bones (such as the tibia and femur) begin to hypertrophy, and by 6.5 to 7 days of incubation the initial development of the bone collar begins with the deposition of osteoid below the perichondrium. Mineralisation begins after around 7 to 7.5 days of incubation with the formation of lamellae which eventually fuse to form osteons that make up the compact bone of the periosteal bone collar (Hall, 1987; Nowlan *et al.,* 2008).

1.3.3. Collagen in Bone

Bone is hierarchical in structure so, to fully understand its mechanisms, composition and structure, its tissues must be studied at a variety of levels (Tzaphlidou, 2005). The mechanical properties of bone are determined, to one extent or another, by its three main constituents: mineral, water and organic material (Currey, 2003). Sommerfeldt and Rubin (2001), estimated that calcified bone consists of around 70% inorganic material, 5% water and 25% organic matrix, and that freshly synthesised matrix (before mineralisation) is around 94% collagen. Collagen is the predominant structural component of bone and correct formation of new bone, both during growth and turnover, is highly dependent upon the collagen molecules themselves, their alignment, their intermolecular cross-linking and their ability to support hydroxyapatite crystals during mineralisation (Knott *et al.,* 1995). As the mineral phase of bone is the component responsible for compressive stiffness, collagen is accountable for the mechanical properties of bone both directly through formation of matrix that provides tensile strength and through the ability of the bone to mineralise properly. Together these aspects allow bone to respond to different stresses and loading conditions and retain its hardness without becoming brittle (Knott *et al.,* 1995).

Collagen is the most abundant protein found in the extracellular matrix (ECM) of bone, and the main structural element of connective tissues (Gelse *et al.,* 2003). There are 28 types of collagen, with type I being the most common in bone (Ricard-Blum, 2011; Yue, 2014). Type I collagen is coded for by the genes *COL1A1* and *COL1A2*, and its initial synthesis in bone takes place within osteoblasts (Viguet-Carrin *et al.,* 2006). The structure of type I collagen is complex and highly hierarchical, with each new component being built upon the former components. Its primary structure consists of 5 or 6 amino acid triplets that take the form of a $(Gly\text{-}X\text{-}Y)_n$ sequence, with the residues designated as X and Y most commonly being accounted for by proline or hydroxyproline respectively. These triplets join together to form a peptide of 15 or 18 amino acids in length which then form a tight, left-handed helix known as an α-chain (sometimes referred to as preprocollagen, and can be in the form of an α1 chain or an α2 chain in type I collagen), with one end of the chain possessing an N-terminal propeptide, and the other a C-terminal propeptide (Ricard-Blum, 2011). This α-chain formation constitutes the secondary structure, but before it can progress onto the tertiary structure, it must undergo posttranslational modifications within the endoplasmic reticulum, specifically the hydroxylation of proline and lysine and the glycosylation of some lysine hydroxyl groups (Ottani *et al.,* 2002). The proline and hydroxyproline provide the polypeptide backbone with some rigidity as they possess closed ring structures that limit rotation, while the glycine that sits on every third location on the peptide occupies a very small space and allows a side chain of glycine residues to appear along the outside of the α-chain. It is the hydrogens on the α-carbon of these glycine residues that enables hydrogen bonds to form during assembly of the tertiary structure (Shoulders and Raines, 2009). It is the C-terminal propeptide that initiates the formation of the triple helix inside the rough endoplasmic reticulum, where 2 α1 chains and 1 α2 chain entwine (held together by hydrogen bonds formed between the glycine residues) to form the right-handed triple helix that characterises collagen (Mouw *et al.,* 2014). These strands of triple helix, with the N-terminal and C-terminal propeptides still attached, are known as procollagen, and it

is at this point that the molecule is packaged into vesicles and transported into the extracellular space. Once in the extracellular space, an enzyme known as procollagen peptidase cleaves the propeptides from the end of the molecule resulting in the final collagen molecule (also referred to as tropocollagen). It is from these final collagen molecules that fibrils and fibres later arise (Kruger *et al.,* 2013; Mouw *et al.,* 2014).

Type I collagen is fibril forming, and these fibrils play an important role in the architecture and rigidity of tissues (van der Rest and Garrone, 1991), and in proper skeletal development (Velleman, 2000). When the terminal propeptides where cleaved from the ends of the procollagen molecule, some terminal but non-helical portions remain attached. These are known as telopeptides, and it is these structures that give the collagen its highly reactive nature, causing it to spontaneously undergo fibrillogenesis (Ottani *et al.,* 2002). During fibrillogenesis, collagen molecules arrange themselves parallel to each other with staggered ends (they are generally displaced by around 67nm, or a quarter of their length, which gives the collagen fibrils their striated appearance) and are joined by hydrogen bonds between the terminal hydroxyproline carbonyl oxygen and the terminal hydroxyproline hydroxyl hydrogen. These segments (known as microfibrils) then stack together, with hydrogen bonds forming between the hydrogen of the hydroxyproline and the carbonyl oxygen of the glycine to form a collagen fibril (Kruger *et al.,* 2013). The diameter of the gap by which the molecules are staggered is a defining element of the collagen fibril and is referred to as the D-period. Each different type of collagen exhibits an individual D-periodicity which can be used to help identify it (Gelse *et al.,* 2003).

The monomers within microfibrils are initially joined with hydrogen bonds as mentioned above but, during maturation of the collagen fibres, aldol and aldol-histidine covalent crosslinks are formed via catalysis by lysyl oxidase (Mouw *et al.,* 2014). These mature fibrils are then bundled together to form collagen fibres. The crosslinks within the collagen fibres are immature upon formation, but slowly mature over time due to the actions of several isomers of lysyl oxidase and lysyl oxidase-like proteins. The collagen fibres do not possess full strength until maturation of all the crosslinks is complete (Viguet-Carrin *et al.,* 2006).

1.3.4. Mechanisms for assessing skeletal integrity

Bone plays an essential role in poultry production in providing a framework for supporting muscle mass and protecting vital organs (Korver *et al*., 2004). The various bone parameters that have been used for evaluating skeletal integrity include bone mineral content (Shang *et al*., 2015); bone ash concentration (Cheng and Coon, 1990); bone densitometry (Shastak *et al*., 2012); bone breaking strength (Kim *et al*., 2004; Shaw *et al*., 2010); and bone ash (Atteh and Leeson 1983; Hall *et al*., 2003). According to the review of Shastak and Rodehutscord (2013), bone ash, bone mineral content, bone strength and bone mineral density are the most useful bone criterion used in assessment of skeletal integrity. Another widely used and easily implemented mechanism for assessing skeletal integrity in poultry is gait analysis. This comprises observing the bird's movement, or lack thereof, and then rating various characteristics. These characteristics commonly include latency to lie (the length of time a bird can remain standing) and the favouring of the left or right side when moving. There are numerous techniques available to measure the various characteristics of bone, including

breaking strength, mineral composition and density, and the relative proportions of different types or structures of bone, such as the ratio of cortical:medullary bone, or the abundance of bone cells such as osteoclasts and osteoblasts or bone structures such as osteons . In the past, most methods used to assess skeletal health in poultry were minimalist, invasive and destructive. Bone ash analysis has been widely used to evaluate the skeletal integrity of poultry (Kim *et al*., 2004; Park *et al*., 2003). The ash content of various poultry bones that have been evaluated include the femur (Dickey *et al*., 2012; Hemme *et al*., 2005); toe (Yan *et al*., 2005; Karimi *et al*., 2013), tibia (Onyango *et al*., 2003, Coon *et al*., 2007; Olukosi and Fru-Nji, 2014) and feet (Garcia *et al*., 2006; Shastak, *et al*., 2012). However, the tibia is the most commonly used in evaluating bone mineralisation (Hall *et al*., 2003). The use of the middle toe was proposed as an alternative assay (Baird and MacMillan, 1942) as it reduces time and labour costs associated with traditional bone ash methods and was shown to be similarly as sensitive as tibia ash (Fritz *et al*., 1969; Potter, 1988). However, Shastak *et al*. (2012) observed it is not always clear from published literature which particular toe or joint at which the toes were removed when evaluating bone ash, which could lead to ambiguity in interpreting results. This ambiguity could be avoided by using the whole foot which provides a larger sample volume with similar ease of processing compared to the toe. The whole foot has been investigated as an alternative (Yan *et al*., 2005; Garcia and Dale, 2006) and has been shown to be as reliable as toes and tibia. Despite being one of the most well-established methods (Gillis *et al*., 1954; Nelson and Walker, 1964), the use of bones and ash content as an indicator of mineralisation has been criticised due to the lengthy and laborious preparation processes prior to ash determination (Scholey and Burton, 2017). Bone mineral content is traditionally assessed by ashing the bones and then measuring the Ca and P content via ICP-OES (Hall *et al.,* 2003), however this method requires post mortem sampling and gives no insight into the distribution of mineral within the bone.

Factors affecting bone strength in poultry include inherited genetic traits, infectious disease, ingestion of toxins, growth rate, gender, nutrition, physical activity and hormonal function and are further elaborated by Rath *et al*. (2000).

Bone breaking strength is another post mortem technique used to assess functional properties but, again, it does not provide any insight into specific composition or structure and it is susceptible to variation caused by inconsistent processing of the samples prior to testing (Lott *et al.,* 1980).

Rath *et al*. (2000) defined bone strength as the ability to endure mechanical stress, and it is related to the ultimate load or stress at which bone will break. Breaking strength is the load at break defined as the sum total of all forces and moments applied to a bone (Nigg, 2007). The degree to which a bone mineralizes is known to affect strength (Reichmann and Connor, 1977; Boivin and Meunier, 2002). Increased bone mineralisation is associated with increases in bone strength and *vice versa* (Shim *et al*., 2012). Poor bone mineralisation can increase the incidence of bone deformity and fractures thereby affecting bird welfare (González-Cerón *et al*., 2015). This comes at a cost as fragile bones are correlated with bone fragments in meat products and discoloured meat which is less appealing to consumers (Rath *et al*., 2000). The importance of maximising bone mineralisation for improved bone strength and a reduction in leg problems was noted in the study of Cheng and Coon (1990).

Rowland *et al*. (1967) examined the relationship between bone breaking strength and dietary calcium and phosphorus and found a 0.98 correlation coefficient between average tibia ash and average bone breaking strength, leading the authors to conclude bone breaking strength was as good as tibia ash in indicating phosphorus availability. Bone breaking strength has since been used by various researchers as an indicator of skeletal integrity with good reliability (Rowland *et al*., 1967; Ruff and Hughes, 1985; Sohail and Roland 1999; Coon *et al*., 2007; Rousseau *et al*.,

2012). Interestingly, Korver *et al*. (2004) reported bone breaking strength measurements *ex vivo* may not accurately reflect resistance to fracture *in vivo*. Different assay preparation procedures and instruments are known to affect results (Orban *et al*., 1993) and may explain the differences observed in published literature.

Species differences in bone strength have been reported (Rowland *et al*., 1972), while other authors (Merkley, 1981; Knowles and Broom, 1990; Fleming *et al*., 1994) have reported caged birds have significantly weaker bones compared with floor-reared birds suggesting activity has an additional influence on bone strength. Knowles *et al*. (1993) found that bone strength increased with bird weight, and the tendency of being broken during transportation and handling also increased with weight but decreased with strength. The authors however concluded the increase in bone strength due to weight was not sufficient to prevent additional damage suffered by heavier birds.

The use of invasive techniques for assessing nutrient bioavailability (e.g. bone ash and strength) requires that animals are sacrificed before any assay can be performed. There are now a variety of methods that can be used *in vivo* or, whilst still being destructive, provide a much greater volume of information than is available through the older methods (Korver, 2004). This is particularly useful in studies aimed at age-related investigation of bone development, and in breeding programmes for the identification of genetic traits linked to leg health in live birds.

The improved method of measuring mineral content of bones in humans *in vivo* by direct photon absorption techniques (Cameron and Sorenson, 1963) was adapted to measure bone mineral content in chickens (Babcock *et al*., 1965). Using this technique bone mineral content is evaluated by measuring the transmission of a mono-energetic photon through a bone; and has subsequently been used in both *in vitro* (Cantor *et al*. 1980) and *in vivo* studies (Akpe *et al*., 1987).

Radiography is a less common technique that can be combined with image analysis to provide more complex assessments of bone quality but, due to the need for birds to remain motionless, this technique is also normally conducted post mortem on excised bones using large, specialist equipment (Fleming *et al.,* 1994). However, recent technological developments have led to more widespread use of dual energy x-ray absorptiometry (DEXA), which uses more portable equipment, and has been successfully used to measure bones in live, unanaesthetised laying hens and showed a strong correlation with results from traditional techniques (Hester *et al.,* 2004).

The use of non-destructive methodologies allows the skeletal development of individual birds to be tracked throughout their life, from hatch to slaughter, rather than relying on the presumption that birds culled serially throughout the length of the trial period are representative of one another. A more developed technique, the dual energy X-ray absorptiometry (DEXA), has also been used to measure bone mineral density in meat poultry (Hester *et al*., 2004; Shang *et al*., 2015). Using this technique, Onyango *et al*. (2003) fed broilers varying calcium and phosphorus levels and reported a high correlation coefficient between bone ash, and bone mineral content or bone mineral density (0.92 and 0.93). The authors concluded it was faster than the bone ash methodology. A limitation of the dual energy X-ray absorptiometry method however is that bone mineral density is determined in 2 dimensions (g/cm2).

Quantitative computed tomography (QCT) uses image slices taken in different directions and at different angles within an object to build up a digital image that allows the spatial distribution and radiographic density of the various physical parts of the object to be accurately measured (Kalender, 2011). The QCT software can be used to build a 3D image and calculate a true measurement of bone volumetric density, and allow for the resolution of both high and low

density structures, even when they are in close proximity to each other (Korver *et al.,* 2004). It has been successfully used to measure both broiler and layer bones by numerous researchers (Jendral *et al.,* 2008; Korver *et al.,* 2004; Martnez-Cummer and Leeson, 2005; Shastak *et al.,* 2012).

Quantitative computer tomography (QCT), which measures bone density per unit volume (g/cm^3), has also been used in the study of bone mineral density in poultry (Jendral *et al*., 2008; Silversides *et al*., 2012) and provides more precise details on bone mineral density and cross-sectional images compared to DEXA measures (Kim *et al*., 2011). Shastak *et al*. (2012a) used QCT in broilers and reported tibia ash was well correlated with total bone mineral density in 3 weeks old but not in 5 weeks old broilers (r^2 = 0.78 and 0.39 respectively).

1.4. Skeletal issues in modern broilers

1.4.1. Lameness and leg weakness

One of the most significant issues in modern broilers is lameness, with some studies suggesting as many as 90% of birds display some abnormality in gait and at least 26% of birds suffered such severe abnormalities that their welfare was impinged (Kestin *et al*., 1992). The same authors also suggested that there was some evidence of a correlation between live weight and leg weakness with many suffering from conditions such as tibial dyschondroplasia and gastrocnemius tendon rupture.

Enhanced growth is largely from developments in the understanding of quantitative genetics of breeding companies, and to a lesser extent the increase in understanding of nutrition (Mebratie *et al.*, 2017). Broiler chickens are subject to intense genetic selection, which has resulted in up

to 300% increase in the slaughter weight (from 25g per day to 100g per day) of fast-growing strains (Knowles *et al.,* 2008). Selection processes favour accelerated growth, weight at slaughter, meat yield (particularly of the breast) and metabolic efficiency relating to feed conversion ratio (FCR) and body weight gain (BWG), and these parameters are under continual evaluation in order to meet commercial objectives (Zuidhof *et al.*, 2014). The success of these phenotypic attributes is a reflection of genotypic capability, yet the association with the prevalence of leg disorders is high.

The measure of performance is often by FCR, to determine the efficiency with which livestock convert feed into a desired property, in the case of broilers this is muscle yield. Aside from genotype, sex and age are also considerable factors in performance. It is known that males have increased performance over females, and maturation of both sexes will influence FCR. Hancock *et al.* (1995) concluded from an evaluation of six commercial broiler strains that during maturation there is no significant difference in growth rate between genotypes, but upon reaching mature weight, growth rate between genotypes was highly significant, indicating a difference in growth rate relating to both strain and age.

An extensive report by Havenstein *et al.* in 2003 compared the live body weight of 2001 broilers and 1957 broilers, each provided a diet with specifications for 2001 birds. The 2001 strain was nearly five times larger at days 42 and 56, showing large improvements in feed conversion, compared to the slight improvement seen in 1957 birds with a 2001 diet. The links between fast and slow growing strains have been a driver for genotypic selection, however, this increase in body weight is not seen in conjunction with an increase in skeletal size, leaving young broilers with up to 5 times the weight on their predominantly unaltered skeletal system when compared to their historic counterparts (Napolitino *et al.,* 2013).

The association between rapid weight gain and walking ability is well established (Kestin *et al.,* 1992; Knowles *et al.,* 2008; Tickle *et al.,* 2018). The most common leg deformities found in commercial broiler chickens are tibial dyschondroplasia, chronic painful lameness, chondrodystrophy or angular bone deformities, valgus-varus deformities, spondylolisthesis, rickets, femoral head necrosis, curled toes and ruptured gastrocnemius tendon (Angel, 2007). Causative agents are often multifactorial, relating to infectious, developmental and degenerative indispositions, thus treatment requires a holistic approach and prevention is often faced with difficulties.

The understanding of how genotype affects lameness is well established, with developmental and metabolic processes often impaired in fast-growing birds. Nutrient provision, rearing system and stocking density are among the most common origins of lameness. Production system heavily influences skeletal development, with arguments between organic and conventional systems producing confliction when considering economic efficiency and welfare. A 2009 assessment conducted by Brandciari *et al.* reviewed the behaviour and muscle fibre of slow-growing (Leghorn), medium-growing (Kabir) and fast-growing (Ross 208) genotypes in conventional and organic rearing systems. It showed fast and medium-growing flocks exhibited more behaviours associated with lameness in both systems. This suggests the persistence of leg abnormalities is independent of housing in birds with more rapid growth. Interestingly slow-growing breeds exhibited an increase in impaired movement in conventional housing systems only. Therefore, factors other than genetics, such as previous experience in the rearing environment, environmental conditions during embryonic development, and epigenetic effects, cannot be dismissed (Janczak *et al*., 2007; Lindqvist *et al*., 2007).

1.4.2. Causes of lameness

Factors influencing lameness are often a combination of clinical and morphological disorders. The associated pathologies of bacterial, viral and fungal infections of the bone, joint or integument are only partially elucidated (Butterworth, 1999). Detailed knowledge of morphology and nutrition are of vital importance in promoting positive leg health.

Metabolic disorders commonly associated with intensive rearing systems, and nutritional provision where efficiency considerations are at the forefront, primarily occur in two systems; cardiovascular, and musculoskeletal (Julian, 2005). Although cardiovascular disorders contribute to flock mortality, musculo-skeletal disorders have much higher incidence and a greater impact on profit and welfare loss due to the impaired growth and other health conditions with which they are commonly associated (Gocsik *et al.,* 2017). A major cause of leg problems is that modern broilers reach expected slaughter weight in approximately 36 days. With the selection for large muscle, bone development has failed to keep pace with rapid body growth, therefore failing to support the overdeveloped body, particularly relating to juvenile rapid growth rate (Bessei, 2006). Skeletal abnormalities appear to result in less severe lameness when compared to those of infectious origin; still, it is responsible for the majority of lameness in commercial flocks (Kieronczyk *et al.,* 2017).

1.4.3. Skeletal factors

The effect of enhanced growth rate and body mass on broiler anatomy and physiology requires further exploration (Tickle *et al.,* 2014). The trade-offs seen in maximising nutrient absorption and pectoral mass are often associated with the decrease in size and functioning of other organs and tissue due to the relocation of resources (Schmidt *et al.,* 2009). Perhaps the greatest trade-

offs are in the musculoskeletal system, causing both respiratory and leg health problems. The effect of skeletal impairment directly affects locomotion and gait, with the mechanisms for this highly influenced by anatomical and biomechanical traits, particularly relating to collagen and hydroxyapatite (Rath *et al.*, 2000).

The high incidences of bone defects relate to the inexact regulation of bone maturity and strength within adolescent birds. The earliest stage of bone development, chondrogenesis, involves progenitor cell specification, cell migration, epithelial-to-mesenchymal transition, and differentiation and maturation of chondrocytes (Pines and Rashef, 2015), with systemic and local hormones influencing its proficiency. Stimulation of parathyroid hormone causes osteoclast bone resorption and directly inhibits osteoblast collagen and mucopolysaccharide synthesis (Canalis *et al*., 1988).

Collagen formation requires a functioning relationship between the inorganic matrix and active living phase of bone via capillary blood vessels and lacunae in order for ossification via osteoblast proliferation to occur. Disruption of such events appears as a consistent factor for leg abnormalities that share strong nutritional links. Following collagen formation, mineralisation occurs, with the rate of mineral deposition thought to influence the normality of bone surface ossification, meaning that erosion and remodelling are required for optimum bone shape, formation and density. Consequently, the decreasing age of broilers inhibits erosion of bone surface, and the pace of bone growth in the modern broiler does not allow enough time for this remodelling and erosion to take place at an optimal rate, and so may be linked to skeletal disorders.

Lameness is one of the most commonly reported maladies seen in modern broiler birds. Some of the most commonly reported leg and musculo-skeletal disorders in broiler birds are rupture of the gastrocnemius tendon, tibial dyschondroplasia, angular bone deformities and rickets.

Tibial dyschondroplasia (TD) is characterised by abnormal changes in the cartilage mass in the proximal head of the tibiotarsus (Leeson, 2016). Some studies suggest that fast grown birds are more than twice as likely to suffer from TD than their slow grown counterparts (Leeson, 2016). TD results in decreased ossification and deficiency of minerals (such as calcium) which affect bone strength have been implicated in the prevalence of TD (Waldenstedt, 2006).

Although the reasons for these problems may be multi factorial, the overall strength of skeletal bone and surrounding tendons are likely to play a role in preventing or limiting foot and leg disorders. Morbidity and mortality from leg disorders lead to production losses (Sokale *et al*., 2013; Waldenstedt, 2006), however, welfare implications for the bird are also of concern. Danbury *et al*. (2000) demonstrated that chickens are able to experience pain, indicating that lameness (an often-painful condition) negatively affects the welfare of broiler birds and, therefore, should be a priority for poultry scientists. In recent years, there has been an influx of research into both the causes and possible methods of preventing and treating broiler lameness. Whilst there has been some positive progress in identifying the causal agents of the major lameness conditions, a completely effective method of prevention and treatment has yet to be identified.

1.4.4. Tendons

It has long been noted that the physical and mechanical properties of the skeleton are not only genetic, but also respond to the strains and stresses placed on the bones by movement and exercise (Rosa *et al.,* 2015). Tendons are responsible for the transmission of the mechanical forces caused during muscle contraction to the bone (Romero Nakagaki *et al.,* 2010), and serve

to attach muscle tissue to the bone (Koob and Summers, 2002; Moussa *et al.,* 2007). It follows that tendon strength and health should play a key role in skeletal development and integrity, as has been suggested in previous work (Riddell *et al.,* 1983).

The gastrocnemius tendon is subject to heavy stress (Hae Yoon *et al.,* 2003) and is particularly prone to rupture in broiler chickens (Riddell, 1983). Gastrocnemius tendon rupture is recognised as a relatively common cause of lameness in broilers (Sokale *et al*., 2013). Although the cause of rupture is multifactorial, including pathogenic infection and dietary deficiencies, increased growth rate and higher live body weights are considered major causal factors (Hill *et al*., 1989; Morris, 1993; Sokale *et al*., 2013; Sorensen *et al*., 1999). Clinical symptoms include lameness, swelling to the posterior surface of the tibiotarsal joint, localised haemorrhaging visible through the skin and hard masses around the tibiotarsal joint where scar tissue has been laid down (Dinev, 2012).

1.4.5. Nutritional factors effecting the musculo-skeletal system

Bone mineralisation involves the local regulation of the systemic hormone prostaglandin insulin-like growth factors, which are influenced by genetic potential, environment and nutrient provision. The skeletal afflictions of poultry can be mitigated by bone remodelling and by promoting the resorption and formation of mineralized tissue. These processes are more efficient, and issues less likely to occur, in birds fed a nutritionally appropriate diet. However, the Nutrient Requirements of Poultry (NRP), the industry accepted guide for broiler producers and breeders, has only been updated a handful of times since its original publication in 1944. The most recent update was in 1994, meaning that the abundance of nutritional research which has taken place in the last 24 years is too recent to be included in the publication that parent

flock companies use to provide nutritional advice to broiler farmers. The industry has begun to recognise this and has diverged from the 1994 recommendations for amino acids, energy and some minerals, but not for trace minerals (Applegate and Angel, 2014). Due to the vast differences in modern broilers when compared to historic strains, and the continual improvement of FCR ratios, it is not unlikely that the birds these recommendations were based on are physiologically and morphologically very different than the birds being fed these diets today. Of the major revisions made in the NRP since 1944, the majority have focussed on macronutrient provision, with some updates to the provision of essential vitamins and minerals, but no changes (or additions) have been made to reflect the growing body of work surrounding trace and ultratrace elements. These elements are presented as tentative inclusion values if mentioned at all, rather than specific requirements, and interestingly there is no mention of Si despite references to some of the original papers by Carlisle being cited in regard to bone mineralisation (Subcommittee on Poultry Nutrition, National Research Council, 1994).

Supplementation acts as a preventative measure for inadequate bone formation and leg health, otherwise caused by mineral imbalances (Waldenstedt, 2006). Nutrients of major concern include calcium (Ca) phosphorous (P) and Vitamin D, which are often discussed in tandem, as their primary role in bone formation accounts for 45% of the mineral content within adult bone (Li *et al.,* 2017). Whitehead *et al.* (2004), stressed the importance of Vitamin D as a limiting factor along with Ca and P regarding occurrence of tibial dyschondroplasia. The latter study examines the effects of vitamin D_3 with suggested µg/kg ranges per life stage for improved cortical bone quality, yet in 2014, leading feed manufacturer's specifications still advised reduced ranges that may need review (Aviagen, 2014a).

Minor nutrients, disassociated from many of the key vitamins and minerals, can also influence bone development, particularly relating to the influencing factors of thyroid hormone

metabolism. Additionally, zinc (Zn), copper (Cu), selenium (Se), fluoride (F), magnesium (Mn), and cadmium (Cd) are key associates to skeletal problems. Zinc is important in promoting live performance, with increased efficiency seen in organic or chelated supplements. It is necessary for chondrocyte differentiation and proliferation, promoting cartilage development and inducing apoptosis of epiphyseal growth plates of young chicks (Ohyama *et al.,* 1997). A significant reduction of angular defects have been seen in zinc supplemented chicks where problems of mineral absorbance at hatch were observed (Kidd *et al.,* 1992).

Selenium holds an essential role in bone formation, in relation with the aforementioned importance of thyroid hormone T3 and T4, and in chondrocyte maturation (Bassett and Williams, 2018). Proper regulation of thyroid hormone supports cartilage growth by various mechanisms such as; stimulation of resting zone cells to promote differentiation, chondrocyte hypertrophy, stimulating vascular embedding of the growth plate and metaphyseal trabecular bone formation (Oviedo-Rondón and Ferket, 2005).

Additional supplements recommended include nickel and fluoride as weight gain is observed, along with an increased bone breaking strength and bone density, due to the promotion of osteoblast activity to enhance mineralisation. Boron, along with adequate or high vitamin D_3 concentrations, has been shown to increase Ca content, resulting in stronger bones. While reduction of magnesium supplementation and exclusion of aluminium has been linked to twisting, shortening and bowing of the tibiotarsus from the reduction in osteoblast formation (Oviedo-Rondón and Ferket, 2005), limiting magnesium is recommended if the formulation of commercial broiler diet contains high levels of dolomitic (Mg rich) limestone. It must be noted that not all minerals have only positive impacts on health, and so dosage must be carefully monitored and controlled. For example, the provision of appropriate levels of copper (a mineral long known to play an important role in bone health, with deficiencies reportedly leading to

fragile, deformed bones (Rucker, 1969)) have been linked to improved collagen cross-linking formation and mineralisation (Ciuresu *et al.,* 2014), but in excess, negative impacts such as developmental issues and severe oxidative brain damage are seen (Oguz *et al.,* 2014).

1.4.6. Non-siliceous additives for lameness

As well as multiple husbandry techniques, such as light schedule manipulation (Olanrewaju *et al.,* 2006), UV light increase (Kristensen *et al.,* 2006) and feed restriction (Brickett *et al.,* 2007) which have been investigated with regards to reducing lameness, there are multiple supplements available that claim to help mitigate this issue.

Calcium supplemented at high levels has been shown to reduce lameness, and is routinely supplemented in poultry diets, but dosage and correct balance of Ca:P is essential, as over supplementation can cause decreased growth and low levels decrease growth while increasing lameness (Li *et al.,* 2017). Phosphorus when given at a lower Ca:P ratio has also been shown to reduce lameness, and is also routinely supplemented in diets, but there is a lot of conflicting evidence regarding appropriate levels, as availability varies and supply of this nutrient is expensive and limited, with over supplementation leading to negative environmental impacts (Abdel-Megeed and Tahir, 2015). Phytase is used routinely to reduce the need for additional P in poultry diets, but it has not solved the lameness issues seen in the industry.

Vitamin D_3 is supplemented in multiple formats. As cholecalciferol it has been shown to reduce rickets and increase bone strength, and is routinely added to diets, but has no effect when added in supplemental quantities when adequate levels of Ca and P are present. It can also increase incidence of some leg abnormalities if dosed incorrectly. 1,25 – Dihydroxycholecalciferol, an analogue of Vitamin D_3, has also been shown to be effective in preventing lameness, but can

slow growth rate if oversupplied (and therefore reduce profitability), and interacts with Ca in the diet leading to an oversupply that can cause hypercalcaemia (Garcia *et al.,* 2013; Kumar *et al.,* 2017). Another analogue of Vitamin D_3, 25 – Hydroxycholecalciferol, can reduce the severity of some lameness, but only when Ca levels are deficient which is an unlikely scenario in modern broiler diets (Han *et al*., 2016). 1,25 – Dihydroxy-16-ene-23yne-cholecalcferol (another Vitamin D_3 analogue) has shown positive effects *in vitro* but has shown no effects on lameness or bone mineralisation in broiler chickens *in vivo* (Farquharson *et al.*, 1996).

Ascorbic acid has been reported to prevent skeletal abnormalities in some avian species, however, it has not been shown to prevent lameness in broilers but there is limited evidence to suggest it may enhance the action of altered lighting regimes (Yildiz *et al.,* 2009) .

Vitamin B (particularly folate, biotin, B_6 and B_{12}) has been shown to reduce lameness in some cases, but only in lameness specifically caused by a Zinc deficiency (Oviedo-Rondón and Ferket, 2005).

The only currently proven method of reliably reducing lameness in commercially produced broiler chickens is by reducing growth rates, and therefore, the strain on the juvenile musculo-skeletal system. Financially, this is not an attractive solution for producers due to the loss of efficiency in a very low margin sector.

1.5. Silicon

1.5.1. Silicon as a mineral

Si is the second most abundant element in the earth's crust, making up 27.7% by weight, and the most abundant component of minerals, with only 8% of the crust being composed of non-

silicate minerals (Milone and Wilson, 2014). Naturally occurring as oxides or silicates, Si is a non-metallic element and has an atomic weight of 28 (Birchall, 1995). Silicon is highly chemically reactive and has a high affinity for oxygen. Due to this it is not generally found in its elemental form, but instead as silica and silicates, quartz, micas, opal and aluminosilicate alkalines, such as plagioclase and feldspars, which are stable in combination with clays, sands and aluminosilicates and which can only normally be broken down via extreme weathering (Exley, 1998). Although not generally found in the hydrosphere, silica broken down from its stable form via biochemical reactions during weather or plant contact may become soluble and therefore bio-available (Jugdaohsingh, 2007). Once in solution, Si from soil minerals forms soluble species by way of hydrolysis, the most stable of which is monomeric silica that is water-soluble and has a pH of 9.6. Known as monosilicic acid or orthosilicic acid, this form of Si was until recently thought to be inert within the body despite knowledge that various plants and lower organisms can utilise Si to form exoskeletons and biogenic silica. Kinrade *et al*. (2004) demonstrated that soluble silicic acid [$Si(OH)_4$] interacts with alkyl diols of sugar, forming five and six-coordinate Si complexes, which suggests that silica can indeed interact with bio-molecules.

Silicon is the most taxonomically diverse biomineral (Knoll and Kotrc, 2015). It is a commonly found, neutrally charged non-metallic element, but despite its great abundance in the Earth's crust (28%) it is rarely found in its elemental form. It appears mostly as silicon dioxide (silica) or silicate compounds due to its high affinity for oxygen. Silica (SiO_2) occurs throughout nature in many forms; but for the most part, its bio-availability is low as it is highly stable in rocks and soil minerals., Increased bio-availability is achieved by chemical and biological weathering (Jugdaohsingh, 2007).

Generally, silica follows a tetrahedral molecular structure (figure 1.7), with a central silicon atom surrounded by four oxygen atoms. Silica has three main crystalline varieties; quartz, tridymite

and cristobalite, accordingly forming a typical lattice structure of considerably strong bonds, contributing to its naturally reduced bio-availability. There are multiple water-soluble forms of silica; ortho, meta and tri-silicates, often referred to as silicic acid (Martin, 2007). Orthosilicic acid $Si(OH)_4$ is the predominantly absorbed form in the body for bone, tendon, aorta, liver and kidney functioning, through hydrolysis prior to gastrointestinal absorption.

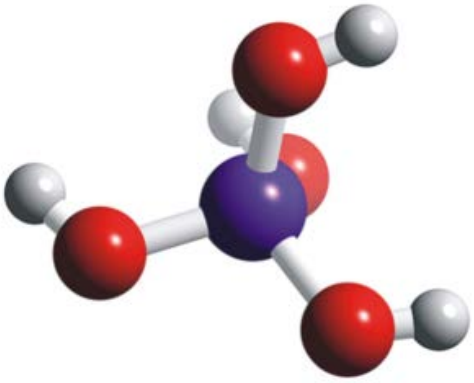

Figure 1.7. Chemical 3D structure of $Si(OH)_4$.

As the simplest and most common form, orthosilicic acid is found universally and can be taken up and utilised by all organisms, being particularly studied in silicified organisms such as diatoms, sponges and higher plants. However, the mechanism of deposition in the majority of higher organisms is largely unknown (Jugdaohsingh, 2007).

1.5.2. The biological importance of Si

The chemistry of Si dictates its bio-availability, depending on the solubility of the compound, with absorbance varying greatly (Martin, 2007). This has caused the biological role of Si to be

only partially discovered, however the known absorbance of monomeric Si within the gastrointestinal tract can be demonstrated due to its penetration of body fluids and tissues, with elevated concentrations found in bone, and the parenchymal tissues. The role of Si in bone formation is the most investigated and is central to current research, often stemming from the removal of Si from feed (Zou *et al.*, 2009; Incharoen *et al.*, 2016; Sgavioli *et al.*, 2016). From this, the essentiality of Si in the diet is well ingrained due to the negative outcomes associated with deficiency, such as abnormal skull and long-bone formation, and decreased cartilage content resulting in ankylosis. As yet formal recommendations for dietary inclusion levels of silica in commercial diets are not apparent.

The gastrointestinal tract is the main entry route of Si into the body, but the mechanisms involved in absorption, metabolism and excretion are generally poorly understood (Jugdaohsingh, 2007). It is known that absorption requires reduction into the considerably smaller, soluble orthosilicic acid, forming when porous Si is dissolved in aqueous solution. As mentioned, orthosilicic acid is the most common absorbed species, as its uncharged nature causes weak interactions within the intestine, thus increasing mobility and permeability across the mucosal layer of the intestine. The rate and efficiency of absorption will depend on concentration, as higher concentrations permit polymerisation and reduced permeability (Anderson *et al.,* 2003).

Systemic circulation inhibits Si distribution to osseous tissue (Mehard and Volcani, 1975). By its binding to glycosaminoglycans and extracellular matrix complexes, as reported by Carlisle (1984; 1986), roles in the formation of cross-links between collagen and proteoglycans are established, although the mineralisation process is unknown (Price *et al.,* 2013). It is speculated, that Si supports calcification of the bone matrix, therefore promoting bone strength and density, as its electrical potential plays a part in electrochemical mineralisation (Heinemann *et al.,* 2011).

The benefits to bone formation are well documented, particularly to osteoblast cell culture across paired tibial cartilaginous epiphyses, chondrocytes and paired frontal bones of broiler chicks (Carlisle and Alpenfels, 1984 and 1978; Carlisle and Garvey, 1982; Carlisle and Suchil, 1983). Support for Si supplementation benefits includes increased bone mineral density, reduction in bone fragility and promotion of bone resorption (Hott *et al.*, 1993). In 2009, Kim *et al.* investigated the relationship between calcium and silicon in ovariectomized rats. By administering diets; (1) Ca-deficient group (0.1% Ca); (2) Ca deficient with Si supplementation group; (3) adequate Ca group (0.5% Ca); (4) adequate Ca with Si supplementation group; (5) high Ca group (1.5% Ca), and (6) high Ca with Si supplementation group, the bone metabolism parameters relative to calcium were assessed. It was concluded that there was an increase in bone mineral density with Si supplementation, but only in calcium-deficient rats as no change was observed in the adequate or high Ca groups. This also supports the positive role of Si in bone formation, particularly when individuals are exposed to additional nutrient deficiencies that are known to cause skeletal disorders.

The importance of silicon was first established by deprivation studies of Carlisle (1972) and Schwarz and Milne (1972). As previously mentioned, preliminary results show effects are related to the formation of organic matrix components and subsequent mineralisation. In the leg bones of silicon deficient chicks, a reduced circumference, thinner cortex and decreased flexibility are seen (Carlisle, 1972). In the skull, abnormal, flattened cranial bones appear, along with nodular arrangement throughout the skeletal system.

