THE DEVELOPMENT OF NOVEL APPROACHES TO ASSESS AND IMPROVE SKELETAL INTEGRITY IN LAYING HENS

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Abstract

High demand for egg products over the last couple of decades has increased the need of supply in egg production. As a consequence, modern laying hens are being put under increased pressure from extended periods of egg production causing increased susceptibility to skeletal disorders such as osteoporosis and skeletal damage. Assessment of skeletal health through measuring bone parameters such as bone strength or bone mineral density is common practice, however, the methods used in previous research vary between research groups and studies. The overarching aim of this study was to assess the effect of different housing systems on skeletal health and egg qualities of laying hens over the period of lay, using new methodology and modelling parameters to help maintain hen welfare whilst possibly increasing the laying cycle of future flocks. A series of trials were carried out to achieve this aim.

Three pilot studies were carried out in the initial stages to determine the optimal bones to be measured in subsequent trials. These studies concluded that multiple bones showed differences due to differences in form and function, and that future studies would therefore need to measure several bones to get a true picture of the birds' skeletal health. Using methodologies influenced by the pilot studies a longer study, split into two parts was carried out to assess the effect of housing system on skeletal integrity and egg quality over the laying period. After the larger trial, another trial was performed using the previously established methodologies to see if they could be used to assess the effectiveness of a novel supplement.

Part one focussed on the effects on bone parameters while part two focussed on the effects on egg quality. The longitudinal study showed that bone strength of the keel, tibiae and humeri were significantly affected by age ($p < 0.001$), housing system ($p < 0.001$), and the interaction effect between age and housing system ($p < 0.001$) with the caged system showing some of the poorest results ((18 weeks MT keel = 173.13N (13.542), 18 weeks C keel = 105.38N (13.542), 72 weeks MT keel = 130.80N (13.542), 72 weeks C keel = 81.74N (14.363)). Bone ash content of the keel, humerus and tibia was also significantly affected by age ($p <$

0.001), housing system ($p < 0.001$) and age*housing system ($p < 0.001$). Caged bone ash content showed some of the lowest values though organic some of the highest ((24 weeks O tibia = 44.36% (0.677), 24 weeks C tibia = 41.91% (0.677), 60 weeks O tibia = 44.28% (0.742), 60 weeks C tibia = 42.73% (0.697)). It was highlighted that bone strength may be more informative than bone ash content when assessing skeletal integrity, as bone ash content results are thought to be more influenced by the presence of medullary bone than bone strength. Furthermore, the modelling estimates of bone data in this chapter showed that bone weight was a significant predictor of bones strength ($p < 0.001$; estimate = 23.60), showing as bone weight increases, bone strength would increase at the estimated rate. Model parameters also showed free-range flat deck ($p = 0.020$; estimate 27.37), free-range multi-tier ($p < 0.001$; estimate = 38.30) and organic bone strength were significantly stronger than caged tibia strength ($p < 0.001$; estimate = 56.78). Barn ($p = 0.024$; estimate = -10.64) and free-range flat deck ($p = 0.025$; estimate = -10.92) bone strength declined significantly faster than caged bone strength between 18-72 weeks of age, with free range flat deck declining slightly faster than barn. Humerus bone strength declined significantly faster than tibia bone strength ($p < 0.001$). Keel bone strength also declined significantly faster than tibia bone strength ($p < 0.001$).

In part two of the longitudinal study - investigating the effect of housing system on egg quality traits over the laying period - results showed that all egg quality parameters were significantly affected by age, housing system and the interaction effect ($p = 0.007$ or less). Egg weight increased from mid lay onwards (36 weeks onwards) with organic showing the heaviest weights of all systems. Egg height showed an increase over age ($p < 0.001$) from 24 weeks of age to 36 weeks of age and then another increase from 48 weeks to 72 weeks of age. Organic egg height was the largest towards the end of lay with multi-tier and cage being some of the smallest. Eggshell strength of the multi-tier and flat deck systems were highest at the beginning of lay, though declined quicker than other systems between weeks 24 and 36. In barn systems, eggshell strength was some of the weakest results during mid to late lay, whilst organic showed some of the highest breaking strengths in the same period. Results for eggshell ash content were somewhat unclear as barn data was significantly higher at 72 weeks compared to all other systems, where in other measurements barn data showed some of the

lowest results. It was shown that in late lay (between 60-72 weeks) eggshell thickness is greatly influenced by age ($p < 0.001$), however multi-tier eggshell thickness and caged eggshell thickness increased slightly while other systems declined sharply.

Regarding egg modelling data, ash weight was a significant predictor of eggshell strength ($p < 0.001$; estimate = 3.73), showing as ash weight increases eggshell strength would increase at the estimated rate. Furthermore, model parameters showed as age increased eggshell strength decreased when using caged eggshell strength as the baseline ($p < 0.001$; estimate = -0.16). Barn eggshell strength was also shown to decline significantly faster over the laying period than caged eggshell strength ($p = 0.036$; estimate = -0.29). When assessing if a relationship was present between egg and bone strength residual data, no relationship was found. It may be that the lack of interaction between data sets were caused by how broadly egg and bone strength data was collapsed at farm level to enable a comparison.

The last part of this project was to determine if these methodologies created from the pilot studies and longitudinal study could identify differences in the effects of a novel supplement. This was done by investigating the effects of a novel silicon supplement on skeletal health and egg quality traits post laying cycle. The study found little effect of the diet on any bone parameters of any bone throughout (diet effect - bone length; $p = 0.099$ or higher, bone width; $p = 0.285$ or higher, bone weight; $p = 0.157$ or higher, bone strength; $p = 0.083$ or higher), making it difficult to the determine the useful of the methods developed previously. It was suggested that the effect of changing housing system may have concealed the effect of silicon supplementation, due to the change in exercise which is known to improve skeletal health.

It was concluded from the project that when assessing skeletal health, multiple bones be used to ensure all areas of the skeletal system are assessed due to form and function varying between bone. For example, a wing bone performs a different locomotory movement compared to a leg bone. It would be recommended from work in this project that utilising the keel, humerus and tibia would be adequate to cover all aspects of skeletal form and function

in the bird, in relation to assessing skeletal health. Measuring bone geometry along with strength was also considered important as these parameters were found to be sensitive to factor effects even after sexual maturity. It was unexpected that results for bone lengths and widths in pilot studies 1 and 2 were still affected by housing system, post maturity. Additionally, egg parameters were not considered meaningful data for skeletal assessment as it was somewhat evident egg production likely takes precedent over skeletal maintenance. Model parameters to assess skeletal health in laying hens would be beneficial in future work. Ultimately, the modelling of skeletal data could act as an early warning system to indicate any potential decline in skeletal parameters within the laying period and allow producers to be proactive in their response. An early response to a skeletal problem could help maintain or possibly improve productivity at a time when there is a drive to produce more eggs per bird, whilst simultaneously maintaining a high standard of hen welfare.

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List of Abbreviations

ANOVA – Analysis of Variance

ANCOVA – Analysis of Covariance

BMC – Bone Mineral Content

BMD – Bone Mineral Density

DEXA – Dual Energy X-ray Absorptiometry

DM – Dry Matter

FCR – Feed Conversion Ratio

FSH – Follicle Stimulating Hormone

FTU – Quantity of enzyme that can release ~ 1μmol of inorganic phosphorus per minute

GnRH-1 – Gonadotrophin Releasing Hormone 1

IU – International Unit

KBD – Keel Bone Damage

LCaP – Low Calcium Phosphorus Diet

LH – Luteinising Hormone

LMM – Linear Mixed Model

NCS – Non-Cage System

nPP – Non-Phytate Phosphorus

PTH – Parathyroid Hormone

QCT – Quantitative Computed Tomography

RANKL – Receptor Activator of Nuclear Factor Kappa-B Ligand

SKAP – Simplified Keel Assessment Protocol

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Chapter 1: Literature Review

1.1 The poultry industry

In recent times there have been major changes in food-consumption patterns across the world, driven by increasing disposable incomes, public perception, food tastes and evolving health concerns (Triall *et al*., 2014; Jones *et al*., 2018). With an efficient and quick turnaround of animal protein sources, the poultry industry now represents the largest animal production sector across the globe. It is also the fastest growing animal production industry worldwide with the past decade seeing an annual growth of between 3-5% (Käpelli *et al*., 2011; Alexandratos and Bruinsma, 2012). The demands for poultry products have boomed over the last 50 years, data from 2018 showed the chicken population was 23.7 billion birds worldwide, with Asia being the largest producer followed by the Americas then Europe. The same year, just below 104 million tonnes of eggs were produced with Asia again leading in production. Statistics for production of chicken meat showed just under 129 million tonnes were also produced in 2018. The Americas were the largest producer chicken meat followed by Asia (FAOSTAT, 2020). Figures from 2011 also revealed poultry meat constituted to 31% of overall meat consumption in the UK and 43% in the USA (FAO, 2016).

Originating from jungle fowl, and being first domesticated over 8000 years ago, chickens have become the most important poultry species globally (Sawai *et al*., 2010). Starting off from small farms, hatcheries, and processing facilities the poultry industry has grown into an industry where relatively few large companies control the sector.

As technologies and the industry have developed, the methods for intensively rearing birds have also changed (Jones *et al*., 2018). Chickens are now separately bred depending on what purpose they are intended to be used for – either meat or egg production (Mueller *et al*., 2018). Production of both poultry eggs and meat requires a highly technical understanding of nutrition among other aspects to fully gain the maximum potential. In terms of welfare, the UK and EU have some of the highest standards globally (Van Horne and Achterbosch, 2008). Less developed countries often rely on dual purpose birds to provide a source of animal

protein. In developing countries, dual purpose birds have been trialled by many companies but have been deemed inefficient for supplying such great demands. Up to 80% of households in less developed countries keep dual purpose birds, as they are better adapted for the environment and require less care so have a lower financial impact for small holdings and families that own them.

1.1.1 The history of poultry

Domestic chickens (Gallus gallus domesticus) predominantly originated from the red jungle fowl (Gallus gallus gallus) (Sawai *et al*., 2010) with minor traits crossing over from the grey jungle fowl (Gallus sonneratii) and the Ceylon jungle fowl (Gallus lafayetti) (Nishibori *et al*., 2005; Eriksson *et al*., 2008). Mitochondrial DNA analysis revealed southern Asia and China to be the first areas of domestication (Miao *et al*., 2013; Chambers *et al*., 2017). Production and consumption of poultry products on an industrial scale became significantly more common during the Second World War, where other animal sources such as beef and pork were in limited supply. From 1945, methods for storing and distributing poultry products have been improved and have helped to stimulate consumption of these foods (FAO, 2009). The present integrated poultry production systems around the globe evolved from small farms, hatcheries, processing plants and feed mills combining under single ownership.

1.1.2 Global context

1.1.2.1 Trends in production

The global meat output for 2017 was approximately 330 million tonnes, which is a one percent increase over 2016. This shows that there is a deceleration in the growth of per capita meat consumption (Godfray *et al*., 2018). In developed countries or countries with high incomes, meat consumption is suggested to have almost peaked. Whereas low-income countries do not always have the demand for animal protein sources due to low wages. It is developing countries which seem to be influencing the trends in meat product, as rising incomes in developing countries are becoming more numerous (Delgado, 2003).

Due to the growing global population, overall production of meat is increasing to supply the demand. However, the consumption of some different sources of meat is reducing per capita.

All meats except poultry are expected to account for a declining share of total meat consumption (Henchion, *et al*., 2014). Other than affordability, there are also religious or cultural factors that may affect the consumption of different types of meat. Country by country, meat consumption varies, however increases do not always relate to global population increase (Speedy, 2003). Poultry has increased three times the level that of what it was in 1960. As such, the poultry share in the meat market has more than doubled (Allievi *et al*., 2015). Alongside poultry meat, poultry eggs are also an extremely viable way of incorporating animal protein sources in the diet.

1.1.2.2 Meat production

Given that the human population is expected to rise to around 9 billion by 2050 and global wealth is on the climb, the demand for poultry products is increasing (Godfray *et al*., 2010; Smith, 2013; Hartmann and Siegrist, 2017). The highest population growth rates are expected to occur in regions suffering most from food insecurity (Croft *et al*., 2018). Global poultry production is shown in Table 1.1, with broiler chickens accounting for the majority of poultry produced. Within 6 years from 2010 to 2016 broiler numbers have increased by over 15%. Other poultry production such as ducks has increased roughly 13% in 6 years whilst other species have smaller increases around 5%.

Table 1.1 Global poultry meat production

	Birds placed per year (millions)						
Species	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**
Broilers	56651	58265	59495	60417	61753	64289	65847
Turkeys	631	637	653	631	631	634	673
Ducks	2685	2783	2901	2876	2833	2972	3056
Geese and Guinea fowl	631	650	692	684	695	639	659

Source: FAOSTAT (2020)

1.1.2.3 Egg production

Eggs now contribute a large proportion of the animal protein in the human diet, as they are a relatively economical source of nutrients (Elson and Tauson, 2011). Since 1970, the global output of eggs has more than tripled, with consumers demand for quality increasing just as

rapidly (FAO, 2009; Windhorst, 2011). The industry maintained an estimated 21.2 billion laying hens worldwide during 2012, a figure which has likely been surpassed in recent years. In 2016, approximately 73.6 million metric tonnes of egg were produced globally (FAO, 2016). Of the 73.6 million tonnes produced, it has been estimated that around 60% of the eggs were produced by only five countries (China, US, India, Japan and Mexico) (Chambers *et al*., 2017). The Food and Agriculture Organisation has predicted that by 2030, 89 million tonnes of eggs will be produced globally. Developing countries will contribute a large proportion of this increase (Conway, 2012). China, Russia and Brazil are also key contributors to this predicted increase as they have shown major boosts in egg production since 2008 (Chambers *et al*., 2017). Global egg production (per egg) between 2010 and 2016 is shown in Table 1.2. Similar to poultry meat production, an approximate 15% increase has been seen over 6 years.

Table 1.2 Global egg production

	Annual egg production (billions)						
	2010	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**
Hen eggs (in shell)	1205	1229	1256	1282	1274	1348	1387
Other bird eggs (in shell)	83	85	87	89	91	94	108

Source: FAOSTAT (2020)

The annual consumption of eggs per country is largely determined by the wealth of the country, ranging from 300g to 19kg consumed per annum (Chambers *et al*., 2017). The USA, EU and Asia consume on average 11.4kg, 12.7kg and 9.2kg respectively (FAO, 2012). The global demand can therefore drastically effect bird welfare, making it a significant issue particularly in laying hens. Whilst all hens from various systems are affected by skeletal damage in their lifetime, up to 85% of free-range laying hens are affected. Over the past two decades research has focused on modifying housing systems and husbandry practices to increase welfare (Sirovnik *et al*., 2018). Furthermore, pressure to extend the laying cycle to 100 weeks with birds producing 500 eggs in their lifetime will lead to welfare challenges which must be addressed (Bain *et al*., 2016).

1.1.3 Layer housing systems

1.1.3.1 Evolution of the housing systems

Towards the end of the 1960s, poultry production had evolved from a small-scale enterprise into a vital part of agriculture. Flock sizes had increases dramatically whilst production became more intensive, to meet the newly found demand of poultry products (Fröhlich *et al*., 2012) During this time laying hens were predominantly reared and housed intensively in conventional cages. EU Council Directive 1999/74/EC states that a conventional cage must provide 550cm^2 cage per hen, 250cm^2 of litter area, a nest box per 7 birds, 10cm long feeder space and 2.5cm drinking space (10 birds to one nipple if nipple drinkers are installed). Perches must be 20cm away from a wall, 20cm above the litter and 30cm apart from one and other (Sherwin *et al*., 2010). By 2012, welfare concerns surrounding conventional cages increased and were no longer permitted in the EU. As a consequence, hens could only be kept

in enriched cage systems or alternative systems, for example, barn or free-range. These systems have been developed, and continue to evolve, to provide greater freedom of movement and to facilitate more natural behaviours in hens (LAYWEL, 2006; Sherwin *et al*., 2010; Regmi *et al*., 2017). Some EU member states banned the use of furnished cages, but the general consensus in the UK is that there is currently a lack of evidence to support a full ban. There has been research undertaken about welfare of hens housed in furnished cages (Jendral *et al*., 2008; Rodenburg *et al*., 2008a; Barnett *et al*., 2009; Tactacan *et al*., 2009), though research using fully commercial conditions is still limited. The poultry industry still remains divided on opinions over cage or cage free, a main factor influencing opinions is consumer pressure (Fiks-van Niekerk and Elson, 2005; Janczak and Riber 2015; Stadig *et al*., 2016), with animal welfare charities such as the RSPCA giving their backing on the move (Farming UK, 2016) and some large supermarkets already moving to cage-free ahead of schedule (Grant, 2020).

1.1.3.2 Housing system changes and requirements

Generally, non-cage systems (NCS) can be divided into two groups: (a) aviary systems and (b) floor housing systems. Both types of NCS are capable of keeping large flocks, between 5000-30,000 birds per system (Rodenburg *et al*., 2008a). Each system must have access to nesting areas, perches, areas of litter to scratch, drinkers, and feeders. Floor housing systems are kept on a single level whereas aviary systems or multi-tier systems have access to a maximum of four tiers, containing all the same facilities (Lay *et al*., 2011) These two major groups can be further divided depending on the availability of an outdoor run. These groups include: (a) barn systems, (b) free range systems and (c) organic systems (Rodenburg *et al*., 2012). Together with furnished cage systems, these groups regulate how eggs in the UK are marketed, (0 = organic, 1 = free range, 2 = barn, 3 = furnished cage). Each egg is stamped with a code to indicate farming method (as above), country of origin and production establishment (DEFRA, 2012).

1.1.3.3 System space and stocking density

Although following the prohibition of conventional cages in the EU in 2012 by the Council directive 1999/74/EC (1999), caged systems still exist as furnished cage systems (Rodenburg *et al*., 2012). After the ban in European countries, furnished caged systems became more prevalent. Switzerland have opted for a full cage ban, where other countries such as Germany and the Netherlands only use large furnished cages. A major difference in the four sub-systems is the space provided for the birds. Furnished cages allow 750cm^2 per bird, where both barn and free range allow for 1111cm^2. Organic birds receive the most space per bird at 1667cm^2 (Steenfeldt and Nielsen, 2015). In terms of outdoor space, both free range and organic systems are required to allow 4m^2 of space per bird for a minimum of 8 hours per day. Barn and caged systems do not have access to outdoor space (Rodenburg *et al*., 2012). In addition, any outdoor space within an organic system must be cared for and maintained under the organic production guidelines of the commission regulation EC No 889/2008 (2008) (Acamovic *et al*., 2008; Bestman *et al*., 2009). Flock size also varies between systems, furnished cage systems can house between 5-100 birds per cage. Free range and barn group sizes are not limited but cannot exceed the maximum stocking density of 9 birds m^{-2}. Group sizes in organic are limited to a maximum of 3000 (Steenfeldt and Nielsen, 2015). A comparison table between systems can be seen below (Table 1.3).

1.1.3.4 System environmental and husbandry requirements

Beak trimming is prohibited in all systems unless authorised by the correct authority to prevent further welfare issues caused by feather pecking and is usually dealt with on a case-by-case basis (Hartcher *et al*., 2015). In addition to space usage and stocking density other environmental parameters have to be monitored for efficient laying hen performance in all types of systems. These include the temperature of the house, humidity, light intensity, ammonia concentration and CO_2 levels (Table 1.4). All systems must also have adequate ventilation and litter coverage (Thiele, 2012). In non-caged systems, 250cm^2 of litter per hen is to be provided whilst in cage systems it is stated that litter must be provided so pecking and scratching are possible. Light intensity should be measured at feed trough level and follow a lighting programme similar to Table 1.5. A gradual dimming of light in a twilight period should also be provided to prevent disturbance (The Welfare of Farmed Animals (England)

Regulations, 2007). Feed and water must be readily available to all hens. Where possible noise must be kept to a minimum and sudden noises should be avoided (DEFRA, 2018).

1.1.4 Comparing welfare between systems

It is generally perceived by the public that non-caged systems are more welfare friendly than caged systems and produce healthier eggs (Miao *et al*., 2005). To what extent this statement is true is dependent on the parameters used to indicate welfare and health (Rodenburg *et al*., 2008a). There are many measurements which can be used to assess welfare; physiological and behavioural measurements are used primarily by farmers and scientists alike. In some animals it is also useful to measure chemical changes within the body, such as hormone changes (Van Goor *et al*., 2016). Recently, studies have focussed on advancing and validating more animal-based measures of welfare to improve methods of which animal welfare can be maintained (Blatchford *et al*., 2016). This is particularly useful in production animals, as greater welfare can often produce greater production and yield better profitability (Hemsworth *et al*., 2015; Averós and Estevez, 2018).

1.1.4.1 Measuring hormones

As previously mentioned, a common way in which welfare can be measured is by monitoring hormone regulation. Commonly, external stimuli play a role in effecting hormone regulation. In farm animals these external stimuli can be attributed to flock/herd mates' behaviour, food competition and environmental stimuli as examples (Palme, 2012). These external stimuli or "stressors" can cause an imbalance of homeostasis and cause the brain to stimulate a stress response. Responses can vary, from behavioural changes, immune system changes and activation of the hypothalamic pituitary adrenal axis or autonomous nervous system (Moberg, 2000). In poultry corticosterone is a hormone commonly used to assess welfare, often measured from the feathers (corticosterone detected in blood of feather stems) and is a relatively non-invasive procedure (Bortolotti *et al*., 2008; Fairhurst *et al*., 2011; Weimer *et al*., 2018; Palme, 2019). Assessing corticosterone levels in hens can therefore be a tool to indicate how well a system is developed for providing suitable hen welfare, when measuring key stressors and stress responses (Häffelin *et al*., 2020).

1.1.4.2 Effect of outdoor access on welfare

Previous studies comparing different housing systems stated that access to the outdoors increases the number of environmental stimuli and allows more freedom for the birds to display natural behaviours (Knierim, 2006; Mench *et al*., 2011). Interestingly, a study by Patzke *et al*., (2009) showed cell size were significantly larger in the dorsomedial hippocampus in free-range birds than caged birds. The outcome of the study was thought to be related to the increased spatial complexity that free range systems offer compared to cages. No differences were found when comparing barn systems to the other systems. Furthermore, free range access has been shown to improve short term learning performance in young chicks (Krause *et al*., 2006). Outdoor access in free-range systems have also shown increased foraging behaviours (Newberry *et al*., 2007; Shimmura *et al*., 2008; Lambton *et al*., 2010).

Although outdoor access has positive effects on welfare, it also increases the risk of disease and predation (Pettersson *et al*., 2016; Singh *et al*., 2017). Bacterial infections are more common in free range flocks than indoor flocks. Red mite (*Dermanyssus galinae*) infestations are common among all laying housing systems but are particularly prominent in free range systems, with an average prevalence of 83% in flocks across the EU (Sparagano *et al*., 2014). Red mites can cause anaemia, stress, and higher mortality rates (Camarda *et al*., 2018). In organic flocks, the risk of disease and other health issues are even greater than in free range as the use of antibiotics is restricted (Rodenburg *et al*., 2012). To reduce bacterial presence and keep parasitic breakouts to a minimum, organic flocks are encouraged to use the entire outdoor area provided. Rotating access to particular parts of the outdoor area at one time can also be used to prevent the spread of disease (Knierim, 2006). In terms of predation, a study by Hegelund *et al*. (2006) reported an average of 6.4% mortality in organic flocks from predation alone.

1.1.4.3 Feather pecking and plumage

Feather pecking in commercial flocks can be prevalent and caused by varying factors, such as environmental stress, lack of enrichment, management issues, hierarchical problems, and poor nutrition (Cooke, 1992; Hartini *et al*., 2002). Feather pecking is common in both caged and alternative systems (Appleby and Hughes, 1991). Interestingly, results from Patzke *et al*.,

(2009) showed that free-range had worse feather plumage than hens in barn or caged systems. Though in other studies, free-range flocks showed less feather pecking behaviour and better plumage score than conventional or furnished cages (El-Lethay *et al*., 2000; Nicol *et al*., 2013). It may be the case that access to free-range in the Patzke *et al*., (2009) study was at a time of high light intensity outside, which is known to increase aggression (Parvin *et al*., 2014; Barros *et al*., 2020), or the lack of stimuli within the housing could cause boredom. However, feather pecking is still present in all systems albeit in different severities, challenging the ethological viewpoint of hen welfare by being still present after intervention (Bestman and Wagenaar, 2014; Elkohoraibi, *et al*., 2014; van Staaveren *et al*., 2020).

1.1.4.4 Hierarchical problems

Hierarchical problems within laying hens have been widely monitored in previous research (Nicol *et al*., 1999; Keeling *et al*., 2003; Carvalho *et al*., 2018). Previously, the general assumption was hierarchical imbalances and the associated problems such as cannibalism and feather pecking become more prevalent as group size increases (Hughes 1975; Bilcik and Keeling, 1999). A study by Keeling *et al*., (2003) investigated the effect of group size (15, 30, 60 and 120 birds) on hierarchical problems and found confounding results. The study found that although in smaller groups (15 birds) aggression started off high and then declined, there may a limit for social hierarchical stability. In the 30-bird group the authors suggested that this group size caused social instability, as there were more dominant hens competing in the group with higher chances of re-interacting. In the 60-120 bird groups, hens were reported to be relatively non-aggressive – suggested to be caused by the reduced chances of interaction between familiar dominant birds in larger groups. Nicol *et al*., (1999) also supported the findings from Keeling *et al*., (2003) indicating that birds in larger flocks appear to adopt more non-social, non-aggressive behaviours. It has been proposed that hens can adjust their behaviour according to group size (Pagel and Dawkins 1997), perhaps supporting why hierarchical problems can be so inconsistent within a flock made up of many different sized groups. Furthermore, the stocking densities varying between systems could also affect the chances of hierarchical problems occurring, thus impacting the welfare reported in each (Keeling *et al*., 2003).

1.1.4.5 Mortality

Considerable differences in mortality have been reported across organic, free range and barn systems. On average barn and free-range flocks have shown lower mortality figures than organic in previous studies. Furnished cage systems have been shown to have the lowest mortality at around 3% (Fossum *et al*., 2009). Barn and free-range mortality can range from around 7-10% (Whay *et al*., 2007; Rodenburg *et al*., 2008b), whilst organic flocks reported an average of 23% mortality with some flocks having very high mortality levels (50-65%) (Hegelund *et al*., 2006). A more recent study by Weeks *et al*., (2016) also found organic flocks to have a higher mortality over the other systems and found barn systems to have an increased mortality over data previously seen.

Organic regulations prohibit beak trimming in organic flock which may contribute to risk of high mortality levels in these systems (Hegelund *et al*., 2006). It has also been suggested methionine levels can often be low in organic diets before supplementation due to methionine not being available in an organic form and the lack of methionine leading to feather pecking occurring more regularly (van Krimpen *et al*., 2005) Combined with the prohibition on beak trimming in organic systems, non-supplemented diets could impact welfare. Methionine is known to be the first limiting amino acid in poultry (Agostini *et al*., 2016; Burley *et al*., 2016). However, lack of methionine in the diet can often be compensated by access to outdoor foraging (Kjaer and Sørensen, 2002; Acamovic *et al*., 2008).

Furthermore, in non-caged systems flocks have been observed to express an unusual behaviour known as 'piling', where many birds congregate in one area at randomly throughout the day and flock cycle (Hegelund *et al*., 2006). The piling behaviour can cause welfare concerns as hens piled up on one and other may inflict physical damage to themselves or others around them. Further research is needed into piling and the welfare implications linked with this behaviour, although it has been observed to occur most frequently at peak dust bathing periods (Campbell *et al*., 2016). A UK based survey showed that piling in organic flocks contributed up to 25% of flock mortality (Sparks *et al*., 2008).

When comparing non-caged systems to caged systems via computer welfare models, De Mol *et al*., (2006) found that feeding level, space per hen, water availability and the presence or absence of perches and nests had the greatest impact on welfare scores. Providing access to outdoor access only slightly improved welfare scores using the computer model (FOWEL). A more in-depth study by Rodenburg *et al*., (2008b) found similar results. Birds in non-caged systems within this study were stated to be less fearful, used more of the resources provided (i.e., perch and nest boxes), were more active and had stronger bones compared to their caged counterparts. On the other hand, birds in furnished cages showed less keel bone damage (KBD), lower mortality rates and lower dust concentrations in the air (Rodenburg *et al*., 2008c). The overall analysis of the welfare scores in Rodenburg *et al*., (2008c) found no significant differences between the non-caged and cages systems involved. These results give the indication that non-caged and caged systems both have strengths and weaknesses in terms of their impact on animal welfare. Comparable results were found by Shimmura *et al*., (2010), who stated that in both types of systems there is potential for improvement. However, in caged systems the cage environment restricts possible improvements, whereas in non-caged environments, housing design and management can be improved to greater extents, resulting in a potential major increase in animal welfare (Rodenburg *et al*., 2012).

1.1.5 Consumer perceptions

Over the past 15 years, the poultry meat and egg industries have faced many challenges. A major challenge being the change in consumer perception over this time. In egg production specifically, evidence from a multitude of studies using consumer surveys found that production systems were the biggest factor in influencing consumer decisions (Jones and Parrot, 1997; Parrot, 2001; Hingley and Parrot, 2008; Parrot *et al*., 2013; Walley *et al*., 2014; Parrot *et al*., 2016). Other factors included animal welfare, food safety, product quality, price and origin. It was suggested that animal welfare was heavily related to production system as a factor and that the consumers see the two factors intertwined. In the past, due to possible lack of information or misperception, the UK egg production industry has been seen in negative light in terms of the production systems used (Van Horne and Bondt, 2013) In 2012, the industry abolished conventional caged and moved to alternative systems in order to align itself better with consumer perceptions and leading to the movement of cage free by 2025.

From a recent study in the series published by Parrot *et al*., (2016) and other collaborators, animal welfare as a factor influencing consumer decisions was ranked as the 3rd highest factor. Animal welfare in meat chickens was only ranked at 9th place out of a possible 20. A shift in preference of eggs from different housing systems was also recorded. In 1997, only 16% of consumers indicated they disagreed with the use of hens in cages, by 2012 that figure was up to 71.4%. Interestingly however, there is a declining trend to pay more for better perceived quality. Overall, it could be suggested that having a more open approach to how poultry are produced for egg or meat would help connect with consumers to where their food comes from and the choices they make (Parrot *et al*., 2016).

The next section will focus on the skeletal growth of laying hens and the challenges that the demands of the consumers create, such as maintaining high animal welfare and negating problems such as osteoporosis and other skeletal defects.

1.2 Skeletal growth in layer hens

A laying hen has a mature skeletal system at the age of 16-18 weeks, after which point, the birds will start to lay eggs (Toscano *et al*., 2020). Calcium from the skeletal system is used to create an egg every 25 hours. Hens are depleted around 80 weeks, with some producers pushing further, trying to reach 100 weeks (Bain *et al*., 2016; Molnár *et al*., 2016). This may cause some additional challenges and skeletal problems, which will be discussed in section 1.3. Bone is a reservoir for calcium and phosphorus and approximately 99% of total body calcium is stored within the skeletal system. The remaining 1% can be found intra- or extracellularly (de Matos, 2008). Bone is composed from crystallised hydroxyapatite [$Ca_5(PO_4)_3(OH)$] derived from calcium phosphate. The crystals are deposited on an organic collagen matrix (Whitehead and Fleming, 2000).

1.2.1 Types of bone tissue

There are 3 main types of bone tissue in laying hens, cortical, trabecular, and medullary (Van De Velde *et al*., 1985; Dacke *et al*., 1993; Hester, 2017). The following sections below will describe each bone tissue type.

1.2.1.1 Cortical bone

Cortical bone is the strongest outer-most layer of bones forming a solid osseus shell around the bone consisting of dense parallel lamellar units (Orsterhoff *et al*., 2016). Cortical bone provides much of the skeletal systems' structural support and is the most abundant bone tissue type. In hens, cortical bone is primarily produced up until the point of lay (Whitehead, 2004), formation of new lamellar cortical bone ceases at this point (Fleming, 2008).

1.2.1.2 Trabecular bone

Trabecular, also known as spongy bone, is the bone tissue found on the inside of cortical bone. Trabecular bone also consists of lamellar units of bone plates but are much less organised in structure than cortical bone and is supplied by diffusion from bone marrow (Nordin *et al*., 2012). Though cortical bone can withstand higher ultimate stresses, due to a spongier structure trabecular bone can withstand higher physical strain depending on its density than

cortical bone. The strength of trabecular bone is more dependent on the level of connectivity within the bone tissue (Keaveny and Hayes, 1993). Although cortical bone is more prevalent that trabecular bone, trabecular bone has a higher bone turnover as it is more metabolically active. In hens, trabecular bone also stops being produced at the point of lay, switching to the production of medullary bone (Fig. 1.1) (Whitehead, 2004).

1.2.1.3 Medullary bone

As a large source of calcium, medullary bone is a specialised type of bone tissue developed on the endosteal surface of the medullary cavity (Fig. 1.1) (Schraer and Hunter, 1985). Medullary bone is a non-structural bone type and has woven structure created from randomly weaved collagen fibres and is only found in female avian species before and during egg production (Whitehead and Fleming, 2000). The mass of medullary bone acts as a calcium reservoir for eggshell development (Dacke *et al*., 1993; Prondvai and Stein, 2014). This type of bone starts being produced at the point of lay or sexual maturity, due to increased oestrogen (Webster, 2004). Whitehead and Fleming, (2000) stated that medullary bone could act as a mesh between structural bone types such as cortical or trabecular bone as the spicules increases fracture resistance. In their study, humerus breaking strength showed a correlation with the humeral medullary bone present. However, the structural support from medullary bone is much less than compact or trabecular bone. Reproductively active hens have developed this bone to avoid a negative balance in calcium, though the quantity of medullary bone can vary over breeds, flocks, and age (Hester, 2017). This type of bone has also been found in bone fossils of egg-laying dinosaurs suggesting medullary bone has long had an important role in eggshell production (Canoville *et al*., 2020). Throughout the laying period resorption of all bone types occur. This means cortical or trabecular bone can be resorbed alongside medullar bone possibly leading to a progressive weakening of the skeletal system (Whitehead and Fleming, 2000; Whitehead, 2004; Fleming *et al*., 2006).

Pneumatic bone

In addition to these three types of bones, some bones in the avian skeletal system can be specialised and become pneumatic (Neijat *et al*., 2019). The sub-type of pneumatic bones is often hollow and are connected to air sacs of the respiratory system, allowing for the passage of air through the bones (Dale *et al*., 2015). The pneumatic bones can contain medullary bone but are frequently non-medullary. Examples of these bones include but are not limited to; keel, coracoid, some ribs, pelvic girdle, some vertebrae, humerus and skull bones (King, 1957). Bones such as the humerus and keel can be pneumatic but also contain medullary bone tissue (Whitehead, 2004; Hester, 2017).

1.2.2 Bone Formation and Transformation

At a cellular level there are three major cells involved in bone metabolism, one for bone formation, one for transformation and one for maintenance. Osteoblasts are the cells which deposit and form bone tissue, osteoclasts are the cells which resorb and breakdown bone tissue for transformation, and osteocytes maintain the bone structures (sections 1.2.2.1 – 1.2.2.3). It is known that remodelling of bone tissues always occurs after formation, leading to a dynamic environment of continuous remodelling (Dacke *et al*., 2015). Osteoclastic degradation is directly followed by osteoblastic regeneration in bone tissue (Hester, 2017). The net effect during the laying period is the gradual loss of structural bone as osteoclasts are recruited to resorb and mobilise Ca from cortical and trabecular bone to produce medullary bone (Whitehead, 2004; Fleming, 2008). As structural bone types are depleted, medullary bone is higher in abundance but is of less structural value unless present in large quantities – therefore skeletal issues can begin to arise (Sandilands *et al*., 2005). When a hen goes out of lay the processes are reversed, and medullary bone gradually disappears as structural bone formation recommences via osteo-mechanism. Structural bone formation can be demonstrated by the creation of new layers atop the medullary which previously coated the surface of structural bones (Whitehead, 2004). The cycle of egg production and bone regeneration is normal and allows for bone quality to be maintained over the hen's lifetime. However, skeletal problems become common when selection of modern-day hens are based upon the ability to remain in a continuous reproductive state over vastly extended periods, causing an inability to undertake natural bone regeneration (Fleming, 2008; Bain *et al*., 2016; Rajput *et al*., 2018).

1.2.2.1 Osteoblasts

Osteoblasts are uni-nucleated cells which produce and release osteoid, a molecule derived from type 1 collagen and non-collagenous protein. Once released into the extracellular matrix, the osteoid is mineralised with hydroxyapatite to form bone (Whitehead, 2004). Vitamin D metabolites, parathyroid hormone (PTH), adrenal and gonadal steroids, specific growth factors and cytokines can act as bone-active mediators to monitor the amount of bone formation (Bloom *et al*., 1942).

1.2.2.2 Osteoclasts

Unlike osteoblasts, osteoclasts are multi-nucleated and are created from hematopoietic marrow precursors (Whitehead, 2004). The structure of osteoclasts allows for the attachment to bone via adhesion molecules, so bone catabolism and resorption can take place (Hester, 2017). The osteoclasts secrete hydrogen and chloride ions to assist in the process of dissolving the bone. A variety or proteinases and proteases are also released at a low pH to dissolve the organic matrix originally laid down as a foundation for the bone to develop. Osteoclasts have fewer receptors compared to osteoblasts, though they do have calcitonin receptors (Dacke *et al*., 2015). Avian calcitonin circulates in relatively high levels, creating a dynamic equilibrium with PTH to ensure a balance. Apoptosis occurs once the correct amount of bone resorption has been completed (Stanford, 2006).