The biochemical influences of silicon regarding bone formation ultimately affect cartilage composition and calcification of active growth sites, typically within the osteoid layer (Birdi *et al.*, 2016). This indicates a direct link to osteoblast proliferation (Shie *et al.*, 2011). Additionally, osteoclast formation and bone resorption are inhibited by silicon caused by interactions of intra

and inter-cellular signalling pathways (Mladenović *et al.*, 2013). This evidence supports the idea that orthosilicic acid directly effects osteoclastogenesis to promote ossification.

1.5.3. The absorbance and utilisation of Si by broilers

Absorption refers to the rate and method of how external molecules and atoms pass through the gastrointestinal tract to the blood, with the small intestine being the most prominent site of absorption (Goodman, 2010). Up- and down-regulatory processes are governed by homeostatic feedback control, which can be expressed by the control of intraluminal binding ligands, cell surface receptors, intracellular carrier proteins, intracellular storage proteins, or the energetics of the transmembrane transport (Pang *et al.*, 2014). Even so, there are many factors that may affect the intestinal absorption process (Said, 2011). While there is no definitive mechanism that has been confirmed for Si absorption, as a water-soluble mineral, it is likely to be similar to that of elements possessing similar properties.

Silicon holds a strong interrelationship with aluminium (Al), calcium (Ca) and molybdenum (Mo) (Jurkic *et al.*, 2013), as it is a dietary cation. At a cellular level, aluminium is toxic and associated with a plethora of pathological symptoms (Elliot and Edwards, 1991). The affinity between aluminium and silicon is uniquely high, in both solid and aqueous states. When in dilute solutions (<10-5M), the synthesis of zeolite from aluminate and silicate anions forms hydroxyaluminosilicate species that suppress Al bio-availability and toxicity (Birchall, 1992). This suggests that high levels of Aluminium could reduce Si availability, and potentially contribute to a deficiency.

Calcium forms the basis of many physiological processes and biological formations (Valable *et al.*, 2018). The separate roles of silicon and calcium in bone formation have previously been

discussed, yet when at low Ca levels, Si uptake is enhanced, suggesting that calcium and silica compete for the same absorption pathway, or that luminal calcium silicates are formed, reducing bio-availability due to its insoluble characteristics (Neilsen, 1991). This is also true for magnesium orthosilicics, suggesting an interaction between silica with both calcium and magnesium (Charnot and Perez, 1971).

There is a lack of research into the specific mechanisms of metabolism and excretion of silica, although it is widely accepted that urinary excretion is the best marker of silica absorption as direct correlations between dietary intake and excretion have been demonstrated (Jugdaohsing, 2007).

Si has many uses in human medicine, for example, Autograft™, used for human bone grafts, is silica based. Chosen for its osteoconductive, osteoproductive and osteoinductive properties (Arcos and Vallet-Regi, 2010) the Si was observed to undergo chemical reactions with sodium and unbound calcium in bone to form a scaffold on which bone is able to grow. A mesoporous silica-based antibiotic has also been developed that builds scaffolds allowing bone repair (Shi *et al.,* 2009). Since the 1970's, there have been numerous studies examining the role of Si within the body and, in particular, how silica affects bone. It has long been suspected that Si may have a role in increasing bone strength, however, results from studies examining this have been varied. Carlisle performed a number of studies in the 1970's and 1980's that found Si to be important in the development of collagenous tissue and that Si increased bone strength in poultry. However, another study by Carlisle (1980), as well as studies by Elliot and Edwards (1991) and Seaborn and Nielsen (1994), all found contradictory results. These variable results have prompted further research to try and elucidate the actual function of Si and whether or not it may be beneficial in preventing lameness in broilers.

Research supplementing poultry diets with Si (both in available, mineral and zeolite forms) in attempts to alleviate some of the skeletal disorders has begun to uncover some interesting results (Scholey *et al.,* 2018), but a clear understanding of the mechanisms by which Si acts and an effective delivery method for supplements have yet to be discovered.

1.5.4. Nutritional Requirement for Silicon in Broilers

Si was initially thought to be inert and pass through the digestive tract with little biological or toxicological impact (Nielsen, 1991), and was recommended for addition to poultry diets only under specific experimental conditions (NRC, 1977). However, bio-available Si has been implicated in a variety of important roles within the body. It potentially contributes to growth and skeletal development (Demiraslan *et al*., 2014), calcification and mineralisation (Carlisle, 1970), formation and maintenance of connective tissue (Carlisle, 1976), wound healing, the immune system and the prevention of aluminium toxicity (Carlisle, 1986; Carlisle, 1980a; Seaborn and Nielsen, 2002). Unfortunately, information regarding the specific mechanisms by which it acts are sparse and lack clarity (Perry and Keeling-Tucker, 2000). It has long been recognized that Si is an essential ultra-trace element, acting in the normal metabolism of higher animals (Carlisle, 1982; Schwarz, 1972). In studies conducted on chicks and rats it was found that Si was essential for normal development, but in particular for normal development of collagenous tissues (Stripanyakorn *et al*., 2005). It has been demonstrated that Si is actually a cellular component of connective tissue. Jugdaohsingh (2007) reported that in rats the highest levels of Si were found in bone and connective tissues such as tendons, nails and skin. It could be assumed that this pattern would be similar in other species, however, Si levels within the various tissues of the chicken have not yet been examined. Jugdaohsingh *et al*. (2008) suggested that the biochemical role of Si is its role in DNA synthesis of osteoblasts and the extracellular

matrix and having a structural function crosslinking pro-collagen during collagen production as well as bone mineralisation and prevention of aluminium toxicity. This hypothesis is partially reinforced by earlier work from Carlisle (1972) and Schwarz and Milne (1972). Both studies found Si to be important in bone and connective tissue development in rats and chickens and suggested that Si may play a role in the synthesis and/or stabilisation of the collagen matrix. Other studies reported that Si stimulates osteoblast proliferation and differentiation (Mladenovic *et al*., 2013).

Deficiencies of Si have been shown to cause a significant decrease in growth rate and weight gain in chicks. Abnormal skeletal development was also displayed, with subjects appearing to have stunted growth and malformations alongside decreased growth of the long bones (Carlisle, 1972). In three separate studies, Carlisle (1972; 1980; 1981) reported that chicks displayed skulls that were of abnormal shape and beaks that were softer and displayed less calcification. Although similar results have also been seen in other studies (Schwarz, 1972; Carlisle, 1976; Brossart *et al*., 1990; Seaborn and Nielson, 2002), results have been inconsistent, with other studies reporting no significant differences (Carlisle, 1980; Elliot and Edwards, 1991; Seaborn and Nielsen, 1994).

No immune reaction was recorded when Si was injected directly into the bone of rabbits, and excess Si has been shown to be excreted from the body via urination without harmful effect (Lai *et al*., 2002). A study on the effect of colloidal acid on the nails and skin of humans showed that the silicic acid thickened the dermis and improved nail and hair condition (Lassus, 1993). Dietary supplementation of Si has not been widely studied. This may be due to previous studies looking at the toxicity of inhaled crystalline Si and resulting silicosis in humans (Martin, 2007), and a lack of understanding of the diversity in chemical forms of Si, many of which are both non-toxic and naturally occurring. However, in the last decade there has been an increase in supplementation

of Si, possibly due to the increase in understanding of medical uses and implications of improved bone health (Martin, 2007), but also in the cosmetic supplements market due to its perceived ability to improve skin, nails and hair. A 2008 study supplementing rats with water-soluble Si, observed that femoral bone mineral density was increased (Bae *et al*., 2008). It was also shown in a different study looking at Si supplementation in cattle that calcium concentrations were increased in the muscle and tendons of the cattle that had been supplemented with Si (Turner *et al*., 2008).

There has been huge variation in the results of previous work looking at the efficacy of Si supplementation, particularly with regard to the effect on bone. This may be in part due to the method and levels of dosage, as well as the many different types and forms of supplement. The early studies of Carlisle (1972, 1976, 1980) that showed beneficial results for Si supplementation all used sodium metasilicate at a dose of either 100 or 250 mg/kg. Later studies by Elliot and Edwards (1991) used 50, 150 or 250 mg/kg of sodium metasilicate and more recently Kayongo-male and Julson (2008) used 500 mg/kg of tetraethylorthosilicicate for supplementation. This study found the most contradictory results with supplemented rats and turkeys displaying decreased bone size and strength parameters.

Alongside dosage and form of Si supplemented, the bio-availability must also be considered as the various forms of Si all vary in their bio-availability. Tetraethylorthosilicicate is hydrolysed during digestion to form monosilicic acid (Kayongo-Male and Julson, 2008). This monomeric form of Si has been shown to be more readily absorbed in the gastrointestinal tract of humans when compared to oligomeric Si, although this has not been confirmed in other species (Jugdaosingh *et al*., 2000). Other variables that may influence the possible bio-availability of Si include species, sex (Nielsen, 2008) and age (Jugdaosingh *et al*., 2000) of the animal, due to the rate of GIT development. It is possible that secondary factors may also influence the function of

Si. Spector *et al*. (2008) found that supplementing humans with Si (choline-stabilised orthosilicic acid) positively influenced the effect of vitamin D and calcium on bone density. A further study in 2006 concluded that supplementation of quail in lay with arginine silicate inositol complex (49.5% arginine, 8.2% silicon and 25% inositol) resulted in a significant increase in bone density (Sahin *et al*., 2006).

A number of Si supplements have been available to the poultry industry, however, a recent review of the available supplements concluded, "silicon supplements are of limited efficacy due to their low absorbance unless they are presented in a caustic, unpalatable form" (Jugdaosingh, 2007, Shariatmadari *et al*., 2008). The bio-availability of these supplements has also been questioned and confirmed in a study by Nottingham Trent University that found none of the supplements contained high levels of bio-available Si (Scholey *et al.,* 2018).

Recent studies at Nottingham Trent University have looked at supplementing the diets of commercial broilers with highly bio-available Si. These studies have indicated that they may benefit from additional Si via improved tibial strength. However, the role silicon plays on tendon strength has not yet been assessed, although various studies have suggested that reducing biomechanical properties of tendons has a negative impact on the overall biomechanical properties of the musculoskeletal system of the leg (Foutz *et al*., 2007), and on the biomechanical properties of bone (Ruiz-Feria *et al*., 2014). Therefore, any improvement in tendon and/or bone strength may be beneficial to the entire musculoskeletal system. The morphometrics of bone are also of consideration as the size is likely to impact the strength. Mutus *et al*. (2006) found there was a strong correlation in bone yield stress parameters (N/Kg) and bone size (particularly bone width).

1.5.5. Supplementation of Silicon

Zeolites are a source of Si that has been used with some degree of success in the broiler industry. Zeolites are porous minerals composed of hydrated aluminosilicates and can accommodate a wide variety of cations including sodium, potassium and calcium. Zeolites possess an infinite, three-dimensional crystalline structure and are able to interchangeably dehydrate and rehydrate and exchange ions without major structural change, and due to this they have been extensively used as adsorbents within numerous industries (Shariatmandari, 2008). They can be both naturally occurring or synthetically produced and vary in Si to aluminium ratio from 2.5-5:1 for naturally occurring zeolites and 1:1 for synthetic forms (Shariatmandari, 2008). The high ion exchange is associated with enhanced calcium utilisation as discussed below (Watkins and Southern, 1991). Clinoptilolite is the most common naturally occurring zeolite that has been used in the broiler industry as a feed additive (Evans, 1989), whereas Zeolite A (sodium aluminosilicate) is the most commonly used synthetic zeolite (Leach *et al*., 1990; Roland *et al*., 1993). Zeolite A has been shown to solubilise in the digestive tract and both the Si and the aluminium within the compound are partially absorbed (Roland *et al*., 1993). It has been observed that supplementation with Zeolite A has significantly increased the oral and intramuscular absorption of calcium which has decreased the severity and occurrence of tibial dyschondroplasia in broilers (Ballard and Edwards, 1988). It has also been shown that Zeolite A has been found to increase the bone ash percentage of broilers and has the secondary effect of decreasing litter moisture, thus reducing the severity and occurrence of hock and breast burn (Leach *et al*., 1990). Of the three minerals within zeolites, it has been suggested that Si is the most likely to have caused the beneficial results found (Roland *et al*., 1993; Rabon *et al*., 1995). It is likely that this is due to the zeolite selectively binding to calcium, which is partially bound to phytate. It is, therefore, thought that this improves the actions of the phytase by allowing it to release the phosphate from the phytic acid more effectively, increasing phosphorus availability

(Edwards 1988). Unfortunately, Zeolites are also high in aluminium, which has been found to be toxic, both to the bird upon ingestion, and, potentially, to the environment upon excretion. Huff *et al*. (1996) found that aluminium toxicity in broilers led to significantly reduced BWG, bone ash percentage and reduced serum phosphorus levels, and concluded that aluminium toxicity should be avoided in broilers. The amount of aluminium found in litter substrate is unlikely to be problematic.

Research regarding Si and poultry initially focussed on its inclusion in diets as a mineral, but issues with bio-availability made identification of its mode of action and, therefore, production of a viable supplement difficult (Carlisle, 1986; Carlisle, 1984). Subsequently, multiple studies investigated its addition to diets in the form of zeolites (mainly a synthetic zeolite with the chemical composition $Na_{12}[(AlO_2)12(SiO_2)12)].24H_2O$ and a natural zeolite, clinoptilolite, with the chemical composition $CaNa_4K_4(AlO_2)_5(SiO_2)_{30}.24H_2O$ (Evans, 1989)), in order to improve litter quality and feed efficiency. However, variability in results, concerns over adverse effects and problems with diet formulation raised doubts as to its usefulness as a feed additive (Shariatmadari, 2008). There is also a lack of understanding of the interactions between the various minerals contained within zeolites and those already present in the digestive tract of poultry (Watkins and Southern, 1991).

Horsetail (*Equisetum arvense*) is often used as a source of Si in human supplements but has been shown to be non-bioavailable when tested at NTU (Scholey *et al.,* 2018). Choline stabilised orthosilicic acid and colloidal silica are available as supplements and claim to be bio-available. However, when compared at NTU to the levels naturally available in tap water (approximately 13ppm), they contained only 26ppm and <10ppm respectively, making their levels of bio-available Si extremely low and unlikely to yield a biological response when consumed.

1.5.6. Developing bio-available Si supplements

The determination of silicon by absorption spectroscopy is routinely used, often referred to as silicomolybdic acid methods (Belton *et al.*, 2010). These colourimetric analyses utilise the formation of heteropolyacid yellow molybdosilicic acid, which can be used as a light absorbing species as it is; or after reduction to heteropoly blue silico-molybdenum species. Although a reputable and widely used method, it does suffer from interference from other cations, however even with its lack of selectivity, the formation of molybdosilicic acid complex is reliable to the extent that 1 silicon atom will consistently bind to 12 molybdenum atoms, therefore silica content can be assessed even in trace amounts (Basak *et al.*, 1996; Motomizu 1989). Similarly, these specific interactions allow for the determination of silicon at low concentrations, such as those found in plasma and digesta samples.

Whilst the total Si content of a sample can be quantified with relative ease, the bio-availability is more difficult to estimate due to high variability between species and indeed variability within different samples of the same ingredient. Dietary Si is found in tap water, although levels vary by geographic location (Jugdaohsingh, 2007). It can also be found in lower levels in a wide range of foodstuffs including fruits, vegetables and grains (Jugdaohsingh *et al*., 2002). The bio-availability of silica ingested by humans is considered to be around 40%, however, the most bio-available Si is considered to be in fluid form, from sources such as water and beer.

Orthosilicic acid, the fundamental building block of biosilicas, is thought to be readily absorbed from the small intestine as its small molecular size and lack of charge allow it to pass easily through the mucosal layer of the gastro-intestinal tract (Rabon *et al.,* 1995). Transit time for food in chickens can vary depending on intake rate but in general when fed *ad libitum* the retention time in the proventriculus and gizzard is about 2 hours (the crop is generally bypassed altogether) and the middle of the duodenum is reached after around another 0.5 hours. The

ileum will be reached in around 5 – 6 hours (Svihus, 2014). By the time the digesta moves into the ileum the vast majority of digestion and absorption is complete, so the rate of release of orthosilicic acid from feed or supplements is critical. Orthosilicic acid is water soluble and weakly alkaline (pK_a 9.8) (Iler, 1979; Perry, 2009) but at neutral pH and concentrations greater than 2 mM, the monomer readily condenses to form insoluble polymers which eventually aggregate to form amorphous gel precipitates (Iler, 1979; Perry, 2009). This precipitation and the increased molecular size and charge reduce its ability to pass through the mucus layer of the gastrointestinal tract and hence decreases its bio-availability (Jugdaohsingh, 2007). Recent investigations in humans indicate a synthetic analogue of orthosilicic acid, monomethylsilanetriol, a monomeric, organosilicon molecule [$Si(OH)_3CH_3$] appears to be a non-toxic form of silicon that retains monomeric form in solution, but there is uncertainty over the *in vivo* biological capacity for cleavage of the Si-CH_3 bond, which may limit bioconversion to the putative bioactive form, $Si(OH)_3OH$ (Pruksa, 2014).

In vitro studies investigating collagen synthesis through culturing of human osteoblast-like cells show physiological concentrations of orthosilicic acid, the monomeric form of silica, increase collagen type 1 synthesis (Reffitt *et al*., 1999), with smaller increases also seen in skin fibroblast cells. While these studies reinforce the idea that silicon may lead to improved skeletal integrity, this cannot be explored further until a form of silicon is produced that is bio-available, non-toxic and affordable. The aim of this study was to determine the bio-availability and potential efficacy of a newly developed (Belton and Perry, 2016), pH neutral form of silicon supplement using meat-type poultry as a model to observe skeletal effects.

The potential for silicon supplementation to reduce the incidence of poultry lameness has been considered previously (Lynch *et al.,* 1992), but difficulties with presenting the silicon in a form which is both bio-available and non-toxic means these have shown limited progress to date

(Shariatmadari, 2008). In animal studies, synthetic silicon forms such as zeolites, alkoxy silanes, amorphous powders and highly caustic/acidic preparations have been adopted as a silicon source, with varying results (Ballard and Edwards, 1988; Evans, 1989; Leach *et al.,* 1990; Eliott and Edwards, 1991). The contrasting reported conclusions on the efficacy of silicon supplementation in reducing the incidence and severity of lameness may be due to differing physico-chemical composition and properties of different silicon supplements, which were not fully described in the published studies. Silicon (no source description) supplemented via the water available to broiler chickens has been shown to have no significant effect on bone breaking strength and bone density but did result in a change in the mineral profile of the bones with increases in phosphorus, zinc, copper, manganese and ash being observed (Sgavioli *et al*., 2016).

Its bio-availability refers to the amorphous silicon form (H_4SiO_4) releasing trace amounts of silicon in the gastrointestinal tract during contact with water and physiological fluids at neutral pH. However, the rate at which Si dissolves depends on phase and surface area (Jurkic *et al.,* 2013; Birchall, 1995). The dissolution and deposition of silica in water can be expressed as:

$$\mathbf{(SiO_2) + 2H_2O \leftrightarrow (SiO_2) + Si(OH)_4}$$

Reduction in bio-availability is presented when the silicon is unstable and polymerized, therefore not only potentially affecting biological parameters negatively but causing health implications (Calomme and Vanden Berghe, 1997; Perry and Keeling- Tucker, 2000).

The soluble and bio-available form of Si, the monomer orthosilicic acid ($Si(OH)_4$), is found universally but in very low concentrations of <100 ppm and attempts to increase this concentration for dietary addition leads to the Si monomer undergoing autopolymerisation and the formation of insoluble, and therefore predominantly non-bio-available, polymers (Perry and

Keeling-Tucker, 2000). Chemists at NTU have produced a bio-available form of Si that is combined with a quaternary ammonium compound and/or a group IA hydroxide and an organic acid (Perry and Belton, 2009), enabling it to be incorporated into diet formulations at a predictable dosage and non-caustic pH and utilised by poultry. The resulting increase in Si absorption and utilisation should provide a better opportunity for understanding the mechanisms and functions of Si as an essential, ultratrace element and allow for a commercially viable supplement to be produced that can improve welfare and production of poultry.

1.6. Aims and objectives

The overarching aim of this study was to assess the effects of adding a bio-available Si supplement to the feed of broilers on their skeletal integrity. For the purposes of this study, the term skeletal integrity was defined as the ability of the bones and joints to properly support the weight of the carcass while allowing the bird to move freely and without discomfort. The objectives set out to meet this aim were:

1. Optimisation of the delivery method of the Si supplement, both in terms of dosage and particle characteristics.
2. Investigation of the potential site of absorption of the Si supplement within the chicken gastrointestinal tract.
3. Evaluation of the effects of the Si supplement on the gross morphology and mechanical characteristics of the leg bones and tendons of meat type chickens.
4. Investigation into the effects of the Si supplement upon the presence of biomarkers and bone cells associated with bone growth and turnover.
5. To examine the effects of time and temperature on the bio-availability of the Si supplement, both alone and once incorporated into feed, when different sources of raw materials are used.

Chapter 2. Materials and methods

2.1. Introduction

This chapter provides an account of the general materials and methods employed throughout the studies featured in this thesis. Three bird (feeding) trials and one supplement development trials were completed (details in table 2.1). Trial 1 observed the effects of supplemented Si from various sources to establish the most effective supplement to use in future trials. Trial 2 compared a high dose of the most effective Si supplement from trial 1 to a control to enable a clear comparison between supplemented and non-supplemented birds. Trial 3 observed the effect of different doses of the same Si supplement as trial 2, to try and establish an optimal inclusion level of the supplement.

Table 2.1. Outline of the trials conducted as part of this thesis, which chapter they can be found in, and their key focus.

Trial	Chapter	Focus
Supplement development trial 1	3	Effects of manufacturing method on particle size and investigation of key properties of a variety of particle sizes of the NTU supplement.
Feeding trial 1	4	A comparison of a variety of commercial supplements with the NTU supplement, when manufactured at two frequencies.
Feeding trial 2	5	The effects of the NTU supplement at a variety of dosages, compared to both an un-supplemented diet and a diet supplemented with sand.
Feeding trial 3	6	Comparison of a supplemented and non-supplemented diet to assess mechanisms of action and absorption in broiler chicks at a young age.
Supplement development trial 2	7	Effects of storage conditions and time on the stability of the NTU supplement when produced in a different manner.

2.2. Birds and husbandry

Institutional and national guidelines for the care and use of animals (Animal Scientific Procedures Act, 1986) were followed and all experimental procedures involving animals were approved by the School of Animal, Rural and Environmental Sciences Ethical Review Group. All bird trials used Ross 308, male broiler chicks, supplied within 24 hours of hatching by PD Hook, Cote Hatchery, Oxfordshire. Birds used in the trials were within the weight range of 38-45g and were from breeder flocks aged between 40-45 weeks. Birds were weighed using dynamic weighing which measured the average weight over a period of 3 seconds (Mettler Toledo International). The chicks were randomised by weight and housed in preheated 0.64m^2 pens in a purpose built, insulated poultry house. The birds were bedded on clean wood shavings (approximately 3cm) and fresh shavings were added into the pens as required. Birds were always allowed *ad libitum* access to the treatment diets and water for the duration of the trial. Commercial guidelines for the care and husbandry of Ross 308 broilers were followed in all studies (Aviagen, 2007). The room was thermostatically controlled to produce an initial temperature of 32°C reduced to 21°C by day 21 using heating fans and supplementary heat lamps. The lighting regimen used was 24 hours light on d1, with darkness increasing by 1 hour a day until 6 hours of darkness was reached and this was maintained throughout the remainder of the study. Birds were checked twice daily to monitor the environmental conditions; heating and ventilation were adjusted accordingly. Any mortalities were recorded along with the date and weight of the bird and reason if culled. All birds sampled were euthanised by cervical dislocation as determined by DEFRA (DEFRA, 2007) and the Animal Scientific Procedures Act (ASPA, 1986).

2.3. Diet formulation

2.3.1. Basal diet manufacture

All trial diets were manufactured on site and fed as mash. The particle size of each diet was uniform, consistent and typical for broiler diets, averaging at approximately 1mm. The composition and analysis of all the trial diets are detailed in the corresponding chapter. When making the diets, each ingredient was individually weighed out and mixed dry for five minutes in a ribbon mixer (Rigal Bennett, Goole, UK) before addition of oil. The diets were then mixed for a further five minutes. The mixer was brushed down at various stages throughout the mixing process to ensure oil clumps were removed. Titanium dioxide (TiO_2) was carefully incorporated into every diet as an inert marker. It was added at 5g/kg to ensure there was sufficient TiO_2 in the digesta samples to determine diet digestibility and was mixed with a small amount of the dry mix prior to inclusion to ensure homogeneity. For each diet the TiO_2 level was analysed. In all studies, diets were randomly allocated to pens within the room, to eliminate any effect of room position. A grab sample was taken during the feed weighing prior to the trial to allow for proximate analysis at a later date. Diets were weighed into bags (new individual bags for each feeding phase; starter, grower and finisher) for each pen to allow intake to be measured. Bags were topped up with feed as required and added feed weights recorded.

2.3.2. Si supplement manufacture

For the purposes of this study, the patent currently filed for this supplement (Belton and Perry, 2016) was used as a guide for manufacture. The group IA silicon salt used is sodium metasilicate in a molar ratio of Na_2SiO_3. Carboxylate groups on the organic acid is 1:2. Citric acid was added in appropriate volumes to make a pH neutral solution, which is a ratio of 1:1.05 Na_2SiO_3 : Citric acid. These components were ground to a homogenous powder using a planetary ball mill

(Across International, NJ, United States), further discussed in section 2.5.12, and mixed together. The supplement for addition to diets requires oil as a carrier. In order to ensure its even distribution in the diet, and to avoid altering the macronutrient profile of the diets, a portion of the soya oil measured for use in the diet was removed and had the Si supplement added at the appropriate rate of inclusion. To ensure the Si supplement was well dispersed within the oil, it was agitated vigorously until evenly suspended, with no visible clumps. The Si enriched soya oil was added to the diet at the same time as the rest of the soya oil and mixed thoroughly in the ribbon mixer as described above.

2.4. Trial period

2.4.1. Feed intake

Each pen of chicks was fed exclusively from an individual experimental bag of diet that was pre-weighed prior to the trial. Any additional feed was weighed into the bags, and weight was recorded. Troughs were positioned horizontally to minimise spillage. On sampling days remaining feed in the trough and bag, and any spilt feed if able to be collected, were weighed. In Trials 1 and 3 feed intake was measured on day 7, 14, 21, 28 and 35. In trial 2 feed intake was measured on d7, 10, 14, 17 and 21. Feed intake was measured as total intake per pen then the average amount consumed per bird calculated.

2.4.2. Bird weights

For all bird trials, chicks were weighed on arrival, and any outside the range of 38-45g were not included in the trial. Birds were distributed into pens based on average weight per pen, ensuring there were no significant differences in starting pen weight between dietary treatments. In Trial

1 and 3 birds were weighed on day 7, 14, 21, 28 and 35. On trial 2 birds were weighed on d7, 10, 14, 17 and 21. Bird weights were measured by weighing the whole pen, and then calculating the average bird weight, unless stated in the specific trial methodology. The increase in average bird weight was used, alongside the average feed intake value, to calculate the average feed conversion ratio (FCR) per pen.

2.4.3. Digesta sample collection

On days requiring digesta sample collection, birds were sequentially fed at timed intervals, ensuring each bird had a minimum of 1 hour feeding prior to being euthanised, to ensure sufficient gut fill. Birds were euthanised in a separate room via cervical dislocation by trained persons. The gizzard was removed and sliced open, and contents were gently scraped into a pot. The duodenal loop (referred to as the duodenum) and the area of the tract from the duodenal loop to the Meckel's Diverticulum, referred to as the jejunum (proximal small intestine), were removed and digesta collected by gentle digital pressure along the piece of tract, to avoid disrupting the mucosal lining. The ileum (distal small intestine) was categorised running from Meckel's Diverticulum to the ileal-caecal-colonic junction. Digesta samples were collected into labelled pots; for all trials, digesta samples were pooled into one pot per pen/plot for each section of the tract. Digesta samples were weighed and immediately frozen and then freeze dried (LTE Scientific, UK) for 5 days. Once the samples were dried the pot was reweighed so that digesta moisture content could be determined. The samples were then ground to fit through a 1mm screen and mixed to ensure homogeneity.

2.4.4. Tibia and femur collection

Tibia bones were separated at the tibiotarsal junction, where the feet were removed, and the tibiofemoral junction. Femur bones were separated at the tibiofemoral junction and the hip. Care was taken to ensure there was operator consistency with bone removal. Both the left and right tibia and femur from at least 2 birds per pen were collected and put in labelled bags per pen per bone. Details on the analysis conducted on these bones can be found in the appropriate sections, and specific numbers of bones collected detailed in the individual trial chapters.

2.4.5. Blood plasma collection

Post mortem blood samples were collected immediately post euthanasia into EDTA coated tubes from 2 birds per pen/plot. Samples were a centrifuged at 3000rpm for 5 minutes to separate the plasma which was collected and stored at -20^{o}C for analysis of Si content and ELISA analysis for the presence of biomarkers.

2.4.6. Tendon collection

The right, distal gastrocnemius tendon was removed from one bird per plot from trial 2. Dissected tendons were placed in labelled bags and frozen at -20°C until analysis could take place. Tendon moisture was maintained by immersion in 0.9% NaCl solution prior to freezing.

2.5. Analytical procedures

2.5.1. Dry matter determination of feed

Dry matter content of the diet was analysed by accurately weighing approximately 5-10g of finely ground sample into pre-weighed crucibles. The crucibles were then dried in a drying oven set at 105°C for approximately 4 days, until a constant weight was reached. The dried samples were cooled in a desiccator and reweighed. Digesta dry matter content was analysed by weighing the digesta samples immediately after collection, freezing them, then freeze-drying them to a constant weight in a Lyotrap freeze drier (LTE Scientific, Oldham, UK). The samples were reweighed once dried, after approximately 5 days in the freeze drier, or until completely dry.

2.5.2. Ash determination in feed and digesta

Ash content of diet and digesta was analysed by accurately weighing approximately 2-5g of sample into a pre-weighed ceramic crucible. The crucibles were then placed in a muffle furnace (Nabertherm, B180) for on a program that brought them from room temperature up to 650°C over a two-hour period, then maintained them for 14 hours at 650°C, before automatically shutting off and allowing them to cool back to room temperature. The ashed samples were then cooled in a desiccator and reweighed.

2.5.3. Titanium dioxide determination

Titanium dioxide (TiO_2) was added into all diets as an inert marker at an inclusion rate of 5g/kg. It was measured in the diets and digesta by the UV-spectrometry method developed by Short *et al*. (1996). TiO_2 standards (0.5mg/ml) were prepared prior to analysis by dissolving 250mg of TiO_2 in 100ml of H_2SO_4 (Fisher Scientific, UK) and bringing the solution up to a volume of 500ml

with distilled water. 100ml volumetric flasks were labelled and 1-10ml of TiO_2 solution was added to each flask. Concentrated H_2SO_4 was then added to each flask to reach a combined volume of 10ml, followed by 10ml of 30% hydrogen peroxide (Fisher Scientific, UK). The flasks were then brought to volume with distilled water and the solutions were stored in glass vials in darkness.

0.3-0.5g of feed or freeze dried digesta was weighed into ceramic crucibles in duplicate and ashed in a muffle furnace (Nabertherm, B180) set at 650°C for approximately 14 hours. Once cooled, 10ml of 7.4M H_2SO_4 was pipetted into each crucible and they were heated using a hotplate until the sample had completely dissolved (approximately 2 hours); 5ml extra acid was added if required and re-heated to ensure complete dissolution of the sample. Once cooled, the sample was quantitatively transferred into a 125ml beaker and then filtered through Whatman 541 hardened, ashless filter papers into 100ml volumetric flasks. 10ml of 30% hydrogen peroxide was added to each volumetric flask, and the flasks were brought to volume with distilled water and mixed. Absorbance of the samples and standards was measured on a UV spectrophotometer (Unicam Helios, USA) set to 410nm. The coefficient used to determine TiO_2 concentration was derived from the regression analysis of the standard curve. The amount of TiO_2/mg in the solutions was calculated by:

$$\frac{\text{Absorbance}*100}{\text{Coefficient}*\text{sample weight (mg)}}$$

2.5.4. Gross energy

Gross energy (GE) of the feed and excreta was measured using a bomb calorimeter (Instrument 1261, Parr Instruments, Illinois, USA) (Rutherfurd *et al*. 2007; Woyengo *et al*., 2010). Pellets of feed and excreta sample, weighing approximately 1g, were made by adding a small amount of water to the sample before pelleting it with a pellet press (Parr Instruments, USA). The pellets were dried overnight at 105°C, before being weighed into tin crucibles (Sartorius CP1245) and

placed in the bomb. The bucket in the bomb jacket was filled with 2 liters of water. 10cm of fuse wire was threaded through the holes in the bomb which the electrodes attach, ensuring the wire touched the pellet. The bomb was then assembled, ensuring the top was tightly screwed on, and then filled with oxygen. Once filled, the bomb was put into the bucket of water, the electrodes were pushed into the bomb, and the lid of the bomb jacket was shut. Sample weight was entered, and the process was started; the calorimeter measures the energy produced (in MJ/kg) when the pellet is exploded.

2.5.5. Tibia and femur ash determination

Tibia and femur bones had the flesh and connective tissue removed manually with scalpels before drying at 110°C for approximately 4 days. Bones had fat removed by the Soxhlet method (AOAC official method 2003.05). The bone was placed into an extraction thimble. A flat-bottomed flask, containing a small amount of anti-bumping granules was accurately weighed, had 150ml of petroleum ether (grade 40/60) added. The thimble was inserted into the bottom of the distillation unit, the distillation apparatus was connected to the condenser and the flask was attached to the apparatus and seated in the heating mantle (set to 40-60°C). The samples were left to extract for approximately 18 hours, then the remaining ether was boiled off on a hotplate and left to evaporate overnight. The dried and de-fatted bones were then weighed into pre-weighed ceramic crucibles and ashed for approximately 14 hours at 650°C (Hall *et al*. 2003). The crucibles were then left to cool in a desiccator and reweighed, so ash content could be derived. Bone ash was calculated as ash weight as a percentage of dry bone weight.

2.5.6. Tibia and femur bone strength

Bone strength of the tibia and femur was analysed using a TA.XT plus texture analyser (Stable Microsystems, Guildford, UK) set up with a 50kg load cell and 3 point-bend fixture (Park *et al*.

2003, Taylor *et al*. 2003; Shaw *et al*. 2010). Firstly, the bones were defleshed of muscle and tissue by hand using a scalpel. The length and width (measured at the central point along the length of the bone) of each bone was measured using digital calipers and recorded. The texture analyser was set to measure force in compression. Test speed was set at 1mm/sec with trigger force set at 7g (0.069N). Supports of the fixture were set at an appropriate distance apart to accommodate for the length of the bones, so that the bones rested on the vertical supports where the metaphysis met the diaphysis at each end. The texture analyser was calibrated using a 5kg weight. The defleshed bone was placed on the fixtures, a test was run and the peak force in Newtons was recorded.

2.5.7. Tendon strength

A pilot study was conducted to ensure optimal methodology for testing tendon tensile strength (appendix I).

Tendons were wrapped at each end so that a 10mm section was bound with Henry Schein Spool Suture Supramid suture material (figure 2.1), to prevent damage to the tendon ends by the tensile grips and to allow the grips to increase hold on the tendon to prevent slippage.

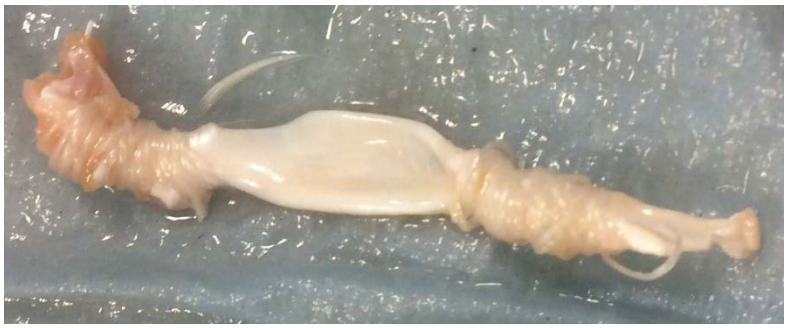

Figure 2.1. The ends of a gastrocnemius tendon wrapped in suture material.

Tensile strength of the tendons were analysed using a TA.XT plus texture analyser (Stable Microsystems, Guildford, UK) set up with a 50KG load cell and a set of compatible tensile grips, as advised by the manufacturer (figure 2.2). The texture analyser was set up to quantify force to

rupture and, prior to use, it was calibrated with a 5kg weight. The test speed was set to 1mm per second and a trigger force of 7g (0.069N) was applied. The tensile grips were lined with helicopter servo tape (a padded double-sided tape used for model helicopters) and lined with 240 grit sandpaper. This method was found to be optimal in reducing slippage, and tendon damage that could confound the results, following the pilot study. The texture analyser was set to measure the force (N) applied to the tendon until failure.

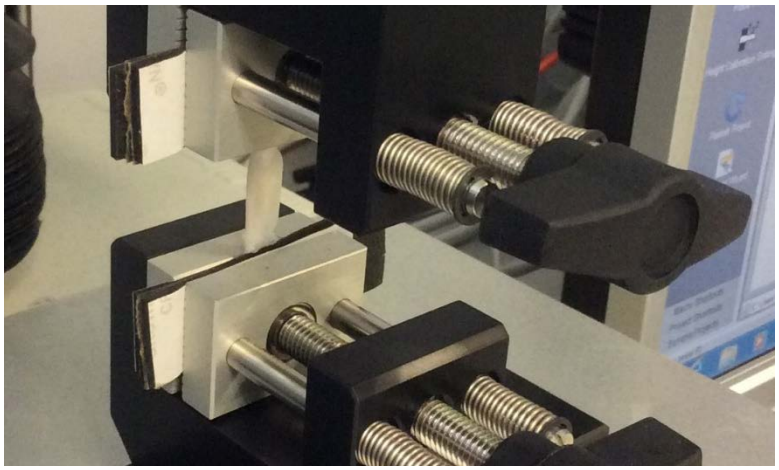

Figure 2.2. The grips of the texture analyser lined with servo tape and sandpaper, holding the suture material wrapped gastrocnemius tendon.

The same settings were used for all tendon tensile strength testing (show in figure 2.3). After mounting in the texture analyser, but before testing, digital callipers and a 4 d.p. balance were used to take width, length and weight measurements of the tendons, as well as cross sectional diameter. After testing, the maximum force (N) applied to the tendon was recorded, along with the time (seconds) taken for the tendon to fail and the distance (mm) stretched before failure (see figure 2.4 for measurements taken).

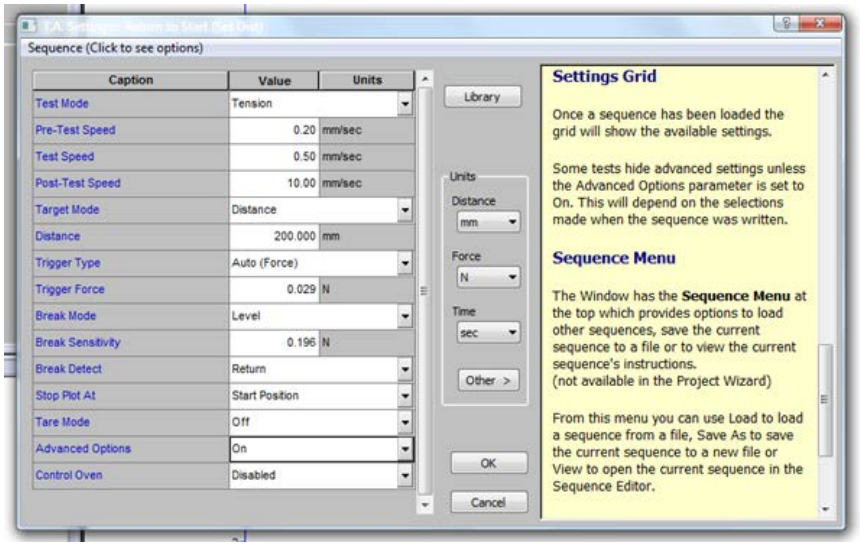

Figure 2.3. Settings of the texture analyser used to test tensile strength of gastrocnemius tendons.

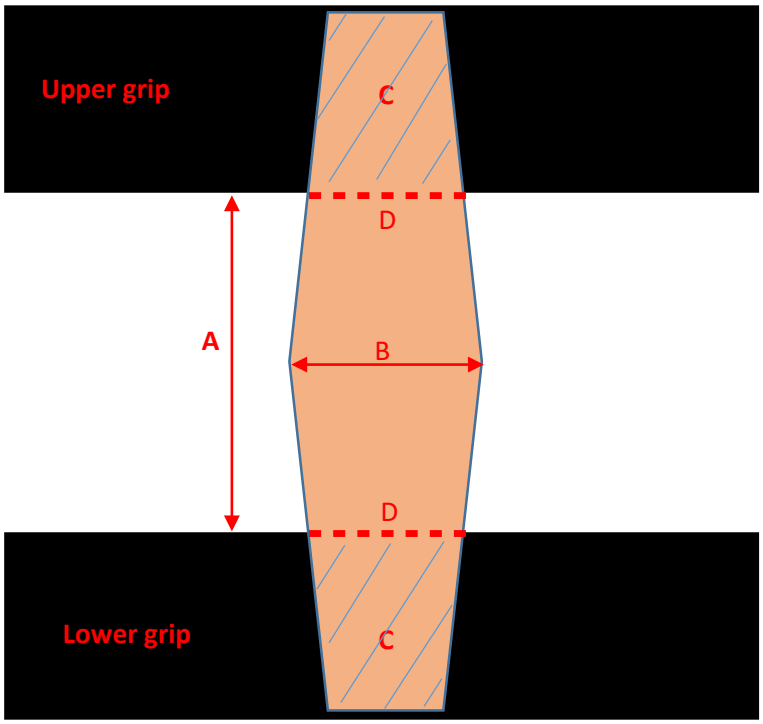

Figure 2.4. The tendon was mounted between the upper and lower grips, with the proximal end being fixed in the upper grip, and the distal end being fixed in the lower grip. Before tensile testing the section visible between the grips was measured (arrow A) along with the thickest point (not shown) and the widest point (arrow B). After tensile testing the damage to the sections of tendon within the grips (shaded area C) was scored, along with the damage at the pinch point (dashed line D).

2.5.8. Inductively coupled plasma - optical emission spectroscopy (ICP-OES) determination of calcium, phosphorus and silicon

Diet, digesta, tibia and femur ash were analysed for silicon (Si), calcium (Ca) and phosphorus (P) by Inductively Coupled Plasma mass spectroscopy with Optical Emission Spectrometry (ICP-OES) (ICP-MS model PQ Excell, VG Elemental, USA). Prior to the assay, all glassware was acid washed for a minimum of 12 hours, rinsed with ultra-pure water and dried, to ensure there was no cross contamination. Approximately 0.5g of sample was weighed in duplicate into 50ml conical flasks. The samples were then incubated for a minimum of 16 hours with 10ml of aqua regia (1 part nitric acid and 3 parts hydrochloric acid) before heating until dissolved (approximately 90 minutes) in a fume cupboard. If necessary, an extra 5ml of aqua regia was added and an additional 30 minutes of heating was carried out to ensure complete dissolution. One blank flask containing just aqua regia was prepared for each 5 sample. The samples were then cooled before the flask contents were diluted with ultra-pure water and filtered into 50ml volumetric flasks through Whatman 541 hardened, ashless filter papers. The volumetric flasks were then brought to volume with ultra-pure water, and the contents were mixed and transferred into 15ml, duplicate tubes per sample. ICP-OES standards were prepared with differing levels of Ca, P and Si (dependent on the predicted levels of the sample being analysed) using 1000ppm ICP-OES grade standards (Fisher Scientific, Loughborough, UK) diluted in ultra-pure water. The samples were analysed on the ICP-OES, set to analyse Ca at wavelength 317.933nm, P at wavelength 213.617nm and Si at wavelength 251.611nm. The readings on the ICP-OES are presented as concentration in mg/L; and the following equation used to convert to g/kg:

(Ca, P or Si in sample (mg/L))*(volume of sample (ml)/weight of sample (g))/1000

2.5.9. Crude protein determination

Samples of diet and digesta were analysed for nitrogen content using the Kjeldahl method (AOAC official method 2001.11) (Tahir *et al*. 2012, Pintar *et al*. 2005; Peter and Baker 2001). Approximately 1g of sample was accurately weighed into distillation tubes (Foss Cat No. 10000155) in duplicate. Both a copper and selenium catalyst tablet (Fisher Scientific, UK) was added to each tube. 12.5ml of concentrated nitrogen-free sulphuric acid was then added to each tube, and they were heated in a digestion unit (1007 Digester, Foss Tecator, UK) set at 450°C. for 45 minutes. Once digestion was complete, the distillation tubes were left to cool for a minimum of 20 minutes and 75ml of distilled water was added to each tube. The tubes were then distilled in a distillation unit (2100 Kjeltec, Foss Tecator, Cheshire, UK) which added 50ml of 10M sodium hydroxide to the samples, distilled them for 3 minutes, then expelled the resulting ammonia into conical flasks containing 2ml 4% boric acid with indicator, causing a colour change from orange to blue. The boric acid was then titrated back to original colour using 0.1M HCl in a burette and the volume of acid used was recorded. Starch was used as a blank. % nitrogen was calculated by:

$1.4 \times (V1-V2) \times M / W$

where:

W= Original weight of sample

V1= Volume of acid to titrate sample

V2= Volume of acid to titrate blank

M=Molarity of acid

6.25 x % Nitrogen = % crude protein

2.5.10. Extractable fat determination

Samples of diets were analysed for extractable fat content by the Soxhlet method (AOAC official method 2003.05). Approximately 5g of sample was accurately weighed into an extraction thimble. A flat bottomed flask containing a small amount of anti-bumping granules was accurately weighed. 150ml of petroleum ether (grade 40/60) was added to the flask. The thimble was inserted into the bottom of the distillation unit, the distillation apparatus was connected to the condenser and the flask was attached to the apparatus and seated in the heating mantle (set to 40-60°C). The samples were left to extract for approximately 18 hours, then the remaining ether was boiled off on a hotplate and left to evaporate overnight. The flasks were reweighed, and extractable fat content was calculated by:

M_2-M_1 / M_0 x 100 = % extractable fat

where:

M_0=Original weight of sample

M_1= Flask plus anti-bumping granules

M_2=Flask plus fat and anti-bumping granules

2.5.11. Biomarker analysis

A chicken procollagen type I N-Terminal Propeptide (PINP) ELISA kit (MyBioSource Inc., CA, USA) was used to analyse blood plasma samples. The sensitivity of the kit is 1.0 ng/ml, with the detection range being 6.25 ng/ml - 200 ng/ml.

Plasma was collected as detailed in section 2.4.5, and the wash solution (1x) was prepared, as per the kit instructions, by diluting one volume of wash solution (20x) with nineteen volumes of distilled water.

All reagents and samples were brought to room temperature (18°C - 25°C) naturally for 30min before starting assay procedures. 50µl of standard was added to each standard well, and 50µl of sample added to each sample well, with 50µl of sample diluent being added to each blank/control well. All standards, samples and sample diluent were added in duplicate to the plate. 100µl of HRP-conjugate reagent was then added to each well, and the plate covered with a closure plate membrane. The plate was incubated for 60 minutes at 37°C, then washed 4 times by dumping the incubation mixtures of the wells into a sink or proper waste container then using a squirt bottle to fill each well completely with wash solution (1×), and after about one minute's standing, inverting and hitting the plate onto absorbent paper towels until no moisture appeared. Chromogen solution A (50µl) and chromogen solution B (50µl), were added to each well successively. Then the plate was protected from light by wrapping in aluminium foil and incubated for 15 minutes at 37°C. 50µl of stop solution was added to each well, and the optical density read at 450nm using an ELISA plate reader within 15 minutes. Results were calculated by averaging the duplicate readings for each standard and sample then subtracting the average optical density of the blank/control ($V_{B/C}$). Standards were then plotted on a standard curve and used to determine the concentration of PINP present in the samples.

2.5.12. Milling

The mill used in this study was the PQ-N2 series planetary ball mill (Across International, NJ, United States). It was supplied with 4x 500ml capacity tungsten carbide milling jars and 4x sets of tungsten carbide milling balls in the following diameters: 6mm, 10mm and 20mm (Across International, NJ, United States). Tungsten carbide was chosen due to its high resistance to wear as Na_2SiO_3 has previously proven to be highly abrasive during milling. For all milling, the factory default settings were adhered to with the only alterations being the diameter of the milling balls used. A protocol for using the mill can be found in appendix II.

2.5.13. Determination of particle size

2.5.13.1. Sieving

Milled Na_2SiO_3 was placed into the top of a stack of test sieves (Endecotts, UK) with the following aperture sizes: 50µm, 80µm, 100µm, 200µm, 250µm, 300µm and 400µm. The lid was firmly affixed, and the sieves placed onto an automated sieve shaker (Sieve Shaker MINOR 200, Endecotts, UK) for 10 minutes. The particles remaining in each layer of the sieve were weighed.

This method is only viable for larger particle sizes (>50µm) as, due to the nature of the equipment, the Na_2SiO_3 suffers considerable exposure to moisture in the air. This causes large aggregates to form which are unable to pass through the sieves, and therefore prevents smaller particles from being accurately measured.

2.5.13.2. Scanning electron microscopy (SEM)

For Na_2SiO_3 particles that were too small to be measured with the test sieves, SEM was used. Samples of Na_2SiO_3 were distributed onto double-sided sticky tape and mounted on aluminium stubs. All loose aggregates were removed by tapping the stub before gold coating with an argon plasma at 1.2 kV and 4 mbar pressure for 2 minutes using an Edwards S150B sputter coater. Images were acquired using a JEOL JSM-840A scanning electron microscope with an accelerating voltage of 20 kV. Images were taken at a magnification of x3000. Image J software was used to measure primary particles. See figure 2.5 for an example of SEM images.

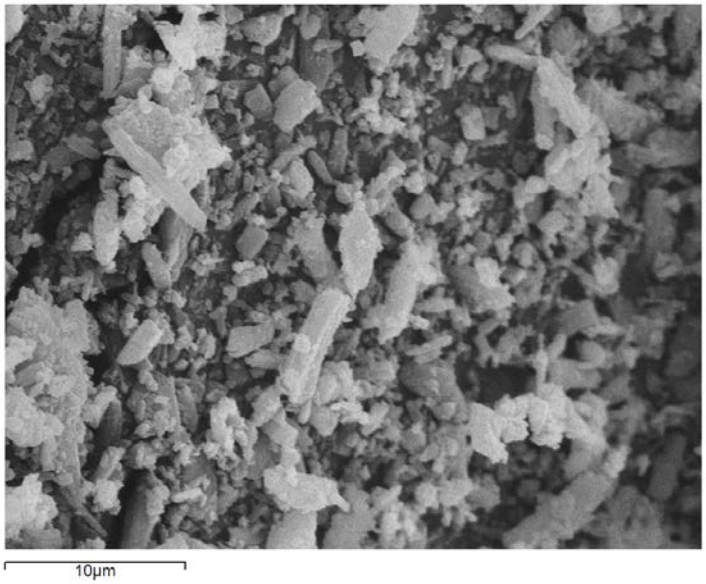

Figure 2.5. Example of an image acquired via SEM of the Na_2SiO_3 at a magnification of x3000.

2.5.13.3. Dynamic light scattering (DLS)

Due to the time-consuming nature of SEM particle measurement, Na_2SiO_3 with particle sizes too small for sieving was also analysed using DLS to assess if this could provide a more time efficient method. Initially, Na_2SiO_3 was thoroughly suspended within soya oil at a concentration of 1mg/ml, and an aliquot placed into a disposable plastic cuvette and run through the DLS (Zetasizer Nano S, Malvern Instruments, UK). However, when the results of this were compared with a soya oil blank it demonstrated that there was interference present, possibly from impurities within the oil, and the measurements were invalid. The oil was centrifuged in an attempt to remove any impurities, but this still yielded the same. A polyethylene glycol 400 (PEG) blank was run, to see if this could provide an alternative medium for suspension during DLS analysis. The PEG showed no interference so Na_2SiO_3 was suspended in this medium, also at a concentration of 1mg/ml, and analysed. Unfortunately, the overall level of back scattered light (derived intensity) for the PEG was very low, suggesting that there was a possibility the samples had dissolved on standing. A further attempt was made using sec-Butyl alcohol as the medium. This yielded promising results that supported findings from SEM measurement and showed

appropriate levels of derived intensity. DLS was used to measure the intensity of particles as a percentage to show the sizes of particles present, and the number of particles present in the sample as a percentage. Two measurements were taken. One immediately after the sample was put into the cuvette, to give a measurement for all the particles present, and one after the sample had been stood in the cuvette for an hour. The second measurement allowed an assessment to be made regarding the suspension of the particles, as it showed if any particles had fallen out of suspension during the standing time.

2.5.14. Ability of particles to suspend within soya oil

2g of Na_2SiO_3 was added to 40g soya oil in a 50ml conical-bottomed centrifuge tube and vortexed to agitate to ensure full suspension. The time it took for particles to sediment at the bottom was documented initially using video, then via photographs when the sedimentation rate slowed. Video was taken for the first 3.5mins, with photographs then being taken at increasing time increments over the next 23hrs at the following times post suspension: 8min, 13min, 18min, 28min, 38min, 48min, 58min, 1hr 18min, 1hr 38min, 2hr 18min, 2hr 58min, 3hr 58min and 22hr 48min. At each time increment, it was noted which, if any, particle sizes had cleared the top third of the tube, the middle third of the tube, or reached a state of full sedimentation. Full sedimentation was defined as the point at which all the particles had settled in the bottom of the tube, with no free-floating particles remaining.

Due to the hygroscopic properties of Na_2SiO_3 it is extremely important that particles have minimal exposure to the air. Excessive exposure leads to moisture being absorbed, and aggregates forming that will not suspend. This can be avoided by preparing all equipment prior to weighing, in order to allow the Na_2SiO_3 to be transported immediately from the scales into the oil, with as little contact with the air as possible.

2.5.15. Bio-availability

2.5.15.1. The molybdenum blue method

For the purposes of this study, bio-available Si is defined as dissolved silica which is molybdenum active in the molybdenum blue assay i.e. it is a monosilicic species, or dissociates rapidly to form a monosilicic species, during the timeframe of the complexation process. The molybdenum blue method was used to assess the levels of bio-available Si present due to its sensitivity and ability to detect only the bio-available monomer, monosilicic acid ($Si(OH)_4$) (Belton *et al.* 2010). The molybdic acid reagent was produced by dissolving ammonium molybdate tetrahydrate (20g) in deionised water (500ml). Concentrated hydrochloric acid (60ml) was then added and diluted after cooling to 1000ml with deionised water. The reducing agent was produced by dissolving oxalic acid (20g), 4-methylaminophenosulphate (6.67g) and sodium sulphite (4g) in deionised water (500ml). Concentrated sulphuric acid (100ml) was then added to the solution and diluted after cooling to 1000ml with deionised water. To produce a standard calibration graph, 1.5ml of molybdic acid reagent was diluted with sufficient deionised water to give a total volume of 16.5ml when 1 – 10ml of 10ppm aqueous solution of SiO_2 was added. The solution was then left to develop for 15 minutes to allow the monosilicic acid to react with the molybdate ions to form the yellow silicomolybdate acid complex. After 15 minutes, 8ml of the reducing reagent was added and the solution left for a further 2 hours to allow reduction of the yellow silicomolybdate acid complex to a blue silicomolbdous acid complex. This method was subsequently adapted to address issues that arose, as described in section 2.5.15.3.

After reduction was complete, but within 48 hours, the absorbance was measured at a wavelength of 810nm. Concentration was plotted against absorbance to determine the relationship and its linearity over the range of concentrations of the standards.

2.5.15.2. Sample analysis

To measure the bio-available Si within the Na_2SiO_3 suspended in soya oil, the Si must first be liberated from the oil and put into solution. This was achieved by vortexing the Na_2SiO_3 and soya oil mixture to ensure thorough and even suspension, then taking a 1g aliquot and mixing it with 50ml deionised water and vortexing again to agitate. This was performed in triplicate for each sample. 100µl of this solution was then analysed using the molybdenum blue method and compared to the calibration standard.

2.5.15.3. Sodium hydroxide digest

At smaller particle sizes, it was not possible to fully liberate the Si from the oil in the manner used in section 2.5.15.2, so a sodium hydroxide digest was used. This method is predominantly similar to the standard assay, with the Na_2SiO_3 suspended in soya oil being dispersed in 50ml 2M sodium hydroxide rather than in 50ml deionised water. 100µl is then sampled for the molybdenum blue assay, as with the standard assay, but with the addition of 200µl of 1M hydrochloric acid to the molybdic acid reagent to re-adjust the pH during complexation. The addition of the reducing reagent and measurement of the absorbance are the same as with the standard method.

The molybdenum blue method needs further modifying and re-validating for use at smaller particle sizes due to issues separating these smaller molecules from their oil coating.

2.5.16. Histology

2.5.16.1. Tibia preparation for staining

After thawing, adherent connective tissue was removed manually, using a scalpel, and bones were fixed in 10% phosphate buffered formalin (Fisher Scientific, UK) for 5 days at 4^0C. A 15mm

section was cut from the centre of the diaphysis by measuring to the centre of the length of the bone, then cutting 7.5mm either side with a Dremel (Multitool 4000, DREMEL®, Middlesex, UK) fitted with a diamond cutting wheel (Diamond Cutting Wheel (SC545), DREMEL®, Middlesex, UK). Proximal and distal epiphyses were removed where the epiphysis met the metaphysis, also using the diamond cutting wheel. The epiphyses were then cut in half longitudinally using a scalpel. A neutral ethylene-diamine tetra acetic acid (EDTA) solution was made up by dissolving 500g of EDTA in 5000ml distilled water, then neutralising with approximately 50g of sodium hydroxide to pH 7.4. All samples were placed into plastic histology cassettes (Simport Macrosette®, Fisher Scientific, UK), and then into the EDTA solution. They were left in the EDTA for approximately 6 weeks, or until they tested as fully decalcified. The EDTA solution was completely changed twice a week to ensure that decalcification continued at an optimum rate.

After 6 weeks of EDTA decalcification, the ammonium oxalate test for decalcification was carried out by combining 5ml of used decalcification EDTA solution with 5ml of ammonium hydroxide and 5ml of ammonium oxalate. The solution was mixed and, after 30 minutes, was checked for the presence of a calcium oxalate precipitate. If the solution was cloudy, or an obvious precipitate had formed, the bones were not fully decalcified, as the tissue was still releasing calcium into the EDTA solution, allowing the calcium oxalate to form. If the solution was clear, the bones were classed as fully decalcified. After full decalcification had occurred, the samples were placed into running tap water for 24 hours, and then transferred into 70% ethanol.

Samples were saturated in paraffin using a Leica ASP300 S tissue processor which moved them sequentially through solutions of 70% ethanol, 90% ethanol, 100% ethanol and xylene before placing them into melted paraffin wax maintained at 60°C. They were then removed from the tissue processor and embedded into paraffin wax blocks using a Leica embedding station with paraffin dispenser and cold plate. Once the blocks were fully set, they were trimmed to an appropriate size for the microtome (Leica RM2235 manual rotary microtome) and cut into 5µm sections. The cut sections were mounted onto Superfrost Plus™ Adhesion Microscope Slides

(ThermoFisher Scientific, UK) using a Leica water bath set at 45^{0}C and then baked on a Leica hotplate set at 45^{0}C. Two slides were mounted per sample, per staining method, to allow for a spare. Sections were then left overnight in a drying oven set at 45^{0}C to fully bond sections to the slides.

2.5.16.2. Method development for histological staining

Due to the inherent difficulties associated with histology, and to ensure the staining procedure was as robust as possible, a pilot study was undertaken to develop the method used in section 2.5.16.1. Six staining methods were selected, based on their affinity for different types of structure, and tested. Details of these stains can be found in table 2.2. and protocols for stains not used in this study, but tested in the pilot study, can be found in appendix III.

During the cutting and mounting process 3 levels of section thickness were tested; 5µm, 6µm and 8µm. Upon visual analysis, using brightfield microscopy (Olympus BX51), it was determined that the 5µm sections showed the best detail for the measurements needed. For the mounting of samples, two methods were tested. Initially cut samples were placed directly onto the slides and then a pasture pipette was used to apply a 70% ethanol solution to allow for adhesion. This method was unreliable as factors such as room temperature and humidity had an impact, leading to variable success rates. Cut samples were then placed in a water bath at 45^{0}C and a slide was placed into the water at a 45^0 angle, under the sample, and slowly pulled out to allow for adhesion along the slide as it was removed from the bath. This method proved more reliable with consistent results, so it was selected for use in the trial analysis.

Table 2.2. Stains tested and their expected visual results.

Stain	References	Expected visual result
Haematoxylin and Eosin (H&E)	Aly *et al*., 2011 and Suvarna *et al*., 2013	Cortical bone stained pink, trabecular bone stained red and medullary bone stained purple, along with well-defined cells
Tartrate-resistant acid phosphatase (TRAP)	Stickens *et al*., 2004 and Suvarna *et al*., 2013	Osteoclasts stained red with surrounding tissue green
Toluidine blue (TB)	Boudenot *et al*., 2014 and Suvarna *et al*., 2013	Bone stained blue, with white holes indicating lacunae with osteocytes present as dark blue structures within
Picrosirius red with (PRFG) and without (PR) fast green counterstain	Ko *et al*., 2013 and Suvarna *et al*., 2013	Cartilage stained red with collagen fibres visible under polarised light and bone counterstained green if fast green used
Periodic acid Schiff with Alcian blue (PAS)	Ko *et al*., 2013 and Suvarna *et al*., 2013	Developing cartilage stained blue, surrounding areas stained red
Masson's Trichrome (MT)	Aly *et al*., 2011 and Suvarna *et al*., 2013	Osteiod stained blue and lamellar bone stained red

An issue with the de-calcification of the bone samples was observed at this stage, as samples were proving difficult to cut and appeared to be compromised. Due to this, all samples were removed from the paraffin blocks by back-processing them in the tissue processor and placed back into the EDTA for a further 2 weeks to allow for further de-calcification. Samples were then re-tested using the calcium oxalate test (section 2.5.16.1), and re-embedded for further cutting, with better results.

Two different mounting mediums were tested for adhering coverslips to the slides; DPX and Entellan (both Sigma Aldrich, UK). Entellan was both easier to apply, and had clearer, more consistent results with less air bubbles and sample distortion, making it most suitable for use.

Three slides were mounted per sample, per stain and run through the various staining methodologies (table 2.2). H&E staining allowed visualisation of individual cells most clearly, along with their nuclei, which made it the most suitable stain for use in samples that were to be used for cell counting. For growth plate measurements, Toluidine blue was selected as the different phases of the growth plate were most clearly distinguishable using this stain.

2.5.16.3. Staining

Sections cut from the epiphysis, showing the growth plate, were stained using a Toluidine blue method taken from Suvarna *et al*. (2013). Slides were placed into metal slide racks, and all staining took place in glass staining troughs. All chemicals for staining were purchased from Fisher Scientific (UK).

A 1% Toluidine blue (CI 52040) in 50% isopropanol (aka Propan-2-ol or Iso-propyl alcohol) was made up, along with solutions of ethanol at 95%, 90%, 70% and 50%.

Cut sections were de-waxed by soaking in Xylene for 15 minutes. Sections were then re-hydrated by moving them successively through solutions of decreasing ethanol strength, then into running tap water for 5 minutes. After draining, they were placed into the 1% Toluidine blue solution at 37°C for 5 minutes. They were then drained and held briefly in isopropanol, before being cleared in xylene and mounted onto slides using Entellan and baked in a drying oven overnight at 45°C to ensure proper drying of the mounting medium. Full details of the time spent in each solution can be found in table 2.3.

Sections cut from the diaphysis, showing the central cross section of the bone, were stained using a H&E method taken from Suvarna *et al*. (2013). Slides were placed into metal slide racks, and all staining took place in glass staining troughs.

A pre-made solution of Mayers haematoxylin was used for this stain. A 0.5% solution of Eosin in 0.05% glacial acetic acid was made up by mixing 0.5ml of glacial acetic acid with 1000ml of distilled water, then adding and dissolving 5g of Eosin Y (CI 45380). Scott's Tap Water Substitute was used as the blueing agent, and mas made by dissolving 8.75g of sodium hydrogen carbonate and 50g of magnesium sulphate in 2500ml of tap water.

Cut sections were de-waxed by soaking in Xylene for 15 minutes. Sections were then re-hydrated by moving them successively through solutions of decreasing ethanol strength, then into running tap water for 5 minutes. After draining, they were placed into the Mayers haematoxylin, washed well in running tap water until "blueing" had occurred (or for a maximum of 5 minutes), then, after draining, differentiated with Scott's tap water substitute. Samples were then placed, after rinsing again and draining, into the 0.5% Eosin solution. After a further rinsing in running tap water, samples were rapidly de-hydrated (to prevent excess leaching out of the Eosin), cleared in xylene and mounted onto slides using Entellan. They were then baked in a drying oven overnight at 45°C to ensure proper drying of the mounting medium. Full details of the time spent in each solution can be found in table 2.4.

Table 2.3. Steps taken to stain tibial growth plate sections with Toluidine blue, detailing the different solutions, the amount of time spent in each one and any additional information needed.

Trough	Solution	Time/mins	Additional notes
1	Xylene	3	In fume cupboard
2	Xylene	3	In fume cupboard
3	Xylene	3	In fume cupboard
4	Absolute ethanol	2	Room temperature
5	95% ethanol	2	Room temperature
6	90% ethanol	2	Room temperature
7	70% ethanol	2	Room temperature
8	50% ethanol	2	Room temperature
9	Running tap water	5	Gentle, indirect flow to protect samples
10	1% Toluidine blue	5	In drying oven set to 37°C
11	Absolute ethanol	0.5	Room temperature
12	Absolute ethanol	0.5	Room temperature
13	Xylene	1	In fume cupboard
14	Xylene	1	In fume cupboard
15	Xylene	1	In fume cupboard

Table 2.4. Steps taken to stain tibial diaphysis cross sections with H&E, detailing the different solutions, the amount of time spent in each one and any additional information needed.

Trough	Solution	Time/mins	Additional notes
1	Xylene	3	In fume cupboard
2	Xylene	3	In fume cupboard
3	Xylene	3	In fume cupboard
4	Absolute ethanol	2	Room temperature
5	95% ethanol	2	Room temperature
6	90% ethanol	2	Room temperature
7	70% ethanol	2	Room temperature
8	50% ethanol	2	Room temperature
9	Running tap water	5	Gentle, indirect flow to protect samples
10	Mayers haematoxylin	3	Room temperature
11	Running tap water	5	Gentle, indirect flow, remove samples when "blueing" has occurred
12	Scott's tap water	2	Room temperature
13	0.5% Eosin	1.5	Room temperature
14	Running tap water	0.5	Gentle, indirect flow to protect samples
15	70% ethanol	0.5	Room temperature
16	90% ethanol	1	Room temperature
17	95% ethanol	1	Room temperature
18	95% ethanol	1	Room temperature
19	Absolute ethanol	1	Room temperature
20	Absolute ethanol	1	Room temperature
21	Xylene	1	In fume cupboard
22	Xylene	1	In fume cupboard
23	Xylene	1	In fume cupboard

2.5.16.4. Imaging and Measuring

All samples were analysed using an Olympus BX51 microscope fitted with an Olympus DP71 camera. Olympus Cell F software was used to capture images, which were then analysed using Image J software. For growth plates, the widths of the resting, hypertrophic and proliferative zones (shown in figure 2.6) were measured. The growth plate was sectioned longitudinally into four equal sections and then captured at a x4 objective to ensure measurements covered the entire growth plate. Measurements for each zone of the growth plate were taken at each longitudinal line, and an average calculated (figure 2.7).

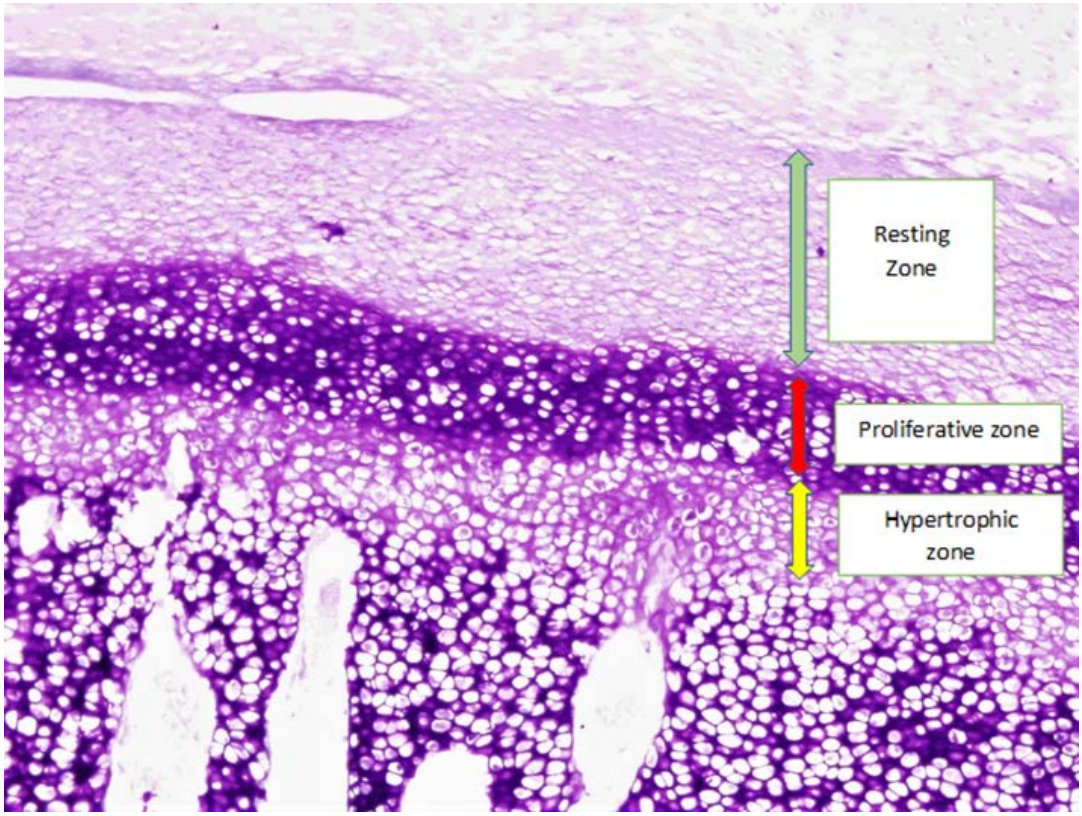

Figure 2.6. The areas measured during analysis of the tibial growth plates.

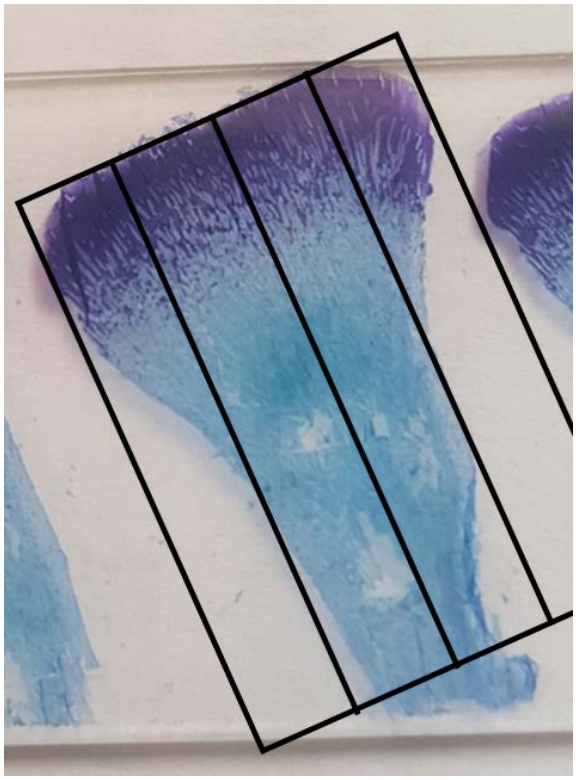

Figure 2.7. The longitudinal lines measured at equal distances along the growth plate, where the width of the various zones was measured.

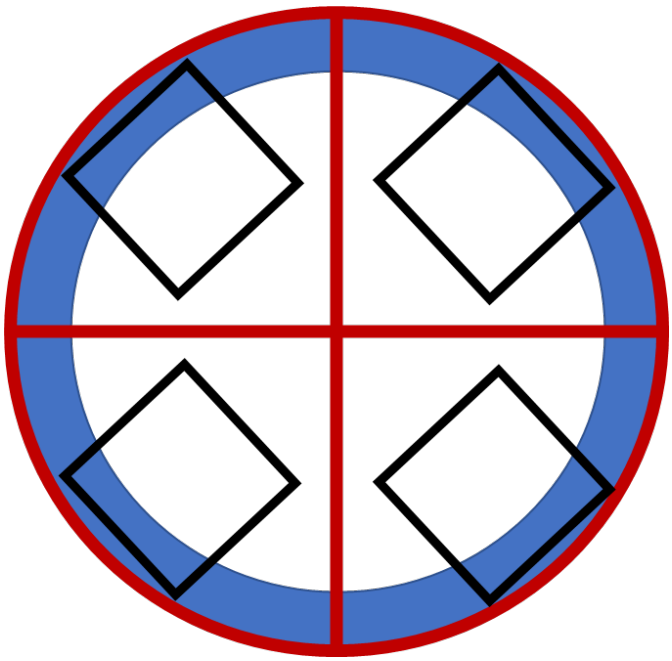

Figure 2.8. A diagram of the cross section of a tibial diaphysis (blue circle), showing how it was divided into 4 quadrants (red lines) and then a 450µm^2 section (black square) was selected to be imaged for cell counts.

For the cross sections of the diaphysis, the sample was sectioned into 4 equal quadrants. A section of 450µm^2 was then selected from the same relative area of each quadrant and image captured at a x20 objective (figure 2.8).

Cells were identified through morphological structure, size, stain intensity and placement within the osteons. Osteoblasts stain a darker shade of purple/pink compared to surrounding tissue, have distinct cuboid nuclei and smooth edges, and occur on the inner edge of osteons (Figure 2.9). Osteoclasts have distinct ruffle borders on the edge in contact with bone tissue, and are multi-nucleated, along with being considerably larger than other bone cells (figure 2.10). Cell and osteon counts were done manually in image J and an average was then recorded.

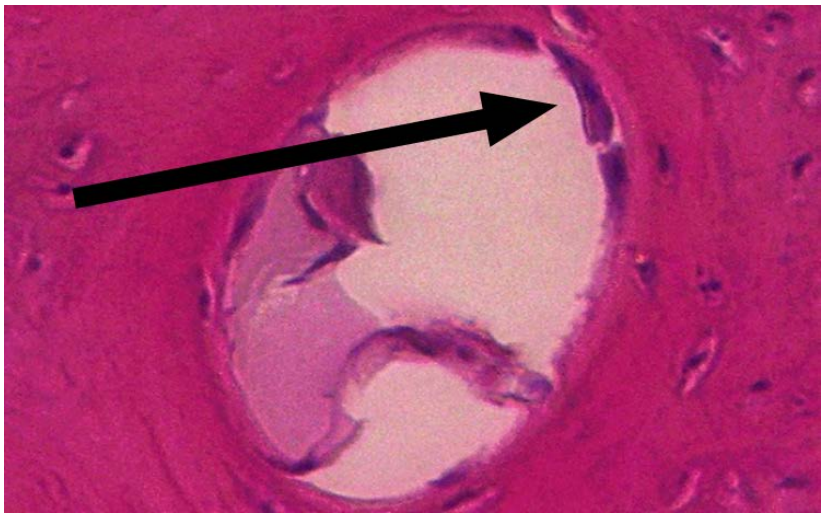

Figure 2.9. An example of an osteoblast found on the inner edge of an osteon, showing a clear nucleus, stained a darker shade than surrounding tissue and demonstrating smooth edges.