1.2.2.3 Osteocytes

Osteocytes are created from osteoblasts on a bone surface when it is fully surrounded by mineralised bone. The shape differentiates, and the cell develops processes, starts to secrete osteoids, regulates osteoid mineralisation and acts as communicator between other cells (Bonewald, 2011). In essence, osteocytes are the regulators of bone remodelling and act as a network of communicating cells that respond to vitamins, hormones, cytokines and mechanical forces (Dacke *et al*., 2015). Osteocytes develop and use cytoplasmic processes to communicate through narrow channels called canaliculi with other cells in the vascular structure of bone tissue. The extensive network of canaliculi allows for bone mineral exchange during periods of high metabolic activity (Hester, 2017). As previously stated, osteocytes are involved in mechanical loading by responding to movement and exercise which stimulates bone remodelling (Dallas *et al*., 2013).

To maintain a balance between bone formation and bone resorption, it is crucial that these bone cells communicate between on another. Cytokines act as a mediator for communication (Hester, 2017) The mechanism of communication in bones cells in mammals is suggested to be similar to the mechanism for communication in avian bone cells. In bone formation or resorption, there are two key cytokines which control when each process occurs (Dacke *et al*., 2015). A cytokine named Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL), located on osteoblast membranes, binds to a receptor found on osteoclast precursor cells – RANK (Wang *et al*., 2008). The combination of RANKL and RANK receptors triggers an osteoclastic differentiation and bone resorption begins (Khosla, 2001; Dale *et al*., 2015). As a balanced system, a cytokine named osteoprotegerin (OPG) competes for the RANK receptors and prevents excessive bone resorption. OPG is stimulated by oestrogen and has been considered as a possible treatment for human osteoporosis (Hamdy, 2007; Jurado *et al*., 2010).

1.2.3 Bone Maturity

Bone maturity can be defined as the finishing point of basic structural development and mineralisation of the skeletal system, where optimal mechanical strength is attained. Maturation consists of many molecular and biochemical changes involving the processes of the osteoblasts, osteoclasts and osteocytes in the bone (Whitehead, 2004). These processes achieve the optimal bone properties in collagen formation such as collagen fibre diameter, collagen crosslink content, and deposition of lamellar bone. All of which have been used as indictors of bone maturity in mammals (Boskey *et al*., 1999; Ristelli and Ristelli, 2006) and can also be useful markers in poultry bone maturity (Knott and Bailey, 1999). Bone maturity in laying hens reached at around 16-18 weeks in the rearing period as they are about to start laying.

1.3 Skeletal problems in layers

As demand for egg products has increased over the past two decades the egg industry has had to evolve to meet this demand. It has been considered that extending the period of lay closer towards 100 weeks may be beneficial for producers and use less replacement birds (section 1.2) (Bain *et al*., 2016). However, with the push for an extended laying period this may create more skeletal problems or animal welfare concerns (Toscano *et al*., 2020). The skeletal problems present in the egg industry currently varies between production system. There are three major skeletal problems; osteoporosis, keel bone damage (KBD) and cage layer fatigue (Lay Jr *et al*., 2011). Cage layer fatigue is uncommon in today's laying flocks as diets are better formulated and inclusive of all the hen's nutritional needs, but in some instances can still occur in poorly managed flocks (Hester, 2017).

1.3.1 Osteoporosis

Osteoporosis is a non-infectious disease caused by decreasing quantities of mineralised structural bone in the skeletal system, in poultry this occurs during a period of lay (Whitehead and Fleming, 2000). The reduced quantities of mineralised structural bone present cause an increased risk of skeletal damage as the bones become more brittle (Whitehead and Wilson, 1992; Whitehead, 2004; Hester, 2017) (Fig. 1.2). Osteoporosis is systemic and is often widespread in current commercial laying hens and contributes to approximately 20 to 35% of all mortalities in the laying industry (Anderson, 2002; Lay Jr *et al*., 2011). Osteoporosis and osteomalacia are likely to occur in combination with one another but are not the same. Osteomalacia is a skeletal problem where defective bone mineralisation occurs, leading to abnormal bone structures forming (Whitehead and Fleming, 2000). The older the bird the more osteoporosis is likely to affect the skeleton. As a hen ages, the osteoblastic oestrogen becomes increasing downregulated, lowering osteoblast count and therefore reducing new bone formation (Dacke *et al*., 2015).

It is well known that genetics effect bone strength and modern egg layers are selected for this trait (Bishop *et al*., 2000; Hester, 2017). After genetics, exercise has the next best effect of reducing osteoporosis. Exercise reduces the number of osteoclasts present in the bone and reduces bone resorption. This means birds in alternative systems often have higher bone breaking strength than those in cage systems (Lay Jr *et al*., 2011) The suppression of osteoclastic activity does not persist throughout life and as hens age the difference in number of osteoclasts present in bone of active and non-active hens begins to diminish (Fleming *et al*., 2006). In the most severe cases of osteoporosis and subsequent bone loss, cage layer fatigue can occur. However, severe cases of osteoporosis in modern hens are not as common as in flocks 20 years ago and cage layer fatigue is no longer commonly seen (Lay Jr *et al*., 2011).

1.3.2 Cage layer fatigue

Cage layer fatigue was first noticed in the mid-20th century in hens that had a high rate of lay (Webster, 2004). As the name indicates, it only affected birds in caged systems. Birds were willing to still eat and drink but had leg problems meaning they could not reach food or water without difficulty. Brittle bones and thin-shelled or shell-less eggs were also a symptom of cage layer fatigue (Hester, 2017). The most severe cases of hens with cage layer fatigue often died from starvation (Bell and Siller, 1962). A deficiency of calcium and phosphorus or an incorrect ratio of the two minerals is thought to be the primary cause, also effected by the lack of movement in cages (Couch, 1955; Webster, 2004). As previously mentioned in section 1.3, cage layer fatigue is not common in modern day flocks, thanks to improved genetic lines and advancements in nutritional management (Hester, 2017).

1.3.3 Keel Bone Damage (KBD)

Keel bone damage (KBD) is a major issue in the laying hen flocks. It is a general term which refers to any damage present in the keel bone (Harlander-Matauschek *et al*., 2015) The keel is extended from the sternum, which in avian species acts as an anchor for flight muscles and has a crucial function in expanding and contracting the thoracic cavity during breathing (Codds *et al*, 2005; Lamberts and Perry, 2015) (Fig. 1.3). Ossification of the keel begins at the cranial end of finishes at the tip, with skeletal development still ongoing during the stages of early lay (Casey-Trott *et al*., 2017b). Ossification of the keel may take place until 28-40 weeks of age (Buckner *et al*., 1949).

KBD can include keel fractures, unnatural deviations from the normal structure, bending of the bone and the creation of bone shards (Wilkins *et al*., 2004). The majority of KBD occurs on the spine or ventral part of the keel or at the caudal tip (Casey-Trott *et al*., 2015) (Fig. 1.4 and 1.5). Beyond obvious skeletal deformation, a reason KBD is being investigated so intensively in the egg industry is because high levels of pain can be experienced by hens suffering from KBD, indicated by a decreased period of time spent perching (Nasr *et al*., 2012a; Nasr *et al*., 2015). Hens that are suffering are likely to be laying at a lower productivity level. Unlike some other health issues faced by laying hens, KBD is found in all housing systems and breeds of hen meaning it is not system or breed specific. KBD is widespread with similar results throughout different countries (Rodenburg *et al*., 2008a; Wilkins *et al*., 2011; Petrik *et al*., 2014).

Despite the ubiquity of KBD, the causes and factors of KBD are mainly still unknown. It has been speculated that a predominant cause could be the movement of hens and the interactions with their environment, for example jumping from perches, knocking into other birds, climbing ramps and resting on wire mesh. Most of these interactions do occur in all housing systems, but more commonly in free-range systems (Heerkens *et al*., 2015; Harlander-Matauschek *et al*., 2015). The prevalence of KBD in laying flocks ranges from 5% to 97%, depending on housing system and age and seems to increase over time (Table 1.6) (Gregory *et al*., 1990; Wilkins *et al*., 2004; Rodenburg *et al*., 2008b; Käppeli *et al.,* 2011; Habig and Distl, 2013; Casey-Trott *et al*., 2017a).

Table 1.6 Prevalence found across different studies in laying hens

Study	KBD Prevalence (%)
Gregory *et al*., (1990)	5-25
Wilkins *et al*., (2004)	50-80
Rodenburg *et al*., (2008b)	62-97
Käppeli *et al*., (2011)	35-44
Habig and Distl (2013)	23-34
Casey-Trott *et al*., (2017a)	42-60

1.3.4 Methods for reducing KBD in laying hens

There are a multitude of different approaches that have been used over the past two decades in order to try and reduce the occurrence of KBD in laying hens. These include dietary changes, changing housing design, tweaking genetic lines, and increasing the potential for exercise (Jendral *et al*., 2008). All approaches are viable, though catering for the hens need for exercise, most often through changes in housing design has been most successful in recent studies (Shipov *et al*., 2010; Casey-Trott *et al*., 2017c).

1.3.4.1 Exercise and housing system

The availability to perform more load bearing exercises in a system can result in an increase in bone strength and mineral composition (Casey-Trott *et al*., 2017b). Results from past studies have shown that there are beneficial effects of exercise on the long bones of adult laying hens between systems (Leyendecker *et al*., 2005; Jendral *et al*., 2008; Shipov *et al*., 2010). Results from Leyendecker *et al*., (2005) revealed that humerus strength of birds housed in an avairy system (247N (±2.9)) had more than double the strength of those birds housed in conventional cages (104.5 (±2.9)), and almost double the strength compared with bones from birds kept in furnished cages (129.6 (±2.9)). Tibia breaking strength was also higher in the aviary system compared to the conventional and furnished cage systems, 175.4 (±2.1), 116.7 (±2.0) and 121.6 (±2.1) respectively.

Improvements in the long bones can also be correlated to improvements in composition and breaking strength of the keel bone, however few studies have directly assessed the effects of exercise on keel bone damage (Regmi *et al*., 2016). The investigation into the effects of exercise on KBD is often confounded, as the addition of extra space or furnishings and housing in extensive systems, can aid with increased exercise but also increase the risk of collisions and injuries (Fleming *et al*., 2006; Scholz *et al*., 2009).

Many rearing systems are now more streamlined to mirror the environment in the laying systems to prevent skeletal damage and health issues when transferred (Hester *et al*., 2013). By targeting the rearing stage and increasing the space or adding apparatus into the system

for the hens to use for exercise at this critical growth period, many studies have reported an improvement in bone breaking strength (Casey-Trott *et al*., 2017b), mineral content, bone geometry (Regmi *et al*., 2015), muscle growth and lower KBD scores (Hester *et al*., 2013). These beneficial effects are known to be sustained throughout the period of lay (Regmi *et al*., 2016). Housing pullets in a rearing system that allows for a more diverse exercise not only improves motor skills, reduces KBD and increases keel development but could also reduce the time it takes for the pullets to adjust to the new system when moved on to a laying system (Gunnarsson *et al*., 2000; Casey-Trott *et al*., 2017a).

1.3.4.2 Genetic lines

As well as exercise, another potential solution to reducing KBD in laying hens would be to breed birds with characteristics such as high bone mechanical strength, which would be more resistant to bone fractures (Whitehead, 2002; Harlander-Matauschek *et al*., 2015). Different genetic lines of laying hen do differ in susceptibility to KBD, and it is suggested that the characteristics of the breed should be evaluated to help reduce the effect of KBD, such as behavioural attributes. The flightier a hen, the more possibility it has of doing damage to itself within the housing system (Candelotto *et al*., 2017). Furthermore, egg production characteristics could affect KBD, though some lower producing lines have shown to have poorer bone quality than higher producing lines, indicating that not only genetics influences the prevalence of KBD and that a multitude of entangled factors are at play (Toscano *et al*., 2020). Although, results from Candelotto *et al*., (2017) do indicate a strong tendency of genetic regulation for reducing KBD susceptibility.

1.3.4.3 Dietary interventions

Dietary changes can help reduce KBD in layers (Jendral *et al*., 2008) and nutritional deficiencies have long been recognised as a problem for bone quality in laying hens (Bain *et al*., 2016). Although not a direct cause of KBD or bone fractures, poor dietary provisions during the laying stage may increase the incidences of bone damage (Toscano *et al*., 2020). A poor calcium-phosphorus ratio is often thought to be a major contributor to this and can lead to osteoporosis (section 1.3.1) (Whitehead and Fleming, 2000). More recently, studies have investigated supplementing layer diets with ingredients such as fatty acids (Tarlton *et al*.,

2011), phytase (El-Hack *et al*., 2018), vitamin D_3 super dosing (Matila *et al*., 2004) and silica (Burton *et al*., 2020 known to help skeletal health in broilers – tested but not reported in layers yet). Tarlton *et al*., (2011) reported a substantial reduction of keel bone fractures in flocks receiving short chain omega-3 fatty acids. A mixture of long and short chain has been subsequently tested but found little benefit (Toscano *et al*., 2012). Some other dietary changes include altering the time at which mineral supplements are added in the laying cycle to make sure they are best utilised, such as increasing calcium supplementation at first light rather than at first egg (Fleming *et al*., 2003; Fleming *et al*., 2006). The dietary requirements of laying hens will be discussed further in section 1.5.

1.3.5 Measuring and evaluating for KBD

As KBD is an issue affecting a large proportion of hens in the industry with the potential to cause considerable pain, many research groups have developed methodologies to measure the severity of KBD (Wilkins *et al*., 2004; Toscano *et al*., 2013; Casey-Trott *et al*., 2015; Jung *et al*., 2019). As stated previously, KBD is an umbrella term and there are many different aspects of damage that KBD can refer to. The main two types of KBD are: fractures and deviations. Fractures are generally classed as any sharp bends, fragmentation and/or shearing of the keel bone (Buijs *et al*., 2019). Deviations can be classed as any abnormality in the keel structure that varies from the theoretical perfect shape in the ventral plane or the straight 2-dimensional plane, not created by fracturing (Casey-Trott *et al*., 2015). Studies on KBD consistently assess fractures, though deviations are often disregarded (Petrik *et al*., 2013), not distinguished from fractures (Scholz *et al*., 2008), or only scored in the absence of a fracture (Stratmann *et al*., 2015). The causes of keel fractures and deviations have been assumed to differ in previous research (Buijs *et al*., 2019). Fractures are believed to be the result of short-term, high-energy collisions, whereas deviations are the likely result of long-term, low energy pressure on the keel. For example, perching is a likely source of long-term low pressure and has been shown to be associated with deviations. Any system with perches available may increase the incidences of keel deviation, though cage systems seem to show more incidences of deviations due to less space available to the hens causing them to perch more often (Pickel *et al*., 2011; Regmi and Karcher, 2013; Harlander-Matauschek *et al*., 2015). The effects on bird welfare between the two types of damage also differ and even once

healed, a fracture could still cause pain and hinder mobility (Nasr *et al*., 2012b). The effects on welfare from deviations without fractures are a more unclear, it has been suggested that deviations could have an effect on balance and lead to unequal loading during movement and therefore increase the risk of fractures to occur (Harlander-Matauschek *et al*., 2015). As the causes and effects of the two types of KBD damage are different, it is important that they are assessed independently of one and other so that preventative measures used in future are as effective as possible (Buijs *et al*., 2019).

1.3.5.1 Palpation

By far, the most commonly used and validated technique to assess KBD is palpation of the keel (Buijs *et al*., 2019; Chargo *et al*., 2019). Palpation is carried out by a trained assessor, carefully running fingers down the sides of the keel bone, feeling for calcium deposits. Calcium deposits are formed due to previous breakages as a result of KBD. Particular attention is normally given to the caudal tip as it is the part of the keel which is most frequently damaged (refer back to Fig. 1.5) (Wilkins *et al*., 2004). A benefit of palpation is that the process can be carried out on live birds as well as assessing post-mortem, so it is not necessary to cull birds. However, post-mortem analysis of the keel is often used in addition to palpation, to ensure the accuracy of the technique (Wilkins *et al*., 2004). More recent studies have used multiple observers with varying levels of training to ensure the palpation technique is viable (Petrik *et al*., 2013; Casey-Trott *et al*., 2015; Heerkens *et al*., 2016). The study by Petrik *et al*., (2013) which aimed to validify the methods by Wilkins *et al*., (2004) found that increased practice led to increased accuracy in categorising KBD via palpation. The average accuracy of true prevalence of KBD in experienced handlers was 91.3% and only 89.0% in inexperienced. Data was also recorded in intervals to show the effects of practicing (Table 1.7). However, there are limits to what palpation can reveal, for example using palpation alone without post-mortem analysis cannot distinguish the severity of deviations or confirm if the damage is new or old. The caudal tip of the keel can also be assessed incorrectly through only palpation as it is under much more muscle tissue (Sherwin *et al*., 2010; Buijs *et al*., 2019).

1.3.5.2 Other technologies and methods used to assess skeletal health and KBD

Palpation is considered to be a keystone method for assessing KBD, but with great advances in technology over the past 20 years, new technologies have emerged or become viable for use in the poultry industry (Casey-Trott *et al*., 2015). It is possible that other methods could give different or superior insights into KBD (Rufener *et al*., 2018). Examples of other methods include X-rays, ultrasonography and variations of computed tomography (Sandilands *et al*., 2010; Richards *et al*., 2011; Regmi *et al*., 2013; Baker *et al*., 2017).

X-ray

X-rays have been commonly used in human health care for many decades and have been assessed as a useful technique to assess KBD. X-rays use electromagnetic radiation directed through a sample to a detector to create an image which can then be analysed (Casey-Trott *et al*., 2015). A benefit of using X-rays to measure KBD is that bone is effective at absorbing radiation which allows a clear picture to be created. X-ray machines are also widely available and can be portable. In addition, X-rays can measure new breaks (before an inflammatory response is seen) and scan parts of the keel otherwise inaccessible by palpation (Richards *et al*., 2011). On the other hand, too much user exposure is a health risk to both handler and hen, analysis can only take place in a 2D plane - meaning multiple x-rays are required to form clear images (Širovnik and Toscano, 2017 in Eusemann; Eusemann *et al*., 2018).

Quantative Computed Tomography

Quantitative Computed Tomography (QCT) has also been used extensively in human health care (CT scans) and is considered a more recent advancement to the traditional X-ray. Computed tomography uses narrow, low-level radiation and can create a complete 2D scan in a few minutes, allowing for minimal restraints of the bird or no use of anaesthetics. The 2D images can then be converted into a 3D model of the sample using specialised software. These models can be rotated on all planes to give a more holistic view of the keel and to indicate minor damage which may be missed using other techniques (Donkó *et al*., 2018) Computed tomography can also differentiate body tissues (Regmi *et al*., 2013). It is also possible for newer machines with higher resolutions to distinguish between bone tissues (Kim *et al*., 2012). Limitations include a lack of portability of clinical machines, samples have to be

stationery and machines are expensive to buy and run. Many studies using QCT to assess KBD still used samples post-mortem for better accuracy (Silverside *et al*., 2012; Regmi *et al.,* 2016). The study by Regmi *et al*., (2016) found that genetic strains of hens that showed more severe deformity scores or incidences of via QCT also showed poorer bone density and less ash content, implying that using both QCT and post-mortem samples can work hand in hand to identify and categorise KBD.