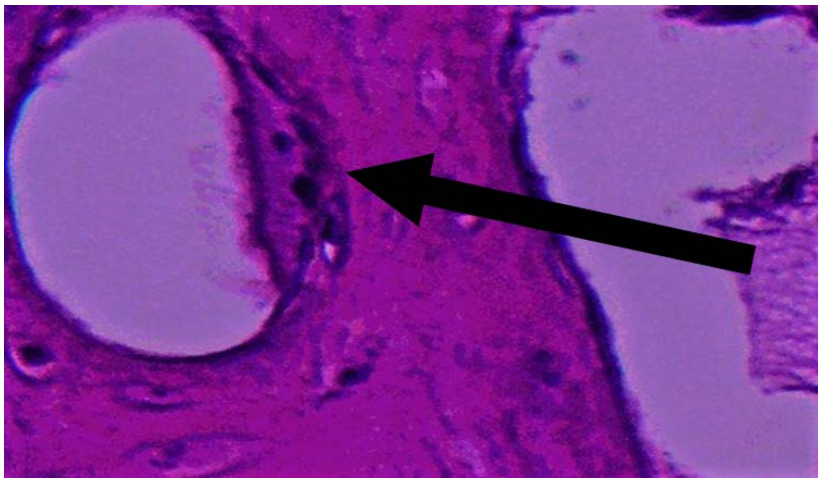

Figure 2.10. An example of a large, multinucleated (black dots) osteoclast with a distinct ruffle border on the edge in contact with the bone tissue.

2.6. Data analysis

All data was analysed using SPSS software version 19, 20 or 21 for Windows (IBM Statistics, 2013). After KS testing to confirm normality, statistical analysis was carried out using appropriate tests to differentiate means, with specific testing identified where used in each chapter. Treatment means were separated using a Duncan post hoc test, chosen because it selects protection level for error rate based on the collection of tests as opposed to the error rate for the individual tests. Correlations were analysed by bivariate correlation using Pearson correlation, chosen because it computes based on true values and depicts linear relationships. Interpretations of the strength between relationships were based on those of Cohen (1988): small r = 0.1-0.29, medium r = 0.30-0.39 and large r = 0.50 to 1.0. Statistical significance was declared at $p<0.05$.

Chapter 3: Effects of manufacturing method on silicon particle size and a comparison of the key properties of a variety of particle sizes

3.1. Introduction

The NTU in house method of preparing the sodium metasilicate (Na_2SiO_3) (described in section 2.3.2) which forms the basis of the patented Si supplement, is a limiting factor in commercialisation due to its small scale. Using the equipment currently available (a domestic electric blender), only small batches (around 20g) can be produced. In order to increase the number and size of studies, and to move towards a commercial scale, an effective method of producing larger quantities of the supplement needs to be developed. With this in mind, a new planetary ball mill with a higher capacity was purchased (Across International, NJ, United States), and the size and volume of the particles produced was examined. How particle size effects the ability of the supplement to suspend within the soya oil is of importance as excessive sedimentation could impact upon the ability of the product to be thoroughly mixed with poultry feed. Investigations were conducted to establish the optimal particle size for suspension within the soya oil. Alongside this, there was also a possibility that changes in particle size could affect the bio-availability of the supplement, both initially and over time. Bio-availability is of major importance as the supplement must continue to deliver optimum levels of bio-availability to ensure birds are receiving the correct dose throughout trials, and to ensure that dose can be accurately calculated. Work was conducted to assess which particle sizes retained the highest levels of bio-availability, and to ensure the optimum particle size for suspension within the oil also demonstrated good retention of bio-availability over time.

The key aims of this study were as follows:

- Identify the range of Na_2SiO_3 particle sizes the ball mill was capable of producing.
- Assess how long these particles remained suspended within soya oil.

- Measure the percentage of Na_2SiO_3 that remains bio-available after addition to soya oil at various particle sizes.
- Evaluate how time affects the bio-availability of the Na_2SiO_3 once suspended in soya oil.

3.2. Procedure

The mill used in this study was the PQ-N2 series planetary ball mill (Across International, NJ, United States). It was supplied with 4x 500ml capacity tungsten carbide milling jars and 4x sets of tungsten carbide milling balls in the following diameters: 6mm, 10mm and 20mm (Across International, NJ, United States). Tungsten carbide was chosen due to its high resistance to wear as Na_2SiO_3 has previously proven to be highly abrasive during milling. For all milling, the factory default settings were adhered to with the only alterations being the diameter of the milling balls used.

3.2.1. Milling run 1

The milling jars were loaded with 100g of Na_2SiO_3 and the 20mm diameter balls. The fastest milling speed (45Hz) was selected and the Na_2SiO_3 milled for a total of 10 minutes at this speed. Due to the large amounts of heat generated by the milling process it was necessary to leave the jars for 60 minutes to cool and to allow the Na_2SiO_3 to settle before opening.

The particles produced in milling run 1 were large enough for use of the sieving method detailed in section 2.5.12. As this method was deemed to have produced a reliable assessment of the range and mass of different particle sizes produced, no further analysis of particle size (SEM or DLS) was conducted on Na_2SiO_3 from milling run 1. Particles were stored separated into size fractions until suspension in soya oil.

The fractions of Na_2SiO_3 in the following particle size ranges were suspended in soya oil: <50µm, 50-80µm, 80-100µm, 100-200µm and 200-250µm. The ability of the different particle sizes to remain suspended within the soya oil was assessed, using the method in section 2.5.13.

After assessment of the ability of the various particle sizes to suspend within the soya oil had been completed, the Na_2SiO_3 and soya oil mixtures were tested for bio-availability. The Si in the Na_2SiO_3, was liberated and dissolved as per the method in section 2.5.14. Then the molybdenum blue assay was carried out (see section 2.5.15) in order to compare the bio-availability of the Si in different sizes of Na_2SiO_3 particles.

3.2.2. Milling run 2

The milling jars were loaded with 100g of Na_2SiO_3 and the 10mm diameter balls. The fastest milling speed (45Hz) was selected and the Na_2SiO_3 milled for a total of 10 minutes at this speed. Due to the large amounts of heat generated by the milling process it was necessary to leave the jars for 120 minutes to cool and to allow the Na_2SiO_3 to settle before opening.

The particles produced in milling run 2 were notably smaller than the particles from milling run 1. When sieving was attempted, the excessive exposure to air during the process caused formation of large aggregates that made it impossible to accurately measure particle size via this method. Instead, SEM was used as per the method in section 2.5.12. 19 images were taken at a magnification of x3000 and 50 distinct primary particles measured on each image. A mean particle size was calculated, along with the number of particles falling within the following ranges of particle size: <0.5µm, 0.5 - 1µm, 1 - 1.5µm, 1.5 - 2µm, 2 - 2.5µm, 2.5 - 3µm, 3 - 3.5µm, 3.5 - 4µm, 4 - 4.5µm, 4.5 - 5µm, 5 - 5.5µm, 5.5 - 6µm and >6µm. Na_2SiO_3 from milling run 2 was also measured using dynamic light scattering (DLS) using the method in section 2.5.12.

Due to the difficulties with sieving particles produced using the 10mm balls, it was not possible to fractionate it, so it was suspended as a whole sample. 2g of Na_2SiO_3 was suspended in 40g soya oil. The ability of the Na_2SiO_3 to remain suspended was assessed visually. on an hourly basis, and then 4 times a day for the next week, and then daily for the remainder of the study and the point when it reached a state of full sedimentation recorded. To assess if the addition of citric acid (a stabilising agent used to produce the supplement) to the Na_2SiO_3 and soya oil had an effect of the ability of the particles to remain suspended, 2g of Na_2SiO_3 and 2g of citric acid milled under the same conditions were suspended separately into 2 tubes of 20g of soya oil, then combined to give a suspension containing 40g soya oil, 2g Na_2SiO_3 and 2g citric acid. As with the suspension containing only Na_2SiO_3, suspension of this blend was assessed visually over the duration of the study as before. The method for assessing their ability to remain suspended (section 2.5.14) was not completed for these samples as the particles remained suspended for the remaining duration of the study, so it was not possible to estimate at what point they reached a state of full sedimentation.

The combined Na_2SiO_3 and citric acid in soya oil was tested (using the standard molybdenum blue method in section 2.5.14.) immediately post-suspension to give a baseline for bio-availability. This method was repeated at 21 days and 30 days post-suspension to evaluate the retention of bio-availability over time and ensure there are no deleterious effects on bio-availability when the Na_2SiO_3 is combined with citric acid. Due to concerns that the smaller particle size was not being fully liberated by the standard assay, a sodium hydroxide digest was used to ensure that the oil was not affecting recovery rates (as per section 2.5.14.). Data was taken via molybdenum blue analysis after 30 minutes in the digest, and again after 24 hours to show the total Si recovered, and how much recovered Si remained bio-available. This method was also repeated at 21 days and 30 days post suspension to compare with previous data.

3.3. Results

3.3.1. Milling run 1

Milling run 1 produced particle sizes ranging from <50µm to >400µm, with the majority being less than 200µm. Table 3.1 shows the mass of each size fraction that was recovered, and Figure 3.1 shows the percentage each size fraction made up of the total mass recovered.

Table 3.1. The mass and volume of each size fraction of Na_2SiO_3 recovered after sieving.

Particle Size/µm	Weight/g	Volume/%
<50	38.1069	19.05345
50-80	24.529	12.2645
80-100	15.8979	7.94895
100-200	61.5034	30.7517
200-250	19.7764	9.8882
250-300	14.3729	7.18645
300-400	17.1691	8.58455
>400	4.4956	2.2478
Total	195.8512	97.9256
Material lost	4.1488	2.0744

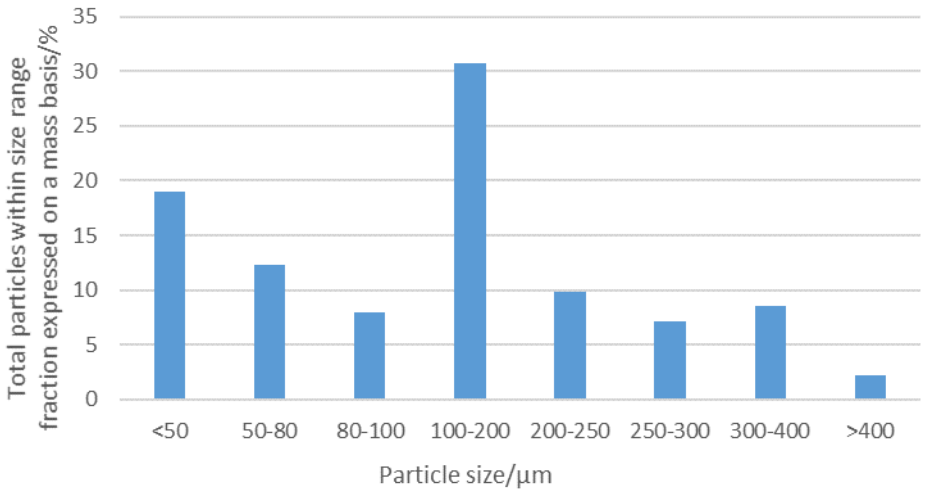

Figure 3.1. The percentage each size fraction of Na_2SiO_3 recovered after sieving.

The time it took for particles from milling run 1 to clear each third of the tube and reach a state of full sedimentation (as defined in section 2.5.14) are detailed in table 3.2. The photographs of the sedimentation are documented in appendix II. The particles that measured <50µm are listed as > 1368 for time to fully sediment, as when measurement ended, they were still partially suspended within the soya oil. Smaller particle sizes showed the best recovery levels for bio-availability, with percentage recovered decreasing as particle size increased over 100µm (Figure 3.2).

Table 3.2. Sedimentation time, after suspension in soya oil, for Na_2SiO_3 particles of different sizes.

Na_2SiO_3 particle size/µm	**Time by which top third of tube was clear of particles/min**	**Time by which middle third of tube was clear of particles/min**	**Time by which full sedimentation had occurred/min**
200-250	1	2	3
100-200	3	8	13
80-100	8	18	28
50-80	28	58	98
<50	238	1368	>1368

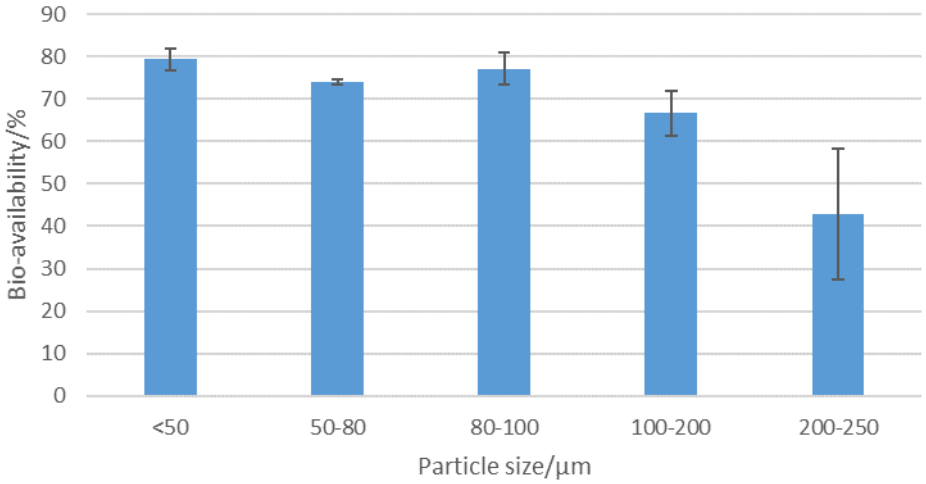

Figure 3.2. Bio-availability retained at various particle sizes, expressed as percentage of $Si(OH)_4$ detected via Molybdenum blue analysis as a proportion of the volume added.

3.3.2 Milling run 2

All Na_2SiO_3 from milling run 2 was measured via SEM so no mass data could be obtained as had been previously achieved for Milling run 1. Mean particle size was 0.96 (±0.631), with of the majority of the particles below 1µm. Figure 3.3 shows the particle size distribution between the ranges stated in section 2.5.13.1.

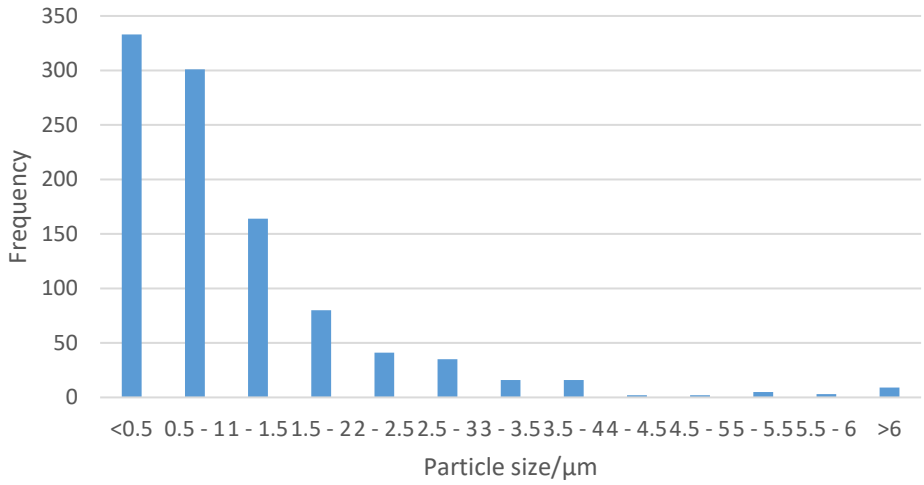

Figure 3.3. The distribution of Na_2SiO_3 particles measured, via SEM from milling run 2 (n=950).

Na_2SiO_3 from milling run 2 was also measured using DLS. The data from the DLS shows that there is a stable population of particles of 800-1000nm radius and shows the presence of larger, sedimenting aggregates. Figures 3.4 and 3.5 show the situation immediately after suspension of the Na_2SiO_3 within sec-Butyl alcohol, and figures 3.6 and 3.7 show the particle size after 1 hour without agitation. The larger spread on figures 3.6 and 3.7, compared to figures 3.4 and 3.5 indicate that there is a considerable population of aggregates which eventually sediment upon standing.

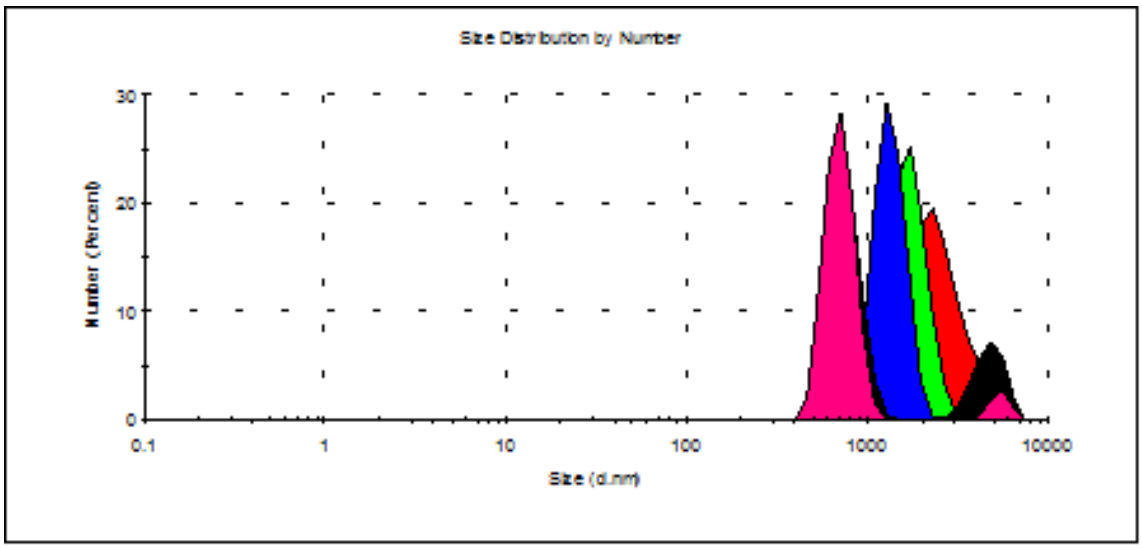

Figure 3.5. Particle size immediately after mixing with sec-Butyl alcohol by number.

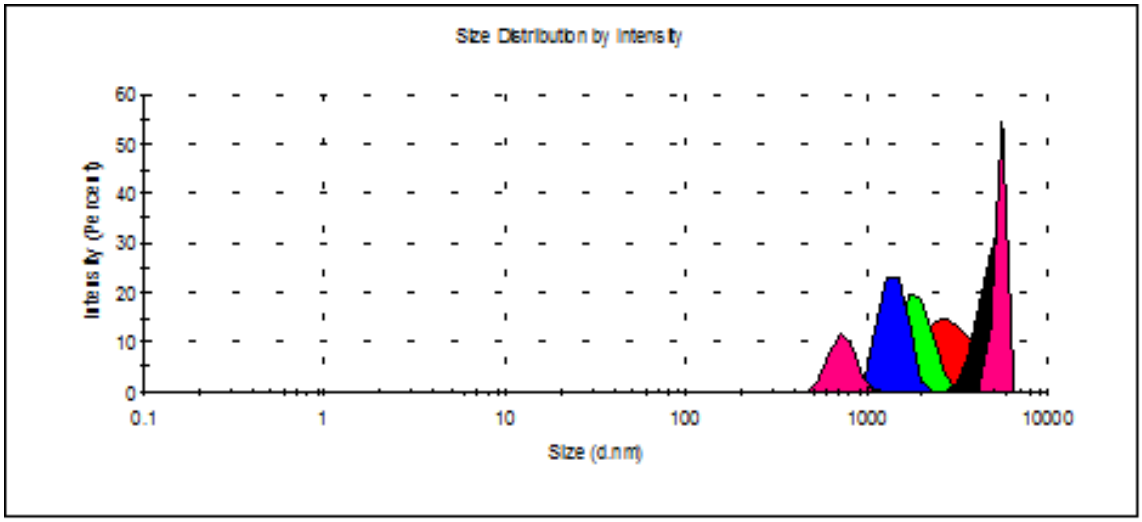

Figure 3.6. Particle size immediately after mixing with sec-Butyl alcohol by intensity.

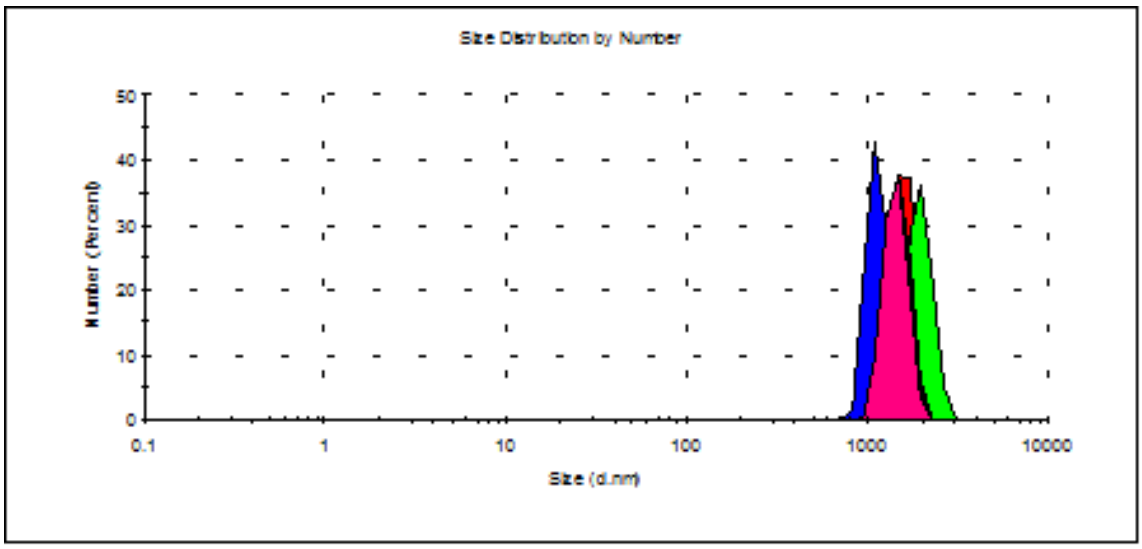

Figure 3.7. Particle size after 1 hour in sec-Butyl alcohol (without agitation) by number.

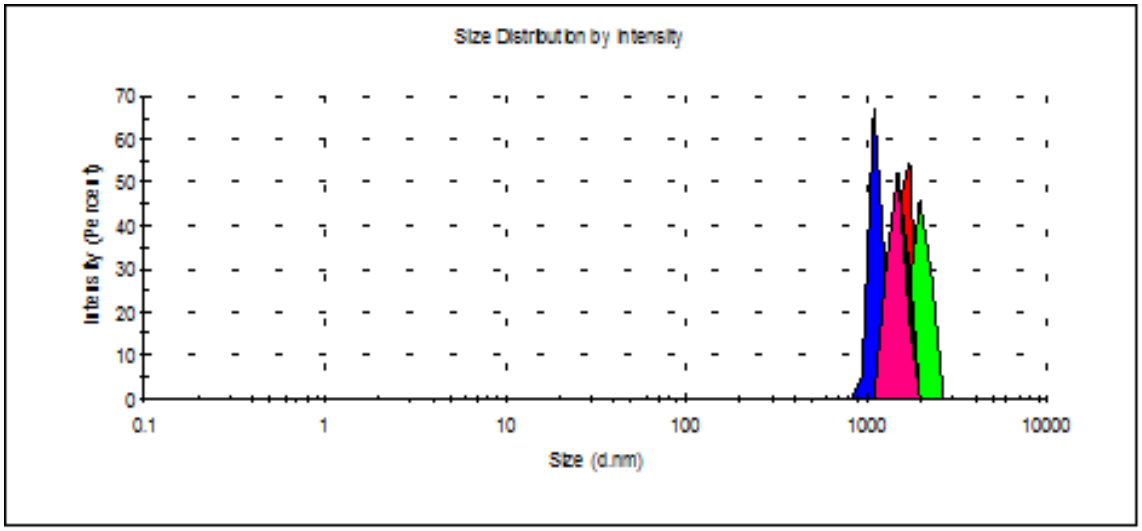

Figure 3.8. Particle size after 1 hour in sec-Butyl alcohol (without agitation) by intensity.

The Na_2SiO_3 from milling run 2 remained suspended within the soya oil for approximately 1 week when suspended without citric acid. After a point of full sedimentation had been reached, considerable agitation (vigorous shaking for >5 minutes) was required to re-suspend the particles in the soya oil. Once the Na_2SiO_3 was re-suspended there did not appear to be any visible aggregates formed, which suggests that reaching a state of full sedimentation does not

cause aggregation. However, when it was combined with citric acid, there was only a negligible amount of sedimentation at approximately 3 weeks post suspension. At three weeks post suspension, the sample appeared to have gelled slightly, and did not appear fully fluid on inversion of the sample tube. Agitation via gentle shaking returned it to its original, fluid state. The Na_2SiO_3 combined with citric acid stayed suspended longer than the Na_2SiO_3 without citric acid. It was also much easier to return the Na_2SiO_3 combined with citric acid to a fully suspended, fluid state after it had gelled slightly (via gentle shaking), than it was to re-suspend the Na_2SiO_3 without citric acid after it had reached a point of full sedimentation.

Na_2SiO_3 from milling run 2 appeared to show a loss of over half its bio-availability after 21 and 30 days posts-suspension (figure 3.9). As solutions in water, the samples of Na_2SiO_3 in soya oil from milling run 2 were returning only approximately 20% as bio-available (much lower than in samples previously milled via the old methods). A 30 minute sodium hydroxide digest was performed and found only about 30% of the expected amount. This could be due to the oil in combination with the much smaller particle sizes making it much harder for water to penetrate and liberate the Si so it can move into solution and be detectable via molybdenum blue assay. Due to this figure still appearing lower than expected, a further 24 hour sodium hydroxide digest was performed. Figure 3.10 shows bio-available Si recovered after extraction in water (via the standard molybdenum blue method) after 30 minutes and 24 hours, and the total and bio-available Si recovered with extraction via the sodium hydroxide digest after 30 minutes and 24 hours.

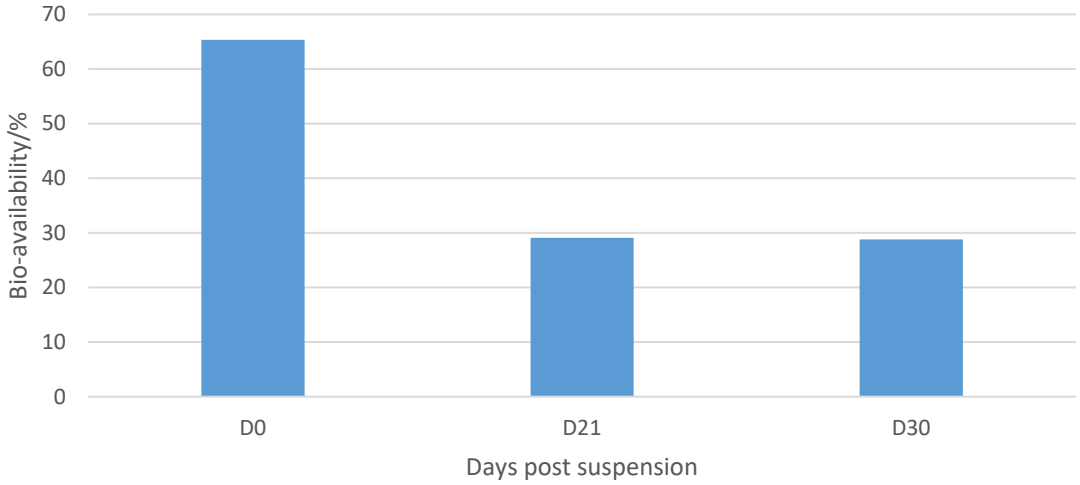

Figure 3.9. Bio-availability retained at various times posts-suspension, expressed as percentage of Si detected via Molybdenum blue assay as a proportion of the volume added to the initial suspension.

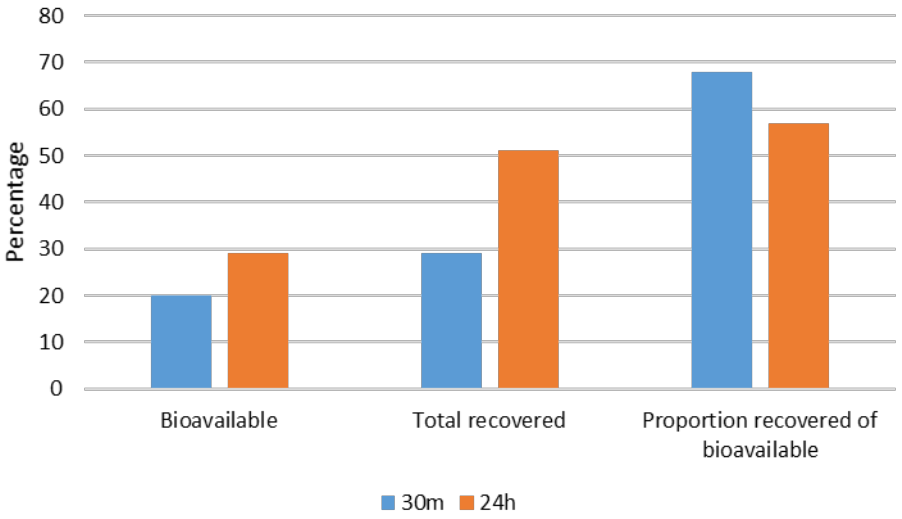

Figure 3.10. Bio-available silica recovered via agitation of oil formulation in water, and via a sodium hydroxide digest at 30 minutes and 24 hours. The columns labelled as '% Bio available' were recovered via agitation in water, and the column labelled '% total recovered' via the sodium hydroxide digest. The column labelled '% of total recovered bio available' shows how much of the Si recovered via sodium hydroxide digest was bio-available as a percentage of the volume added to the initial suspension.

3.4. Discussion

Whilst this study has gone some way to define the abilities of the ball mill to produce particles of various sizes, and to begin to define the properties of these particles with regards to their ability to suspend within soya oil and retain bio-availability, there are still many questions to be considered going forward. The key factor in the milling procedure is the difficulty in removing the Na_2SiO_3 from the mill while limiting its exposure to moisture. While this concern can be mitigated when smaller quantities of Na_2SiO_3 are required, it may present an issue at larger quantities, as would be required in commercial production. This is because small quantities of the Na_2SiO_3 can be weighed directly from the milling jar into oil to stop condensation of the material and the associated reduction of bioavailability. However, if the total contents of the milling jar are required, then the milling balls must be removed from the Na_2SiO_3 by sieving and, even when the largest possible aperture size sieve is used, this process can cause significant exposure of the Na_2SiO_3 to air. The addition of soya oil to the mill before milling may result in the particles being coated with oil as they are ground, thus preventing any unnecessary exposure to moisture. This method would need to be evaluated however to ensure that there are no adverse effects on the ingredients, or on the mill itself. The effects of adding oil to the mill on the particle sizes produced and the milling parameters needed would also need to be examined, as addition of oil may cause the Na_2SiO_3 to behave in a different manner than when milled alone. Mukhtar *et al*. (2013) examined the effects of wet milling on Zeolite particle size and found that the size first decreased with time, then increased again due to agglomeration. However, it is unknown whether a similar effect would be seen when milling the Na_2SiO_3 particles with the oil, or the effect on bio-availability and therefore more investigation would be required.

This study also only examined one speed setting and one time setting on the ball mill. Further examination of the effects of milling at different speeds and timescales may allow the creation of a protocol which reliably produces specific particle sizes. For example, research has shown that the speed, time and milling ball to powder ratio all have a dramatic effect on the final

particle size achieved, as well as altering the structure of the particles themselves (Biyik and Aydin, 2015; Mukhtar *et al*., 2013; Rizlan and Mamat, 2014). It is not yet known how the NTU Si supplement would respond to these parameters, and if their alterations would have a negative effect on the efficacy of the product.

The parts of this study relating to sedimentation and re-suspension would suggest that although smaller particles suspend better, larger particles are easier to re-suspend after a state of full sedimentation has occurred. This indicates a need to evaluate in the future which of these properties is of greater importance. It has been suggested that the effect of particle size on bio-availability is also affected by the encapsulation of the particle, and the individual chemical properties of the substance being supplemented (Acosta, 2009) as well as the size and shape of the particle. As the NTU supplement is novel, much more research into its potential absorptive properties are required, and if further work to assess the best particle size for retention of bio-availability indicates there are several sizes providing similar results, an investigation into whether it would be preferable to have a well suspended or easily re-suspended particle would be necessary. The ease with which a particle suspends, or sediments, is affected by the viscosity of the substance it is suspended in as well as its temperature (Oliver and Ward, 1959; Tsubaki *et al*., 2000; Ghawi and Kris, 2008). This is particularly relevant in feed, as if sedimentation occurs within the oil, before the supplement is fully mixed within the feed, then the supplement may not be evenly distributed throughout the diet. Bellamy *et al*. (2008) noted that the greater the difference in particle sizes, the greater chance there was that sedimentation within a powder would occur with agitation (such as might be encountered during transport of the supplement), which suggests that a more uniform particle size would perform better in terms of homogeneity and create a consistent dosage within feed. This study looked superficially at sedimentation and ease of re-suspension, but further, more detailed study is needed to provide reliable data, and to investigate the potential impact of factors such as agitation, temperature and viscosity of the oil.

There are numerous areas surrounding bio-availability that would benefit from further investigation. More frequent testing over the initial post suspension period to give greater insight into specific timescales for loss of bio-availability is a key factor. As the first tests were performed after a number of days, it would be valuable to test first hourly, and then daily, to assess exactly when the initial loss of bio-availability occurs, and if this is related to the rate of sedimentation. Once this has been established, different particle sizes combined with citric acid should be tested for retention, as currently only the smallest particles have been assessed regarding this. The effects of temperature of the retention of bio-availability also needs investigating, as do the effects of repeated sedimentation and re-suspension as these areas all need to be clarified before the product could be commercialised. These factors are particularly relevant for commercialisation as during manufacture it is likely that the product will be produced and combined into feed at different locations, and undergo transport during different weather conditions and climates, with hot or humid climates presenting a particular challenge.

The soya oil has demonstrated a strong ability to protect the small particle sizes from water, which is a positive feature in terms of product efficacy but seems to have affected the ability of the *in vitro* assay to accurately assess bio-availability. Due to the issues encountered liberating the bio-available Si from oil at smaller particle sizes, the assay procedure may need to be developed in order to show that it accurately reflects the presence of Si which is bio-available in the sample. Investigation into the effects of the addition of a surfactant to the process may prove useful in order to more effectively remove the oil from the Si, but again, this would need to be carefully evaluated as addition of new components to the assay may affect the accuracy of the readings or the properties of the supplement itself. The assay also needs evaluating to ensure that the *in vitro* values produced are truly representative of the *in vivo* rates of uptake.

It is necessary to assess the sampling methods for *in vitro* bio-availability testing via the molybdenum blue assay, as it is possible that taking an aliquot is not representative of the whole sample. This is particularly true when assessing the larger particle sizes, as the speed of

sedimentation makes it difficult to acquire a truly representative aliquot and therefore bio-availability may have been under-reported for larger particle sizes. Even if the product is well agitated to re-suspend the particles, they still sink so rapidly it is difficult to accurately obtain a sample. This is particularly important as the whole product would be added to feed and so, once mixed, the full volume of Na_2SiO_3 and oil would be present in the feed. This means that by using an aliquot, rather than the full volume of Si in oil, this is not representative of what would actually be introduced into poultry diets.

An improved methodology for assessing the rate of sedimentation should be developed in future work, to allow accurate and precise quantification of the rate the Na_2SiO_3 settles as, currently, all timescales are estimated. Further investigation is also needed to assess the accuracy of DLS for assessing particle size, as only a limited number of samples were tested via this method, and therefore its comparability to SEM cannot yet be confirmed. The only method found to keep the particles suspended for sufficient time to allow the DLS to measure them was to suspend in sec-Butyl alcohol, but even using this procedure, they rapidly sediment, potentially affecting the reliability of the results. This sedimentation is not observed when the particles are suspended in soya oil, suggesting better distribution using the oil formulation, most likely due to the increased viscosity of the oil compared to the sec-Butyl alcohol although this requires confirmation.

Due to the hygroscopic properties of Na_2SiO_3, it is important that the samples have minimal contact with air. The maximum time the Na_2SiO_3 samples from milling run 2 in this study were exposed to air was less than 2 minutes when weighing from the milling jar into the oil. If, in other studies, Na_2SiO_3 is allowed contact with the air for longer than this time, its properties may differ when compared to the Na_2SiO_3 used in this study.

There are several limiting factors of this investigation. The aliquots taken may not have been representative, and a better method for assessing the whole sample needs to be developed. This may involve producing much smaller representative samples which could be tested complete (although smaller samples are harder to produce accurately) or scaling up the

molybdenum blue assay so larger samples can be tested. Given the short time scale it took for some particles to fall out of suspension, it is unlikely that these will remain suspended for long enough to be evenly distributed in feed. This makes these particles unattractive from a commercial perspective as they are both unlikely to retain efficacy and impossible to dose reliably in feed. It appears that the oil is successfully fulfilling its intended role of aggregation prevention and protection from moisture, which is a positive factor, however, the potential impact of using oil on the reliability of the molybdenum blue method is of concern. The citric acid does not appear to be having a negative effect on the ability of the Na_2SiO_3 to remain suspended in the soya oil, and it seems likely that the addition of citric acid suspends the Na_2SiO_3 for a longer period, while also aiding the re-suspension of the Na_2SiO_3 after sedimentation has taken place. Citric acid is a necessary addition to the supplement to neutralise the caustic properties of the Na_2SiO_3, and so means that the supplement can be fed safely to animals without causing issues within the digestive tract.

The ball mill was able to produce batches of 800g of Na_2SiO_3 ground to a particle size of 1-10µm with a milling time of 10 minutes and a post-milling cooling and settling time of 120 minutes. Smaller particle sizes (<100µm) demonstrate better retention of bio-availability immediately post-suspension (~10% higher). Smaller particle sizes (<50µm) demonstrate better suspension within the soya oil, remaining suspended indefinitely (when combined with citric acid). Once Na_2SiO_3 particles <10µm are combined with citric acid and suspended in the soya oil, they demonstrate a loss of bio-availability over the initial 3 weeks post suspension using the standard molybdenum blue assay. Further development of the assay suggests that this may be a false result. The oil appears to be more effective in protecting smaller particles, and there need to be further steps added to the assay in order to fully liberate the bio-available silica and enable an accurate reading. Further work is needed to assess in greater detail the timescales for loss of bio-availability, and the effects of other variables such as temperature in these timescales. An attempt at milling the Na_2SiO_3 with oil to limit unnecessary exposure to water is recommended.

Further development of the *in vitro* assay procedure is needed to insure it is providing accurate predictive results. All future work needs to be carried out using Na_2SiO_3 combined with citric acid, as this is likely to be how the product will be manufactured commercially, and therefore data collected on this combination will be far more relevant than data collected on the Na_2SiO_3 alone.

The next steps in this project examine the supplement, with the Na_2SiO_3 milled a uniform size, and test its effects *in vivo*, with broiler chicks.

Chapter 4: A comparison of commercially available Si supplements with the NTU Si supplement

4.1. Introduction

As the 10mm diameter milling balls produced the least variable particle sizes (discussed in chapter 3), this method was used to produce the Si supplement for the trial in this chapter. A series of commercially available Si supplements were tested via the molybdenum blue method outlined in section 2.5.15, and those with the highest bio-availability used to compare to the NTU supplement (Scholey *et al*., 2018). A team of chemists at NTU, who specialise in the production of nanoparticles, produced a Si nanoparticle coated in lysine which was also used for comparison. The methods used to create the Si nanoparticles are not addressed as they are chemistry based, rather than biology, and are outside the scope of this thesis.

This study was designed to determine which silica supplement resulted in the greatest uptake of Si in the blood plasma and had the greatest positive impact on tibia bone parameters at bird age d14, d21 and d35. It was also used to determine if the NTU supplement is more efficacious when produced in one batch at the beginning of the trial, or in weekly batches throughout the trial.

The key aims of this study were as follows:

- Identify the Si supplement that shows the greatest levels of absorption into the blood plasma.
- Assess which Si supplement shows the greatest effects on tibia bone parameters.
- Determine whether it is necessary to manufacture the NTU supplement weekly, or if it is just as efficacious when manufactured in one batch.

4.2. Trial Procedure

4.2.1. Husbandry conditions

Birds were sourced from PD Hook Cote hatchery, Oxford, from a flock aged 43 weeks. Birds were feather sexed on day of hatch and poor birds discarded on arrival.

315 day old male Ross 308 were weighed before random allocation to 45 mesh sided pens bedded on wood shavings. Feed and water were available *ad libitum*, with care taken to ensure the birds ate and drank as soon as possible. Husbandry guidelines were followed as stated in section 2.2.

4.2.2. Diet formulation and condition of animals

Birds were fed a basal diet of generic commercial starter diet in mash form from d0-21, and generic commercial finisher diet in mash form from d21-35, alone or with one of 4 sources of Si. Table 4.1 shows details of each experimental diet.

Table 4.1. Dietary treatments for the product comparison trial

Diet	Treatment
A	Basal diet only (Control)
B	Basal diet with lysine coated Si nanoparticles (Si-Lys)
C	Basal diet with NTU Si made in weekly batches (NTU-W)
D	Basal diet with NTU Si made in one batch (NTU-1B)
E	Basal diet with Bamboo silica (Bamboo)

Diets were manufactured in house as per the method in section 2.3. All diets were produced in one batch at the beginning on the trial, with the exception of diet C, which was manufactured

weekly. This study allowed for 9 replicates per treatment. Calculated values for basal starter and finisher diets are shown in table 4.2. Analysed values for the individual diets are shown in table 4.3.

Table 4.2. Basal diets with rates of inclusion (%)

Ingredient	Starter	Finisher
Wheat - Feed	63.20%	71.73%
Rapeseed Solv Ext	4.00%	4.00%
Soybean meal 48	25.97%	18.30%
Soy oil	3.56%	3.45%
Salt	0.30%	0.30%
Sodium Bicarbonate	0.10%	0.10%
DL Methionine	0.28%	0.15%
Lysine HCl	0.26%	0.21%
Threonine	0.07%	0.04%
Limestone	0.91%	0.87%
Dicalcium Phos	0.87%	0.37%
Vitamin premix	0.50%	0.50%

Table 4.3 Analysed content of diets for the product comparison trial.

	Starter				Finisher				NTU-W				
Diet	**Control**	**Si-Lys**	**NTU-1B**	**Bamboo**	**Control**	**Si-Lys**	**NTU-1B**	**Bamboo**	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**
DM (g/kg)	864.97	858.43	848.58	870.99	867.62	876.07	848.9	824.04	861.63	853.1	860.38	842.65	853.18
Ash (g/kg)	3.77	4.24	4.39	4.45	3.45	3.73	4.15	4.17	4.65	3.51	5.17	3.86	3.96
Protein (g/kg DM)	208.07	210.78	192.78	214.97	201.49	218.31	178.35	174.58	225.69	219.43	205.67	194.41	172.9
GE (MJ/kg DM)	19.98	19.99	20.01	20.11	19.45	19.35	19.45	19.5	20.12	19.88	19.5	19.42	19.37
Total P (g/kg DM)	4.67	4.07	4.78	4.66	3.34	3.25	3.25	3.29	4.9	4.69	4.17	3.03	3.17
Total Ca (g/kg DM)	8.57	8.08	9.68	9.26	5.92	6.96	6.75	6.89	8.1	9.91	7.61	6.31	6.77
Total Si (g/kg DM)	0.47	0.53	1.1	0.69	0.64	1.01	1.24	1.02	1.16	1.43	1.12	1.33	1.27
Fat (g/kg)	68.82	74.06	73.26	73.75	64.05	65.97	62.69	66.48	76.36	74.1	71.69	70.47	66.87

4.2.3. Treatment schedule / randomisation plan / condition of animals

One replicate was a pen containing 7 individually weighed chicks and only birds between 38g and 46g were placed. Chicks were weighed by pen on d0 and allocated to a dietary treatment on arrival. Diet allocation was randomly allocated around the trial room, to reduce the effect of ventilation and possible environmental differences around the room.

4.2.4. Observations during the study

Bird observations were used to monitor the environment and if the birds appeared uncomfortable, the temperature and/or ventilation was altered accordingly. Birds were observed twice daily during the trial and any observations related to health recorded in a trial diary. Any dead birds were weighed, and reasons recorded if culled.

Feed intake was calculated as per the methods in section 2.4.1. Birds were weighed by pen on arrival and on sampling days 7, 14, 21, 28 and 35 as per section 2.4.2. Weight and feed intake were used to calculate feed weekly feed conversion ratio (FCR). On each sampling day, 2 birds per pen were individually weighed and marked with a coloured marker to ensure that all measurements taken from that bird were identifiable.

Post mortem blood samples were collected (as per section 2.4.5) on d14, 21 and 35 for post-trial analysis pooled from 2 birds per pen, and plasma stored at -20^{o}C for analysis of Si. The left tibia bones were collected (as per section 2.4.4) from the 2 individually weighed birds per pen and frozen. Bones were manually cleaned of tissue, weighed and the length and width of each bone was measured using digital callipers. Bone strength was analysed using a TA.XT plus texture analyser (Stable Microsystems, Guildford, UK) set up with a 50kg load cell and 3 point-bend fixture, as per the methods in section 2.5.6. After breaking, bones were dried in a drying oven set at 105°C for approximately 4 days until the weight was constant, then cooled in a

desiccator and underwent ether extraction to remove fat. They were then placed in a muffle furnace (Nabertherm, B180) for approximately 14 hours at 650°C (see section 2.5.5). The ashed samples were then cooled in a desiccator and the ash prepared for mineral analysis via ICP-OES to assess Ca, P and Si content, as per section 2.5.8.

4.2.5. Statistical analysis of data

Statistical analysis was carried out using SPSS v.22. After KS testing to confirm normality, data were analysed using one way ANOVA to investigate the effect of dietary treatment on bird performance, bone length, width, strength and mineral content and of Si uptake measured in serum. Bonferroni post hoc testing was used to elucidate differences between diets.

4.3. Results

4.3.1. Environment

No environmental anomalies occurred during this trial.

4.3.2. Health and Condition

Mortality figures are shown in table 4.4 and demonstrate that there was no cause for concern regarding the overall health of this flock. No differences were seen across treatments in terms of mortality at any age, and total mortality for the study was 2.5% which is standard for NTU studies and lower than would be expected in commercial settings.

Table 4.4. Bird Mortality for the product comparison trial by week and treatment.

Diet	d0-7	d7-14	d14-21	d21-28	d28-35	Total by Diet
Control	0	1	0	0	0	1
Si-Lys	0	2	0	1	0	3
NTU-W	1	1	0	0	0	2
NTU-1B	0	0	0	0	0	0
Bamboo	0	1	1	0	0	2
Total by Week	1	5	1	1	0	8

4.3.3. Bird Uniformity

Birds were individually weighed and only birds between 38 and 46g were used in the study. Mean start weights for each treatment are shown in table 4.5. There was no significant difference in start weight across the dietary treatments.

Table 4.5. Start weights for chicks in the product comparison trial (±SE).

Treatment	d0 BW/bird (g)
Control	43.57 (0.73)
Si-Lys	43.21 (0.75)
NTU-W	42.84 (0.73)
NTU-1B	43.35 (0.55)
Bamboo	43.03 (0.78)
p value	0.481

4.3.4. Performance

Table 4.6 shows that there was no significant effect of silica supplement on feed intake (FI), bodyweight gain (BWG) or feed conversion ratio (FCR) in the Starter phase (d0-21). Table 4.7

shows BWG was significantly higher in birds fed diet B (lysine coated nanoparticles) than those fed diet A (control), D (NTU made in one batch) or E (bamboo silica) in the Finisher phase (d21-35). Table 4.7 also shows that FCR was significantly better in birds fed diet B than those fed diet C , D or E in the Finisher phase (d21-35). Table 4.8 shows BWG was significantly higher in birds fed diet B than those fed diet A or E, and that FCR was significantly worse in birds fed diet C and E than those fed diet B over the duration of the trial (d0-35).

Table 4.6. Performance data from the Starter phase (d0-21) in the product comparison trial (±SE).

Treatment	d0-21 FI/bird (g)	d0-21 BW/bird (g)	d0-21 FCR
Control	1095.67 (21.65)	799.22 (27.10)	1.38 (0.04)
Si-Lys	1149.52 (34.51)	857.13 (34.51)	1.35 (0.04)
NTU-W	1120.94 (23.48)	830.69 (12.03)	1.35 (0.04)
NTU-1B	1155.23 (21.66)	887.42 (22.14)	1.31 (0.02)
Bamboo	1200.44 (31.10)	860.73 (20.51)	1.40 (0.03)
p value	0.131	0.111	0.422

Table 4.7. Performance data from the Finisher phase (d21-35) in the product comparison trial (±SE).

Treatment	d21-35 FI/bird (g)	d21-35 BW/bird (g)	d21-35 FCR
Control	2043.93 (63.92)	1176.13 (46.55)^{b}	1.75 (0.05)^{ab}
Si-Lys	2121.43 (51.83)	1372.35 (48.77)^{a}	1.56 (0.05)^{b}
NTU-W	2317.25 (53.71)	1209.87 (36.24)^{ab}	1.92 (0.02)^{a}
NTU-1B	2080.88 (60.92)	1167.93 (38.17)^{b}	1.79 (0.04)^{a}
Bamboo	2117.46 (94.95)	1089.91 (24.47)^{b}	1.94 (0.08)^{a}
p value	0.083	0.001	<0.001

Table 4.8. Cumulative performance data from d0 to d35 in study Product comparison trial (±SE)

Treatment	d0-35 FI/bird (g)	d0-35 BW/bird (g)	d0-35 FCR
Control	3139.60 (79.42)	1975.36 (67.12)[b]	1.60 (0.04)[ab]
Si-Lys	3270.95 (58.11)	2229.49 (58.18)[a]	1.47 (0.03)[b]
NTU-W	3438.19 (74.45)	2040.56 (33.54)[ab]	1.68 (0.02)[a]
NTU-1B	3236.11 (66.08)	2055.34 (51.69)[ab]	1.58 (0.03)[ab]
Bamboo	3317.01 (120.99)	1950.63 (26.26)[b]	1.70 (0.05)[a]
p value	0.203	0.006	0.001

4.3.5. Serum Si content

Table 4.9 shows that SiO_2 levels in the serum were significantly higher in birds fed diet C and D than those fed any other diet, at all ages measured.

Table 4.9. SiO_2 in serum in the product comparison trial (±SE).

Treatment	d14 SiO_2 in serum/ppm	d21 SiO_2 in serum/ppm	d35 SiO_2 in serum/ppm
Control	2.67 (0.09)[b]	2.25 (0.07)[b]	2.04 (0.04)[b]
Si-Lys	2.44 (0.07)[b]	2.04 (0.09)[b]	2.09 (0.09)[b]
NTU-W	5.86 (0.31)[a]	5.92 (0.14)[a]	5.30 (0.32)[a]
NTU-1B	6.19 (0.42)[a]	5.55 (0.18)[a]	5.11 (0.30)[a]
Bamboo	2.64 (0.08)[b]	2.34 (0.08)[b]	2.44 (0.09)[b]
p value	<0.001	<0.001	<0.001

4.3.6. Tibia length, width and strength

Table 4.10 shows there was no significant effect of silica supplement on the measured bone parameters at bird age d14. Table 4.11 shows tibia strength was significantly higher in birds fed diet D than those fed diet C or A at d21. Table 4.12 shows tibia strength was significantly

higher at d35 in birds fed diet D than those fed diet A. Tables 4.10, 4.11 and 4.12 all show the silica supplements had no significant effect on individual bird tibia strength:bodyweight ratio.

Table 4.10. Tibia length, width and strength on d14 in the product comparison trial (±SE).

Treatment	Length/mm	Width/mm	Strength/N	Strength/N:BW/kg
Control	58.02 (0.97)	4.08 (0.06)	70.58 (3.01)	194.34 (15.51)
Si-Lys	56.19 (0.58)	3.91 (0.06)	65.32 (3.49)	182.94 (10.07)
NTU-W	56.15 (1.15)	3.90 (0.15)	71.80 (4.42)	185.56 (21.53)
NTU-1B	55.27 (0.71)	3.99 (0.06)	67.41 (3.19)	172.62 (10.49)
Bamboo	56.16 (0.40)	3.84 (0.05)	61.85 (3.77)	170.69 (11.90)
p value	0.260	0.355	0.369	0.500

Table 4.11. Tibia length, width and strength on d21 in the product comparison trial (±SE).

Treatment	Length/mm	Width/mm	Strength/N	Strength/N:BW/kg
Control	68.99 (0.78)	5.09 (0.09)	190.99 (7.87)^{b}	272.36 (27.36)
Si-Lys	67.78 (1.38)	5.44 (0.20)	223.22 (9.45)^{ab}	294.75 (33.00)
NTU-W	67.90 (0.83)	5.07 (0.12)	195.93 (9.07)^{b}	266.63 (23.17)
NTU-1B	70.87 (0.82)	5.54 (0.14)	242.96 (12.69)^{a}	249.64 (18.10)
Bamboo	68.28 (0.75)	5.48 (0.16)	213.52 (10.22)^{ab}	254.00 (17.14)
p value	0.188	0.105	0.008	0.590

Table 4.12. Tibia length, width and strength on d35 in the product comparison trial (±SE).

Treatment	Length/mm	Width/mm	Strength/N	Strength/N:BW/kg
Control	96.67 (0.68)	7.11 (0.16)	344.00 (11.78)^{b}	187.27 (18.34)
Si-Lys	99.55 (0.89)	7.37 (0.14)	389.42 (10.17)^{ab}	209.49 (10.70)
NTU-W	98.54 (0.89)	7.24 (0.15)	389.48 (22.07)^{ab}	213.61 (15.43)
NTU-1B	97.18 (1.44)	7.21 (0.15)	419.95 (12.52)^{a}	195.42 (11.02)
Bamboo	96.94 (1.34)	7.33 (0.17)	379.13 (19.28)^{ab}	197.51 (12.59)
p value	0.482	0.815	0.049	0.142

4.3.7. Tibia ash and mineral content

Table 4.13 shows the tibia ash percentage for d14, 21 and 35, where no significant differences were recorded between diets.

Table 4.13. Tibia ash for d14, 21 and 35 in the product comparison trial (±SE).

Diet	d14 Tibia ash (%)	d21 Tibia ash (%)	d35 Tibia ash (%)
Control	44.01 (0.742)	49.16 (0.530)	48.94 (0.555)
Si-Lys	45.16 (0.877)	49.65 (0.619)	49.01(0.416)
NTU-W	45.73 (0.983)	50.20 (0.542)	48.63 (0.599)
NTU-1B	45.85 (0.612)	50.48 (0.542)	49.27 (0.449)
Bamboo	44.51 (0.948)	49.64 (0.633)	48.10 (0.600)
p value	0.485	0.501	0.585

Tables 4.14, 4.15 and 4.16 show the Ca, P and Si content of the tibia ash, expressed as per gram of dry bone weight for d14, d21 and d35 respectively. There were no significant differences measured at d14 or d21, or for Ca and P at d35. Silica content tended to be higher in Diet D at d35 compared with the control diet (p=0.063).

Table 4.14. Ca, P and Si content of tibia ash (per gram dry bone weight) on d14 in the product comparison trial (±SE).

Diet	d14 Si (mg/g)	d14 Ca (mg/g)	d14 P (mg/g)
Control	0.183 (0.0188)	154.43 (2.379)	70.20 (1.525)
Si-Lys	0.233 (0.0225)	159.12 (3.033)	71.26 (1.460)
NTU-W	0.191 (0.0121)	159.11 (4.421)	71.14 (2.028)
NTU-1B	0.203 (0.0113)	163.27 (3.073)	72.71 (1.539)
Bamboo	0.192 (0.0117)	157.57 (3.426)	71.26 (1.416)
p value	0.200	0.496	0.877

Table 4.15. Ca, P and Si content of tibia ash (per gram dry bone weight) on d21 in the product comparison trial (±SE).

Diet	d21 Si (mg/g)	d21 Ca (mg/g)	d21 P (mg/g)
Control	0.159 (0.0100)	175.37 (2.618)	76.81 (0.936)
Si-Lys	0.178 (0.0135)	182.93 (3.448)	80.37 (1.383)
NTU-W	0.170 (0.0103)	179.42 (2.169)	78.22 (0.854)
NTU-1B	0.186 (0.0102)	180.23 (2.579)	78.89 (1.102)
Bamboo	0.161 (0.0100)	177.78 (2.806)	77.92 (1.062)
p value	0.354	0.221	0.391

Table 4.16. Ca, P and Si content of tibia ash (per gram dry bone weight) on d35 in the product comparison trial (±SE).

Diet	d35 Si (mg/g)	d35 Ca (mg/g)	d35 P (mg/g)
Control	0.153 (0.0080)	184.88 (2.451)	76.71 (0.920)
Si-Lys	0.180 (0.0080)	185.88 (1.932)	78.11 (0.926)
NTU-W	0.183 (0.0115)	183.04 (2.093)	76.49 (0.912)
NTU-1B	0.203 (0.0157)	188.26 (1.659)	78.91 (0.760)
Bamboo	0.181 (0.0097)	181.90 (3.153)	76.05 (1.280)
p value	0.063	0.339	0.201

4.4. Discussion

The three silica supplements (lysine coated nanoparticles, the NTU Si and bamboo silica) had no deleterious effect on feed intake, bodyweight gain or feed conversion at d14, d21 or d35. Birds fed the diet containing lysine coated Si had the greatest bodyweight gain and feed conversion ratio. This is likely due to the fact that lysine is the first limiting amino acid in poultry and, therefore, supplementation of this amino acid will have had a growth promoting effect independently of the Si (Baker and Han, 1994).

SiO_2 levels in blood plasma were significantly higher in birds fed diets containing the NTU supplement (both made weekly and as one batch) than those fed any other diet. This suggests that these supplements are successfully being absorbed and entering the bloodstream of the birds, whereas the lysine coated nanoparticles and the bamboo Si are not. This indicates that the NTU supplement possesses greater bio-availability and is maintaining its monomeric form even after addition to feed. This monomeric form of Si has been shown to be more readily absorbed in the gastrointestinal tract of humans when compared to oligomeric Si, although this has not been confirmed in other species (Jugdaosingh *et al*., 2000). A number of Si supplements have been available commercially, however reviews of other supplements concluded that they were of limited efficacy due to low absorbance (Shariatmadari *et al*., 2008). The bio-availability of these supplements has also been questioned and confirmed in a study by Nottingham Trent University that found none of the supplements contained physiological levels of Si (Scholey *et al.,* 2018). It is generally accepted that, due to the hygroscopic and caustic nature of stable monomeric Si, operations to increase the palatability of Si by adjusting its pH and exposing it to moisture in feed generally causes high levels of aggregation and condensation leading to it losing bio-availability. This was discussed in chapter 3 and is covered in greater detail in chapter 7 of this thesis.

It was surprising that the Si nanoparticles did not show greater levels of absorption, as recent research by Gopi *et al*., (2017) and Gangadoo *et al*., (2016) has demonstrated the usefulness of this method in poultry nutrition, in particular with trace minerals that traditionally exhibit lower levels of bio-availability. It may be that, due to the issues with condensation at lower pH and with exposure to moisture, the Lysine coating on the Si nanoparticles did not provide enough protection from the adverse conditions in the gut, and bio-availability was not retained.

Tibia strength was significantly higher at d21 and d35 in birds fed the diets supplemented with the NTU Si made as one batch compared to those fed the control diet. As this was one of the

diets that demonstrated improved absorption, it follows that these findings may be a result of the Si supplementation. It is interesting that the differences only appear later in life. This may be due to the strain on the bones from increased body weight being greater as the birds age, or it could be due to developmental processes that occur later in life being the ones effected by the Si supplementation. This is a less likely explanation as skeletogenesis takes place at a young age, beginning during embryonic development, with the major alterations to bone in later life being repair and maintenance (Weaver and Fuchs, 2014). There is also the possibility that the effect takes place at an early age, during initial bone formation, but does not become measurable or statistically significant until later in life when the bones are larger and more mineralised. As the formation of long bones involves the conversion of the initial cartilage anlage into a collagen matrix, which is then subsequently mineralised (Mackie *et al.,* 2008; White and Wallis, 2001), it may be that the Si is influencing the production or arrangement of collagen that makes up this matrix, and thus its ability to be effectively mineralised, which would have a long-term effect on the mechanical properties of the bone. It may be that the effects do not translate into a measurable structural difference until the bone has matured further. This seems the more likely explanation as the highly hierarchical nature of type I collagen (the major type found in bones) means that the initial stages of development must be completed successfully, and to a high level of integrity, in order to facilitate the successful completion of the further steps (Ricard-Blum, 2011). Any improvement in the initial stages of bone formation could, therefore, be presumed to have a positive effect on later development.

Silica content of tibia bone tended to be higher in birds fed the NTU Si produced in one batch compared to those fed the control diet, which suggests that the NTU supplement is not only being absorbed, but also remaining in the bone for a period of time. Although this is only significant at d35, it is supported by numerical increases in P and Ca, suggesting there may be a possible silicon effect on bone mineralisation but it is likely that other supporting mechanisms relating to non-mineral elements of bone formation are also initiated by silicon

supplementation. The results from this trial also demonstrate that making the NTU supplement in weekly batches does not provide any significant advantage in terms of absorption or efficacy of the product. This is a beneficial finding, as it dramatically reduces the amount of labour involved in producing the supplements and makes it more marketable from a commercial standpoint. For a supplement to be commercially attractive it needs to have a long shelf life and remain stable as feed is typically bought in bulk quantities, and so the supplement needs to retain its efficacy for long periods.

Chapter 5: Si supplement dose response trial in broiler chicks up to slaughter at D35

5.1. Introduction

In the last chapter (Chapter 4), it was demonstrated that the Si supplement produced by NTU was successfully absorbed by the birds, and that a biological response to the supplement was observed with regards to bone strength and body weight gain. This chapter describes a trial which was designed to examine how the absorption of the supplement, and the resulting effects, changed with dosage. Dosing birds with the minimum possible volume of a supplement to gain the desired effects is not only more cost effective but reduces the impact of manufacture and transport on the environment while mitigating any impact of any potential negative responses (if any). A negative control was also included in this study in the form of sand. This was to further investigate the requirement for a bio-available form of Si, as it is widely known that sand is predominately composed of Si (SiO_2), but in its inert, highly aggregated form (Iler, 1979). If sand is able to achieve the same results as the NTU produced Si supplement, it is likely to be a much more stable supplement with far fewer environmental and economic costs associated with its manufacture and distribution.

The key questions this chapter aims to investigate are as follows:

- Investigate the minimum dose of the Si supplement needed in diets to elicit a biological response in the birds.
- Assess whether using sand as a source of Si shows the same level of absorption of Si into the blood when compared to the NTU produced supplement.
- Assess whether using sand as a source of Si shows the same level of biological response when compared to the NTU produced supplement.

5.2. Trial Procedure

5.2.1. Husbandry conditions

Birds were sourced from PD Hook Cote hatchery, Oxford, from a flock aged 43 weeks. Birds were feather sexed on day of hatch and poor birds discarded on arrival.

336 day old male Ross 308 were weighed before random allocation to 48 mesh sided pens bedded on wood shavings. Feed and water were available *ad libitum*, with care taken to ensure the birds ate and drank as soon as possible. General husbandry conditions were followed as in section 2.2.

5.2.2. Diet formulation and condition of animals

Birds were fed a generic commercial starter diet in mash form with Phytase added to all diets at 500FTU (Quantum Blue, AB Vista), as is considered industry standard. They were fed a starter diet from d0 - 21 and grower from d21 – 35. There was a total of 6 dietary treatments; basal diet without supplement, basal diet with one of 4 different doses of Si added, or basal diet with sand added. Diets were manufactured in house following the method detailed in section 2.3. The dietary treatments are shown in table 5.1. This study allowed for 8 replicates per treatment. Calculated values for starter and grower diets are shown in tables 5.2 and 5.3. Analysed values for individual diets are shown in table 5.4.

Table 5.1. Dietary treatments for the dose response trial

Diet	Treatment
A	Standard Broiler Mash + Phytase
B	Standard Broiler Mash + Phytase + 250ppm Si
C	Standard Broiler Mash + Phytase + 500ppm Si
D	Standard Broiler Mash + Phytase + 750ppm Si
E	Standard Broiler Mash + Phytase + 1000ppm Si
F	Standard Broiler Mash + Phytase + 1000ppm Sand

Table 5.2. Experimental diets with rates of inclusion (%) of ingredients in the starter phase.

Ingredient	Diet A	Diet B	Diet C	Diet D	Diet E	Diet F
Wheat	60.67	60.645	60.62	60.595	60.57	60.57
Rapeseed Solv Ext	4.00	4.00	4.00	4.00	4.00	4.00
Soybean meal 48	28.70	28.70	28.70	28.70	28.70	28.70
Soya oil	3.98	3.98	3.98	3.98	3.98	3.98
Salt	0.30	0.30	0.30	0.30	0.30	0.30
Sodium Bicarbonate	0.10	0.10	0.10	0.10	0.10	0.10
DL Methionine	0.25	0.25	0.25	0.25	0.25	0.25
Lysine HCl	0.24	0.24	0.24	0.24	0.24	0.24
Threonine	0.06	0.06	0.06	0.06	0.06	0.06
Limestone	0.43	0.43	0.43	0.43	0.43	0.43
Dicalcium Phos	0.81	0.81	0.81	0.81	0.81	0.81
Vitamin premix	0.49	0.49	0.49	0.49	0.49	0.49
econase	0.01	0.01	0.01	0.01	0.01	0.01
phytase	0.01	0.01	0.01	0.01	0.01	0.01
silica	0	0.025	0.05	0.075	0.1	0
sand	0	0	0	0	0	0.1

Table 5.3. Experimental diets with rates of inclusion (%) of ingredients in the grower phase.

Ingredient	Diet A	Diet B	Diet C	Diet D	Diet E	Diet F
Wheat - Feed	64.38	64.36	64.33	64.31	64.28	64.28
Rapeseed Solv Ext	4.00	4.00	4.00	4.00	4.00	4.00
Soybean meal 48	23.47	23.47	23.47	23.47	23.47	23.47
Soya oil	6.19	6.19	6.19	6.19	6.19	6.19
Salt	0.30	0.30	0.30	0.30	0.30	0.30
Sodium Bicarbonate	0.10	0.10	0.10	0.10	0.10	0.10
DL Methionine	0.14	0.14	0.14	0.14	0.14	0.14
Lysine HCl	0.07	0.07	0.07	0.07	0.07	0.07
Threonine	0.00	0.00	0.00	0.00	0.00	0.00
Limestone	0.51	0.51	0.51	0.51	0.51	0.51
Dicalcium Phos	0.39	0.39	0.39	0.39	0.39	0.39
Vitamin premix	0.49	0.49	0.49	0.49	0.49	0.49
econase	0.01	0.01	0.01	0.01	0.01	0.01
phytase	0.01	0.01	0.01	0.01	0.01	0.01
silica	0	0.025	0.05	0.075	0.1	0
sand	0	0	0	0	0	0.1

Table 5.4. Analysed values for the dietary treatments at each feeding phase.

Diet	DM g/kg	Ash g/kg	Fat g/kg DM	Protein g/kg DM	GE MJ/Kg DM
Starter A	85.49	3.93	5.85	31.07	19.90
Starter B	86.22	4.09	4.59	34.43	19.38
Starter C	87.41	4.23	4.72	30.60	18.90
Starter D	86.91	4.38	4.75	30.35	19.07
Starter E	86.08	4.48	4.72	31.87	19.34
Starter F	86.36	3.70	6.32	31.34	18.90
Grower A	86.83	3.63	7.88	24.56	20.16
Grower B	86.97	3.42	7.94	24.51	20.01
Grower C	86.55	3.48	7.96	23.68	20.17
Grower D	87.13	3.53	7.49	24.40	20.08
Grower E	86.01	3.58	7.89	25.40	20.38
Grower F	87.31	3.44	8.37	23.11	19.96

5.2.3. Treatment schedule / randomisation plan / condition of animals

One replicate was a pen containing 7 individually weighed chicks, and only birds between 38g and 46g were placed. Chicks were weighed by pen on d0 and allocated to a dietary treatment on arrival. Diet allocation was randomly allocated around the trial room, to reduce the effect of ventilation and possible environmental differences around the room.

5.2.4. Observations during the study

Bird observations were used to monitor the environment and if the birds appeared uncomfortable, the temperature and/or ventilation was altered accordingly. Birds were observed twice daily during the trial and any observations related to health recorded in a trial diary. Any dead birds were weighed, and reason for dispatch recorded if the bird had to be culled.

Bird weights and feed intake were calculated weekly throughout the trial as described in sections 2.4.1 and 2.4.2 respectively. Additionally, 2 birds per pen were individually weighed and marked with a different coloured pen, for identification purposes, on sample collection days.

Post mortem blood samples were collected (as per section 2.4.5) on d21, 28 and 35 for post-trial analysis, pooled from the 2 marked birds per pen, and plasma stored at -20^{o}C for analysis of Si. The left and right tibias and femurs were collected (as per section 2.4.4) from the 2 marked birds per pen and frozen. Bones were manually cleaned of tissue, weighed and the length and width (calculated by marking the centre point along the length of the bone, then measuring width at that point to standardise the measure across samples) of each bone was measured using digital callipers. Bone strength was analysed using a TA.XT plus texture analyser (Stable Microsystems, Guildford, UK) set up with a 50kg load cell and 3 point-bend fixture, as per section 2.5.6. After breaking, bones were ashed following the method in section 2.5.5, and the total percentage of bone mineral content calculated. Bones from day 21 were not analysed for ash content, as not enough sample was present after other analyses had been conducted.

5.2.5. Statistical analysis of data

Statistical analysis was carried out using SPSS v.22. After Kolmogorov–Smirnov testing to confirm normality, data were analysed using a one way ANOVA to investigate the effect of dietary treatment on bird performance, bone length, width, strength and mineral content and of Si uptake measured in plasma. Tukey's post hoc testing was used to elucidate differences between diets.

5.3. Results

5.3.1. Environment

No environmental anomalies occurred during this trial.

5.3.2. Health and Condition

Mortality figures are shown in table 5.5 and demonstrate that there was no cause for concern regarding the overall health of this flock. No differences were seen across treatments in terms of mortality at any age, and total mortality for the study was 2.5% which is standard for NTU studies and lower than would be expected in commercial settings.

Table 5.5. Bird Mortality for dose response trial by week and treatment

Diet	d0-7	d7-14	d14-21	d21-28	d28-35	Total by Diet
Control	0	1	0	0	0	1
250ppm Si	0	2	0	1	0	3
500ppm Si	1	1	0	0	0	2
750ppm Si	0	0	0	0	0	0
1000ppm Si	0	1	1	0	0	2
Sand	1	0	0	0	0	1
Total by Week	2	5	1	1	0	9

5.3.3. Bird Uniformity

Birds were individually weighed and only birds between 38 and 46g starting weight were used in the study. Mean start weights for each treatment are shown in table 5.6. There was no significant difference in start weight across the dietary treatments.

Table 5.6. Average body weight (BW) for chicks in the dose response trial at d0 (±SE).

Treatment	d0 BW/bird (g)
Control	44.0 (0.84)
250ppm Si	43.8 (0.81)
500ppm Si	43.6 (0.66)
750ppm Si	43.6 (0.77)
1000ppm Si	44.1 (0.65)
Sand	44.2 (0.70)
p value	0.756

5.3.4. Performance

Table 5.7 shows that there was no significant effect of the dose of silica supplement on feed intake (FI), bodyweight gain (BWG) or feed conversion ratio (FCR) from d0-35 of the trial, although diet D (Si supplemented at 750ppm) did demonstrate a numerically lower FCR. There was also no significant difference in BWG, FI or FCR in birds fed the diet containing sand at 1000ppm (diet F) when compared to the control or birds supplemented with the NTU Si.

Table 5.7. Average body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) per bird from d0 – 35 of the dose response trial.

Diet	BWG (±SE)	FI (±SE)	FCR (±SE)
Control	2089 (60.7)	3494 (39.4)	1.68 (0.038)
250ppm Si	2057 (80.7)	3402 (100.1)	1.66 (0.030)
500ppm Si	2131 (50.4)	3470 (52.2)	1.63 (0.032)
750ppm Si	2249 (77.1)	3591 (80.8)	1.60 (0.031)
1000ppm Si	2114 (61.3)	3512 (78.3)	1.67 (0.035)
Sand	2183 (67.7)	3564 (60.3)	1.64 (0.035)
p value	0.409	0.494	0.642

Between d0-7 (table 5.8) birds fed sand exhibited a significantly higher BWG than those fed 250ppm Si, and between d7-14 (table 5.8) there was a similar pattern, with birds fed sand exhibiting significantly higher FI than those fed 250ppm Si. However, there were no significant differences in performance data from d14-21 (table 5.10), d21-28 (table 5.11) or d28-35 (table 5.12). There were no other significant differences between diets at any other time point.

Table 5.8. Average body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) per bird from d0 – 7 of the dose response trial.

Diet	FI/bird D0-7 (g) (±SE)	BWG/bird D0-7 (g) (±SE)	FCR D0-7 (±SE)
Control	171 (7.6)	103 (3.9)^{ab}	1.65 (0.053)
250ppm Si	166 (3.3)	99 (3.5)^{b}	1.61 (0.072)
500ppm Si	172 (8.4)	108 (3.8)^{ab}	1.60 (0.081)
750ppm Si	172 (3.3)	113 (3.5)^{ab}	1.53 (0.059)
1000ppm Si	168 (5.4)	107 (3.1)^{ab}	1.59 (0.097)
Sand	184 (7.2)	117 (4.0)^{a}	1.58 (0.082)
p value	0.501	0.016	0.916

Table 5.9. Average body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) per bird from d7 – 14 of the dose response trial.

Diet	FI/bird D7-14 (g) (±SE)	BWG/bird D7-14 (g) (±SE)	FCR D7-14 (±SE)
Control	492 (10.4)[ab]	279 (6.8)	1.77 (0.058)
250ppm Si	447 (9.8)[b]	277 (11.3)	1.64 (0.056)
500ppm Si	496 (20.2)[ab]	288 (7.0)	1.71 (0.085)
750ppm Si	486 (12.4)[ab]	300 (9.1)	1.64 (0.059)
1000ppm Si	483 (10.3)[ab]	295 (9.1)	1.65 (0.078)
Sand	511 (11.6)[a]	300 (7.1)	1.71 (0.051)
p value	0.022	0.271	0.696

Table 5.10. Average body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) per bird from d14 – 21 of the dose response trial.

Diet	FI/bird D14-21 (g) (±SE)	BWG/bird D14-21 (g) (±SE)	FCR D14-21 (±SE)
Control	461 (17.5)	661 (14.9)	1.44 (0.039)
250ppm Si	453 (25.7)	619 (19.7)	1.38 (0.037)
500ppm Si	481 (26.8)	651 (19.0)	1.37 (0.046)
750ppm Si	506 (17.9)	684 (19.1)	1.36 (0.021)
1000ppm Si	459 (25.1)	650 (20.9)	1.43 (0.045)
Sand	466 (24.7)	681 (15.7)	1.48 (0.057)
p value	0.618	0.158	0.262

Table 5.11. Average body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) per bird from d21 – 28 of the dose response trial.