Ultrasonography

Widely used in human treatments ultrasound was suggested to have some benefits in laying hens when determining skeletal damage. Ultrasonography uses high frequency sound waves that reflect off structures and tissues to generate images, similar to X-rays and QCT. Unlike X-rays, using an ultrasound gives no exposure to ionising radiation to both handler and recipient, so is deemed to be safer by medical professionals (Casey-Trott *et al*., 2015).
Sandilands *et al*., (2010) used ultrasound to scan the ventral edge and the lateral side of the keel to detect KBD in hens every 8 weeks. Hens were housed in pens with or without perches and with differing heights of ceiling. Results showed that 36% of the birds with perches and 32% without perches had KBD. 37% of the hens provided with a high ceiling showed KBD, whereas only 30% showed KBD with a low ceiling by 50 weeks of age. These results suggested that birds apply greater force the higher the jumps and allowing extra movement space increases the risk of of KBD.

Depending on feather coverage and breast tissue mass, the clarity of the scans could vary and effect the efficacy of ultrasonography in poultry (Casey-Trott *et al*., 2015). There is also no distinction between the types of bone when using ultrasound techniques, meaning bone descriptions made prior to the scanning are essential when comparing the data (Martinez-Cummer *et al*., 2006). An upside of ultrasonography is that if the bird is restrained correctly the technique could potentially be used on live hens reducing the need for post-mortem sampling (Fleming *et al*., 2004; Casey-Trott *et al*., 2015).

1.3.5.3 Differences in criteria for assessing KBD

Although there is a common goal to assess KBD in the poultry industry, there have been multiple methodologies created to assess KBD (Casey-Trott *et al*., 2015). There are two seminal works which focussed on measuring KBD, The first study Wilkins *et al*., (2004) and the second, Scholz *et al*., (2008). Both methods have been used and modified in recent research by many research groups. A difference between these two studies is how they quantified KBD. In the study by Wilkins *et al*., (2004) only the presence or absence of old breaks were recorded, while the later study by Scholz *et al*., (2008) ranked the severity of the damage using a 1 – 4 scale system (4 = no deformity, 3 = slight, 2 = moderate, 1 = severe). Both studies did not distinguish or record the presence of keel deviations though used both the palpation technique and port-mortem examinations for KBD analysis. Deviations were not analysed for in Wilkins *et al*., (2004) and in Scholz *et al*., (2008) damage was only grouped by 'deformity' meaning fractures and deviations could not be separated in the results. Keel deviations were observed in Wilkins *et al*., (2004) method but not included in the scoring system. It was suggested that the alteration in the shape of the keel was a result of the normal remodelling process in response to consistently low pressures being applied to the keel when hens perch (Barnett *et al*., 2009; Pickel *et al*., 2011; Regmi and Karcher, 2013). Furthermore, by using a scale system, the method by Scholz *et al*., (2008) showed that the further analysis of 'slight deformations' (of which wouldn't have been recorded in Wilkins *et al*., (2004)), may actually show evidence of microscopic fractures. Similar findings of microscopic fractures were also reported by Fleming *et al*., (2004). 51% of 'slight deformations' were revealed to contain microscopic fractures (Scholz *et al*., 2008). Using the Wilkins *et al*., (2004) method, these fractures would have been missed. Additionally, the Wilkins *et al*., (2004) method only analysed for old breaks, whereas the method in Scholz *et al*., (2008) assessed bone histology to measure old and new breakings, therefore giving a more comprehensive insight into KBD. The disparity in how KBD criteria was being assessed in past research has led to concerns over misinterpreting KBD. This has led to other researchers incorporating both fractures and deviations into their assessments of KBD, alongside more in-depth bone analysis and more thorough scaling systems or grouping criteria. (Käppeli *et al*., 2011; Gebhardt-Henrich and Frölich, 2013; Habig and Distl, 2013; Stratmann *et al*., 2015; Heerkens *et al*., 2016). The method outlined in the study by Scholz *et al*., (2008) has become the basis of most methods

measuring KBD in present research. A report from Casey-Trott *et al*., (2015) suggests there should be a push for consistency within the methodology used for measuring KBD and also put forward a simplified method based upon the work by Wilkins *et al*., (2004) and Scholz *et al*., (2008) – Simplified Keel Assessment Protocol (SKAP), described in detail in the next section.

1.3.5.4 Simplified Keel Assessment Protocol (SKAP)

In the SKAP method proposed in Casey *et al*., (2015), both fractures and deviations are assessed as separate mutually exclusive damage types with a total of four possibilities – the presence or absence of a fracture and the presence or absence of keel deviation. It is still not clear if deviations without fracture cause pain or affect the animal's welfare, though it is possible that deviations leave the structure of the keel weakened and more susceptible to fractures. In support of this theory, poorer bone mineral density (BMD) was found in hens with severe keel deformities compared the BMD of hens with normal keels (Hester *et al*., 2014). Weakened keels due to deviations is concerning as even 'slight' deviations reported in Scholz *et al*., (2008) contained histological evidence of fractures.

Furthermore, the proposed SKAP method does not use a damage severity grading system, as Casey-Trott *et al*., (2015) suggested that grading the severity of damage to the keel varies vastly between research groups and would use multiple subjective scales. By not including a severity grading system the proposed SKAP method eliminates difficulty and ambiguities in determining whether some fractures are more or less severe than others. In addition, Casey-Trott *et al*., (2015) claimed that damage severity grades from individual studies would not provide much information to other research groups outside of the group creating the report, although deeming severity grades an important tool. This was due to the lack of information available to establish meaningful severity thresholds at the time of the study. In the future with additional information, severity grades may be used more commonly and standardised throughout research groups. Many more recent layer health studies have adopted the SKAP method and added their own damage grading systems implying consistency between research methods are moving toward a similar goal (Riber and Hinrichsen, 2017; Rørvang *et al*., 2018; Jung *et al*., 2019; Stratmann *et al*., 2019).

1.4 Egg production and development

Over the past 3 decades animal sourced proteins, specifically eggs have increased in demand to supply the need to protein in the human diet at low costs. Modern day layers have been bred to be efficient and once sexual maturity is reach around 18-20 weeks of age, can produce nearly an egg a day for 60+ weeks (Hester, 2017). A hen takes around 24 to 25 hours to form an egg and there is hope that in the future, flocks can lay up to 100 weeks or more to reduce the number replacement hens needed, minimise disposal of male chicks, optimise use of land and other diminishing resources and generally lower the poultry carbon footprint (Bains *et al*., 2016). Hens still lay in clutches similar to most other egg-laying species, though due to the demand for egg products these clutches have been extended over time to maximise output via genetic selection (Hester, 2017). In the next 40 years, the global population is set to rise 25% and to meet the food demand, it is suggested that food production needs to increase by 60% (Bains *et al*., 2016).

1.4.2 Egg development process

Simultaneous development of a series of follicles in the left ovary allows hens to produce nearly an egg a day. A newly hatched hen begins life with up to 12,000 oocytes in the ovaries but of these, only 250-500 will develop in the hen's lifetime. Only one follicle with reach maturity every 24 hours (Nys and Guyot, 2011). At ovulation, the follicle which is most mature and contains the most yolk mass is captured by the funnel structure of the infundibulum at the proximal end of the oviduct (Fig. 1.6). Successive deposition of different components of the egg are added while moving down the oviduct (Romanoff and Romanoff. 1949). The albumen, membranes and shell are all deposited by different parts of the oviduct in a pre-set sequence of events. During the first 4 hours in the oviduct, the albumen (egg white) is formed in the magnum section. Shell membranes are then formed in the isthmus within an hour, before passing into the shell gland (Bain *et al*., 2016). The developing egg mass spends 19 hours within the shell gland while the shell formation, occurs in 3 specific stages and is controlled by an array of organic matrix proteins (Nys *et al*., 2004; Mann *et al*., 2006). Some of these proteins become engulfed in the development of the eggshell and can have antibacterial properties (Rehault-Godbert *et al*., 2011). Firstly, the mammillary layer is formed

and then the palisade layer, resulting in an unfinished interwoven fabric of organic and inorganic constituents. Finally, in the last one and a half hours, before oviposition, the shell pigment and cuticle are deposited. The egg is then ready to be laid (Hinke *et al*., 2012). Oviposition usually occurs after dawn if hens are kept on a standard 14L:10D light cycle, whereby a new ovulation takes place straight after or sometimes just before expulsion (Bain *et al*., 2016). The exact process of oviposition is controlled by neurohypophyseal hormones and prostaglandins secreted by the ovary (Nys and Guyot, 2011).

1.4.3 Neuroendocrine system

Reproduction in hens is controlled by the Gonadotropin releasing hormone 1(GnRH-1) neurones in the hypothalamus, part of the brain that controls environmental and internal endocrine signals (Bain *et al*., 2016). Differences in the neuroendocrine system is thought to be what makes some birds capable of laying an increased capacity of eggs in their lifetime, and could be genetically selected for (Dunn, 2013). Stimulation of the left oviduct is controlled by oestrogen and progesterone which are produced by the developing follicles in response to an increase in luteinising hormone (LH) and follicle stimulating hormone (FSH) secreted by the pituitary gland. As a hen ages, the hypothalamus cells may become less efficient and ultimately lead to reduced functionality in the oviduct and therefore poorer egg development (Dunn *et al*., 2009; Bains *et al*., 2016). Oestrogen also plays an important role in the formation and maintenance of medullary bone at the onset of lay (Dacke *et al*., 1993) (section 1.2.1.3).

1.5 Dietary requirements of the laying hen

Modern day hens are highly efficient at producing roughly an egg a day throughout prolonged periods; and therefore, require specific nutrition to provide the resources for growth, maintenance, skeletal health, and egg growth. Nutritionist's design specialised diets that fulfil these nutritional requirements, with the main constituents of a commercial laying hen diet being carbohydrates, proteins, minerals, and vitamins (National Research Council (NRC), 1994).

1.5.1 Carbohydrates

Carbohydrates are important sources of energy for poultry and the majority of carbohydrates are provided from cereal grains in the form of starch, a form of carbohydrate easily utilised by poultry. Energy provided by the carbohydrates is used in all aspects of poultry life, including growth, maintenance, and egg production. Three major cereals used in poultry feeds at the current time are wheat, sorghum and maize with diets containining 60-80% of carbohydrates (Black *et al*., 2005). Not all carbohydrates are easily digestible to poultry, such as non-starch polysaccharides or oligosaccharides and may require other supplements like enzymes to help digest and release the energy of these feedstuffs. (NRC, 1994; van Krimpen *et al*., 2009).

1.5.2 Proteins

Protein sources in poultry provide all the essential amino acids hens need for a range of different functions. Amino acids are the primary constituents of structure and protective tissues such as skin, ligaments, bone matrices and feathers, as well as soft tissues such as muscles and organs (NRC, 1994) Amino acids are also involved in some other system functions, such as cell signalling and hormone control (Wu, 2009). Body proteins are always in a dynamic state between synthesis and degradation therefore, an adequate intake of dietary amino acids is required. If amino acids levels are inadequate, a drop in production can occur. There are 22 amino acids, and all are physiologically essential. Nutritionally, these amino acids can be divided into two categories: essential – those that poultry either cannot synthesize at all or cannot make enough to meet metabolic requirements, and non-essential – which can be synthesized from other amino acids (NRC, 1994). Large quantities of non-

essential amino acids can reduce the use of essential amino acids in new amino acid synthesis, reducing the risk of slower growth, bodily maintenance, or production (Wu *et al*., 2013). Lysine and Methionine are the first and second limiting amino acids in poultry and often must be supplemented into diets to allow for normally functionality (Agostini *et al*., 2016).

1.5.3 Minerals

Hens require multiple minerals within their diet to perform many physiological, metabolic, and neurological functions including but not limited to calcium, phosphorus, sodium, potassium, magnesium chloride and zinc (NRC, 1994; Davies, 2016; Hester, 2017). Calcium and phosphorus have a major role in the skeletal health in both broilers and layers, and in egg development in layers (de Matos, 2008; Plumstead *et al*., 2008).

1.5.3.1 Calcium in layer hens

Calcium plays many vital roles in biological functions, though in hens, its main functions are skeletal maintenance and egg development. Bone is a reservoir for calcium as well as phosphorus, with approximately 99% of total body calcium located in the skeleton. The remaining 1% can be found in intra- or extracellularly (de Matos, 2008). Calcium is used in the skeletal system to mineralise the bone matrix and unmineralized osteoids by forming hydroxyapatite crystals [$Ca_5(PO_4)_3(OH)$] which give the skeleton its structural strength (Hester, 2017). In egg production, calcium is mobilised from the skeletal system in bones such as the medullary bone to create eggshell, via the osteosis process described in sections 1.2.2.1 – 1.2.2.3. Low amounts of calcium in the diet can lead to skeletal problems such as KBD or osteoporosis (section 1.3) and negatively affect eggshell quality in the form of thinner or missing eggshells (Whitehead, 2004).

One eggshell contains 2-2.5g of calcium when formed therefore throughout the year an extraordinary quantity of calcium is needed, up to 30 times total body calcium reserves can be used (Johnson, 2015). Subsequently a laying hen possesses the ability to rapidly respond to hypocalcaemic conditions within minutes, whereas other species can take 24 hours (de Matos, 2008). Without this response and the high inclusion of daily calcium (4.5g per bird) in

modern day diets, present flocks would be susceptible to cage layer fatigue (Hester, 2017). On the other hand, an overdose of daily calcium can depress appetite, ultimately leading to poorer skeletal health if diet adequate diet is not consumed. Egg production and bone metabolism rely on a calcium-phosphorus relationship, interlinked with vitamin D_3 (Table 1.8) (Crespo, 2014; Bello and Korver, 2019).

1.5.3.2 Phosphorus in layer hens

Phosphorus constitutes to a very small percentage of eggshell; however, it is needed in the diet (0.275g per 110g of feed) because of its involvement in calcium metabolism and bone metabolism (Sohail and Roland, 2002; Skřiven *et al*., 2016). In effect, phosphorus regulates Ca absorption. The Ca:P ratio most commonly used is 2:1 and this is usually considered optimal in poultry feed formulations (Anwar *et al*., 2016). Low levels of plasma P stimulate the production of parathyroid hormone (PTH) and 1,25-dihydroxy cholecalciferol ($1,25(OH)_2D_3$) leading to enhanced intestinal Ca absorption (Adedokun and Adeola, 2013). Synthesis of Ca binding proteins is also increased, thus further improving the absorption of intestinal Ca (Anwar *et al*., 2016). Continuous advancements in genetics, nutrition and the environment have led to discussions whether the ratio is correct for current flocks as there have been many improvements in a model laying hen and the overall utilisation of Ca and P (Pelicia *et al*., 2009). Like calcium surplus phosphorus can be detrimental to bone strength, and a deficiency could lead to a drop in egg production (Sohail and Roland, 2002).

1.5.4 Vitamins

Vitamins can be generally classed under two categories: fat soluble (A, D, E, K) and water soluble (B and C). Vitamin C is not classified as a dietary requirement in poultry as they are able to produce it themselves (Pardue *et al*., 1985). All vitamins play a role in biological functions of poultry, however vitamin D, specifically vitamin D_3 plays a crucial role in skeletal health and egg development (Hester, 2017).

1.5.4.1 Vitamin D_3 usage

Vitamin D_3 has been comprehensively studied in chickens. An insufficient supply of Vitamin D_3 can impair the calcium mobilising response towards PTH injections, and as a consequence

the chicken cannot establish a normal plasma Ca concentration (Hurwitz, 1989). This then has an overall negative effect on skeletal integrity and eggshell quality, as bone or eggshell cannot be mineralised (Proszkowiec-Weglarz and Angel, 2013). Adequate vitamin D_3 supplementation (3300 IU/kg of diet) has been known to enhance P utilisation in poultry, thus supporting calcium metabolism by increasing the plasma membrane Ca pump (Hy-Line, 2010; Adedokun and Adeola *et al*., 2013). The effects of vitamin D_3 on calcium and phosphorus absorption are related to one and other in a complex equilibrium. The critical form of vitamin D_3 is ($1,25(OH)_2D_3$) which acts as a steroid hormone (Hester, 2017), ($1,25(OH)_2D_3$) controls the homeostasis of Ca and P through direct actions in the intestines and kidneys as well as bones. Feedback mechanisms are used to inhibit or stimulate the production of PTH (Proszkowiec-Weglarz and Angel, 2013).

1.6 Mitigating effects of egg production on bone via supplements

As described in section 1.3 the biggest factors effecting skeletal health in laying hens are osteoporosis and KBD. This section will focus on dietary supplementations used to try and alleviate the effects prolonged of egg production.

1.6.1 Phytase inclusion in layer diets

Phytase inclusion is one of the most researched areas in the past 20 years. Extensive research has been undertaken on the use of phytase in broiler chickens and it has been shown to improve leg strength and bone quality by increasing the bioavailability of phosphorus and calcium (Scholey *et al*., 2018a). Similar effects have been found in laying hens, with increased bone quality and egg production (Hughes *et al*., 2009; Pelicia *et al*., 2009).

In a study by Bello *et al*., (2019), 20 hens (aged 68 weeks) housed individually were fed one of five diets for 10 weeks, two had a moderate reduction in Ca and P, one with and one without phytase inclusion at 600 FTU and two diets with a severe reduction in Ca and P, one with and one without phytase inclusion at 600FTU and a positive control (NC1, NC2, NC1 + BSP, NC2 + BSP, PC respectively). The study investigated if phytase supplementation could alleviate adverse effects of reductions of Ca and P in the diets on egg and bone qualities as well as general performance. Results showed that NC1 was able to maintain performance throughout the full length of the trial, at the cost of body weight and bone mineralisation. Hens fed NC2 decreased the performance at week 71. Hens on NC1 were able to produce eggs as usual whereas hens on NC2 were forced into a moult to restore structural bone loss. Hens fed diet NC1 + BSP showed no loss of BW or bone mineralisation in relation to the PC and were able to maintain medullary bone mineralisation to support eggshell formation. Furthermore, hens on NC2 + BSP still showed some bone loss in terms of less medullary bone mineralisation but were able to alleviate the decrease in performance, egg quality and bone quality over the 10-week period. Ultimately, egg-laying hens are able to maintain productivity on reduced Ca and P diets, depending on the degree of nutrient deficiency (Silversides *et al*., 2006; Geraldo *et al*., 2014). Supplementation of phytase can reduce the resource usage of developing layer hen diets at no performance cost to the hens (Bello *et al*., 2019).

1.6.2 Omega-3 fatty acids

The natural behaviour of foraging in chickens can allow for a high intake of omega-3 fatty acids from green leaves (Tarlton *et al*., 2013). Therefore, farmed poultry fed mainly on cultivated grains may lack in omega-3 fatty acids whilst being high in omega-6 fatty acids. Polyunsaturated fatty acids and omega-3 fatty acids are the immediate precursors to bone functions among other biological functions (Rahman *et al*., 2009) and a balance between ω3 and ω6 may reduce osteoporosis in laying hens as has been found in women (Moon *et al*., 2012). A study by Tarlton *et al*., (2013) found a significantly reduced occurrence of KBD in laying hens fed diets supplemented with ω3 fatty acids (Alpha linoleic acid) in their diet compared to hens supplemented with ω6 fatty acids (linoleic acid). The occurrence of KBD was 62% lower at 50 weeks and 42% lower at 70 weeks of age. BMD of the humeri and mineral content at 70 weeks in the ω3 group were shown to be significantly greater than those in the ω6 group. These improvements in the ω3 fatty acid group could be as a result of ω3 fatty acids increasing osteoblast activity (Watkins *et al*., 2003).