Diet	FI/bird D21-28 (g) (±SE)	BWG/bird D21-28 (g) (±SE)	FCR D21-28 (±SE)
Control	588 (14.9)	972 (28.2)	1.65 (0.033)
250ppm Si	580 (25.4)	938 (31.3)	1.62 (0.024)
500ppm Si	569 (18.9)	937 (33.6)	1.65 (0.029)
750ppm Si	584 (22.3)	963 (31.0)	1.65 (0.017)
1000ppm Si	590 (29.2)	969 (30.0)	1.66 (0.076)
Sand	570 (20.4)	985 (34.5)	1.73 (0.033)
p value	0.973	0.866	0.549

Table 5.12. Average body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) per bird from d21 – 28 of the dose response trial.

Diet	FI/bird D28-35 (g) (±SE)	BWG/bird D28-35 (g) (±SE)	FCR D28-35 (±SE)
Control	687 (15.4)	1186 (21.5)	1.73 (0.048)
250ppm Si	681 (16.0)	1165 (35.7)	1.71 (0.019)
500ppm Si	684 (20.0)	1181 (28.2)	1.73 (0.036)
750ppm Si	724 (30.5)	1243 (58.9)	1.71 (0.017)
1000ppm Si	716 (30.8)	1242 (48.7)	1.74 (0.042)
Sand	692 (25.4)	1203 (23.8)	1.75 (0.042)
p value	0.72	0.611	0.966

5.3.5. Plasma Si content

Table 5.13 shows that SiO_2 levels in the plasma increased with the amount of the NTU Si in the diet but did not increase with the addition of the sand compared with birds fed the control diet. This is also demonstrated by figure 5.1.

Table 5.13. SiO_2 in plasma at d21, 28 and 35.

Diet	d21 $Si0_2$ in Plasma/mgl^{-1}(±SE)	d28 $Si0_2$ in Plasma/mgl^{-1}(±SE)	d35 $Si0_2$ in Plasma/mgl^{-1}(±SE)
Control	1.28 (0.248)^{d}	1.36 (0.228)^{d}	1.25 (0.193)^{d}
250ppm Si	3.27 (1.267)^{cd}	3.03 (0.505)^{bc}	2.68 (0.634)^{c}
500ppm Si	3.85 (1.113)^{bc}	3.59 (0.497)^{b}	3.79 (0.567)^{b}
750ppm Si	5.73 (1.060)^{ab}	4.07 (1.606)^{ab}	4.20 (0.929)^{ab}
1000ppm Si	5.75 (1.382)^{a}	5.06 (1.127)^{a}	4.95 (0.887)^{a}
Sand	1.95 (0.500)^{d}	1.77 (0.304)^{cd}	1.63 (0.329)^{cd}
p value	<0.001	<0.001	<0.001

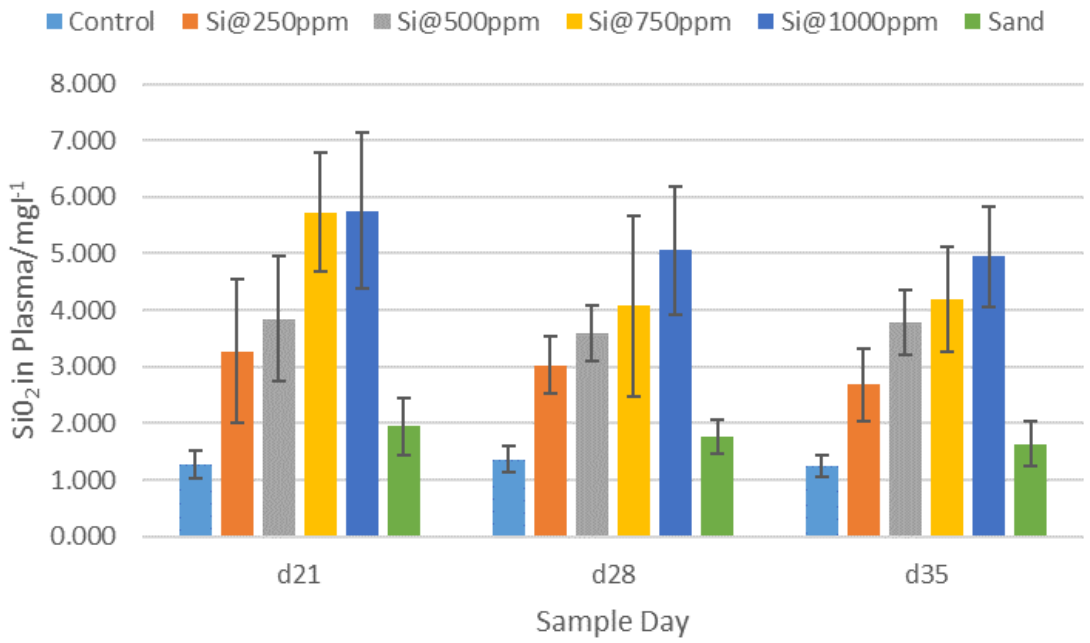

Figure 5.1. SiO_2 in plasma at d21, 28 and 35 (±SE).

5.3.6. Tibia length, width and strength

Table 5.14 shows the length, width and weight of tibias and femurs for each diet for samples collected on days 21, 28 and 35. No Significant differences were seen between any diets at any timepoint.

Table 5.14. Length, width and weight of bones collected from birds in the dose response trial.

		Day 21		Day 28		Day 35	
	Diet	**Tibia**	**Femur**	**Tibia**	**Femur**	**Tibia**	**Femur**
Width/mm (±SE)	**Control**	5.48 (0.073)	7.04 (0.106)	7.37 (0.125)	8.49 (0.192)	7.49 (0.108)	9.69 (0.166)
	250ppm Si	5.32 (0.182)	6.94 (0.121)	7.58 (0.186)	8.40 (0.244)	7.34 (0.152)	9.52 (0.219)
	500ppm Si	5.80 (0.160)	7.36 (0.094)	7.27 (0.116)	8.50 (0.124)	7.64 (0.365)	9.69 (0.219)
	750ppm Si	5.66 (0.084)	7.29 (0.143)	7.56 (0.150)	8.42 (0.161)	7.44 (0.179)	9.69 (0.235)
	1000ppm Si	5.42 (0.115)	7.05 (0.142)	7.47 (0.167)	8.52 (0.196)	7.21 (0.143)	9.61 (0.246)
	Sand	5.55 (0.280)	7.00 (0.141)	7.32 (0.127)	8.38 (0.180)	7.54 (0.145)	9.82 (0.210)
	p value	0.386	0.127	0.608	0.990	0.724	0.955
Length/mm (±SE)	**Control**	75.45 (0.624)	56.77 (0.649)	85.89 (0.902)	62.44 (0.556)	102.77 (0.773)	75.17 (0.731)
	250ppm Si	74.39 (0.703)	56.78 (0.616)	86.76 (1.018)	62.89 (0.492)	101.08 (1.750)	73.88 (0.974)
	500ppm Si	76.77 (0.779)	57.45 (0.768)	89.18 (0.873)	64.23 (0.763)	100.69 (3.668)	75.90 (0.684)
	750ppm Si	75.74 (0.836)	56.52 (0.637)	87.75 (0.733)	63.12 (0.910)	103.95 (0.864)	75.36 (0.706)
	1000ppm Si	75.02 (0.805)	55.98 (0.896)	86.37 (1.592)	62.76 (0.996)	100.59 (2.237)	73.27 (1.489)
	Sand	74.45 (1.007)	56.22 (0.701)	85.71 (1.339)	63.21 (0.550)	103.58 (0.957)	75.04 (0.758)
	p value	0.306	0.764	0.288	0.635	0.720	0.363
Weight/g (±SE)	**Control**	7.56 (0.120)	5.31 (0.246)	10.96 (0.310)	8.55 (0.238)	17.32 (0.490)	12.76 (0.359)
	250ppm Si	7.10 (0.263)	5.21 (0.141)	11.50 (0.460)	8.44 (0.278)	16.00 (0.538)	11.89 (0.373)
	500ppm Si	7.77 (0.298)	5.62 (0.166)	11.56 (0.310)	8.76 (0.300)	16.21 (0.459)	12.69 (0.325)
	750ppm Si	7.59 (0.214)	5.75 (0.164)	11.59 (0.198)	8.58 (0.305)	17.37 (0.658)	12.80 (0.464)
	1000ppm Si	7.46 (0.261)	5.30 (0.238)	11.48 (0.605)	8.96 (0.401)	15.55 (0.917)	11.65 (0.678)
	Sand	7.28 (0.349)	5.22 (0.186)	11.20 (0.259)	8.64 (0.209)	17.41 (0.538)	12.92 (0.395)
	p value	0.534	0.252	0.830	0.867	0.142	0.227

Table 5.15 and 5.16 show tibia and femur breaking strength respectively (corrected for bodyweight), across all diets at d21, 28 and 35. There were no significant differences between any diets at any timepoint.

Table 5.15. Tibia breaking strength/N (±SE) on d21, 28 and 35.

Diet	D21	D28	D35
Control	130.53 (7.018)	162.07 (8.249)	238.94 (7.454)
250ppm Si	126.52 (10.972)	173.65 (12.836)	236.92 (8.476)
500ppm Si	155.73 (7.131)	163.04 (5.597)	247.81 (11.247)
750ppm Si	142.13 (5.207)	179.29 (9.720)	250.99 (10.921)
1000ppm Si	132.18 (6.319)	168.63 (10.112)	221.25 (11.155)
Sand	135.30 (16.160)	155.28 (8.840)	243.26 (6.857)
p value	0.334	0.543	0.316

Table 5.16. Femur breaking strength/N (±SE) on d21, 28 and 35.

Diet	D21	D28	D35
Control	174.94 (12.173)	212.35 (12.587)	279.94 (10.875)
250ppm Si	172.35 (12.833)	213.42 (17.886)	293.48 (8.716)
500ppm Si	199.47 (9.941)	206.40 (6.807)	270.21 (10.350)
750ppm Si	190.98 (4.892)	206.66 (11.599)	287.05 (10.598)
1000ppm Si	170.67 (8.017)	202.05 (12.669)	268.32 (14.024)
Sand	169.94 (16.797)	197.86 (8.231)	283.89 (11.882)
p value	0.333	0.939	0.582

5.3.7. Tibia ash and mineral content

Table 5.17 shows tibia and femur ash content, across all diets at d28 and 35. There were no significant differences between any diets at any timepoint.

Table 5.17. Ash content of tibias and femurs (%) at d28 and 35 (±SE).

Diet	d28 Tibia	d28 Femur	d35 Tibia	d35 Femur
Control	35.2 (0.81)	33.0 (0.77)	36.3 (0.48)	33.4 (1.14)
250ppm Si	36.5 (0.63)	36.7 (1.46)	36.8 (0.35)	32.4 (1.53)
500ppm Si	35.8 (0.42)	33.4 (1.50)	36.0 (0.38)	34.2 (1.16)
750ppm Si	35.9 (0.60)	34.7 (0.97)	37.5 (0.42)	33.4 (0.91)
1000ppm Si	36.3 (0.29)	32.8 (1.09)	36.3 (0.44)	34.89 (0.92)
Sand	35.2 (0.58)	33.9 (0.67)	36.0 (0.51)	35.6 (0.82)
p value	0.523	0.259	0.129	0.400

5.4. Discussion

The silica supplement had no deleterious effect on performance at any dosage or time point, and showed a slight trend for improved performance at higher dosages, although this was not statistically significant. The slight increase in BWG in birds fed the sand at 1000ppm between d0-7 was an unexpected result but is more likely due to the sand aiding feed breakdown, and therefore digestibility, in the gizzard than the birds being able to liberate bio-available Si from the sand, and this suggestion is further supported by the fact that the increase in BWG was not seen alongside an increase in plasma Si concentrations, suggesting a lack of absorption. Increases in dietary fibre have been shown to have the potential to increase retention of feed in the upper GIT and improve gizzard function (Hetland, 2005) while also stimulating hydrochloric acid (HCl) production in the proventriculus (Duke, 1986). This leads to a lower pH in the gizzard which it has been shown increases pepsin activity and mineral absorbance (Guinotte *et al*., 1995; Gonzalez Alvarado *et al*., 2008). It has been established that dietary fibre can have a positive effect on gizzard development and nutrient digestibility (Mateos, 2002), and that insoluble particles which are resistant to grinding can result in stimulation of gizzard activity and an improvement in the development of the muscular layers of the gizzard, thereby increasing gizzard size (Rogel *et al*., 1987; Gonzalez Alvarado *et al*., 2008), and the retention of coarse

particles in the gizzard may cause reflux of digesta from later in the GIT back to the gizzard thereby improving nutrient utilisation (Rogel *et al*., 1987). It has been noted in previous studies, that adding additional fibre (Hetland *et al.*, 2003) and/or grit (Garipoglu *et al*., 2006) to poultry diets increased the size of the gizzard significantly, along with the size of edible organs and the overall length of the gut, so it may be that the increase in BWG is due to an increase in the size of the organs due to the sand increasing gizzard and gut development, rather than an increase in muscle mass. This would not be a desirable outcome for producers, who would be looking for an increase in muscle mass and carcass yield specifically, rather than an increase in overall bodyweight.

The correlation between the concentration of the NTU Si added to diets with the volumes found in the plasma is shown in figure 5.2 and suggests that the Si is absorbed in proportion to its concentration in the diet. The fact that the concentration of Si found in the plasma of birds fed the sand supplemented diet was not significantly different to the concentrations found in the birds fed the control diet further supports the suggestion that birds cannot liberate bio-available Si from sand.

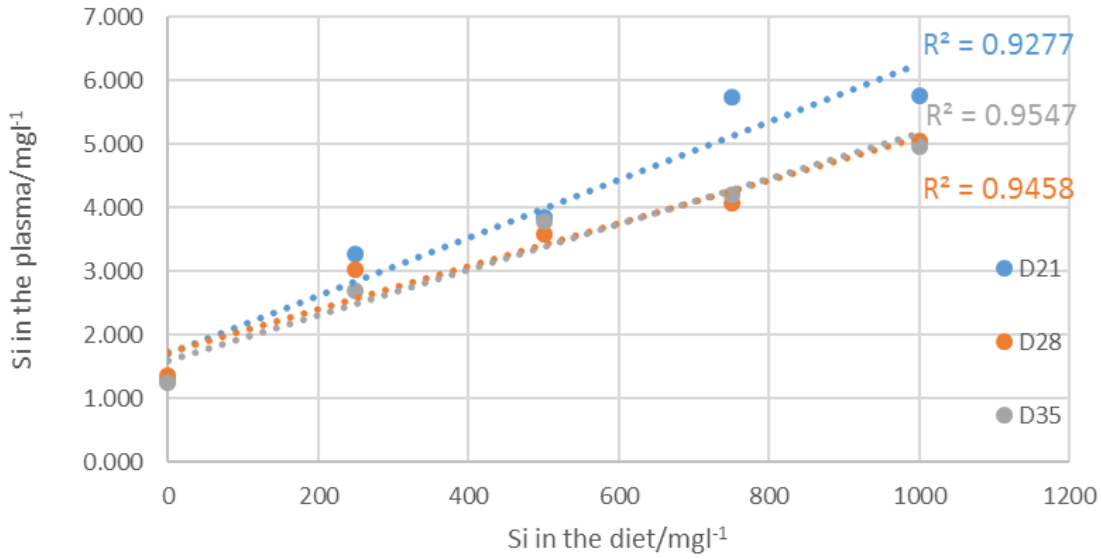

Figure 5.2. Correlation between the volume of the NTU Si added to diets and the volumes of Si found in the plasma at d21, 28 and 35.

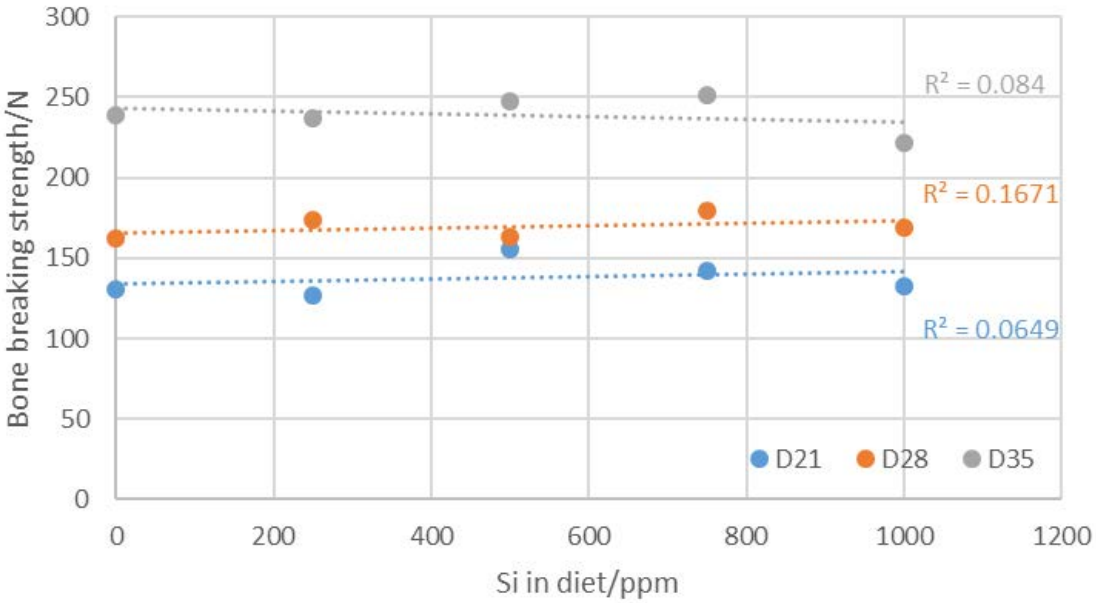

Figure 5.3. Correlation between the breaking strength of tibias and dose of Si in the diet.

Figures 5.3 (tibias) and 5.4 (femurs) show correlations between breaking strength of the bones and the dosage of Si in the diets. There were no strong correlations observed for either bone, which was unexpected as it contradicts the results from the last trial (chapter 4), and from previous trials conducted at NTU with Si (Scholey *et al.,* 2018).

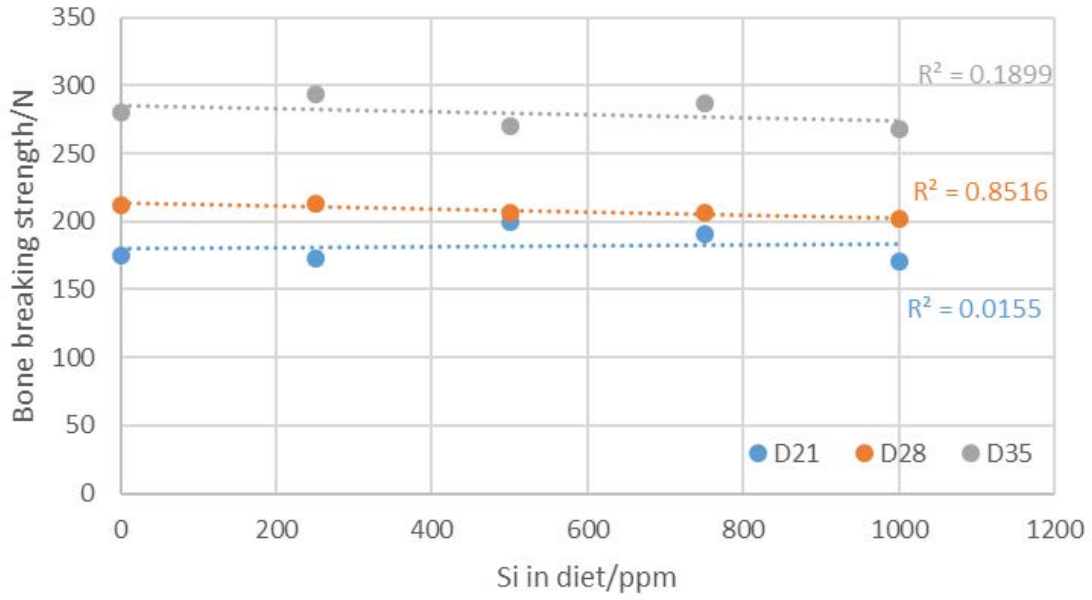

Figure 5.4. Correlation between the breaking strength of femurs and the dose of Si in the diet.

There was no statistically significant effect on any bone parameters for any diets at any time point. This was also an unexpected result as the supplement appears to have been absorbed at similar rates to previous studies (chapter 4), so a similar biological response was expected. This may be due to the fact that the Si supplement was produced by multiple different individuals due to staff absences during the trial preparation period, which may have resulted in operator differences, as not all staff are equally familiar with the machinery used in milling the Si, or the methodologies for suspending it in the soya oil (discussed in chapter 3). Although the presence of a standard SOP for the milling and suspension of the supplement should have insured that it was produced consistently, there is still a possibility that human error occurred. It was noted that the supplements that were added to the diets were visibly different in some weeks which may have affected their efficacy.

If some of the doses of the supplement that were given were less efficacious, due to poor manufacturing, it would indicate that a vital treatment point may have been missed, indicating that the role of Si in bone development may be focussed more at one timepoint than another. This may indicate that while the Si had been absorbed in to the plasma, the role for which it was needed had already been completed, or had not yet occurred, and so it was not utilised by the bird. This would go some way to explaining the lack of biological response observed, as most of the formation of bone occurs in early life, beginning during embryonic development, with the major alterations to bone in later life being repair and maintenance (Weaver and Fuchs, 2014). It is considered more likely that the effect takes place at an early age, during initial bone formation, but does not become measurable or statistically significant until later in life when the bones are larger and more mineralised. As the formation of long bones involves the conversion of the initial cartilage anlage into a collagen matrix, which is then subsequently mineralised (Mackie *et al.,* 2008; White and Wallis, 2001), it may be that the Si is influencing the production or arrangement of collagen that makes up this matrix, and thus its ability to be effectively mineralised, which would have a long-term effect on the mechanical properties of the bone. It

may be that the effects do not translate into a measurable structural difference until the bone has matured further. This seems the more likely explanation as the highly hierarchical nature of type I collagen (the major type found in bones) means that the initial stages of development must be completed successfully, and to a high level of integrity, in order to facilitate the successful completion of the further steps (Ricard-Blum, 2011). Any improvement in the initial stages of bone formation could, therefore, be presumed to have a positive effect on later development, so it seems more likely that the earlier supplementation would have a greater effect on the bone parameters. If the Si was not dosed in the early phase correctly (up to d14) this may have affected the development of the collagen matrix, leading to the lack of effect seen in the older birds. The birds in this trial were similar weights at the same age as in the previous trial (chapter 4) so it is unlikely that a difference in body weight contributed to the lack of biological response, which supports the suggestion that it was due to a lack of bio-availability at a significant developmental stage. In order to examine this idea further, more work is needed to investigate the specific mechanisms by which the Si may be operating.

In conclusion, while this trial may not have truly fulfilled its role as a dose response trial, due to the lack of biological response observed in the birds, it has further supported that the NTU supplement is absorbed by the birds and shown that the absorption is correlated with the dose of Si in the diet. This suggests that the Si is more likely to be absorbed via passive mechanisms, such as diffusion, rather than active transport but further investigation is needed into its passage through the GIT in order to support this statement. It has also provided some useful insight into the times at which correct dosage of Si may be most important but again, much more thorough investigation into the potential mode, or modes, of action of the Si supplement is needed in order to elucidate this area further.

Chapter 6: Absorption and utilisation of Si by young broiler chicks

6.1. Introduction

The trial in this chapter was designed to examine the possible absorption site of the Si within the gastrointestinal tract (GIT), and to examine its biological effect on numerous different tissues in young broilers. The data from the bird trial reported in chapter 5 associated with early post-hatch time points suggested that early bone development may be a key developmental stage for impact of the Si supplement: therefore, the next trial focussed on this life stage. The concentration of Si in the plasma was measured, along with the presence of biomarkers for bone formation and histological parameters of different site on the bones. Effect on gross morphology (length, width, weight) was measured, as was bone breaking strength and mineral percentage, and tendon strength. In the previous chapter (chapter 5), it was shown that the Si supplement produced by NTU was successfully absorbed by the birds at a similar level when supplemented at 750ppm and 1000ppm. While this suggested that the lower dosage (750ppm) may be more economically viable from a commercial standpoint, the lack of biological response in that trial was cause for concern. Due to this, and the fact that a biological response was observed at the higher dosage (1000ppm) in the product comparison trial (chapter 4), the supplement was given at the higher rate for the trial described in this chapter, in order to ensure that there was the best possible chance of a biological response which could be measured effectively.

It was hoped that by measuring many different tissue types and comparing the morphology from a structural level down to a cellular level, a better understanding would be gained into the possible mode, or modes, of action of Si *in vivo*. An increased understanding of how Si is affecting specific tissues will not only further knowledge in this area of poultry nutrition, which is currently lacking, but also offers the potential for production of an efficacious supplement that may offer greater benefits and lend itself to application in other fields.

The key questions this chapter aims to investigate are as follows:

- Investigate how is Si partitioned within the solid and aqueous phases of digesta, and what this suggests about possible sites of absorption.
- Assess how Si effects the gross morphology, histological characteristics and breaking strength of bones.
- Assess how Si effects the gross morphology and breaking strength of tendons.
- Investigate if Si supplementation increases biomarkers for bone formation?

6.2. Trial Procedure

6.2.1. Husbandry conditions

Birds were sourced from PD Hook Cote hatchery, Oxford, from a flock aged 43 weeks. Birds were feather sexed on day of hatch and poor birds discarded on arrival.

576 day old male Ross 308 were weighed before random allocation to 48 mesh sided pens bedded on wood shavings. Feed and water were available *ad libitum*, with care taken to ensure the birds ate and drank as soon as possible. General husbandry conditions were followed as in section 2.2.

6.2.2. Diet formulation and condition of animals

Birds were fed a generic commercial starter diet in mash form with Phytase (Quantum Blue, AB Vista) added to all diets at 500FTU, as is considered industry standard. They were fed a starter diet for the whole trial, from d0 - 21. There were two dietary treatments; basal diet without supplement (Control) and basal diet with Si supplemented at 1000ppm (Si). Diets were

manufactured in house following the method detailed in section 2.3. This study allowed for 12 replicates per treatment. Calculated values for diets are shown in table 6.1. Analysed values for individual diets are shown in table 6.2.

Table 6.1. Experimental diets with rates of inclusion (%) of ingredients in the starter phase.

Ingredient	Control	Si
Wheat	60.67	60.57
Rapeseed Solv Ext	4.00	4.00
Soybean meal 48	28.70	28.70
Soya oil	3.98	3.98
Salt	0.30	0.30
Sodium Bicarbonate	0.10	0.10
DL Methionine	0.25	0.25
Lysine HCl	0.24	0.24
Threonine	0.06	0.06
Limestone	0.43	0.43
Dicalcium Phos	0.81	0.81
Vitamin premix	0.49	0.49
Silica	0.00	0.10

Table 6.2. Analysed values for the dietary treatments at each feeding phase.

Diet	DM g/kg	Ash g/kg	Fat g/kg DM	Protein g/kg DM	GE MJ/Kg DM	Ca mg/kg DM	P mg/kg DM
Control	88.16	4.53	5.34	24.05	19.20	8.23	7.04
Si	87.21	4.65	5.37	23.58	19.30	8.46	7.45

6.2.3. Treatment schedule / randomisation plan / condition of animals

One replicate comprised two adjacent pens (referred to as a plot) containing 12 individually weighed chicks per pen (24 per plot). Only birds between 38g and 46g were placed. Chicks were weighed by pen on d0 and allocated to a dietary treatment on arrival. Diet allocation was randomly allocated around the trial room, to reduce the effect of ventilation and possible environmental differences around the room.

6.2.4. Observations during the study

Bird observations were used to monitor the environment and if the birds appeared uncomfortable, the temperature and/or ventilation was altered accordingly. Birds were observed twice daily during the trial and any observations related to health recorded in a trial diary. Any dead birds were weighed, and reason for dispatch recorded if the bird had to be culled.

Bird weights and feed intake were calculated on d7, 10, 14, 17 and 21 throughout the trial as described in sections 2.4.1 and 2.4.2 respectively. This measurement was done per pen, rather than per plot, in order to allow for accurate measurement of feed intake, and then the data was combined into plots before statistical analysis to allow for comparison with other parameters.

Post mortem blood samples and leg bones (tibia and femur) were collected (as per section 2.4.5 and 2.4.4 respectively) on d7, 10, 14, 17 and 21 for post-trial analysis, with digesta from the gizzard, duodenum, jejunum and ileum (collected as per section 2.4.3) and the gastrocnemius tendon (collected as per section 2.4.6) being collected only on d21. Table 6.3 shows details of the samples collected, the days they were collected on and whether they were collected per pen or per plot.

Blood samples were analysed for total Si content via ICP-OES (as per section 2.5.8) and for the presence of PINP, a biomarker for bone formation (as per section 2.5.11).

Tibias and femurs collected on days 7, 10, 14, 17 and 21 were first analysed for gross morphology and breaking strength (as per section 2.4.6). After breaking, they were then analysed for ash percentage using the method in section 2.5.5. One right tibia taken from each plot on d21 was used for histological analysis, as per section 2.5.16. The gastrocnemius tendons taken on d21 were analysed for breaking strength and gross morphology as per the method in section 2.5.7.

The digesta collected on d21 was centrifuged for 10 minutes at 3000rpm and supernatant collected (approximately 5cm^3) following procedures by Pang and Applegate (2007). This supernatant was considered the aqueous phase. The digesta remaining in the centrifuge tube was then filtered through a 0.45µl membrane filter and the portion remaining, after as much liquid as possible had been removed, was considered the solid phase. Both solid and aqueous phases were tested for bio-available Si using the molybdenum blue method as detailed in section 2.5.15.

Table 6.3. Sample collection details for each collection day of the trial.

Day	Blood	Tibia and femur	Gastrocnemius tendon	Digesta	Performance
7	8 birds, pooled per plot	Left and right, 8 birds per plot	None taken	None taken	Per pen, all birds
10	6 birds, pooled per plot	Left and right, 6 birds per plot	None taken	None taken	Per pen, all birds
14	4 birds, pooled per plot	Left and right, 4 birds per plot	None taken	None taken	Per pen, all birds
17	2 birds, pooled per plot	Left and right. 2 birds per plot	None taken	None taken	Per pen, all birds
21	All remaining birds, pooled per plot	Left and right, all remaining birds per plot	Left tendon, 2 birds per plot	All remaining birds, pooled per plot	Per pen, all birds

6.2.5. Statistical analysis of data

Statistical analysis was carried out using SPSS v.22 (IBM Statistics). After Kolmogorov–Smirnov testing to confirm normality, data were analysed using a one way ANOVA to investigate the effect of dietary treatment on bird performance, bone length, width, strength and mineral content and of Si uptake measured in plasma.

6.3. Results

6.3.1. Environment

No environmental anomalies occurred during this trial.

6.3.2. Health and Condition

Mortality figures are shown in table 6.4 and demonstrate that there was no cause for concern regarding the overall health of this flock. No differences were seen across treatments in terms of mortality at any age, and total mortality for the study was 2.5% which is standard for NTU studies and lower than would be expected in commercial settings.

Table 6.4. Bird Mortality by week and treatment.

Diet	d0-7	d7-14	d14-21	Total by Diet
Control	4	2	1	7
Si	4	2	1	7
Total by week	8	4	1	14

6.3.3. Bird Uniformity

Birds were individually weighed and only birds between 38 and 46g starting weight were used in the study. Mean start weights for each treatment are shown in table 6.5. There was no significant difference in start weight across the dietary treatments.

Table 6.5. Average body weight (BW) for chicks at d0 (±SE).

Treatment	d0 BW/bird (g)
Control	44.2 (0.15)
Si	43.7 (0.45)
p value	0.235

6.3.4. Performance

Table 6.6 shows that there was no significant effect of the silica supplement on feed intake (FI), or feed conversion ratio (FCR) from d0-21 of the trial. However, there was a significant increase in body weight gain (BWG) in birds fed the Si supplement and a trend for increased FI, when compared to those fed the basal diet alone.

Table 6.6. Average body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) per bird from d0 – 21.

Diet	BWG (±SE)	FI (±SE)	FCR (±SE)
Control	717.56 (25.325)	1051.27 (19.026)	1.48 (0.036)
Si	792.30 (22.712)	1107.70 (21.358)	1.41 (0.028)
p value	0.04	0.064	0.129

6.3.5. Plasma Si content

Table 6.7 shows that SiO_2 levels in the plasma were significantly increased with the addition of the Si to the diet across all ages sampled from d7 to d21.

Table 6.7. SiO_2 in plasma at d7, 10, 14, 17 and 21 (±SE).

Diet	d7 $Si0_2$ in Plasma (mg/l^{-1})	d10 $Si0_2$ in Plasma (mg/l^{-1})	d14 $Si0_2$ in Plasma (mg/l^{-1})	d17 $Si0_2$ in Plasma (mg/l^{-1})	d21 $Si0_2$ in Plasma (mg/l^{-1})
Control	2.33 (0.104)	2.09 (0.117)	1.67 (0.106)	2.05 (0.232)	1.75 (0.104)
Si	6.50 (0.299)	6.49 (0.538)	5.78 (0.535)	6.00 (0.568)	5.16 (0.475)
p value	<0.001	<0.001	<0.001	<0.001	<0.001

6.3.6. Presence of a biomarker of bone turnover

Table 6.8 shows the concentration of PINP measured in the blood plasma at d7, 14 and 21. There was no significant difference seen at d7, but on days 14 and 21, birds supplemented with Si had significantly lower blood plasma concentrations of PINP compared to un-supplemented birds.

Table 6.8. Average PINP concentration (ng/ml) (±SE) in blood plasma.

Diet	d7	d14	d21
Control	0.99 (0.016)	1.03 (0.055)	1.37 (0.146)
Si	0.99 (0.019)	0.85 (0.034)	1.02 (0.063)
p value	0.940	0.007	0.034

6.3.7. Partitioning of Si between the solid and aqueous phases of digesta

The results for the gizzard and duodenum are not included, as there was insufficient aqueous phase digesta present in the gizzard, or solid phase digesta present in the duodenum, to give reliable comparisons. Table 6.9 shows the concentration of bio-available Si found in the feed, and in the solid and aqueous phases of digesta at the various gastrointestinal (GIT) sites. At all sites there was significantly more bio-available Si present in the solid phase of digesta of birds fed the Si supplement. There was also significantly more bio-available Si found in the supplemented feed compared with the control feed. There was significantly more bio-available Si present in the supplemented feed, at the jejunum there was significantly more bio-available Si present in the aqueous phase of digesta of birds fed the Si supplement, and in the ileum there was a trend toward more bio-available Si present in the aqueous phase of digesta of birds fed the Si supplement.

Table 6.9. Concentration (mg/l^{-1}) of bio-available Si found in the feed and in the solid and aqueous phases of digesta at the various gastrointestinal (GIT) sites (±SE).

		Solid phase		Aqueous phase	
Diet	**Feed**	**Jejunum**	**Ileum**	**Jejunum**	**Ileum**
Control	64.4 (7.63)	232.8 (22.88)	63.535 (5.17)	33.308 (3.97)	52.976 (4.23)
Si	105.5 (10.21)	367.5 (26.47)	250.942 (12.46)	50.387 (8.37)	60.765 (4.03)
p value	0.032	<0.001	<0.001	0.006	0.054

Table 6.10 shows the volume of bio-available Si found in each phase, as a percentage of the total volume of bio-available Si detected across both the solid and aqueous phase. Values were converted into percentages before statistical analysis. Bio-available Si was significantly higher in the solid phase of digesta for both the jejunum and ileum in the supplemented birds, and

significantly lower in the aqueous phase. In the jejunum, the partitioning appears to be more equally seen between birds fed supplemented and control diets, however, in the ileum the differences were marked.

Table 6.10. Volume of bio-available Si (%) found in each phase, as a percentage of the total volume of bio-available Si detected across both the solid and aqueous phase (±SE).

Diet	Solid phase jejunum	Aqueous phase Jejunum	Solid phase ileum	Aqueous phase ileum
Control	83.04 (1.9)	16.96 (1.9)	53.80 (2.5)	46.20 (2.5)
Si	85.94 (1.8)	14.07 (1.8)	77.89 (2.9)	22.11 (2.9)
p value	0.036	0.036	<0.001	<0.001

6.3.8. Tendon length, width and strength

There were no differences found between diets for the average length, width or thickness of tendons, or the amount of force, distance or time taken for rupture to occur (table 6.11).

Table 6.11. The average length, width and thickness of tendons along with the amount of force, the distance stretched and the time taken to rupture (±SE).

Diet	Force/N	Length/ mm	Width/ mm	Thickness/ mm	Distance/ mm	Time/Sec
Control	48.08 (4.966)	16.89 (0.766)	6.73 (0.302)	1.40 (0.059)	6.43 (0.688)	12.85 (1.372)
Si	51.13 (3.899)	18.35 (1.046)	6.80 (0.189)	1.39 (0.101)	7.23 (0.698)	14.46 (1.396)
p value	0.634	0.271	0.851	0.888	0.420	0.419

6.3.9. Tibia and femur length, width and strength

Table 6.12 shows the length, width and weight of tibias and femurs on each diet for samples collected on days 7, 10, 14, 17 and 21. On d7, supplemented birds demonstrated significantly wider, longer and heavier tibias than un-supplemented birds. On d10 supplemented birds showed significantly longer tibias and femurs, and heavier tibias. On d14 supplemented birds showed longer tibias. There were no significant differences on d17, but by d21, supplemented birds showed significantly wider, longer and heavier tibias and femurs than un-supplemented birds.

Table 6.13 and 6.14 show tibia and femur breaking strength respectively, across all diets at d7, 10, 14, 17 and 21. No significant differences in tibia or femur breaking strength was seen at d7, 10, 14 and 17. On d21, both tibia and femur of supplemented birds demonstrated significantly higher breaking strength compared to un-supplemented birds.

Table 6.12. Length, width and weight of tibias and femurs on each diet for samples collected on days 7, 10, 14, 17 and 21.

Day	Bone	Measurement	Control Diet	Si Diet	p value
7	Tibia	Width/mm (±SE)	2.64 (0.035)	2.74 (0.026)	0.026
		Length/mm (±SE)	42.15 (0.112)	43.01 (0.202)	0.001
		Weight/g (±SE)	1.06 (0.025)	1.17 (0.028)	0.005
	Femur	Width/mm (±SE)	3.10 (0.041)	3.17 (0.031)	0.150
		Length/mm (±SE)	32.91 (0.154)	32.92 (0.195)	0.998
		Weight/g (±SE)	0.78 (0.017)	0.82 (0.020)	0.161
10	Tibia	Width/mm (±SE)	3.27 (0.074)	3.32 (0.049)	0.537
		Length/mm (±SE)	47.09 (0.468)	49.03 (0.189)	0.001
		Weight/g (±SE)	1.62 (0.052)	1.84 (0.047)	0.006
	Femur	Width/mm (±SE)	3.72 (0.059)	3.77 (0.052)	0.608
		Length/mm (±SE)	34.03 (2.759)	37.77 (0.249)	0.001
		Weight/g (±SE)	1.19 (0.031)	1.27 (0.033)	0.094
14	Tibia	Width/mm (±SE)	4.12 (0.057)	4.20 (0.069)	0.608
		Length/mm (±SE)	57.23 (0.395)	58.45 (0.538)	0.001
		Weight/g (±SE)	2.99 (0.083)	3.21 (0.097)	0.094
	Femur	Width/mm (±SE)	4.82 (0.079)	4.97 (0.073)	0.169
		Length/mm (±SE)	43.99 (0.472)	44.60 (1.104)	0.219
		Weight/g (±SE)	2.19 (0.062)	2.33 (0.067)	0.117
17	Tibia	Width/mm (±SE)	4.84 (0.116)	4.98 (0.124)	0.424
		Length/mm (±SE)	65.15 (0.841)	66.71 (0.796)	0.190
		Weight/g (±SE)	4.31 (0.171)	4.66 (0.192)	0.189
	Femur	Width/mm (±SE)	5.67 (0.131)	6.05 (0.148)	0.067
		Length/mm (±SE)	49.73 (0.593)	50.74 (0.571)	0.233
		Weight/g (±SE)	3.20 (0.841)	3.43 (0.135)	0.231
21	Tibia	Width/mm (±SE)	5.45 (0.089)	5.73 (0.101)	0.041
		Length/mm (±SE)	70.44 (0.617)	74.10 (0.525)	<0.001
		Weight/g (±SE)	5.65 (0.162)	6.59 (0.153)	<0.001
	Femur	Width/mm (±SE)	6.43 (0.101)	6.77 (0.100)	0.017
		Length/mm (±SE)	53.53 (0.450)	56.27 (0.365)	<0.001
		Weight/g (±SE)	4.13 (0.115)	4.92 (0.103)	<0.001

Table 6.13. Tibia breaking strength/N (±SE) on d7, 10, 14, 17 and 21.

Diet	d7	d10	d14	d17	d21
Control	22.64 (0.837)	51.27 (3.053)	93.90 (5.047)	111.35 (8.018)	132.12 (11.554)
Si	23.11 (0.523)	56.07 (3.127)	100.74 (4.781)	118.59 (7.932)	176.20 (9.375)
p value	0.639	0.128	0.336	0.347	0.008

Table 6.14. Femur breaking strength/N (±SE) on d7, 10, 14, 17 and 21.

Diet	d7	d10	d14	d17	d21
Control	34.15 (2.524)	54.91 (3.088)	112.46 (5.681)	144.81 (7.742)	126.07 (8.878)
Si	34.22 (2.110)	62.30 (3.576)	116.79 (7.968)	144.92 (7.774)	177.17 (8.826)
p value	0.887	0.132	0.663	0.713	0.001

6.3.10. Tibia and femur ash and mineral content

Table 6.15 and 6.16 show the ash content of tibias and femurs respectively, across diets and sample collection days. Tibias for the d14 collection day are omitted as samples were lost during processing.

Table 6.15. Ash content (%) of tibia bones (±SE).

Diet	d7	d10	d17	d21
Control	29.96 (0.862)	32.20 (0.499)	35.10 (0.463)	31.69 (0.494)
Si	30.68 (0.727)	33.88 (0.284)	35.36 (0.497)	34.79 (0.496)
p value	0.525	0.008	0.709	<0.001

Table 6.16. Ash content (%) of femur bones (±SE).

Diet	d7	d10	d14	d17	d21
Control	30.87 (0.587)	32.00 (0.518)	35.40 (0.344)	33.85 (0.460)	31.47 (0.520)
Si	32.15 (0.257)	32.57 (0.238)	36.00 (0.562)	34.38 (0.439)	33.86 (0.652)
p value	0.060	0.333	0.600	0.412	0.009

6.3.11. Histological parameters of tibias and femurs

Tables 6.17 and 6.18 show the average measurements of the various growth plate zones and the average number of structures present in the diaphysis of the tibias respectively. Growth plates of birds fed the supplemented diet showed a significant increase in width of the resting zone, compared to birds fed the un-supplemented diet, but no significant differences were observed in the proliferative or hypertrophic zones. A significant increase in the number of osteoblasts and osteoclasts present in the diaphysis was seen in birds fed the supplemented diet, compared to birds fed the un-supplemented diet, and while no significant difference was observed, there was a trend toward an increase in osteon numbers.

Table 6.17. Average widths (μm) of the resting, proliferative and hypertrophic zones of the growth plates of the tibias (±SE).

Diet	Resting	Proliferative	Hypertrophic
Control	569.5 (39.68)	397.8 (12.89)	347.7 (7.76)
Si	712.4 (45.44)	393.5 (9.80)	351.2 (8.02)
p value	0.020	0.782	0.544

Table 6.18. Average numbers of osteoblast, osteoclasts and osteons present in the diaphysis of the tibias (±SE).

Diet	Osteoblasts	Osteoclasts	Osteons
Control	61.96 (2.140)	4.82 (0.368)	24.50 (0.773)
Si	72.50 (2.303)	5.80 (0.296)	26.85 (1.221)
p value	0.002	0.028	0.089

6.4. Discussion

During this study, performance parameters were measured to verify that the Si supplement showed no negative effects that would limit its viability as a feed supplement. While there was no significant effect on feed intake or FCR, 0-21 day body weight gain was significantly increased in birds fed the Si supplemented diets. This is an important factor should the supplement be commercialised, as improvements in bird performance in addition to improved skeletal integrity positively impacts on the economic viability of using the supplement.

The significantly higher concentrations of Si present in the blood plasma throughout the trial indicate that the supplement is being successfully absorbed through the gastrointestinal tract as was seen in previous studies (Short *et al*., 2011; Scholey *et al*., 2018 and chapters 4 and 5). However, from the data gathered here it is not possible to say with confidence at what particular GIT site this is taking place. The findings from the investigation into the partitioning of Si between the solid and aqueous phases of digesta yielded some interesting and unexpected results. In the solid phase the concentration of Si was seen to be much higher in the jejunum than in the feed. This was unexpected, as the concentration appears to more than triple during transit through the GIT to the jejunum. This seems unlikely to be a true representation of an actual mechanism, and there are two possible explanations. The first is that the birds are somehow liberating large volumes of bio-available Si from the feed in the foregut. This could be considered unlikely since the ingredients in the basal diet do not contain enough naturally

occurring Si to cause the kind of disparity observed in this study. Dietary bio-available Si ($Si(OH)_4$) is found naturally in several foods and beverages, including grains and most drinking water (Jugdaohsingh *et al.,* 2002), however, Powell *et al*. (2005) studied the most commonly consumed dietary sources for humans, and found them to contain only very low concentrations. The products which would be of most relevance to the poultry industry have been listed in table 6.19. It can be seen from these values that it is highly unlikely that there was sufficient bio-available Si present in the basal diet for the birds to liberate the amount of Si required to account for the change in concentration from the feed to the jejunum digesta.

Table 6.19. Concentrations of Si found in commonly consumed human food products. Adapted from Powell *et al*. (2005).

Product	Si (mg/l^{-1})
Bran flakes	0.482
Oat bran	2.336
Oats	1.139
Wheat bran	1.098
Puffed wheat	0.219
Shredded whole wheat	0.134
Wholemeal wheat flour	0.304
Brown rice	0.376
Soya beans	0.119
Tap water	0.025

The other possible reason for this apparent dramatic increase in Si concentration from the feed to the jejunum in the solid phase of digesta, is that the measurement of Si within the diet is not truly representative of the concentrations present at the time of feeding. It was found during the testing of the neat Si supplement, both alone and once incorporated into feed, (discussed in chapter 7 in greater detail) that storing the supplement at low temperatures affected the ability

of the molybdenum blue assay to detect the concentration of bio-available Si present. As these feed and digesta samples were stored at -20°C in a freezer between collection and testing, it is possible that this adversely affected the results. It was also demonstrated that adding the supplement into feed reduced retention of bio-availability over time, so the gap of some months between sample collection and testing may also have impaired the readings of both the feed and digesta samples.

In the aqueous phase the concentration of Si reduced as it moved from the feed into the tract (as would be expected), but it is suspected that this reduction may be greater than it appears, due to the potential issues with the Si readings in the initial feed samples suggested above. If this is the case, then a dramatic decrease is seen in Si concentration in the aqueous phase between the feed and the jejunum, followed by an increase in concentration from the jejunum into the ileum, with higher concentrations always being present in the supplemented birds compared to the un-supplemented birds. This could be explained by a combination of factors. The Si may be being predominately absorbed via the aqueous phase in the foregut, accounting for the drop in concentration. This would make sense as it has long been reported that, while mechanical, chemical and enzymatic processes occur throughout the gastrointestinal tract, absorption of nutrients occurs only in the small intestine (Larbier and Leclercq, 1994). The further increase in Si may then be due to Si from the solid phase moving into solution in the aqueous phase, thus causing the concentration to rise again at this point. This theory is supported in the control diet by figure 6.1, which shows the concentrations of Si in the solid and aqueous phases relative to each other. It can be seen that as the Si concentration in the solid phase increases, it decreases in the aqueous phase, and *vice versa*. This suggests movement of Si between phases as it moves through the hindgut. However, the same cannot be said for the Si supplemented diet. As can be seen in figure 6.2, the concentration of bio-available Si in this diet follows a similar pattern to the control, but with far greater volumes remaining in the solid phase, and a much smaller amount moving to the aqueous phase. This may be due to the oil in

the supplement inhibiting the full breakdown of the Si in the tract. It was demonstrated in chapter 3 that smaller particle sizes were harder to liberate from the oil *in vitro*, so it may be the case that the slightly larger particles were passed into the aqueous phase and absorbed (causing the higher plasma Si readings) while the smaller particles remained encapsulated in the oil and could not move into the aqueous phase. This poses an interesting question, as it may be that if the most appropriate particle size for absorption can be further investigated and refined, then the production of an even more efficacious supplement with even higher bio-availability may be produced, further reducing the costs of production and the volume of product required to meet optimum dosage levels.

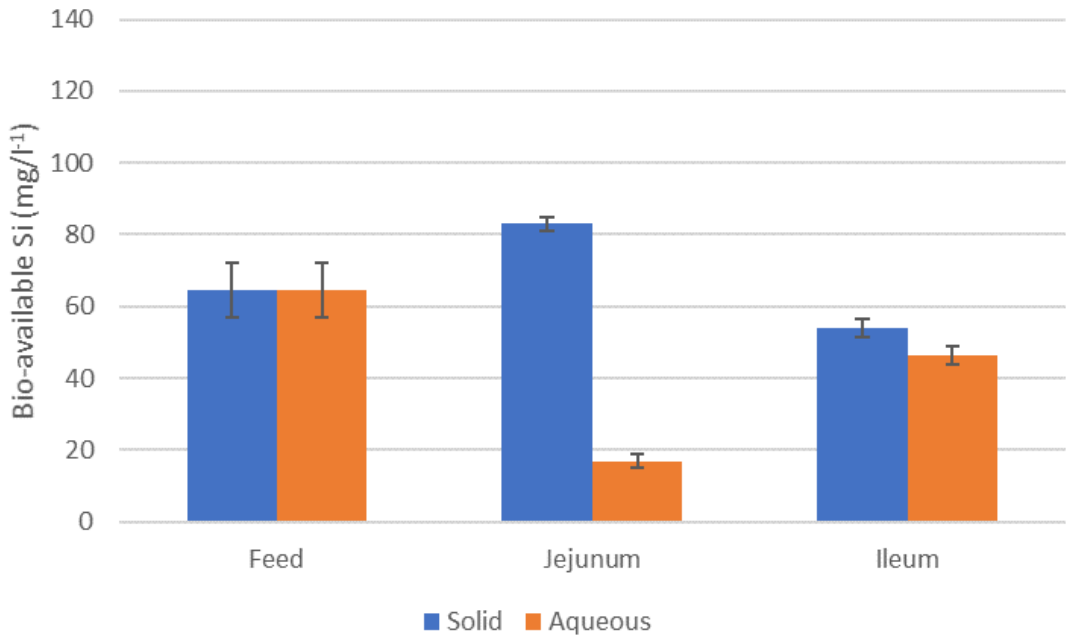

Figure 6.1. Concentration of bio-available Si in the solid and aqueous phases of digesta in birds fed the un-supplemented diet (±SE).

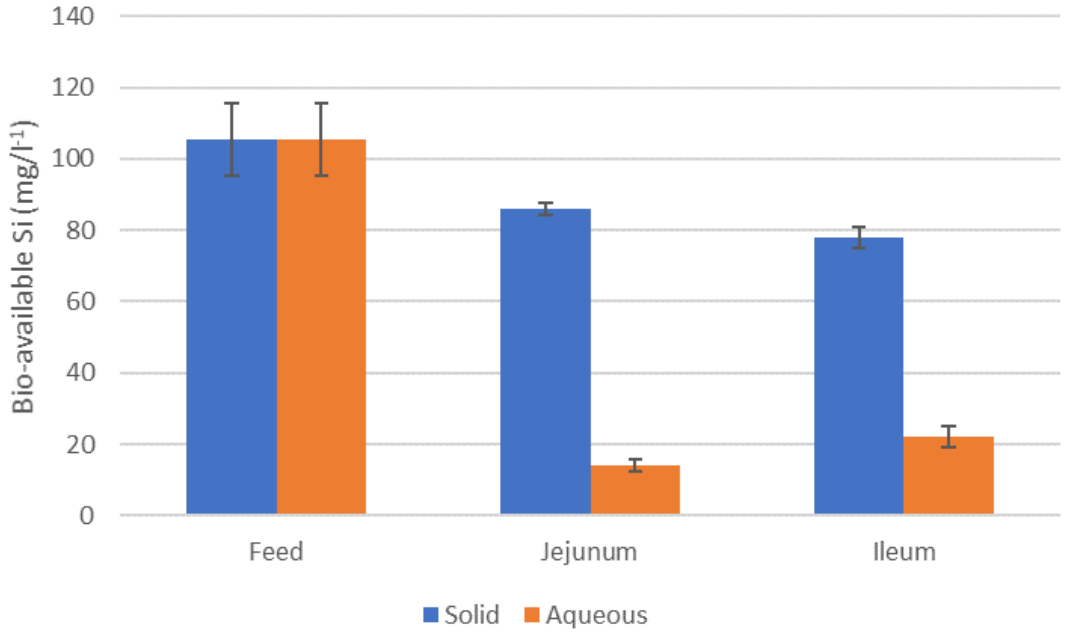

Figure 6.2. Concentration of bio-available Si in the solid and aqueous phases of digesta in birds fed the Si supplemented diet (±SE).

Throughout the literature it is continually indicated that the mechanism for Si uptake is unknown, therefore the limited understanding of the absorbance of bioavailable Si in broiler chickens is novel and difficult to compare. Despite this, through the use of model organisms and known water-soluble vitamins and minerals, the reliability of how and where in the body Si is absorbed can be assessed.

With other water-soluble vitamins and minerals, absorbance is dependent on the intracellular concentration of sodium (Na) in polarised epithelial cells of the small intestine, in order to establish an electrochemical gradient of Na that allows nutrient distribution via channels and transporters to the intestinal lumen and circulatory system. When suspended in a fluid, solutes can cross the capillary bed of the intestinal villi to be distributed where needed. In the case of Si, predominant transport is to biomineralized cellular structures such as the parenchymal and connective tissues (Marron *et al.*, 2016).

For the most part, minerals take the form of water-soluble salts (McCabe *et al.*, 2015), dissociating to aqueous cations and anions when dissolved. Similarly, absorption is dependent on solubility, allowing known mineral absorbance to additionally support suggestions as to the site of Si absorbance.

The majority of water-soluble vitamins (most B vitamins and vitamin C) are absorbed in micelles via simple diffusion, with the exception of B_{12} which is up taken by endocytosis in the ileum. Water-soluble nutrient absorption predominantly takes place in the duodenum and jejunum (Naderinejad *et al.*, 2016), showing similarities towards to absorbance of Si within the body. The fact that the volume of Si dropped dramatically between the feed and the jejunum, and further between the jejunum and the ileum supports the suggestion that Si is also being absorbed via diffusion in these areas of the tract.

The lack of significant difference between diets for tendon parameters suggests that the Si is not acting via feedback from the osteocytes. There was a potential hypothesis that the Si could have been increasing the integrity of the tendons, which in turn improved the interaction of tendon and bone and meant that the osteocytes within the bone where able to better communicate where forces were acting upon the bone and, therefore, the osteoblasts were able to respond and reinforce the bone that was under strain. The lack of differences suggests that this is not the case, and that the Si is more likely to be active via a different mechanism. As mentioned in chapters 4 and 5, it seems more likely that the Si is somehow acting on the bones themselves, rather than the improvement in bones being a secondary effect of improved tendon integrity. This is supported by the data from this trial regarding histological measurements and biomarker presence.

While there were some differences observed at days 7 and 14, with supplemented birds demonstrating wider, longer and heavier tibias, and longer tibias respectively, the data collected for the bone parameters suggest that the Si is having the greatest effect at days 10 and 21. At

day 10, the tibias of supplemented birds were both wider, longer, heavier and contained a higher ash percentage, but did not demonstrate an increase in breaking strength. This suggests that while the bones may be generally larger, and more mineralised, this does not translate to a structural advantage to the birds in terms of bone strength. This is likely due to the fact that the skeleton of the bird at this age is still relatively cartilaginous (Applegate and Lilburn, 2002; Han *et al*., 2015), and so although more mineralised than the tibias of un-supplemented birds, they may not be mineralised enough at this age to withstand greater compressive forces. The larger size of the bones may also be linked to the increase in body weight of the birds fed the supplemented diet as, logically, larger birds will have larger skeletons. The data was corrected for bodyweight (shown in tables 6.20 and 6.21), and still demonstrates the same trends, although the statistical significance only remains in the femurs at d21. This suggests that while body weight may be partially responsible for the changes seen, there are other contributing factors.

Table 6.20. Tibia breaking strength/N:BW/kg (±SE) corrected for body weight on d7, 10, 14, 17 and 21.

Diet	d7	d10	d14	d17	d21
Control	149.2 (2.18)	237.9 (10.28)	238.6 (6.41)	194.6 (8.46)	606.3 (90.54)
Si	153.9 (6.10)	229.0 (10.99)	242.4 (10.19)	201.5 (13.36)	746.1 (72.13)
p value	0.248	0.225	0.729	0.419	0.119

Table 6.21. Femur breaking strength/N:BW/kg (±SE) corrected for body weight on d7, 10, 14, 17 and 21.

Diet	d7	d10	d14	d17	d21
Control	221.8 (14.57)	262.6 (12.56)	276.1 (15.39)	239.6 (8.86)	586.8 (84.05)
Si	231.4 (16.61)	245.6 (11.22)	291.3 (12.49)	263.2 (15.43)	753.2 (62.89)
p value	0.644	0.386	0.686	0.273	0.028

At d21, both tibias and femurs in supplemented birds demonstrated wider, longer, heavier and stronger bones with an increase in ash percentage compared to un-supplemented birds. Again, the generally larger size of the bones may be due to the increase in body weight also demonstrated by these birds, but the increase in ash percentage suggests that these bones are also more mineralised when compared to their un-supplemented counterparts. At this age, the increase in size and mineralisation does seem to translate into a structural advantage, where the bones can resist higher levels of compressive force, so it may be the case that the greatest advantages of Si supplementation only become apparent once the skeleton has reached a certain stage of development. The dramatic increase in the N/kg of bodyweight required to break the bones (tables 6.20 and 6.21) shown between d17 and d21 suggests that this may be the age at which the differences become truly measurable. This supports the suggestion that the action of Si may be on the early stages of bone formation when the cartilage anlage is being replaced by the collagen matrix, which is subsequently mineralised (Mackie *et al*., 2008; White and Wallis, 2001). There is potential that the Si is influencing the production or arrangement of the collagen that makes up this matrix, and thus its ability to be effectively mineralised, which would have a long-term effect on the mechanical properties of the bone. It may be that the effects do not translate into a measurable structural difference until the bone has matured (and mineralised) further. This seems the more likely explanation as the highly hierarchical nature of type I collagen (the major type found in bones) means that the initial stages of development must be completed successfully, and to a high level of integrity, in order to facilitate the successful completion of the further steps (Ricard-Blum, 2011). Any improvement in the initial stages of bone formation could, therefore, be presumed to have a positive effect on later development.

This would mean that while the biological response was taking place at a younger age, this response is not measurable by the methods used in this study until the bones have become almost fully mineralised. Applegate and Lilburn (2002) demonstrated that broiler tibial ash

content peaked at around 21 days post hatch) and this early change to the primary structure of the bone translates into a measurable increase in the mechanical properties of the bone.

The histological data collected at d21 shows an increase in the width of the resting zone in the growth plate of tibias of supplemented birds, occurring in tandem with an increase in the presence of both osteoblasts and osteoclasts in the diaphysis of these bones. As the resting zone of the growth plate is the area associated with the potential for bone growth and the support of the growth plate as a whole (Mizuhashi *et al*. 2018), an increase in its size may mean that these bones have the potential to grow to a greater extent than those with narrower resting zones. It may also indicate that the bones are better able to use the inbuilt feedback loop that exists between the resting and proliferative zones (Mizuhashi *et al*. 2018) to ensure that demands for bone cells are being met. Osteoblasts and osteoclasts are the cells responsible for bone formation and repair, so an increase in these further supports the suggestion that supplemented bones are able to achieve larger sizes and greater strength. Interestingly, the plasma concentrations of PINP showed a decrease in supplemented birds, suggesting a decrease in the levels of bone formation. This was unexpected, as it would seem logical that in bones that were significantly larger and stronger, and also contained more potential for growth and larger numbers of bone forming cells, an increase in bone formation would be seen. This apparent disconnect in parameters could be due to multiple reasons. The most likely are considered to be that the bone that has been laid down is of a higher integrity, and so is sustaining less damage and requiring less new bone to be formed for the purposes of repair, or that the bone formation in supplemented birds is a more efficient process, meaning more bone is able to be formed using the same volume of biological resources. Both of these raise interesting questions in regards to which processes are potentially being improved. It could be the case that the collagenous element of the bones is being laid down in a more structurally sound arrangement, making it able to be mineralised more effectively in a more structurally sound manner. This would seem logical, seeing as Si has also been linked to the processes of improved wound healing and has

been shown, through its use in biological scaffolds, to possess the ability to adhere well to biological tissues, even increasing the ability of bones to re-mineralise and regenerate during healing (Lee *et al*., 2009).

In conclusion, while this trial yielded a huge volume of data that allowed some hypotheses to be discarded, it has not provided insight into the precise mechanisms by which Si increased bone strength in broiler chickens. The data suggest that these mechanisms are most likely to involve the early cellular development of bone, with effects that become measurable later in life. This could be explored further through future investigations into the relative gene expression levels of the various bone cells involved in bone formation (osteoblasts, osteoclasts and the chondrocytes within the growth plate), and also by looking further at collagen content and crosslinking within the organic matrix of the bone. It may also be interesting to look at the crystalline structure of the hydroxyapatite crystals that mineralise the matrix, and investigate further the histological parameters that were covered in this study to assess on a larger scale, and in other bones, what changes to cellular structure and abundance are taking place.

Chapter 7: Discussion, conclusion and recommendations

7.1. Introduction

This chapter is split into three sections to discuss the potential of bio-available silicon supplements as a feed ingredient in the meat poultry sector. Firstly, the success of the investigations undertaken will be discussed alongside their key findings. Secondly, the impact of these conclusions on global poultry production will be discussed alongside possible future directions for developing their application. Subsequently, key areas for future research and development are outlined and finally, key recommendations based on this work are given.

Modern broilers are susceptible to skeletal disorders, particularly those affecting the legs (Kestin *et al.,* 1992; Manohar *et al.,* 2015; Whitehead, 1997), with the main factors contributing to this being intensive genetic selection for growth and the increase in the nutritional quality of feed. These elements have resulted in birds that have a better feed conversion efficiency, reach slaughter weight at a much earlier age and are subject to far more intensive production processes than their counterparts from as little as 50 years ago (Bradshaw *et al.,* 2002; Julian, 1998). Skeletal issues are some of the most severe faced by the poultry sector (with regards to both meat and laying birds) and these issues include, but are not limited to, tibial dyschondroplasia (Waldenstedt, 2006), rickets (Carlisle, 1986), cage layer fatigue, keel bone fractures (Fleming *et al.,* 2006; Whitehead and Fleming, 2000), twisted legs, valgus and varus deformities (Bradshaw *et al.,* 2002) and lameness (Mench, 2004). Issues relating to leg weakness in particular are extremely concerning from a bird welfare perspective, and constitute some of the most costly losses to the poultry industry in terms of both bird mortality and losses at processing (Bennett *et al.,* 1999, Zuidhof *et al.,* 2014).

This project has used nutrition as a tool to counter the physiological challenge imposed on meat poultry by intense selection for growth and meat yield. The focus has been on improving skeletal

integrity: that is the ability of the bones and joints to properly support the weight of the carcass while allowing the bird to move freely and without discomfort. A process for making a bio-available silicon supplement was invented at NTU, and the original aim of this PhD project was to assess the impact of silicon on skeletal integrity in broilers. However, as the project developed, it became clear that additional objectives focussed on optimising the supplement for commercialisation were required. Therefore, the objectives of this PhD were:

1. Optimisation of the delivery method of the Si supplement, both in terms of dosage and particle characteristics.
2. Investigation of the potential site of absorption of the Si supplement within the chicken gastrointestinal tract.
3. Evaluation of the effects of the Si supplement on the gross morphology and mechanical characteristics of the leg bones and tendons of meat type chickens.
4. Investigation into the effects of the Si supplement upon the presence of biomarkers and bone cells associated with bone growth and turnover.
5. To examine the effects of time and temperature on the bio-availability of the Si supplement, both alone and once incorporated into feed, when different sources of raw materials are used.

These objectives were examined in discrete phases, each allowing progression to the next phase of work. First a large capacity ball mill was purchased for a series of investigations into the effect of altered milling parameters on particle size and suspension in carrier oil in the Perry laboratory. These *in vitro* findings were then used to conduct a series of three bird trials in the NTU poultry research unit, exploring the *in vivo* effects of the supplement.

7.2. Conclusions and critiques

The work conducted at the Perry laboratory proved useful in that it allowed the production of much larger volumes of the supplement, enabling testing of particle size and suspension to occur.

Whilst this study went some way to define the abilities of the ball mill to produce particles of various sizes, and to begin to define the properties of these particles with regards to their ability to suspend within soya oil and retain bio-availability, there are still many questions left unanswered. This study only examined one speed setting and one time setting on the ball mill. Further examination of the effects of milling at different speeds and timescales may allow the creation of a protocol which reliably produces specific particle sizes. It is not yet known how the NTU Si supplement would respond to these parameters, and if their alterations would have a negative effect on the efficacy of the product. The parts of this study relating to sedimentation and re-suspension revealed that although smaller particles suspend better, larger particles are easier to re-suspend after a state of full sedimentation has occurred. This indicates a need to evaluate in the future which of these properties is of greater importance. It has been suggested that the effect of particle size on bio-availability is also affected by the encapsulation of the particle, and the individual chemical properties of the substance being supplemented as well as the size and shape of the particle. As the NTU supplement is novel, much more research into its potential absorptive properties are required, and if further work to assess the best particle size for retention of bio-availability indicates there are several sizes providing similar results, an investigation into whether it would be preferable to have a well suspended or easily re-suspended particle would be necessary. The soya oil used in this study demonstrated a strong ability to protect the small particle sizes from water, which is a positive feature in terms of product efficacy but seems to have affected the ability of the *in vitro* assay to accurately assess bio-availability. Due to issues encountered liberating the bio-available Si from oil at smaller particle sizes, the assay procedure may need to be developed in order to show that it accurately reflects the presence of Si which is bio-available in the sample and to insure it is providing accurate predictive results. All future work needs to be carried out using Na_2SiO_3 combined with citric acid, as this is likely to be how the product will be manufactured commercially, and therefore data collected on this combination will be far more relevant than data collected on

the Na_2SiO_3 alone. The next steps in this project examined the supplement, with the Na_2SiO_3 milled to a uniform size, and investigated its effects *in vivo*, with broiler chicks.

The *in vivo* investigations into how the Si supplemented effected the broiler chicks yielded many interesting results. None of the trials suggested that the NTU supplement had any negative effects on any performance parameters, and indeed, some trial showed positive effects on growth rate, meaning that the supplement would be attractive for use in industry as it would not reduce profitability by increasing the time taken to reach slaughter weight, or the efficiency of the bird's growth. The trial that investigated the comparison of commercially available Si supplements with the NTU Si supplement showed that the NTU supplement did indeed present with higher levels of absorption and so, therefore, bio-availability than other commercially available supplements. It also showed that the bio-availability and biological response were not dependent upon it being produced in small, regular batches. The fact that it performed equally as well when produced in one batch at the beginning of the trial bodes well for future commercialisation as it suggests a good level of product stability and shelf life. It also showed that creating a Lysine coated Si nanoparticle did not enhance absorption, which was unexpected due to the successful use of nanoparticle technology to increase bio-availability in other trace minerals, but may be linked to condensation of the silica, as it is well established that only the monomeric form of orthosilicic acid can be absorbed. This trial demonstrated an increase in breaking strength, ash percentage and Si content of tibias by d35 in birds fed the NTU supplement, suggesting that the Si produced by NTU is bio-available enough to elicit a biological response from the birds, while the other supplements were not, and that it is having a positive impact upon the skeletal system, but not at the expense of performance. This work initially looks promising, but further research looking in much greater depth at the biological responses is needed.

The second *in vivo* trial was used to assess the effects of dosage, to establish if there was an optimum inclusion rate for the Si supplement. Results showed a plateau of effects at the

inclusion rate of 750ppm, with no further increase in beneficial biological responses observed when dosage was increased to 1000ppm. However, this trial did not display the same significance of results as the previous trial in this thesis (or in previous studies conducted at NTU), which was thought to be the result of issues manufacturing the supplement for this study. This limits the usefulness of this as a dose response trial, as although the supplement did exhibit a strong correlation between dosage and Si levels in the blood plasma, it cannot be certain that the supplement was correctly produced and there was no correlation between bone strength and dosage. Some interesting results were observed, however, with the diet supplemented with sand showing an increase in early BWG (most likely attributable to an increase in GIT development and weight, as no increase in Si absorption was observed for this diet), and the fact that the supplement was likely to have been produced inconsistently, suggested that a significant timepoint had been missed in supplementation, leading to the suggestion that the Si was acting at a specific stage of skeletal development.

The final *in vivo* trial was a thorough investigation into the potential mechanisms of action of the Si supplement. It assessed the potential absorption site via solid and aqueous partitioning of Si within the digesta, the effects of Si seen in bones and tendons on both a macro and microscopic level, and the presence of biomarkers for bone turnover, alongside performance parameters. It showed increases in bone strength, cell counts, and biomarkers associated with bone formation, and suggested that Si is most likely absorbed via the foregut (prior to the jejunum) via diffusion, similar to other water-soluble nutrients. The partitioning data suggested that the carrier oil may be protecting the Si particles too effectively and prevent some of them passing into the aqueous phase of digesta, which could mean that there is potential that the supplement could be made even more bio-available should an optimum particle size for absorption be elucidated. This trial also demonstrated that Si had no measurable effect on tendons, so it's mode of action is most likely focussed on the bones themselves rather than on the musculo-skeletal interactions between bones and tendons and given the differences in

histological and biomarker parameters this effect is most likely at a cellular level. Given the increase in bone forming cells and the resting zone of the growth plate, alongside the decrease in biomarkers for bone collagen turnover, it seems that the process of bone formation may be more efficient or of a greater integrity in supplemented birds. It was also noted in this trial that the greatest measurable effects were seen on the bones at around d21, which coincides with the timepoint previously reported to be the age at which bone mineralisation peaks in broilers, indicating that the bones must have reached a certain stage of mineralisation before the results of supplementation become apparent. This trial yielded a huge volume of data providing a foundation for further work. In particular, there is scope for deeper investigation into the following areas in this trial as, due to time constraints, each area was covered in much less detail than would have been preferred; bio markers for bone resorption, specific mineral content of the bones and digestibility markers.

7.3. Future work

There are many areas for future work that could follow on from this thesis. The trials conducted as part of this PhD could also all be conducted under commercial conditions as some positive results from field testing would provide greater confidence in the initial findings and help to ensure that the supplement is equally effective and still elicits a biological response in a non-research setting. However, there is a large potential for future work, both in commercial and research settings, expanding on these findings, the key areas of which are explained below.

7.3.1. Skeletal effects of silicon

The work looking at the histological and biomarker effects on the skeleton could be expanded to give further insight. The gene expression of the various bone cells (osteoblasts and osteoclasts

specifically along with gene expression in the growth plates) could be examined in depth to give more information on how exactly their behaviour is being altered, which may give further insight into the mechanisms of action. It has been noted previously that there are specific gene expressions associated with skeletal problems such as tibial dyschondroplasia (Praul *et al*., 2000), so it may well be the case that individual gene expression is responsible for the improved bone strength observed in this study. Other biomarkers could be assessed to offer further insights into cellular activity; as PINP is correlated strongly with bone turnover by measuring the levels of collagen matrix laid down by osteoblasts it could be used alongside other formation markers such as bone specific alkaline phosphatase (BALP), which is a ubiquitous, membrane bound tetrameric enzyme found on the outer cell surfaces of osteoblasts, and so correlates more strongly with number of cells than cellular activity *per se*. This would offer the opportunity to compare cell abundance with cell activity, demonstrating whether the Si was just increasing the number of cells, or increasing their bone formation activity levels as well. It could also be used in conjunction with biomarkers specific for bone resorption, such as ßcarboxyl-terminal telopeptide of type I collagen (ßCTX), which measures the products of collagen hydrolysis performed by osteoclasts during bone resorption. This would give a ratio of bone turnover: resorption and allow conclusions to be drawn regarding whether the bones where being produced in a more structurally sound manner originally, or whether they were simply being remodelled more effectively to withstand the strains placed upon them (Leeming *et al*., 2006).

Another area for exploration in assessing the skeletal impact of Si is to assess its use in egg laying strains. These birds are equally as prone as meat type poultry to skeletal issues, but rather than those affecting the legs, they tend to suffer from osteomalacia and osteoporosis caused by mobilisation of minerals from the bones to form egg shells that, due to the intensive production pressures placed upon them, they struggle to replace in a structurally sound manner. This leads to an increase in bone fragility and fractures, particularly in bones such as the keel and wings, which often bear the brunt of impacts, and in extreme cases can cause cage layer fatigue where

structural loss of bone occurs in the spinal regions, leading to paralysis and death (Whitehead *et al*., 2003). It would be interesting to see if the benefits seen with the bones of broilers are replicated in layers, and if that helps to mitigate the skeletal problems associated with their intensive egg production. As the effects of Si in broilers have been shown to occur during early life (from hatch to slaughter), the effects it could have when fed to young pullets on the much more mature bone of layers (up to three years old) would be interesting to observe. If it is the case that the Si is affecting the early cellular development of bone, which then goes on to translate to greater structural integrity later in life, then it could help prevent some skeletal issues in layers. However, if the effects also occur in bone remodelling, rather than just in the initial bone formation, then it could provide significant benefits for laying birds throughout their life by reducing osteomalacia and osteoporosis and help extend the length of the lay period. This would also be of considerable benefit to producers as it would increase the economic life of the bird, but without the losses associated with skeletal weakness that currently reduce both welfare and profitability. If it can help mitigate skeletal issues in layers, then it may have massive potential in the European poultry market over the coming years with the introduction of the cage free by 2020 initiative. Layers housed in free range systems have been reported to have much higher incidences (some as high as 95%) of fracture than birds kept in cages, due to the more hazardous nature of the environment and their increased ability to move around over different heights (Wilkins *et al*., 2011; Tarlton *et al*., 2013). If many European egg producers and retailers to commit to cage free, then there is likely to be an increase seen in fracture rates among laying hens due to the change in housing systems, and there will be a need for methods to help alleviate this issue.

Completely aside from the poultry sector, there are other areas that would benefit from supplements that have the potential to improve skeletal integrity. Performance and working animals, for example sports horses and racing dogs, could benefit greatly from improved skeletal integrity to improve their performance and reduce the incidence or healing time of injuries such

as fractures. Human athletes could also benefit similarly, as could individuals who have had bone surgery or suffer from chronic bone disorders and conditions. Finally, there is the issue of osteoporosis in humans, which most commonly effects post-menopausal women (approximately 50%), with numbers of osteoporotic fractures expected to reach 230,000 per year, with costs to the NHS of over £2.1 billion per year, by 2020. Osteoporotic fractures have substantial impacts on UK health services, and will continue to do so, unless highly effective preventative interventions can achieve widespread use (Burge *et al*., 2001). If Si can be used in species other than poultry, and retains its beneficial effects, then its usage has the potential to be extremely widespread and impact upon many sectors, both human and animal.