1.6.3 Vitamin D_3 supplementation

As previously described in section 1.5.4.1, vitamin D plays a crucial role egg and bone metabolism of layers by enhancing calcium and phosphorus utilisation. Wen *et al*., (2019) found that pullets fed either 8,348, 18,348, 35,014, 68,348 IU D_3/kg had greater BMD and bone mineral content than a control (1681 IUD_3/kg) (Table 1.9). Though there is a lack of research into vitamin D supplements in pullet laying hens, these results align themselves with studies based in broilers were tibia ash and strength were improved by supplement of vitamin D_3 (Whitehead *et al*., 2004; Khan *et al*., 2010).

Laying hen data from Wen *et al*., (2019) found no differences in KBD between treatments. They also found tibia bone mineral content increased at the maximum inclusion (68,348 IU), compared to 8,348 IU and 18,348 IU, though the authors stated this may be due to the reduced egg production that was present within hens fed the maximum, meaning less Ca and P needed to be utilized. Tibia ash content was also increased in all supplement intervals compared to the control (Table 1.10), unlike similar previous research where no tibia ash differences were found at different intervals (Persia *et al*., 2013).

Furthermore, the tibia mineral content of the layers in all treatments increased compared to the control, though there was no significance difference in tibia breaking strength. The data does show a trend suggesting dietary D_3 can improve tibial breaking strength (p = 0.09), aligning itself with previous studies which did show tibia breaking strength to be stronger with inclusions of D_3 (Mattila *et al*., 2004). Data from this study may confirm the usefulness of vitamin D_3 in mitigating skeletal health problems in layers via providing supplement to pullets before the point of lay (Wen *et al*., 2019).

1.6.4 Use of silica as a supplement

Silicon (Si) is the second most abundant element in the earth's crust. It also exists in all body tissues but is most abundant in bone, hair, skin, nails, and arteries and is an essential element of bone formation, collagen biosynthesis and lipid metabolism (Carlisle, 1981; Jugdaohsingh, 2007; Eremin, 2016). It has long been recognised that Si is an ultra-trace element for normal metabolism of higher animals (Carlisle, 1982). Supplementation in humans and animals have previously shown an increase in BMD and bone strength (Price *et al*., 2013). A deficiency of Si can lead to bone disorders and stimulate poor growth (Bodak *et al*., 1997). More recently nanotechnology has been revealed to be a fundamental aspect of animal nutrition, especially mineral nutrition. This had led to the full potential of minerals such as Si being understood (Faryadi and Sheikhahmadi, 2017).

Silicon supplementation in poultry is a more novel approach to mitigating poor skeletal health (Safaeikatouli *et al*., 2012). Many studies using Si have been performed in broilers, but it has been less reported in layer hens (Faryadi and Sheikhahmadi, 2017). Previous research in

broilers have shown Si to increase gastrointestinal stimulation (Safaeikatouli *et al*., 2012), increase body weight gain (Carlisle, 1972) and improve bone strength and ash (Scholey *et al*., 2018b) while reducing tibial dyschondroplasia (Rabon *et al*., 1995). In laying quail, limited research has shown supplements of Si to increase bone ash and calcium concentration along with egg parameters such as egg mass and eggshell weight, though this is very dose dependent (Faryadi and Sheikhahmadi, 2017). Earlier research into broilers contradicts this study showing percentage of bone ash was not affected by Si supplementation (Carlisle 1976) and did not have an effect on improvement and development of the skeleton (Elliot and Edwards, 1991). It is obvious from the lack of literature in the use of Si in laying poultry that the true effects of silica as a supplement cannot be fully confirmed.

1.6.4.1 How silicon could impact the avian skeleton

For silicon to be bioavailable it must be present in the monomeric form known as orthosilicic acid (Jurkić *et al*., 2013). Many established research has been undertaken into the effect of silicon on bone growth and remodelling in mammals but less so in avian species, as previously stated. In humans, osteoblast-like cells were shown to increase collagen type 1 synthesis (Reffitt *et al*., 2003), indicating that the collagen matrix could be improved by the addition of silicon. Increased synthesis of collagen type 1 therefore promotes more collagen fibrillogenesis – the development of collagen fibres or "strands" within a bone, even at relatively low inclusions of silicon (Eglin *et al*., 2003). The inclusion of silicon and its effects on the collagen matrix may most likely improve bone parameters such as bone strength. Results from Burton *et al*., (2020) found that in broiler chickens bone mineralisation was not affected by the inclusion of silicon, but bone strength was improved. This could indicate that the structure or organisation of the collagen matrix was improved rather than the amount of bone deposited. If similar results were seen in laying hens, an improvement of bone strength could prevent damage, as bones may be more able to withstand collisions or falls. In addition, though osteoporosis still occurs, an improved bone strength could also negate some of the effects throughout lay and increase hen welfare. Within this project, a short trial has been performed investigating the methodologies created throughout the project, utilising Si as a feed supplement. The results may reveal any effects it may have on reducing osteoporosis or bone damage. This may help clarify the effects Si can have in egg production.

1.7 Current practices for assessing skeletal health

Modern day laying hens have been selected for an increased rate of egg production, laying almost daily for consecutive months at a time, which require vast amounts of calcium (Campbell, 2020). Expectedly, this places the hen's skeletal system under much physiological strain (Whitehead and Fleming, 2000; Whitehead, 2004). A disproportion in structural bone resorption and regeneration can have a negative impact skeletal health causing osteoporosis, weaker bones and increase the likelihood of bone damage (Whitehead and Fleming, 2000).

There are methods by which skeletal health can be better maintained. Common solutions as previously mentioned in sections 1.3.4 and 1.6 include increasing the opportunities to exercise (Jendral *et al*., 2008; Regmi *et al*., 2017a), providing additional supplements within a nutritional programme (Tarlton *et al*., 2013) and increasing genetic selection for birds with stronger bone traits (Harlander-Matauschek *et al*., 2015). In addition, other methods focus on altering rearing systems to align with laying systems to improve the locomotive behaviours of pullets to mitigate risk of damage when moving into the laying system (Casey-Trott *et al*., 2017a). Overall, the studies Casey-Trott *et al*., (2017b) and (2017c) found that aviary-reared hens maintained more skeletal health benefits of rearing exercise than did conventional caged-reared hens. Furthermore, it is also becoming more common for farm management to change management methods, such as making modifications to a system to reduce skeletal damage (Campbell, 2020). Modifications can include additional ramps to help hens traverse multiple levels in aviary-based systems (LeBlanc *et al*., 2018). Rubberised or coated perches have also been trialled in multiple systems. Stratmann *et al*., (2015) found that soft perches reduced the prevalence of keel bone fractures and deviations within an aviary system compared to hard perches by ~15%. Other studies also support housing modifications, so that older housing systems are improved by design to help meet the needs of the laying hen (Sandilands *et al*., 2009).

An important aspect of creating these solutions to improve skeletal health is to interpret and evaluate how effective they are. Consequently, the poultry industry has developed many methods to assess skeletal health and therefore the effectiveness of interventions or

management changes. Broadly, these methodologies can be grouped into two categories, *in vivo* and *ex vivo* – with advantages and disadvantages of each methodology. For detailed measurements on bone structural integrity, *ex vivo* or *in vitro* methods are typically used (Campbell, 2021). For this, birds are euthanised and a sample bone or selection of sample bones are extracted so physical measurements can be taken. Multiple types of bones are often used as different bone types are affected differently by amount of physical activity or type of exercise. To determine mechanical properties of a bone, a common ex vivo method is measuring bone breaking strength (Fleming *et al*., 2006; Regmi *et al*., 2016). Bone imaging can be used to also measure structural properties of bone by using technologies such as QCT-imaging and then using precision measurements to measure for bone length, width, cortical thickness, cortical density and trabecular density or total bone mineral density. As previously mentioned in section 1.3.5.2, there are other technologies which are capable of producing a similar output to CT imaging, such as X-rays, ultrasonography and Dual energy X-ray Absorptiometry (DEXA) scans (Casey-Trott *et al*., 2015; Toscano *et al*., 2018). A study by Fleming *et al*., (2004) found that in both caged and free-range birds, ultrasound speeds had a positive correlation with bone strength values. In the absence of expensive equipment, bones can be ashed to provide a value for mineral content (Regmi *et al*., 2016). Recent bone mineral density estimations through quantitative computed tomography are highly correlated with analytical bone ash and mineral content methods (Robinson and Karcher, 2019).

In vivo methods are based predominantly on palpation – most often used to determine the presence or severity of keel bone damage (Casey-Trott *et al*., 2015). It can also be used to check other bones for damage but is less accurate than damage assessment via dissection. Palpation could provide a reliable assessment of a live bird and prevents the need for euthanising but depends on the type and location of the damage (Petrik *et al*., 2013; Buijs *et al*., 2019). In addition to individual bone analysis, QCT, X-rays and DEXA can be used on live birds when restrained, allowing for accurate tracking of damage over or bone regeneration over time (Eusemann *et al*., 2018; Chargo *et al*., 2019). A less commonly used in vivo method used to assess skeletal health would be to use blood samples. Blood samples could be taken throughout the laying period to quantify serum markers for bone formation and resorption (Kerschnitzki *et al*., 2014) as calcium concentration in blood serum fluctuates during different

stages of oviposition (Regmi *et al*., 2015). A more novel approach of assessing skeletal health would be to use biomechanical analysis through micro-CT and beam analysis to measure mechanical bone properties in vivo, though the novelty of this method and the lack of studies suggest a need for future work (Vaughan *et al*., 2016). Table 1.11 provides a summary of methods to assess skeletal health in previous studies.

Table 1.11 Summary of methods used to assess skeletal integrity in laying hens

Reference	Year	Bone	Age (weeks)	Housing System	Strain of bird	Assessment Method*
Newman and Leeson	1998	Tibia	69 to 72	Aviary, Conventional Cage	Brown Strain	Dissection
Fleming *et al*.,	2004	Humerus, Tibia	25 to 70	Conventional Cage	Lohmann Selected Leghorn White	Dissection, Ultrasound
Schreiweis *et al*.,	2004	Humerus, Tibia	24 to 65	Conventional Cage	Hy-Line White Leghorn	DEXA, X-rays
Leyendecker *et al*.,	2005	Humerus, Tibia	24 to 72	Aviary, Conventional caged, Furnished cage	Brown Strain	Dissection
Mazzuco and Hester	2005	Humerus	76 to 125	Conventional Cage	White Leghorn	Dissection, DEXA
Martínez-Cummer *et al*.,	2006	Humerus	54 to 66		White Leghorn	Dissection, Ultrasound
Jendral *et al*.,	2008	Femur, Humerus, Tibia	20 to 65	Cage with or without perches and nest boxes	White Leghorn	Dissection, QCT
Shipov *et al*.,	2010	Humerus, Tibia	104	Conventional Cage, Free Range	Hy-Line W99	Dissection, QCT
Wilkins e*t al*.,	2011	Humerus, Keel, Tibia	End of lay	Barn, Furnished cage, Free Range, Organic	Bovan Goldline, Hy-Line Variety B, Lohmann Brown	Dissection, Palpation
Silversides *et al*.,	2012	Radius, Tibia	0 to 50	Barn (Floor pens), Caged	Lohmann White, Lohmann Brown, H&N White, Rhode Island Plymouth Rock Cross	Dissection, QCT

Habig and Distl	2013	Humerus, Keel, Tibia	24 to 80	Barn Multi-Tier	Lohmann Selected Leghorn White, Lohmann Brown	Dissection, Palpation
Hester *et al*.,	2013	Femur, Fibula, Humerus, Keel, Radius, Tibia, Ulna	17 to 71	Cage	Hy-Line White Leghorn	Dissection, DEXA
Kerschnitzki *et al*.,	2014	Femur				Blood Samples, Dissections, QCT, X-ray
Petrik *et al*.,	2015	Keel	20 to 65	Barn, Cage	Brown strain	Palpation
Regmi *et al*.,	2015	Humerus, Tibia	4 to 16	Aviary, Conventional Cage	Lohmann White	Blood Samples, Dissections, QCT
Regmi *et al*.,	2016	Femur, Keel, Tibia	78	Barn, Barn with range, Cage	Hy-Line Brown, Hy-Line Silver Brown, Barred Plymouth Rock	Dissection, QCT,
Vaughan *et al*.,	2016	Humerus, Tibia	19 to 77	Aviary, Conventional Cage, Furnished Cage	Lohmann White Leghorns	Dissection, QCT
Casey-Trott *et al*.,	2017c	Humerus, Radius, Tibia	16 to 73	Aviary, conventional cage, Furnished cage	Lohman Selected Leghorn Lite	Dissections, QCT
Regmi *et al*.,	2017a	Humerus, Tibia	18 to 72	Aviary, Conventional Cage	Lohmann White	Dissections
Regmi *et al*.,	2017b	Humerus, Tibia	77	Conventional Cage, Furnished Cage, Aviary	Lohmann White	Dissections, QCT
Rodriquez-Navarro *et al*.,	2018	Tibia	56	Aviary, Cage	White Leghorn	Dissection, X-ray
Neijat *et al*.,	2019	Femur, Humerus, Keel, Radius, Tibia	0 to 73	Aviary, Conventional Cage, Furnished Cage	Lohmann Selected Leghorn Lite	Dissection
Robinson and Karcher	2019	Femur, Humerus, Keel, Tibia	85		Hy-Line W36	Dissection, QCT

Qiaoxian *et al*.,	2020	Femur, Humerus, Tibia	32 to 57	Conventional Cage, Flat floor pens	Taihang	Dissections

*Dissections in the assessment methods column include ex-vivo and in vitro methods i.e., physical bone measurements, biomechanical measurements, BMD, or ash content

The decision to use particular *in vivo* or *ex vivo* methods can be determined by factors such as cost, requirement of euthanasia, and method training (Martínez-Cummer *et al*., 2006 Campbell, 2020). Conventional methods are most often destructive and *ex vivo*, usually involving sample dissections and bone measurements. More novel non-destructive *in vivo* methods include Quantitative Computed Tomography (QCT) or Dual Energy X-ray Absorptiometry (DEXA) and can prevent sample destruction (Donnelly, 2010). A strong disadvantage of destructive methods is the fact hens must be euthanised to remove samples. In a commercial setting, this would also affect the profits as the sample hens would no longer be in the system to produce eggs. On the other hand, these methods allow for a much more direct comparison of skeletal health through bone measurements and analysis. Compared to destructive methods, non-destructive methods prevent the need to cull and do not affect farmer profits. If a portable device is available, results could also be obtained quickly and on site. The main disadvantage of non-destructive methods such as QCT or DEXA scans, would be the cost required to purchase the equipment and the time required for training to use it. Some recent studies compare the use of *in vivo* methods to *ex vivo* methods (Hester et al., 2004; Martínez-Cummer *et al*., 2006; Kim, Bloomfield and Ricke, 2011; Regmi *et al*., 2016). A mixture of methods when possible is thought to provide the most comprehensive bone analysis for determining skeletal health, including some *in vivo* and *ex vivo* aspects (Regmi *et al*., 2016).

From the summary of methods previously used to assess skeletal health, the most common methods used were dissections to allow for physical biomechanical measurements and QCT to scan the bones. The most commonly used bones were the tibia and humerus, though other bones such as the keel or femur were also used somewhat less frequently. Age of the birds used, type of housing system, and strain of bird varied to a degree within different studies. Although not always reported, Martínez-Cummer *et al*., (2006) stated that both orientations of the same bone should be accounted for due to the potential asymmetry in growth.

It is known that exercise influences skeletal health and can affect bones different depending on their locomotory function (Casey-Trott *et al*., 2017c). As laying hens particularly in the UK, have long laying cycles and pressures to extend these even further (Bain *et al*., 2016), a

longitudinal approach with sampling at multiple time points, encompassing multiple housing systems and multiple bones is thought to provide a highly comprehensive assessment of skeletal health. Within this thesis, studies were carried out in to order to inform and produce a longitudinal overview of skeletal health, using common UK housing systems, and multiple sample bones at frequent intervals of lay.

1.8 Current methods to assess egg quality

In addition to skeletal health, egg qualities are also important factors within the egg industry. The aim in the industry is to achieve the best skeletal health whilst trying to achieve the best egg quality. Particularly within the UK, with the change in preferred systems and the move away from caged systems, egg qualities are often analysed to ensure production rates and profits are being achieved, but also that egg production is not taking a toll on the hens and reducing hen welfare (Lay *et al*., 2011; Habig and Distl, 2013).

There are many factors which are evaluated using egg qualities such as housing systems, shelf life, effects of diet, genetic differences, and age (Mertens *et al*., 2006; Habig and Distl, 2013; Jones *et al*., 2014) Some of these factors may interact causing egg quality changes to be multifactorial (Lay *et al*., 2011). As such, there are many egg quality measurements that have been used in previous research. These commonly include egg weight, egg size, eggshell breaking strength, eggshell thickness, eggshell density, albumen height, albumen pH, Haugh unit, eggshell colour, egg composition, yolk weight, shell composition, dry matter content, and eggshell ratio (Van Den Brand *et al*., 2004; Mertens *et al*., 2006; Hidalgo *et al*., 2008; Wang *et al*., 2009; Wen *et al*., 2019). Table 1.12 provides a summary of recent studies assessing egg quality and what measurements they used.

Table 1.12 Summary of methods used to assess egg quality in previous studies

Reference	Year	Age	Housing System	Strain	Assessment measurements
Guinotte and Nys	1991	69 to 74 weeks	Individual Cages	ISA Brown	Eggshell strength
Ketelaere *et al*.,	2002	36 to 74	Cage	Hisex White, Bovan White, Lohmann-LSL, 3 experimental breeds (Hendrix Poultry Breeders)	Static and dynamic stiffness, eggshell strength, eggshell thickness
Rodriguez-Navarro *et al*.,	2002	30 to 58 weeks	Individual Cages	ISA Brown	Eggshell strength, eggshell index, eggshell thickness, eggshell crystallisation
Van Der Brand *et al*.,	2004	25 to 59	Cage, Outdoor (free-range)	ISA Warren Medium Heavy	Egg weight, shape index (length and breadth), albumen height, albumen pH, albumen weight, yolk weight, yolk colour, eggshell weight, eggshell thickness, albumen dry matter content, yolk dry matter content
Leyendecker *et al*.,	2005	24 to 72	Conventional Cage, Furnished Cage, Aviary	Brown Strain	Eggshell strength, eggshell thickness, eggshell density
Vits *et al*.,	2005	18 to ~58 weeks	Furnished Cage	Lohmann Brown, Lohmann Selected LSL,	Egg weight, eggshell strength, albumen height, eggshell thickness, eggshell density
Mertens *et al*.,	2006	47 weeks	Battery Cage, Furnished Cage, Aviary, Free-range	Bovan Goldline	Dynamic shell stiffness, crack detection, egg weight
Swiatkiewicz and Koreleski	2008	25 to 70 weeks	Individual Cages	Hy-Line Brown	Eggshell breaking strength, eggshell thickness, eggshell density, shell percentage of egg

Hidalgo *et al*.,	2008		Caged, Free-range, Barn, Organic	Grade A commercial supermarket bought	Albumen, yolk and shell percentages, egg weight, albumen height, air cell height, blood spot percentage, Haugh unit, egg surface area, shape index, egg diameter, egg height, shell index, shell thickness, eggshell strength
Lichovníková and Zeman	2008	19 to 66 weeks	Conventional Cage, Furnished Cage, Floor-based (barn)	ISA Brown	Eggshell thickness, dry eggshell weight, eggshell weight ratio, eggshell strength, calcium content
Olgun *et al*.,	2009	78 weeks		White Leghorn LSL	Egg weight, egg height, egg mass, egg width, shape index, yolk height, albumen height, yolk index, albumen index, eggshell weight, eggshell percentage, Haugh unit
Wang *et al*.,	2009	14 to 50 weeks	Battery Cage, Free-range	Blue-shelled layers - Dongxiang	Egg shape (length and breadth), shape index, eggshell colour, eggshell strength, egg weight, albumen height, Haugh unit, yolk colour, albumen weight, yolk weight, eggshell weight, eggshell thickness, yolk/albumen/shell ratio, yolk cholesterol
Tumová *et al*.,	2011	20 to 64 weeks	Conventional Cage, Furnished Cage Floor housing	ISA Brown, Hisex Brown, Moravia BSL	Egg weight, albumen height, eggshell weight, albumen weight, Haugh unit, eggshell strength, eggshell colour, yolk colour, eggshell thickness, eggshell surface area, eggshell index, pore density,
Nasr *et al*.,	2012	33 to 42 weeks	Free-range with perches	Lohmann Brown	External appearance, egg weight, egg length, egg width, egg shape index, eggshell thickness, eggshell weight, eggshell percentage, eggshell density,

Habig and Distl	2013	24 to 80 weeks	Barn Multi-Tier	Lohmann Selected Leghorn White, Lohmann Brown	Egg weight, eggshell strength, albumen height, Haugh unit, eggshell thickness, eggshell weight
Skrivan *et al*.,	2013	20 to 44 weeks	Furnished Cage	ISA Brown	Egg weight, albumen height, Haugh unit, eggshell strength, albumen weight, yolk weight, eggshell thickness, eggshell weight, eggshell index
Jones *et al*.,	2014	19 to 77 weeks	Aviary, Conventional Cage, Furnished Cage	Lohmann LSL White	Eggshell dynamic stiffness, egg weight, albumen height, Haugh unit, vitelline membrane strength, whole egg total solids
Englmaierová and Tumová	2014	20 to 60 weeks	Conventional Cage, Furnished Cage, Aviary, Litter	Hisex Brown	Shape index, albumen/yolk/shell percentages, egg weight, shell weight, shell strength, shell thickness, shell index, albumen height, Haugh unit, yolk height, albumen index, yolk to albumen ratio
Stefanello *et al*.,	2014	47 to 62 weeks	Experimental Cage	Hyline W36	Egg weight, albumen height, eggshell weight, eggshell percentage, eggshell thickness, eggshell strength, eggshell ultrastructure
Karcher *et al*.,	2015	19 to 77 weeks	Aviary, Conventional Cage, Furnished Cage,	Lohmann LSL White	Shell dynamic stiffness, eggshell strength, egg weight, albumen height, Haugh unit, vitelline membrane strength, eggshell thickness, whole egg total solids
Wen *et al*.,	2017	40 to 48 weeks		Hyline Brown	Eggshell strength, eggshell thickness, egg weight, yolk weight, albumen weight, shell weight, yolk percentage, albumen percentage, shell percentage, yolk fat content
Wen *et al*.,	2019	2 to 68 weeks	Top tier of a multi-tier housing system (no range)	HyLine W36	Yolk vitamin D_3 concentration, eggshell strength

Kraus *et al*.,	2021	34 to 50 weeks	Furnished Cage, Floor pens	CGS hen, OR hen (Native breeds)	Egg weight, shape index, egg surface area, eggshell proportion, eggshell thickness, eggshell strength

From the summary of methods used to assess egg quality, the most common measurements to assess egg quality were egg weight, eggshell strength, egg thickness and albumen height. Other measurements were also used frequently but often varied depending on the factors assessed in each study. Similar to the summary of methods used in assessing bone health, the ages and housing systems used when studying egg qualities vary. As age increases, egg qualities have been shown to decrease (Kraus *et al*., 2021), with worse textural eggshell properties in older hens compared to younger hens causing lower eggshell strength (Rodriguez-Navarro *et al*., 2002). It has been found that the housing systems also effect egg qualities (Mertens *et al*., 2006; Holt *et al*., 2011; Karcher *et al*., 2015). Within the UK there is a push to extend the period of lay further to reduce the need for hen replacement after each laying cycle (Bain *et al.,* 2016). Like bone health, it is thought advantageous to have a longitudinal approach with sampling at multiple age groups, encompassing multiple housing systems to provide a comprehensive assessment of skeletal health. Within this thesis, studies were carried out in to order to inform and produce a longitudinal overview of egg qualities using UK farms. The relationship between bone and egg characteristics were also assessed to determine whether there was or was not a relationship between skeletal health and egg quality.

1.9 Thesis overview, aims and objectives

This project will focus on laying hens and egg production. Skeletal problems in laying hens during egg production have been recorded extensively over the past few decades and many procedures are in place to try and prevent or reduce the occurrence of skeletal problems. Prevention of skeletal problems can be achieved by making nutritional changes, environmental changes, and also making alterations to genetic lines. Assessing efficacy of procedures is made more difficult by the range of production systems in use. Many farms have changed systems to follow trends of consumer preference whilst others have not, leading to diversity in UK production systems. Although breeding companies produce breed standards indicating what might be achieved, very little data exists based on commercial production other than when a disease outbreak occurs. The key novel aspect of this project is that it uses on-farm commercial, healthy bird data from the four main production systems to predict via modelling the skeletal growth of laying hens throughout the laying period. The practical output of this project is a tool similar to a BMI chart used in humans. This will allow farmers to identify whether their birds are within a healthy range for skeletal health parameters and on a trajectory to remain within this range throughout lay. The project also investigates the relationship between skeletal integrity and egg quality throughout the period of lay to determine whether egg quality may be a non-invasive predictive measure of skeletal integrity in later life. An outcome from this project will be the development of an early warning system for the industry to monitor flock skeletal health and prevent or reduce skeletal problems in laying hens.

As such, an overarching aim of this study was to assess the effects of different housing systems on skeletal health and egg qualities of laying hens over the period of lay. Another aim was to be able to model the data for skeletal health and egg quality to help predict future values individual to housing systems used and for different age groups of birds. The relationship between bone characteristics and egg qualities were also assessed to see if the two data sets interacted with one and other. For the purposes of this study, skeletal health was defined as the ability of the hen's skeletal structure to be normally maintained

throughout the period of egg production without showing signs of defects or damage. The objectives to meet these aims were:

1. To investigate which are the optimal bones to use to assess skeletal health accurately.
2. To investigate the effect of age and housing system on skeletal health over a laying period.
3. To examine the effect of age and housing system on egg quality over a laying period.
4. To model the bone characteristics from multiple housing systems over a laying period to help predict future skeletal health.
5. To model egg quality characteristics from multiple housing systems over a laying period to help predict future egg quality.
6. To determine if there is a relationship between bone and egg characteristic to inform on the impact of housing system and age on skeletal health of laying hens.
7. To identify whether the previous objectives can be used to assess whether an intervention influences skeletal integrity.

Chapter 2: Materials and Methods

2.1 Introduction

This chapter provides an overview of the methods used throughout the studies within this thesis. Three pilot studies, a longitudinal on-farm study and an in-house trial were completed for this thesis (Table 2.1).

Table 2.1 An overview of trials undertaken as part of this thesis

Study	Aim	Chapter
Pilot study 1	Investigating the optimal bones to be used to assess skeletal health using broiler chickens	3
Pilot study 2	Investigating the optimal bones to be used to assess skeletal health using end of lay hens	3
Pilot study 3	Confirming the optimal bones used to assess skeletal health in hens at the beginning of lay	3
On-farm study (bone)	Effects of housing system on skeletal health over the period of lay	4
On-farm study (egg)	Effects of housing system on egg quality over the period of lay	5
In-House Trial	Validity of methods for assessing skeletal health	6

In-house bird husbandry was only required for pilot study 1 and the in-house trial, the remaining samples for the other studies were collected off-site. Bird husbandry for the sample birds from off-site sources followed both the health and welfare regulations set by the external collaborator and The Welfare of Farmed Animals (England) Regulations 2007. Birds housed on site were cared for following the institutional and national guidelines of care and use of animals (Animal Scientific Procedures Act, 1986) and all experimental procedures both on and offsite were approved by the NTU School of Animal Rural and Environmental Sciences Ethical Review Committee.

2.2 Pilot study 1

2.2.1 Bird husbandry

This trial used 160 Ross 308 male broiler chicks, collected from PD Hook, Cote Hatchery, Oxfordshire within 24 hours of hatching. The chicks were weighed individually and placed in groups of 10 per pen, across 16 pens (8 per treatment). Each chick used, weighed between 38-45g and each pen weight average was calculated, any birds outside of this range were excluded. Birds and treatments were randomly allocated. The chicks were placed in 0.64m^2 purpose built metal pens filled with approximately 3cm of non-dust wood shavings. *Ad libitum* feed via moveable troughs and water via nipple drinkers (2 per pen) were available throughout the period of the study. The lighting programme was set to 24 hours light on D1 and decreasing by 1 hour per day until 6 hours of darkness was reached, at which point this schedule was followed for the remainder of the trial. The lighting regime also included two dark periods and 22:00 – 00:00 and 02:00 – 06:00 with a 15-minute twilight period either side of darkness. The temperature of the room was set at 32°C for the arrival of the chicks and reduced to 20°C by D21 via computerised thermostat outside of the trial room. Bird health and environmental checks were carried out twice daily from placement to D21, these included bird health, mortality checks, temperature, lighting, nipple drinker cleaning and checks, food checks, bedding quality and ventilation. Any mortalities were recorded for date, possible cause of mortality, pen and weight then stored in a freezer for disposal. If a bird was showing illness or injury, birds were culled by cervical dislocation as determined by the Department for Environmental, Food and Rural Affairs (DEFRA). As with other mortalities the relevant data was recorded before the bird was stored.

2.2.2 Dietary treatments

Diets for this study were manufactured and for the trial by Target Feeds Ltd (Shropshire, UK), comprising of a wheat-soya based basal diet mixed with one of two phytase inclusions (500 FTU or 1500 FTU) (Quantum Blue, AB Vista). The diet was provided in two dietary phases, starter crumb (D0-14) and grower pellet (D14-21). Each treatment was fed to 8 pens in 5kg pre-weighed bags per pen.

2.2.3 Sample collection

Samples collected for this study after dissection included the keel bone, humeri, ulnae, radii, femurs, and tibiae (both sides for all bones where applicable). An image of the left and right bones was taken at the beginning of the trial so bones could be identified in the sample bag (Fig. 2.1). Bones from individual birds were stored individual labelled bags. Samples were collected at two time points, day 14 and day 21. Lengths, widths, weights, and strengths of the bones were measured after the bones had been defleshed. The dissection process and bone analysis methods are explained in more detail in sections 2.7.1 – 2.7.3.

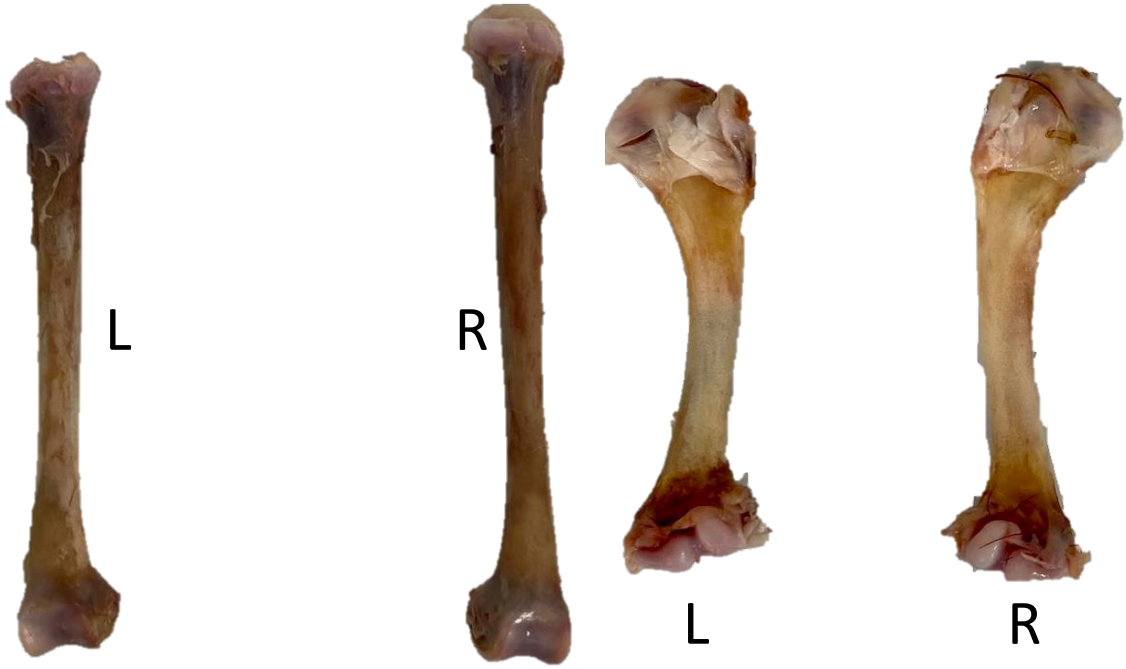

Figure 2.1 Tibia and humerus bone orientation (Tibia on the left, Humerus on the right) (noting the 'kicks' of the tibia angled inwards at the tibia-foot joint and the heads of the humerus facing outwards with the flat surface facing towards the user)

2.3 Pilot study 2

2.3.1 Bird husbandry

For pilot study 2, Lohmann Brown Classic hens were taken at the end of lay from two participating commercial farms, collected from a processing facility. One farm utilised a free-range system (76 weeks old) and one farm utilised a colony caged system (79 weeks old). Both farms complied with the Lohmann Brown management guide irrespective of the system used (Lohmann GB Limited). Standard commercial layer diets and water were provided *ad libitum*. Birds were regularly checked, and environmental conditions were monitored. Any mortalities were recorded, and birds culled if deemed appropriate due to illness or injury.

2.3.2 Dietary treatments

Dietary treatments for this study were manufactured for the participating farms by Noble Foods Ltd (Milling – Bilsthorpe, UK). The feed was manufactured to meet breed nutritional requirements throughout the rearing period and laying period and could be altered for each farm based on egg weight and bird performance over time. Strict compliance with national regulatory guidelines on the safe manufacture of feed such as the Universal Feed Assurance Scheme (2016) and Foods Standard Agency guidelines (1999) were followed.

2.3.3 Sample collection

Fifteen birds from each system were collected from the processing facility post-mortem and body weights were recorded. The keel bone, humeri, ulnae, radii, femurs, and tibiae were dissected from all birds from both systems and flesh was removed. Bones from individual birds were bagged as one group and bones were identified using an image such as Fig. 2.1 (section 2.7.1 and 2.7.2). Bone length, width, weight, and strength were measured and recorded (section 2.7.3).

2.4 Pilot study 3

2.4.1 Bird husbandry

Free range and caged hens were also used in pilot study 3, from two participating commercial farms that were also used in the on-farm project; Welsh's Farm, Boston, Lincolnshire and Longbelt Farm, Bilsthorpe, Newark. The free-range farm contained a multi-tier free-ranged system, and the caged farm used a multi-level colony caged system. Both groups of hens were Lohmann Brown Classics aged 30 weeks, and the farm management systems complied with the Lohmann Brown management guide. A commercial layer diet provided by Noble Foods Ltd, specific to each system was provided *ad libitum* to each group of hens. Water was also provided *ad libitum.* Bird and environmental checks were carried out regularly, mortality was recorded and where necessary birds were culled via cervical dislocation.

2.4.2 Dietary treatments

As in pilot study 2, dietary treatments were manufactured for the participating farms by Noble Foods Ltd – Milling and were made to the requirements and performance of the birds following all relevant guidelines. More information on the dietary treatments provided can be found in Appendix 1.

2.4.3 Sample collection

Twelve hens (6 free range, 6 caged) aged 24 weeks were collected from two collaborating farms (6 birds per farm) and culled via cervical dislocation. Body weight was recorded and then the birds were dissected (section 2.7.1). The bones that were taken were the keel, humeri, radii, ulnae, femurs, and tibias. Bones were de-fleshed and measured as described in sections 2.7.2 and 2.7.3.

2.5 In-house trial

2.5.1 Bird husbandry

Ex-colony caged Lohmann Brown hens aged 72 weeks were used over the seven-week (49 days) trial, collected from Longbelt Farm, Noble Foods Ltd, Bilsthorpe, Newark. Three hens were placed in each of 24 0.64m^2 purpose-built pens, with 2 spare pens of birds. Birds were individually weighed and marked with a colour (blue, yellow, pink) of animal spray with a leg ring to indicate individual birds. Wood shavings were used for litter and spread across all pens at 3cm deep. Food and water were available *ad libitum* via troughs and nipple drinkers. Two 0.027m^3 nest boxes were provided with 1cm of shavings in each box. Temperature was controlled via a thermostat and set at 21°C throughout the trial. Lighting was set as 16 hours light and 8 hours darkness, with a 15-minute twilight period each side of darkness. The light intensity of the room was set to measure 15 lux at feed trough height to reduce aggression. Bird health checks and environmental checks were undertaken twice daily. Checks included, bird health, feed levels, water supply, litter quality, egg counts, lighting, temperature, and leg ring checks. Any mortalities were weighed and recorded along with and pen number and date. Birds showing any illness or injury were culled via cervical dislocation.

2.5.2 Dietary treatments

Birds were fed one of two dietary treatments – GoldNlay (GLW, Shepshed, Leicestershire) with (experimental) or without (control) the inclusion of a silicon supplement at 600ppm and diet allocation was randomised by block for environment. The experimental diet was manufactured by adding a premeasured amount of silicon into a ribbon mixer (Rigal Bennett, Goole, UK) with 100kg of GoldNLay mash and mixing for 10 minutes. The control diet was weighed out into feed bags without further processing. Each diet was fed to 12 pens plus one spare, with each feed bag weighing 6kg per pen. Feed was topped up when necessary and feed bag weight was recorded.