7.3.2. Non-skeletal effects of silicon

Aside from the potential future work involving the skeletal effects of Si, there is also much work that could be undertaken to assess its other impacts. As it appears to be acting via the collagen matrix in bone, there is potential that it could have an impact on other highly collagenous structures such as the skin and muscles. As many of the other areas of concern to the poultry sector also involve the skin and muscle, it is worth exploring further the effects Si is having. Feeding trials could be run and the incidence and severity of conditions such as hock and breast burn and food pad dermatitis measured. These would likely be improved alongside skeletal integrity as birds that are more able to move are less likely to spend extended periods laying in the litter, and if their skin is also improved then it may be that they are more resistant to these conditions. An improvement in skin strength would also have economic benefits for producers as skin tearing during rearing and at processing is responsible for both welfare and economic costs, as not only does it increase incidence of infections and stress in live birds but causes downgrading and trimming of carcasses that reduces profitability (Salim *et al*., 2012). Granot *et al*., (1991) observed that skin tears decreased as collagen content of skin increased, and Smith

et al., (1977) and Leeson and Summers (2005) noted that it seemed directly correlated with collagen content and that collagen synthesis was reliant upon many nutritional factors, particularly the presence of the correct levels of vitamins and minerals. Foot pad dermatitis, hock burn, and breast burn are commonly used as methods to assess welfare of a flock (Haslam *et al*., 2007), and so any reduction in their incidence would be associated with improved welfare for the birds. This is beneficial as it is being increasingly noted that improved welfare goes hand in hand with improved performance and sustainability (Burton *et al*., 2016). These conditions are easily assessed via scoring systems, such as those used by Ask (2010), and can be done on trial birds continually throughout a feeding trial, and with minimal stress to the birds, as they are non-invasive. Their incidence and severity could then be compared to other parameters, such as performance or measurements of skeletal integrity, to determine if there was any correlation between Si supplementation and improved welfare indicators. Another area of concern is the recent increase in breast myopathies being seen in fast growing chickens. Woody breast and white striping both reduce profitability by decreasing the acceptability of the meat to the consumer and affecting its suitability for further processing (Kuttappan *et al*., 2016). Whilst the specific causes of these myopathies are not currently known, it has been postulated that they are the result of the fast growth rates causing a decrease in the integrity of the muscle fibres during their formation, leading to problems with muscle structure and function (Kuttappan *et al*., 2016). Incidences and severity of these myopathies could be examined in feeding trials by using the scoring systems such as those reviewed by Kuttappen *et al*. (2016).

It would also be useful to examine the mechanisms by which Si seems to elicit its performance affects in broilers. Increased body weight would generally be seen as a positive attribute, however, if it is due to overdevelopment of the digestive tract rather than an increase in meat yield, then this would not be the case. This could be easily examined via feeding trails by taking meat cuts, such as breast and thigh, from the birds post mortem, and calculating what proportion of the carcass was saleable meat, and what was offal or waste. Length and weight of

various sections of the GIT could also be examined to try and determine where, if anywhere, the Si was causing the tract to develop.

7.3.3. Development of the supplement

There is still much work to be done to ensure that the supplement is as efficacious and efficient as possible. Further investigation into the particle sizes in the commercially sourced ingredients used in chapter 7 is needed, with SEM being considered the most accurate way to assess this. Once the particle sizes available have been assessed, it would then be prudent to reassess the ability of these particle sizes to retain their bio-availability once added to feed. Another area that needs further development is the *in vitro* assay used to detect the bio-available Si in samples. Whilst it has been shown to provide meaningful results in this project, there are still issues preventing its immediate application as an *in vitro* test. The method appears unable to entirely liberate the full volume of Si from the oil carrier, but this could possibly be mitigated by adding a surfactant to the assay. The method also detects only lower concentrations of Si, which means that highly concentrated samples such as the neat supplement require large dilutions, thus potentially reducing the accuracy of results. The fact that storage of the supplements in freezers at -20°C seemed to confound the results is also of concern, although this issue may possibly be resolved by ensuring all samples are at room temperature before measurements are started. A detailed investigation into the effects of different storage conditions at low temperatures at various time points is recommended. There is also the use of this assay in the field to consider. As it stands, the molybdenum blue assay requires equipment that cannot easily be transported to farms or feed mills, and so the development of a simple test that can be used *in situ* to establish that the correct dosage is present in feed should be a priority. This could potentially be similar in design to the enzyme check assays already in production, but the suitability of Si for methods such as this needs a great deal of further work.

Once the supplement has been standardised using the methods above, feeding trials are needed to ascertain its most effective application. Work should be done looking at the minimum dosage that can be fed, and at what time points supplementation is most effective. Key queries are whether feeding it for just the first two weeks post-hatch provide the same benefits as feeding throughout life, and whether supplementing only at the start of lay or in response injury are still beneficial.

7.4. Recommendations for industry

This project suggests that the production of a bio-available Si supplement that can help to improve the welfare and skeletal integrity of broilers, without reducing performance, is possible. This could help improve the quality of life for meat chickens, and also help improve profitability for producers by reducing the amount of bird losses associated with poor bird health and carcass quality. This in turn indicates that a reassessment of the NRC may be necessary, with a greater emphasis placed upon the importance of supplements and micro/trace minerals. This would provide producers and nutritionists with reliable and accessible information that may allow them to make more informed decisions regarding the supplementation of feed, particularly in response to skeletal issues.

However, before a supplement can be produced there are a number of factors that must be further investigated. As one raw material is primarily a fine Si powder there are issues associated with manufacture. One of these is the risk of silicosis which occurs from exposure to fine Si particles, so an appropriate method of manufacturing the supplement that addresses all the health and safety concerns must be developed.

Secondly, the issues of bio-availability and stability within diets must be addressed to ensure that the product is properly retaining its efficacy, and that there is an assay that can reliably test

this. It would also be prudent to look into the effects of different climates on the supplement, as the rapidly growing markets of India and Africa present challenges with regards to climate that are vastly different from those in the UK.

Finally, there is the cost of production and transport to be considered: the supplement must be produced cheaply enough that it is still economically viable for use on farms. Alongside this are considerations of supply chain and availability of materials, and the need to develop relationships with various supplies and manufacturers in order to protect the supply chain, and the initiation if IP protection to prevent others from copying the methodology. In conclusion, this project has demonstrated that a novel form of bioavailable silicon has positive impacts on skeletal integrity and provides some insights into how the beneficial effects may occur, but further work is required before the product can be commercialised.

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Appendix I

Study title: Investigation into optimal methodology for mounting the Gastrocnemius tendons of broiler chickens when testing tensile strength.

1. Study Objective

The objective of this study was to investigate the best method for mounting the gastrocnemius tendon of broiler chickens in the grips of a texture analyser during testing for tensile strength.

Due to the multiple different approaches employed in the literature for mounting, a pilot study was required to assess the best method for ensuring optimal mounting of the tendons in the grips. The method of mounting the tendons in the grips is important as, if a weak spot is created by damaging the tendons during mounting, failure can occur prematurely and give a false or inaccurate reading. Also, due to the fact that tendons are kept moist during testing, slippage could occur meaning the tendon is released by the grip before a reading can be taken and is then over-extended or irreparably damaged so no further accurate reading can be acquired. Various solutions to these problems have been suggested in the literature, but they vary in the level of success achieved and the species to which they are applied. As such, the aim of this study was to determine the best method of mounting the tendons of broiler chickens in the grips while testing their tensile strength. The data collected during this investigation will be used to create the most reliable method possible for mounting of broiler tendons in grips during tensile testing for future investigations.

The key questions this study aimed to investigate are as follows:

- Which method of mounting the tendons in the grips is least likely to damage to the tendon at the point of attachment?
- Which method of mounting the tendons in the grips results in the least slippage of the tendons within the grips?

2. Methodology

Work was undertaken to assess the best methodology for using a TA.XT. Plus Texture Analyser (Stable Microsystems, Surrey) to test the tensile strength of the gastrocnemius tendon of broiler chickens. Tensile strength was taken to mean the maximum force that could be applied to the tendon, during extension, before tendon failure occurred.

A set of standard tensile grips (Stable Microsystems, Surrey) were used to hold the tendons in the texture analyser on the recommendation of the manufacturer. The grips were tightened around the tendons until firm resistance was felt, and care was taken not to overtighten to avoid pinching the tendon in the grips. See figure 1 for diagram of gripping method.

A range of materials were utilized to protect and secure the tendon in the grips, and the effect of the different methods on the tendon during tensile testing was visually observed and damage was scored on a scale of 0 to 3 (see table 1).

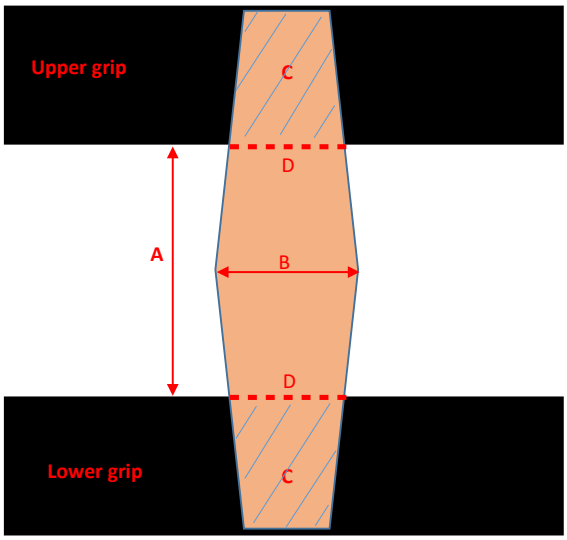

Figure 1. The tendon was mounted between the upper and lower grips, with the proximal end being fixed in the upper grip, and the distal end being fixed in the lower grip. Before tensile testing the section visible between the grips was measured (arrow A) along with the thickest point (not shown) and the widest point (arrow B). During tensile testing the level of slippage was recorded. After tensile testing the damage to the sections of tendon within the grips (shaded area C) was scored, along with the damage at the pinch point (dashed line D).

Table 1. Scoring system used to assess the level of slippage and damage to tendons during tensile testing.

Score	Slippage of tendon	Damage to tendon within grips	Damage to tendon at pinch point
0	No slippage	No damage	No damage
1	Minimal slippage, did not affect testing	Minimal damage, did not affect testing	Minimal damage, did not affect testing
2	Some slippage, may have affected testing	Some damage, may have affected testing	Some damage, may have affected testing
3	Severe slippage, results likely invalid	Severe damage, results likely invalid	Severe damage, results likely invalid

2.1 Sample collection

Birds were humanely euthanized via cervical dislocation at 21 days of age. Legs were removed from the carcass and the gastrocnemius tendon was dissected from the leg with a scalpel. The section of tendon taken ran from the area of attachment to the tibia muscles at the proximal end of the gastrocnemius tendon, past the hock joint (the cartilage sheath was also dissected out intact to avoid damaging the tendon where it attaches to this structure), down to where it began to separate into digital flexor tendons at the distal end. Care was taken not to damage the tendon with the scalpel and not to stretch or over-extend the tendon during dissection as this could affect the readings given during tensile testing. The tendons where wrapped in tissue paper soaked in 0.9% NaCl and frozen immediately at -20°C until tensile testing could begin.

2.2 Tendon tensile strength testing

The texture analyser used for this study was a TA.XT Texture analyser (Stable Microsystems, Surrey) fitted with a 50kg load cell. A set of compatible tensile grips was used to mount the tendons, as advised by the manufacturer. The texture analyser was set to measure the force (N) applied to the tendon until it failed. The same settings where used for all tensile strength testing. The only alterations made were the materials used to line the grips and protect the tendon. After mounting in the texture analyser, but before testing, the length of the tendon visible between the grips was measured, along with the width and thickness of the tendon at its widest and thickest visible point. After testing, the maximum force (N) applied to the tendon was recorded, along with the time (Secs) taken for the tendon to fail and the distance (mm) it stretched before failure.

2.3 Determination of grip lining material

In order to prevent creating a weak point on the tendon during mounting, the grips require lining with a material that will distribute the force applied by the grips as evenly as possible, so the tendon is securely held but not damaged or pinched (Foutz *et al.* 2007).

Current literature has explored various techniques for testing the tensile strength of tendons. Mazurek *et al.* (2011) tested human *profundas* tendons on a similar texture analyser. They found that wrapping the end in suture material then gluing to sandpaper before placing in the metal grips worked best. However, when performing a similar study on rabbit tendons, Jielile *et al*. (2010) tested the tendons by mounting them directly on to the metal clamps. Ruiz-Feria *et al*. (2014) found that wrapping the ends of the dissected tendon in sandpaper helped to secure the tendon in the clamps but prevent damage at the attachment sites. This study used various combinations of sandpaper, padded servo tape and suture material to grip and protect the tendon. It also assessed the difference in damage to the tendon when gripped closer to the ends, compared to when it was gripped closer to the wider and thicker middle section.

3. Mounting Methods

3.1 Method one – No additional materials

The tendon was loaded directly into the tensile grips with no additional materials present. The tendon was secured to the top grip at the point where the tendon started to narrow at the muscle attachment site at the proximal end, and to the bottom grip at the point where it attached to the cartilage sheath at the distal end (Fig.1). The tensile test was then run.

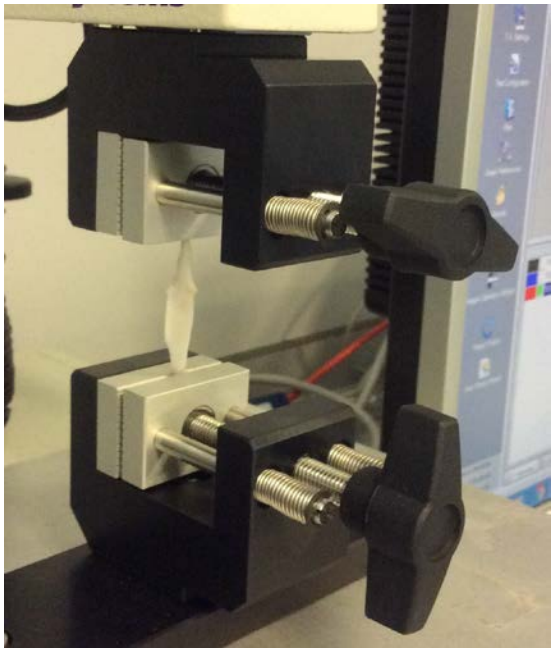

Figure 1. The tendon was mounted directly between the grips with no additional materials present.

3.2 Method two - Sandpaper

The ends of the tendon were wrapped in wet and dry sandpaper, abrasive side facing inwards in contact with the tendon. Wet and dry sandpaper (medium grade) was used due to its ability to withstand the moisture of the tendon without disintegrating and its non-absorbent qualities that would prevent drying the tendon and potentially creating a weak spot. The tendon was secured to the top grip at the point where the tendon started to narrow at the muscle attachment site at the proximal end, and to the bottom grip at the point where it attached to the cartilage sheath at the distal end (Fig.2). The tensile test was then run.

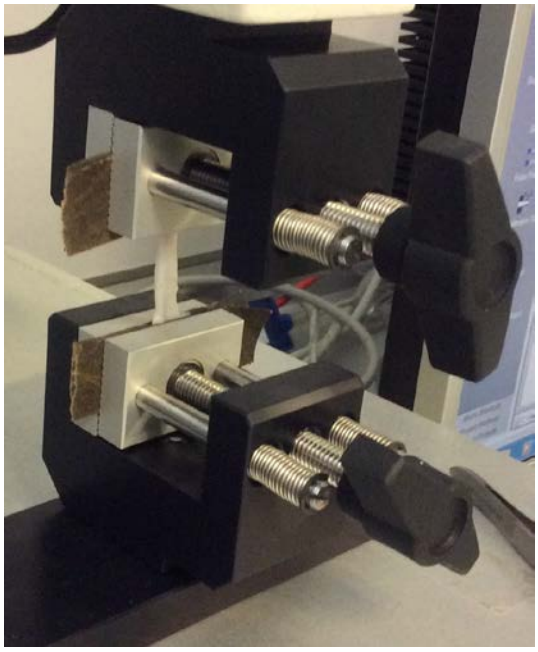

Figure 2. The tendon was placed between two pieces of sandpaper inside the grips, with abrasive side facing inwards toward the tendon.

3.3 Method three – Sandpaper with servo tape

The tendon was placed between two sheets of wet and dry sandpaper (medium grade) as in section 3.2. The smooth back side of the sandpaper was attached to self-adhesive padded servo tape. This was then placed in the grips (Fig.3) so that the servo tape formed a compressible protective layer between the sandpaper and the grips that would distribute the pressure on the tendon more evenly. The tendon was secured to the top grip at the point where the tendon started to narrow at the muscle attachment site at the proximal end, and to the bottom grip at the point where it attached to the cartilage sheath at the distal end (Fig.3). The tensile test was then run.

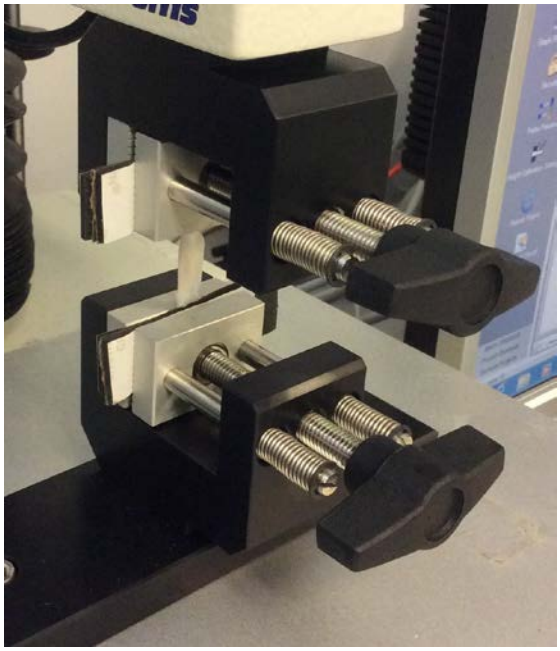

Figure 3. The tendon was placed between two sheets of sandpaper each backed with padded servo tape.

3.4 Method four – Suture material

The tendon ends were wrapped in non-absorbable suture material (Henery Schein Spool Suture Supramid). Wrapping was placed at the point that the tendon started to narrow for the muscle attachment site and the point where it started to narrow for the cartilage attachment site (Fig.4). The tendon was secured to the top grip at the wrapped point where the tendon started to narrow at the muscle attachment site at the proximal end, and to the bottom grip at the wrapped point where it attached to the cartilage sheath at the distal end. The tensile test was then run.

Figure 4. The tendon ends were wrapped in suture material. The ends were then directly mounted between the grips with no additional materials present.

3.5 Method five – Suture material with sandpaper and servo tape

The tendon ends were wrapped with suture material, as in section 3.4, then placed between the two sheets of wet and dry sandpaper lined with the padded servo tape as in section 3.3. The tendon was secured to the top grip at the wrapped point where the tendon started to narrow at the muscle attachment site at the proximal end, and to the bottom grip at the wrapped point where it attached to the cartilage sheath at the distal end (Fig.5). The tensile test was then run.

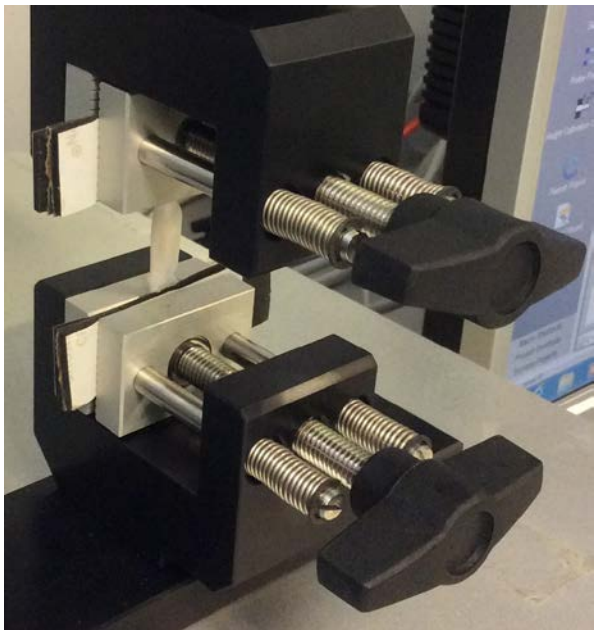

Figure 5. The wrapped ends of the tendon were placed between the sandpaper and servo tape.

3.6 Method six – Grip site

The two remaining tendons were mounted using the method in section 3.5 as this was the method that caused least damage and weak spots at the grip point. However, rather than mounting from the point where the tendon started to narrow at the muscle attachment site at the proximal end to the point where it attached to the cartilage sheath at the distal end, it was gripped at the widest and thickest central portion of the tendon (Fig.6). The tensile test was then run.

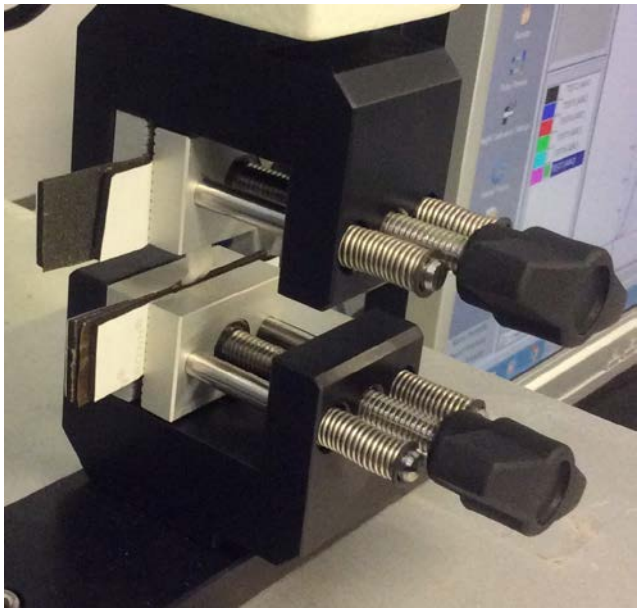

Figure 6. The widest and thickest central portion of the tendon was measured, and then the tendon was wrapped in suture material, and gripped, at the proximal and distal ends of this section.

4. Results

The measurements taken prior to the tensile testing are shown in table 2. The scores given each attachment method for slippage and damage are shown in table 3.

Table 2. Measurements taken from the tendons during the tensile testing.

Method of Attachment	Length visible between grips/mm	Width at widest point/mm	Thickness at thickest point/mm	Distance stretched before failure/mm	Test time until failure/sec	Force needed for failure/N
1	23.23	7.16	1.29	6.615	13.232	70.0309
2	25.99	7.01	1.29	4.409	8.82	72.8842
3	26.98	7.81	1.55	3.604	7.21	36.1692
4	38.61	8.32	1.31	7.892	15.786	37.1771
5	22.99	7.12	1.87	4.467	8.936	21.2335
5	38.41	8.52	1.5	8.774	17.55	24.0095
6	13.06	7.23	1.8	5.591	11.184	46.8166

Table 3. Scores given each tendon to assess the level of slippage and damage.

Method of Attachment	Slippage score	Damage within grips score	Damage at pinch point score
1	1	3	3
2	1	2	3
3	1	2	2
4	0	1	2
5	0	1	0
5	0	1	0
6	0	1	0

As well as the measurements and scores recorded above, the tendon was also visually observed during testing to allow any possible issues to be identified. Slippage was not an issue after the sandpaper was combined with the suture material. Damage at the pinch point was virtually eliminated by the combination of the servo tape with the suture material, and the least damage occurred to the tendon held within the grips occurred once the suture material

was introduced. The measurements taken of the tendons before testing do not provide any insight into the effectiveness of the testing, but they allow some comparison with the tensile test measurements, and no particular method of attaching the tendons to the grips produced unusual or anomalous results.

5. Discussion

It was felt that methods one, two and three were unreliable as the grips of the texture analyser were causing visible damage to the tendons both at the pinch point and to the sections held within the grips. During testing it was observed that the tendon was clearly failing at areas where this excess damage was occurring, and therefore, the results were deemed invalid and the methods unsuitable.

Method four yielded minimal damage to the tendons within the grips, but still caused enough damage at the pinch points that it was felt this may be effecting the validity of the results.

Once the sandpaper and servo tape were introduced for method five, the level of damage at the pinch points decreased and the tendon did not exhibit any obvious signs of mechanically induced weakness during testing. A second tendon was tested using method five to check for repeatability, and the tendon behaved in a very similar manner. While the results were quite different to the previous tendon tested with method five, it was felt that this was caused by biological differences in the size and shape of the tendons, rather than the testing.

In an attempt to reduce the impact of biological variation, it was decided to use only the thickest, widest middle section of the tendon for method six, as this area of the gastrocnemius tendon is far more consistent between birds than the tendon as a whole. The tendon used for method six displayed the same minimal damage as the tendons used for method five, but it developed a more obvious failure point that was clearly not due to mechanically induced weaknesses. This area of the gastrocnemius tendon is also the section most prone to rupture

in broiler birds, as it is the section found adjacent to the hock. This makes it the most relevant site to test, as it is most likely to be representative of the area placed under strain *in vivo*. Due to the factors outlined above, it is felt that that method of attachment most suited for use with the gastrocnemius tendons of broiler chickens is method six.

7. Key findings

- The TA.XT Plus Texture Analyser fitted with tensile grips is a suitable tool for testing the tensile strength of broiler tendons.

- Using suture material in conjunction with sand paper and servo tape caused the least damage to the tendons.

- Slippage is virtually eliminated by the use of sandpaper.

- Testing only the widest, thickest central portion of the tendon should limit biological variation.

- In order to state whether one method is statistically more valid than the other, a larger investigation would need to take place.

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Jielile, J., Bai, J.p., Sabirhazi, G., Redat, D., Yilihamu, T., Xinlin, B., Hu, G., Tang, B., Liang, B. and Sun, Q. 2010. Factors Influencing the Tensile Strength of Repaired Achilles Tendon: A Biomechanical Experiment Study. *Clinical Biomechanics,* 25 (8), 789-795.

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Ruiz-Feria, C.A., Arroyo-Villegas, J.J., Pro-Martinez, A., Bautista-Ortega, J., Cortes-Cuevas, A., Narciso-Gaytan, C., Hernandez-Cazares, A. and Gallegos-Sanchez, J. 2014. Effects of Distance and Barriers between Resources on Bone and Tendon Strength and Productive Performance of Broiler Chickens. *Poultry Science,* 93 (7), 1608-1617.

Appendix II

SOP for milling and sedimentation timeline photographs

SOP for Milling using the planetary ball mill

Introduction

The planetary ball mill can be used for mixing, homogenizing, fine grinding, mechanical alloying, cell disruption, small volume high-tech material production and colloidal grinding. It can be used wet or dry. It has built-in grinding stations comprising of 4x 500ml capacity tungsten carbide jars with 3 varying sizes of tungsten carbide balls. This SOP relates to the procedure for grinding materials in order to reduce, and/or make uniform, their particle size.

Materials

Across International PQ-N2.220 planetary ball mill

Planetary ball mill user manual

Suitable material for grinding (no greater than 3mm particle size to begin with)

Method

1. Ensure machine is correctly connected to a suitable electricity supply, and that any adapters used have had appropriate safety testing.
2. Place material to be ground and balls into the jars, ensuring that they are no more than 3/4 full when both balls and material are inside.
3. Refer to the user manual for guidance on the maximum number of each size of ball that can be placed inside the jars, and for recommendations on which ball size to use to achieve required results.

4. Put the lids on the jars, ensuring rubber seal is in place, and place them into the cradles, ensuring the machine is loaded evenly (never with an odd number of jars, jars are of an equal weight and jars are loaded diagonally opposite each other if machine is not full).
5. Ensure all jars are securely fixed into the cradles using both force applying mechanisms of the jar clamping devices. This means manually tightening both the pressure screw AND the locking screw as far as possible to ensure that sufficient counter-pressure is exerted to keep the jars and lids in place.
6. Never use tools to tighten the jar clamping devices as this may result in over tightening and problems releasing the jars should an error occur.
7. DO NOT run the machine unless all jars are securely clamped! Check this by manually exerting pressure on the jars and feeling for any movement.
8. Fully close the protective cover and ensure that the jars, lids and clamping mechanism are clear of the cover and no friction will occur during grinding.
9. Switch ball mill on at the wall socket and at the circular 'On/Off' button. This button should light up green when machine is on. Wait for LED display to come on.
10. Press the 'Menu/Esc' button until 'Cd01' is displayed, then press the 'Enter/Data' button to check the setting for this function.
11. Check settings are set to factory defaults for codes Cd01 to Cd16, as described in the table on page 8 of the user manual.
12. If they are not press the up or down arrow buttons to alter the settings, until correct setting is displayed, and press the 'Enter/Data' button after each change is completed to ensure it is saved.
13. The display will automatically move to the next function code after saving the previous setting.
14. For information on how to alter the speed of the machine, refer to the user manual.
15. Press the 'Run' button to begin grinding, and the 'Stop'Reset' button to stop the grinding when particles have reached desired size.

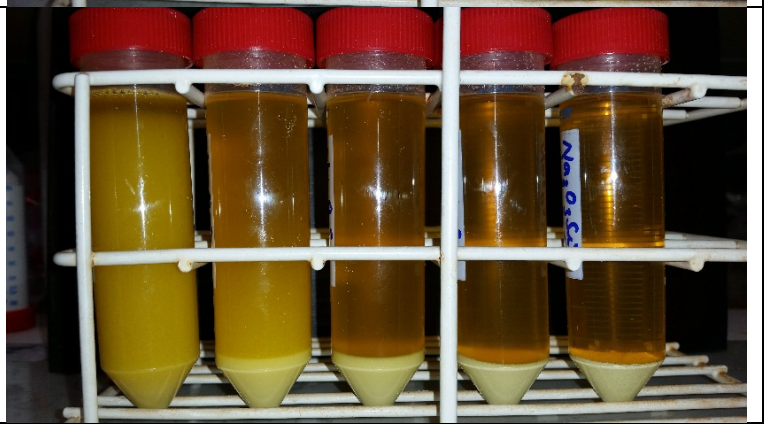
16. Switch off the machine at the source and wait for it to become completely stationary before opening the safety cover.
17. After grinding, temperature and pressure may be very high inside the jars.
18. Leave the machine for a minimum of 30mins after grinding to allow dust to settle and jars to cool. Only open jars in a fume cupboard while wearing appropriate PPE.
19. To remove the balls, pour the contents of the jars through the sieve provided, and allow dust to settle before removing from the fume cupboard.

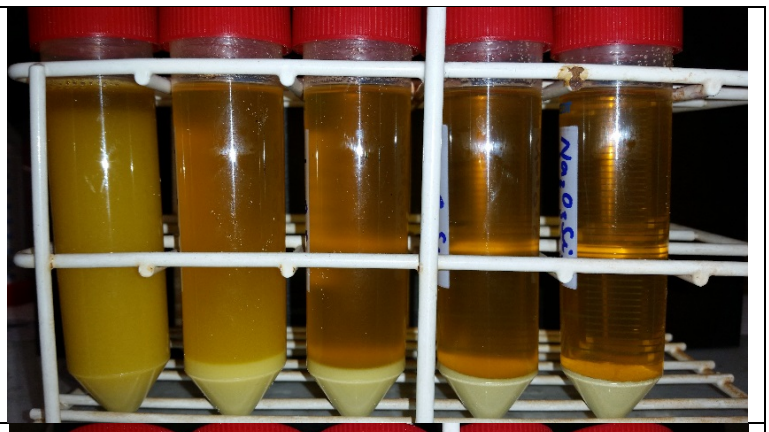
Any problems should be reported to the laboratory technicians or an appropriate member of staff.

Sedimentation timeline photographs

Time post-suspension/mins	Photo
0	
1	

2	
3	
8	
13	

18	
28	
38	
48	

58	
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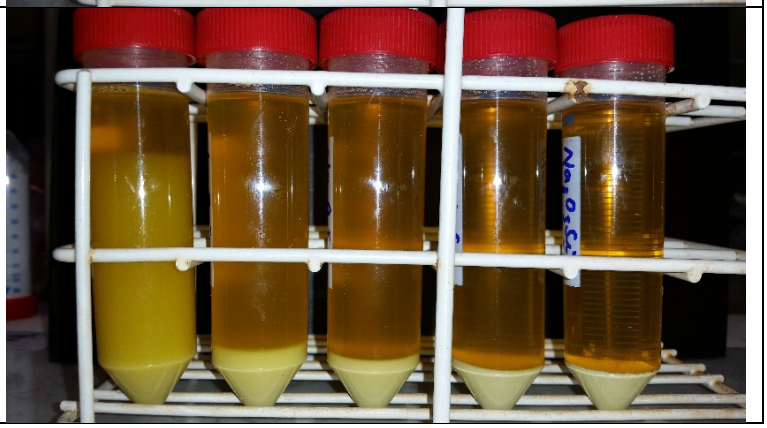
138	

178	
238	
1368	

Appendix III

Staining protocols

1. Toluidine blue

Trough	Solution	Time/mins	Additional notes
1	Xylene	3	In fume cupboard
2	Xylene	3	In fume cupboard
3	Xylene	3	In fume cupboard
4	Absolute ethanol	2	Room temperature
5	95% ethanol	2	Room temperature
6	90% ethanol	2	Room temperature
7	70% ethanol	2	Room temperature
8	50% ethanol	2	Room temperature
9	Running tap water	5	Gentle, indirect flow to protect samples
10	1% Toluidine blue	5	In drying oven set to 37°C
11	Absolute ethanol	0.5	Room temperature
12	Absolute ethanol	0.5	Room temperature
13	Xylene	1	In fume cupboard
14	Xylene	1	In fume cupboard
15	Xylene	1	In fume cupboard

2. Alcian blue + PAS

Trough	Solution	Time/mins	Additional notes
1	Xylene	3	In fume cupboard
2	Xylene	3	In fume cupboard
3	Xylene	3	In fume cupboard
4	Absolute ethanol	2	Room temperature
5	95% ethanol	2	Room temperature
6	90% ethanol	2	Room temperature
7	70% ethanol	2	Room temperature
8	50% ethanol	2	Room temperature
9	Running tap water	5	Gentle, indirect flow to protect samples
10	1% Alcian blue	30	In drying oven set to 37°C
11	Absolute ethanol	0.5	Room temperature
12	Absolute ethanol	0.5	Room temperature
13	Xylene	1	In fume cupboard
14	Xylene	1	In fume cupboard
15	Xylene	1	In fume cupboard

3. Masson's Trichrome

Trough	Solution	Time/mins	Additional notes
1	Xylene	3	In fume cupboard
2	Xylene	3	In fume cupboard
3	Xylene	3	In fume cupboard
4	Absolute ethanol	2	Room temperature
5	95% ethanol	2	Room temperature
6	90% ethanol	2	Room temperature
7	70% ethanol	2	Room temperature
8	50% ethanol	2	Room temperature
9	Bouins solution	5	Mordanting, in vented oven set at 56°C
10	Running tap water	5	Or until yellow colour is gone from slide
11	Weigerts Haematoxylin	5	Room temperature
12	Running tap water	5	Gentle, indirect flow to protect samples
13	Distilled water	0.5	Gentle rinse
14	Panceau Fusin	5	Room temperature
15	Phosphotungstic acid	5	Room temperature
16	Light green masson	3	Room temperature
17	1% acetic acid solution	1 dip	Room temperature
18	Absolute ethanol	0.5	Room temperature
19	Absolute ethanol	0.5	Room temperature
20	Xylene	1	In fume cupboard
21	Xylene	1	In fume cupboard
22	Xylene	1	In fume cupboard

4. Picrosirius red with fast green

Trough	Solution	Time/mins	Additional notes
1	Xylene	3	In fume cupboard
2	Xylene	3	In fume cupboard
3	Xylene	3	In fume cupboard
4	Absolute ethanol	2	Room temperature
5	95% ethanol	2	Room temperature
6	90% ethanol	2	Room temperature
7	70% ethanol	2	Room temperature
8	50% ethanol	2	Room temperature
9	Running tap water	5	Gentle, indirect flow to protect samples
10	Weigerts working solution	8	Room temperature
11	Distilled water	10	Rinse gently
12	Picrosirius red w fast green	60	Room temperature
13	Acidified water	2 dips	Room temperature
14	Absolute ethanol	0.5	Room temperature
15	Absolute ethanol	0.5	Room temperature
16	Xylene	1	In fume cupboard
17	Xylene	1	In fume cupboard
18	Xylene	1	In fume cupboard

5. Haematoxylin and Eosin

Trough	Solution	Time/mins	Additional notes
1	Xylene	3	In fume cupboard
2	Xylene	3	In fume cupboard
3	Xylene	3	In fume cupboard
4	Absolute ethanol	2	Room temperature
5	95% ethanol	2	Room temperature
6	90% ethanol	2	Room temperature
7	70% ethanol	2	Room temperature
8	50% ethanol	2	Room temperature
9	Running tap water	5	Gentle, indirect flow to protect samples
10	Mayers haematoxylin	3	Room temperature
11	Running tap water	5	Gentle, indirect flow, remove samples when "blueing" has occurred
12	Scott's tap water	2	Room temperature
13	0.5% Eosin	1.5	Room temperature
14	Running tap water	0.5	Gentle, indirect flow to protect samples
15	70% ethanol	0.5	Room temperature
16	90% ethanol	1	Room temperature
17	95% ethanol	1	Room temperature
18	95% ethanol	1	Room temperature
19	Absolute ethanol	1	Room temperature
20	Absolute ethanol	1	Room temperature
21	Xylene	1	In fume cupboard
22	Xylene	1	In fume cupboard
23	Xylene	1	In fume cupboard