2.5.3 Data collection

2.5.3.1 Body weights

Hens were weighed on arrival before being placed into pens. Hen weight was recorded weekly for individual hens from D7 to D49. Weights were measured using a 1.d.p top pan balance (Mettler Toledo International).

2.5.3.2 Feed intake

Diets were pre-weighed into 6kg feed bags and provided exclusively to each pen. Every 7 days feed was tipped back from the troughs into the feed bags and bags reweighed to calculate weekly feed intake. Feed intake was calculated per whole pen.

2.5.3.3 Bone samples

One bird at D28 was euthanised via cervical dislocation with the remaining 2 birds being euthanised at D49. The keel left and right humerus and left and right tibia were collected from each bird via the dissection method outlined in sections 2.7.1 and 2.7.2. Bones were then measured for length, width, weight, strength, and height (keel only) as described in section 2.7.3.

2.5.3.4 Egg samples

Eggs were collected daily so weekly egg weight per pen and an average egg weight per bird could be calculated starting from D7 throughout to D49. Using this data, a feed conversion ratio for egg production was also produced (egg production ratio). Two sample eggs were also collected from each pen at D13 every 7 days until D48 (also included in the weekly weights). These sample eggs were analysed for egg weight, shell breaking strength, albumen height, shell weight, shell thickness and Haugh unit. The methods for these measurements are explained in more detail in section 2.7.4.

2.6 On-farm project

2.6.1 Bird husbandry

For the on-farm project, day old hens varying in breed were reared in rearing systems until 16 weeks of age, following the relevant management guideline and then transferred into laying systems for up to 60-80 weeks. The breeds ranged from Lohmann Brown Classics, Bovan Browns and Hy-line Browns, depending on farm choice. The laying systems included free-range multi-tier systems, free-range flat deck systems, colony caged systems, barn systems and organic systems. Individual farm information will be explained in more detail in chapter 4. Management guidelines for breed and system were followed in all participating farms and the Welfare of Farmed Animals (England) Regulations 2007 were adhered to. Individualised diets per farm were provided *ad libitum* as well as water. Feed and water additives or supplements were added into the diets *ad hoc* and recorded when added. Bird health checks and environmental checks were undertaken regularly. Mortalities and culled birds were recorded.

2.6.2 Dietary treatments

Dietary treatments for farms involved in the on-farm project were provided for by Noble Foods Ltd or a private commercial mill depending on the farm. The feed was manufactured to meet breed nutritional requirements throughout the rearing period and laying period and could was altered for each farm based on egg and hen performance. Additives or supplements were added when necessary and recorded. National regulatory guidelines on the safe manufacture of feed such as the Universal Feed Assurance Scheme and Foods Standard Agency guidelines were followed. A dietary regime for each farm and dietary analysis for each diet used can be found in the Appendix 1.

2.6.3 Sample collection

Six hens were collected from participating farms every 6 weeks from 18 to 72 weeks of age. The hens were culled via cervical dislocation upon arrival to the farms. Body weight was recorded and then the birds were dissected for the keel bones, left and right humerus and left and right tibia. Measurements of the bones were taken after they had been defleshed

and included length, width, weight, strength and height (keel only). Thirty egg samples were collected from the farms every 12 weeks from 24 weeks of age until the end of the project. The eggs were measured for egg weight, egg height (tallest measure), breaking strength, shell thickness and shell ash weight. For the methods of these measurements see section 2.7.4.

2.7 Lab Analysis

2.7.1 Bird dissection

Firstly, an incision was made at the groin to reveal the leg structure of the bird. Using the thumb and first two fingers, the leg was then pushed downwards/outwards at the pelvic joint to dislocate the femoral head from the pelvic bone. After the femoral head has been made loose, the surrounding tissues were cut with a scalpel to free the leg from the bird. This process was carried out on both legs. To separate the femur and tibia, the leg was bent at the knee and an incision was made at the tibiofemoral junction through the anterior and posterior cruciate ligament to separate them. An indicator of where to make the incision was to identify an area of fat around the top of the knee where the femur and tibia meet. The tibias and femurs were stored in individual labelled bags and frozen at -20°C until further processing.

Next, the wings were removed from the bird at the humeral head. Firstly, the feathers at the top of the breast (around the shoulder joint) where brushed to one side to give a clearer view of the shoulder. A 4cm incision was then made diagonally outwards from the top of the breast/shoulder area towards the ribs, cutting down to the bone structure beneath but being careful not to cut into the bone. Next, the wing was pulled taught away from the body and the ligaments and cartilage surrounding the humeral head were cut with a scalpel to free the wing. After both wings had been removed, the humerus bones were separated from the ulna, radius and digits by making a cut at the elbow joint. The cut was from the inner elbow to the outer elbow and through the connecting skin tissue and feathers. A gap was created, between the ulna and radius on one side and the humerus on the other. Connective tissues still intact were cut around this area in order to free the humerus from the rest of the ulna, radius and digits. The digits were then cut away from the ulna and radius and discarded. The humerus, ulna and radius from both orientations were bagged individually and stored in a freezer at -20°C until further processing.

To remove the keel bone from the body, the skin surrounding the breasts was cut then pulled up towards the neck area and cut where the wings had been removed to allow for a clearly view of the keel. A small incision was then made underneath the caudal tip of the keel into

the chest cavity. By having removed the skin, a fat line is visible which roughly outlines the keel location and the ribs. Scissors were used to make a cut along this fat line up until the ribs; from here, poultry shears were used to cut through the ribs on either side. The cuts were made to where the humerus had been removed. Afterwards, the scapula bone was cut on either side to help remove the keel from the body (effectively removes the crown from the bird). Any membrane or connective tissue in the chest cavity connecting the keel was also cut. The next step was to prize away the keel bone and adjoining breast meat from the bird. To do this, the thumb was place in the chest cavity and index finger on the peak of the keel and prized away towards the bird's head (caudal tip of the keel was being moved towards the head). Care was taken not to break any part of the keel in the process. Squeezing too hard around the end of the keel could cause parts of the keel to break. Then the keel was careful pulled away from the bird, separating at the coracoid bones. If the keel was difficult to pull away, a cut was made around the breast tissue where the end of the coracoids was to allow for an easier removal. The rest of the bird could then be discarded. The keel bone and connecting tissue were also stored until further processing.

2.7.2 Bone processing

Before processing all samples were left to thaw fully for a day. Starting with the keel bone, an incision is made perpendicular to the furcula – the point at which the two clavicles join (wishbone), deep enough until the keel if felt below. From this incision, another was made on one side, at a right angle under one of the clavicles towards the coracoid-clavicle joint. The same needed to be done on the other side (Fig. 2.2). The clavicles were then loose enough to pull away from the keel and discard.

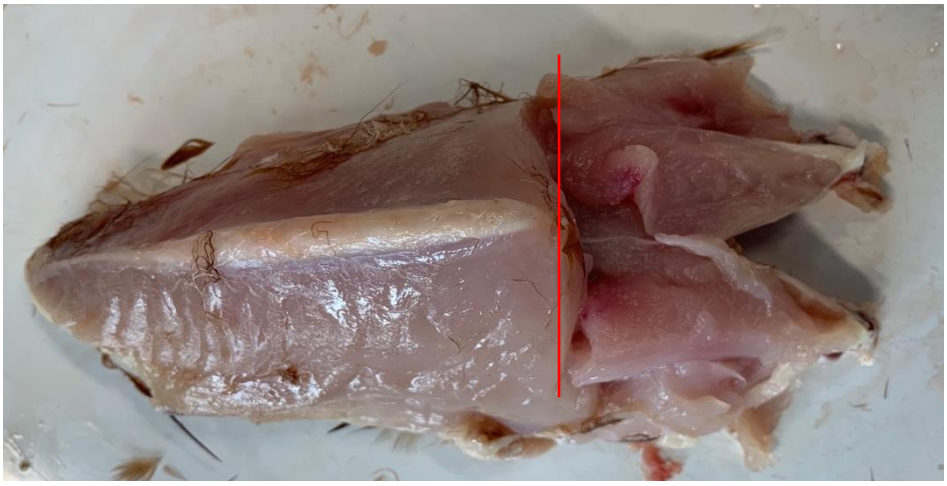

Figure 2.2 Keel bone with the wishbone removed in order to remove the remaining adhering flesh

Next, the keel was turned over so that the chest cavity was facing upwards. Using a scalpel, two incisions were made either side of the keel, from top to bottom to cut through the breast membrane. Care taken not to cut into the keel if samples were from young birds. Using curved dissection scissors, two cuts either side of the keel were made to cut away the ribs, making sure to follow keel shape and not cut away any of the keel head. Once this had been done, the keel was then turned over and ligaments and tendons were cut around the coracoid-clavicle joint in order to free the breast muscle. Following the keel structure with a scalpel or finger, breast tissue connected to the keel plate (curved plate running from the cranial to caudal end of the keel) was loosened on both sides and cut where breast tissue and keel ridge are attached and removed (Fig. 2.3).

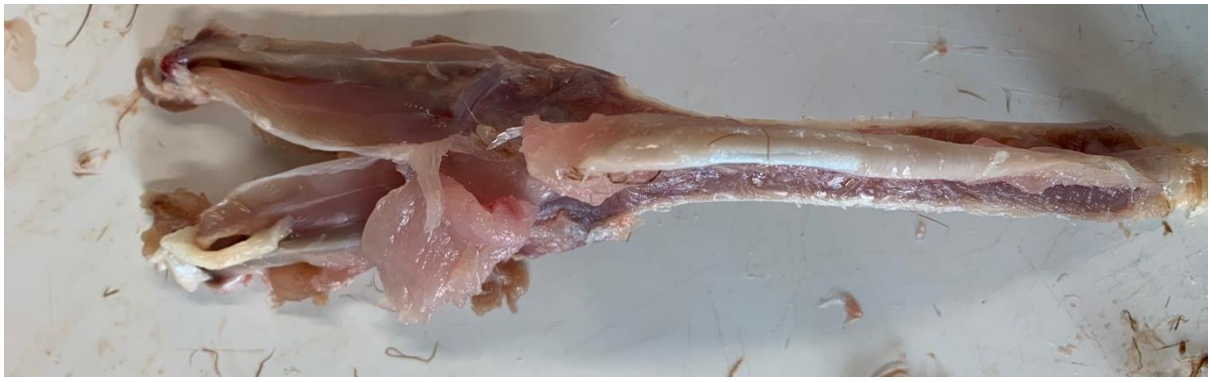

Figure 2.3 Removal of adhering breast tissue from the keel bone

Whilst holding the keel, the coracoids were carefully pushed down in the direction and which they bend until they snapped out of place. Using the curved dissection scissors, they were then cut away from the main cranial end of the keel. Any remaining parts of the ribs were also carefully trimmed off. The remaining flesh on the keel was scraped off with a scalpel and wiped down with blue roll (Fig. 2.4). Some keels had major breaks or damage to them in which case not all the flesh could not be removed. The keel was then stored again at -20°C until measured.

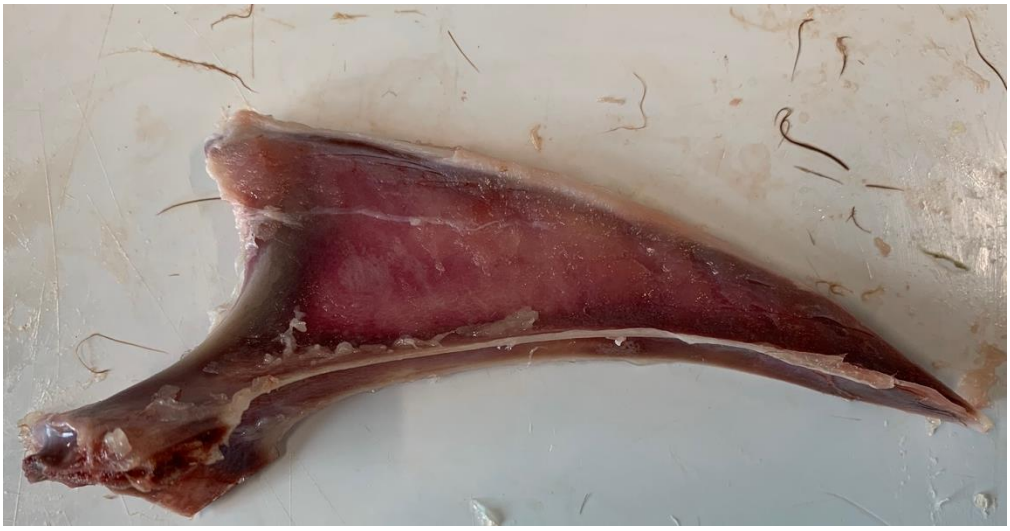

Figure 2.4 Keel bone with all the flesh and coracoids removed

At the tail of the humerus (elbow joint), a scalpel was used to cut and peel away the tendons and flesh around the circumference of the end of the bone. Once the bone was revealed the wing flesh could be pulled by hand towards the head of the humerus (Fig. 2.5).

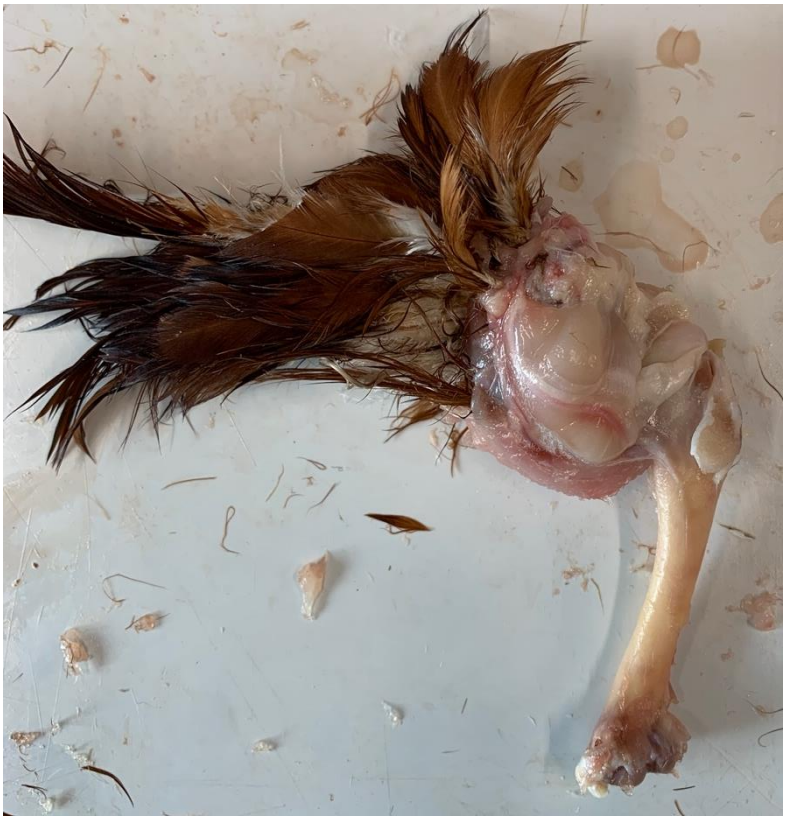

Figure 2.5 Removal of the tendons around the humerus at the elbow joint

Any remaining tissue on the shaft of the humerus was then scraped away with a scalpel. Using a straight pair of dissection scissors, tendons attaching tissue to the humeral head were cut away and discarded. As much flesh as possible was removed but difficulty was experienced due the abnormal ovoid shape of the humeral head. Finally, the bones were wiped down with blue roll to try and take any last flesh off the bones. Next the radius and ulna were separated at the distal and proximal radioulnar joints using a scalpel and connective tissue was removed using a scalpel and scissors similarly to the method used when processing the humerus. All bones were wiped down with blue roll and then stored at -20°C ready for measuring.

The method of stripping a tibia is also similar to the method used on the humerus. Holding the tibia by the distal end, a scalpel was used to cut the flesh and tendons around the circumference of the distal end of the tibia. A slice was made from the distal to proximal end of the tibia to loosen the connecting tissues. The flesh could then be pulled down towards the

proximal end of the tibia revealing the fibula (Fig. 2.6). Using a scalpel, the fibula was detached from the tibia shaft and discarded. The remaining tissue was pulled taught around the proximal end of the tibia and cut away with straight dissection scissors. Any flesh remaining afterwards was scraped off and the bone was then wiped down, then stored for measuring. To remove flesh from the femur, an incision was made from the femoral head to the distal end of the femur (tibiofemoral joint) on the inner side of the femur, following the curve of the bone. Flesh could then be peeled off and connective tissues removed from the bone and the bone wiped down. Not all bones were used in each study, details of which sample bones were used in each study can be found in the relevant chapters.

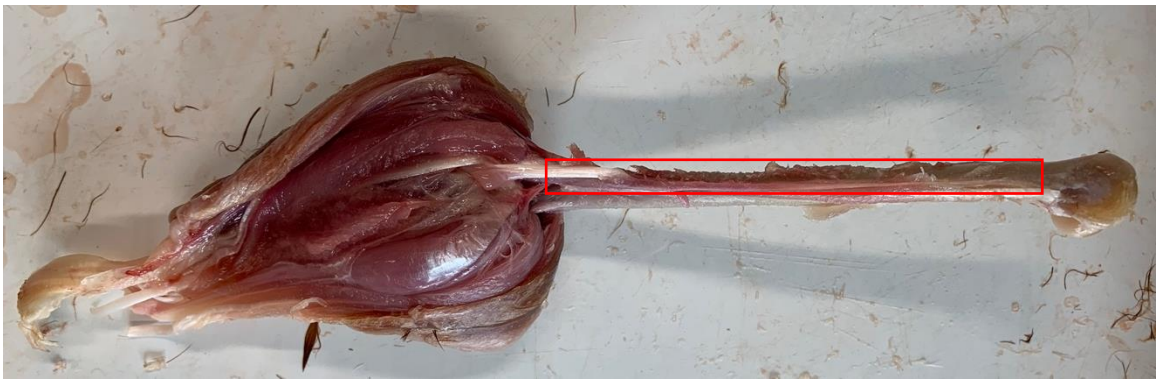

Figure 2.6 Removal of connective tissues from the distal end of the tibia to the proximal end revealing the fibula (highlighted in red)

2.7.3 Bone measurements

Once bone samples had flesh and tissues removed, multiple measures were taken for analysis including length, width, weight, height (keel only) and breaking strength. Keel bone damaged was also recorded using pre-defined groups. Length, width and height were all measured using digital callipers to an accuracy of 2 decimal places. Length of all samples was measured by the most extreme points of the proximal and distal ends of all bones. Width was measured differently depending on bone. Keel width was measured between the two outer points of the keel head where the coracoid bones joined to the keel (Fig. 2.7).

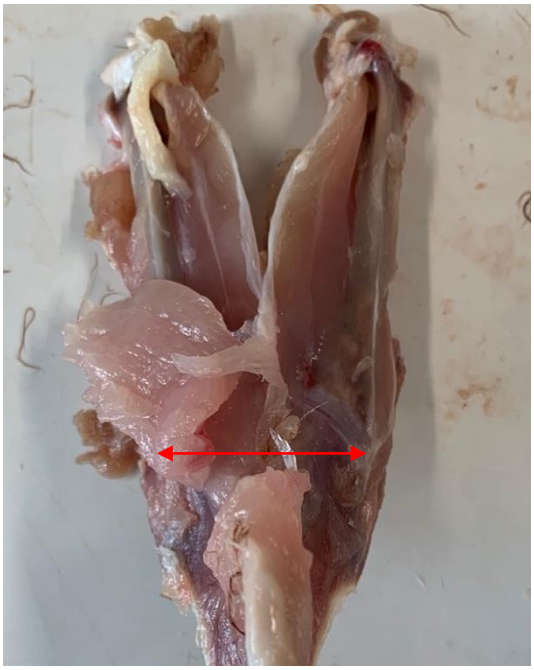

Figure 2.7 Measuring width of the keel

Humerus width was measured at the midpoint in the flattest orientation. Ulna and radius width were also measured in this way. Femur width was measured at the midpoint of the bone with the bulbous part of the joint at the distal end directed towards the floor. The width of the tibia was measured at the midpoint, measuring the thickest orientation (Fig. 2.8). Height of the keel was measured from the top of the keel crest to the base of the keel (Fig. 2.9). All bone weights were record fresh using a 4 decimal place analytical scale (Satorius, UK).

Figure 2.8 Measuring width of the tibia

Figure 2.9 Height of the keel bone

Bone breaking strength was measured using a texture analyser (TA.XT 100; Stable Micro Systems, Guildford) with a 3-point bend attachment (HDP/3PB; Stable Micro Systems, Guildford) and a 100KG loadcell. Bones were placed across the rig ventrally with the proximal ends to the left and the distal ends to the right (Fig. 2.10). The distance between the two resting points on the rig could be altered depending on size of the bone. Bones were all broken at the midpoint and strength was measure to 4 decimal places in Newtons. Table 2.2 summarises the test parameters used in the texture analyser program (Exponent, UK).

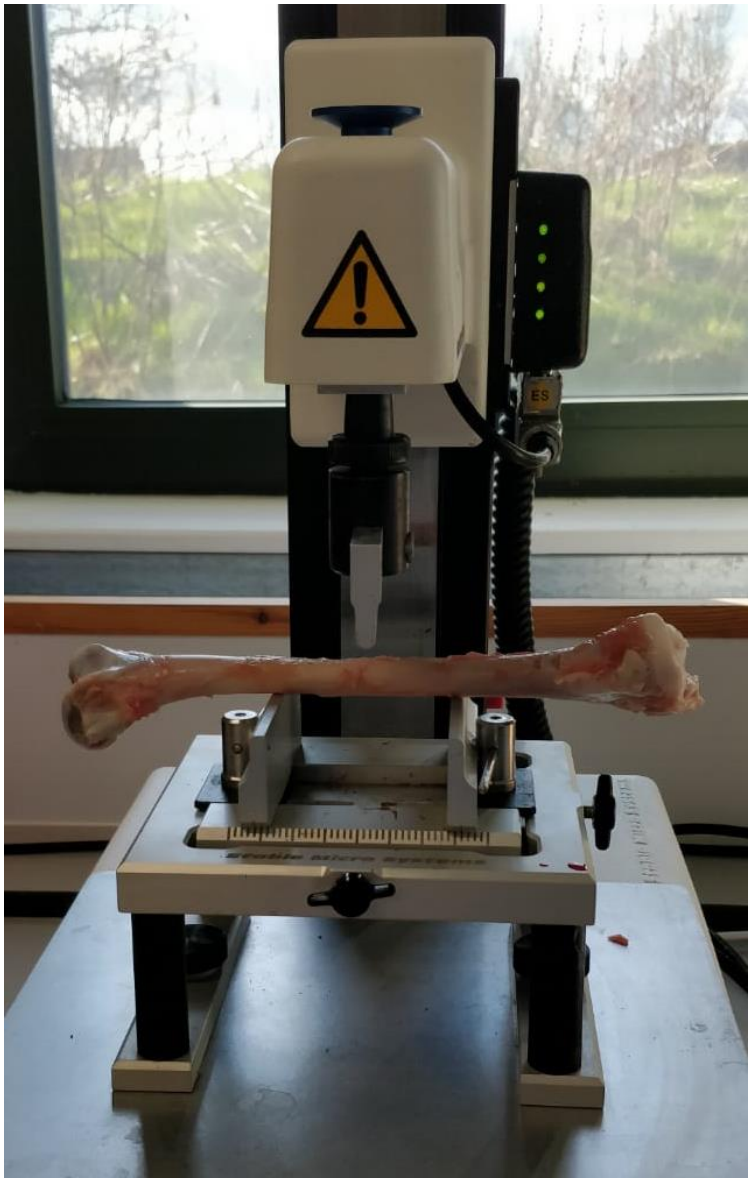

Figure 2.10 TA.XT 100 texture analyser

Table 2.2 A summary of test parameters used to measure bone breaking strength

Settings	Values	
	Long bones (all except keel)	**Keel bone**
Distance between resting points	40 mm	32 mm
Set start height	150mm	160 mm
Pre-test speed	5 mm/s	5 mm/s
Test speed	3 mm/s	3 mm/s
Post-test speed	20 mm/s	20 mm/s
Trigger force	0.05 N	0.05 N
Target distance	10 mm	20 mm

After measuring the bones, all samples were then placed into labelled individual pie trays and dried at 105°C in an oven for 3 days until a constant dry weight was achieved. The bones were then weighed on a 4 decimal place analytical balance (Satorius, UK) and then stored in labelled bags until they were processed for ash weight. Ash content of the bones was determined after the bones were defleshed, measured and dried. Empty crucibles were weighed to 4 decimal places and assigned to an individual bone. The bones were then placed into the crucibles and weighed then placed in a muffle furnace (SNOL 22/1100 LHM01) at 650°C for 12 hours. The crucibles were then taken out of the furnace and once cooled, the crucibles were individually weighed to 4 decimal place using analytical scale (Satorius UK). The ash was then discarded after weight was recorded. Ash weight and bone ash content (Chapter 4 only) for each bone was calculated using the formula below:

$$Bone\ ash\ weight\ (g)$$
$$= Full\ crucible\ weight\ (g)(after\ ash) - Empty\ crucible\ weight\ (g)$$

$$Bone\ ash\ content\ (\%) = \left(\frac{Bone\ ash\ weight\ (g)}{Bone\ dry\ weight\ (g)}\right) \times 100$$

2.7.4 Egg measurements

Eggs were stored for up to 3 days before being analysed. Egg weight was recorded using a 4 decimal place scale (Satorius, UK). The height of the egg was measured from the apex of the egg to the bottom, using digital callipers and recorded to 2 decimal places. Eggs were then marked at 4 points around the mid circumference to indicate where the thickness measurements would be taken from after shell strength had been measured (Fig. 2.11).

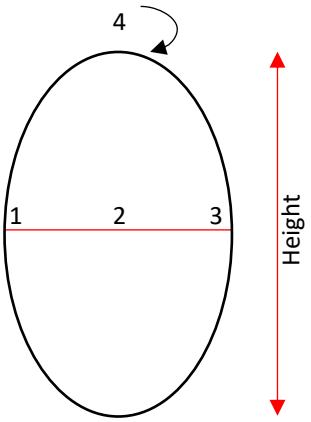

Figure 2.11 Egg shell height and thickness markers

Strength was measured using a cylindrical probe (P36/R; Stable Micro systems, Guildford) attached to the texture analyser (TA.XT 100; Stable Micro Systems, Guildford) breaking the egg a the mid-point in a horizontal position (Fig. 2.12).

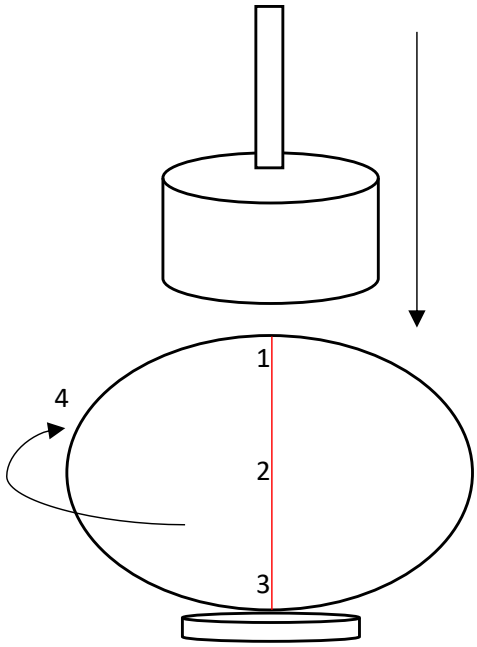

Figure 2.12 Egg breaking setup

Breaking strength was measured in Newtons to 4.d.p and recorded. A summary of test parameters for measuring eggshell strength can be found in table 2.3. Next, the shell was split in two and the albumen and yolk were discarded, and the inside of the shell was wiped out (Egg contents were saved in chapter 6 to measure albumen height).

After eggshell breaking strength was measured, the contents of the egg were then placed on a steel and glass breakout table (QCA-P; TSS, York). Next a height gauge (QCH; TSS, York) was used to measure albumen, it placed over the egg contents with the measuring needle positioned 1cm away from the egg yolk. When correctly positioned the needle was then pressed down through the albumen until it touched the breakout table and then was released. The digital reader (QCD; TSS, York) would then display albumen height in mm. Once albumen height was recorded, the egg contents were discarded. The gauge and breakout table were wiped down after each egg and the gauge reset (Albumen height was only recorded in chapter 6). After being wiped out, shell thickness was measured at the 4 points marked out earlier using digital callipers (2 points in chapter 6). Shells were then left to dry

for 24 hours and afterwards, weighed (Chapter 6) and then stored until being placed in the furnace.

Table 2.3 Test parameters for measuring eggshell breaking strength

Settings	Values
Set start height	120 mm
Pre-test speed	5 mm/s
Test speed	3 mm/s
Post-test speed	20 mm/s
Trigger force	0.05 N
Target distance	3 mm

To measure eggshell ash weight, shells were placed individually into pre-weighed empty crucibles and then placed in a furnace for 12 hours at 650°C. Crucibles were then removed from the furnace and left to cool. Once cooled, each crucible was weighed to 4 decimal places and ash discarded. Ash weight and ash content were calculated using the equations below:

$$Eggshell\ ash\ weight\ (g) = Full\ crucible\ weight\ (g)(after\ ash) - Empty\ crucible\ weight\ (g)$$

$$Eggshell\ ash\ content\ (\%) = \left(\frac{Eggshell\ ash\ weight\ (g)}{Egg\ weight\ (g)}\right) \times 100$$

Haugh unit was calculated (Chapter 6) using albumen height and egg weight, inputted into the equation below:

$$Haugh\ Unit\ (HU) = 100 \times log\ (h\ - 1.7w^{0.37} + 7.57)$$

2.8 Dietary Analysis

Any diets created in-house were analysed using in-house methods and provided as g/kg values (except energy MJ/kg). Diets used in the on-farm project were analysed by Noble Foods Ltd and a dietary analysis was provided. Some farms used were contracted by Nobel Foods Ltd and their dietary analysis were also provided where possible.

For diets used in the in-house trial, diets were analysed for the following constituents: energy, dry matter (DM), ash, fats and protein. For diets used in the on-farm project dietary analysis provided values for calcium, phosphorus, ash, fibre, oil (ether extract) protein and DM.

2.8.1 Determination of Gross energy

Gross energy was determined using a bomb calorimetry method, using sucrose as a standard (sucrose result: 16.493 MJ.kg) via an external company (Pemberton Analytical Services; Shawbury, UK).

2.8.2 Dry Matter

Dry matter content was measured by accurately measuring 5-10g of feed into pre-weighed crucibles. The crucibles were then placed in a drying oven at 105C for 3 days or until a constant weight was reached. Once dry the crucibles were placed in a desiccator, cooled and reweighed. Two repeats per diet were carried out and the results averaged per diet. Dry matter g/kg was calculated using the following equation:

$$= \left(Sample\ weight\ (g) - Dry\ weight\ (g)\right)\ \times 1000$$

2.8.3 Determination of Ash

Ash content was measured by weighing 5-10g of feed into pre-weighed crucibles. The crucibles were then placed in a muffle furnace (SNOL 22/1100 LHM01) at 650°C for 12 hours. Once cooled, the crucibles were reweighed at room temperature. Ash content g/kg was calculated using:

$$= \left(Sample\ weight\ (g) - Ash\ weight\ (g)\right)\ \times\ 1000$$

2.8.4 Fat determination

Fat determination was calculated using a Soxtherm® machine (Soxtherm®, Gerhardt Analytical Systems; Germany) using petroleum ether as a reagent. 3-5 boiling stones were placed in the provided beakers and weighed (m1). 10g of diet or whole samples were then weighed out using an analytical scale and placed in cotton thimbles which were held in the beakers (m0). Cotton wool was then used to plug the thimble tops so the diet would not be lost in extraction. 150ml of petroleum ether was then added to the Soxtherm® and the machine was set to use program “PetEther”. After 2 hours the extraction is complete, the beakers were left to cool for 30 minutes and then removed from the machine. Next, the thimble and cotton wool were removed from each beaker and then the beakers were placed in a fume hood for 1 hour to allow all excess petroleum ether to evaporate. After 1 hour, the beakers were then reweighed to calculate fat content (m2). Settings for the “PetEther” program are summarised in Table 2.4. The following equation was used to calculate fat in g/kg:

$$= \frac{m2 - m1}{m0} \times 1000$$

Table 2.4 Standard operating conditions for program "PetEther"

Operation	Setting
T-Classification	200°C
Extraction Temperature	150°C
Reduction Interval	03.00 mins
Reduction Pulse	3s
Hot Extraction	60 mins
Evaporation A	5.0 x Interval
Rinsing Time	30 mins
Evaporation B	5.0 x Interval
Evaporation C	0.0 mins

2.8.5 Determination of proteins

Protein content was determined via the Dumas method using a Dumatherm® Machine (Gerhardt Analytical Systems; Germany) using an argon atmosphere by an in-house technician (Poultry Research Unit; Nottingham Trent University, UK). 3 replicate samples were used per diet to create an average value. A percentage value of nitrogen was provided (n), the following equation was used to calculate protein content in g/kg:

$$= (n \times 6.25) \times 10$$

2.9 Data Analysis

Data for the pilot studies (chapter 3) were analysed using IBM SPSS 26 (IBM Statistics), utilising a range of independent T-tests, One-way ANOVAs, and univariate analyses. The significance threshold for all tests were set at $p < 0.05$. The statistical methods used are explained in more detail in chapter 3. Data in chapters 4 and 5 were analysed using R and R Studio with two-way ANOVAs and Gaussian LMMs for modelling data. Further explanations of the data analysis can be found in chapter 5 and 6. The data from the In-house trial (chapter 6) was analysed using IBM SPSS 26 (IBM Statistics) and R and R Studio using a mixture of one-way ANOVAs and two-way ANOVAs.

Chapter 3: Pilot Studies

3.1 Introduction

This chapter presents a series of pilot studies developed to establish methods used elsewhere in this thesis. The overall aim of these pilot studies was to determine which bones were optimal for use in assessing skeletal health of laying hens throughout a range of commercial housing systems. In section 3.1, bone parameters were opportunistically measured from broiler chickens from an existing feeding trial, examining efficacy of phytase in altering mineral uptake. Section 3.1 presents findings of which bones (keel, humerus, ulna radius, femur, and tibia) were the most sensitive to highlight differences and which were logistically viable to dissect for future work. Section 3.2 examined these same bones in laying hens at the end of lay (76 and 79 weeks of age). Section 3.2 presents findings about whether the selected bones from section 3.1 are still sensitive and viable to use as samples to assess skeletal health in laying hens. Section 3.3 used the same sample bones as the previous two pilot studies but using laying hens at the beginning of lay (30 weeks), to validate whether sample bones were still optimal for assessing skeletal health in laying hens at different ages. Results from these studies were used to inform data collection in the subsequent data chapters (chapters 4 and 6).

3.2 Pilot study 1: Identifying which bones are optimal to assess skeletal health in broiler chickens

3.2.1 Introduction

In all aspects of poultry, skeletal health is critical to ensuring welfare. Therefore, there is great interest in evaluating and assessing skeletal development in both meat and egg chickens (Williams *et al*., 2000; Campbell *et al*., 2019). In meat chickens, research is often focussed on the development and improvement of leg bone health due to the fast-growing genetics and load bearing capabilities of the bone, which could result in skeletal disorders such as tibial dyschondroplasia and lameness (Pines and Reshef, 2015). On the other hand, laying hens can endure poor skeletal health due to systemic problems such as osteoporosis or keel bone damage (KBD) (Fleming *et al*., 2006). By opportunistically using broiler birds from an ongoing trial, multiple bones were removed and analysed to identify which bones were most logistically viable to remove and which might show the best sensitivity for elucidating differences between dietary treatments. Findings from this study were used in section 3.2 and 3.3 to confirm if the same bones were also optimal for assessing skeletal health in laying hens.

3.2.2 Aim

To determine which bones were the easiest to dissect from day 14 and day 21 broiler chickens and to investigate which bones were the most sensitive at revealing the differences in effects between two dietary treatments.

3.2.3 Methods

Fourteen-day old (n= 16) and twenty-one-day old (n=16) male Ross 308 chickens, fed one of two diets: 'LO PHY': 500 FTU of phytase and 'HI PHY': 1500 FTU phytase (Quantum Blue, AB Vista), were obtained from an in-house trial. A wheat-soya based basal diet meeting the requirements for age and strain of the birds was used throughout the two phases, starter crumb (D0-D14) and grower pellet (D14-D21) (Table 3.1). Energy and fats were calculated in house as in section 2.8. Diets were sent off to Sciantec (Cawood, UK) for dry matter (DM), protein content and calcium and phosphorus content analysis. Each treatment was fed to 8 pens (4 birds per pen). The methods for trial environment and bird husbandry are outlined in section 2.2. One bird per pen was euthanised at D14 and D21 via cervical dislocation and stored at -20°C until they were dissected. The following bones were dissected out of each bird and stored individually: keel, left humerus, right humerus, left ulna, right ulna, left radius, right radius, left femur, right femur, left tibia and right tibia. Each bone per bird was then stripped of flesh prior to recording bone measurements (section 2.7.2 and 2.7.3). Keel bone strength was not measured in this study as the keels were not fully calcified in these young broilers. Independent T-tests (IBM SPSS 26) were used to compare diets and age groups separately for each bone type; $p < 0.05$ was the set significance threshold.

Table 3.1 Analysed content of dietary treatments (starter and grower phases)

	Diet			
Constituents (g/kg)	**'LO PHY'**		**'HI PHY'**	
	Starter	**Grower**	**Starter**	**Grower**
DM	880.0	878.0	880.0	879.0
Protein	223.0	201.0	231.0	201.0
Fats	32.8	37.0	39.1	42.1
Calcium	6.8	6.7	8.5	6.5
Phosphorus	4.8	4.6	5.4	4.5
Energy (MJ/kg)	17.05	17.29	17.07	17.53

3.2.4 Results

Table 3.2 showed that at D14 only the width of the right femur was significantly affected by dietary treatments (p = 0.001), with the 'HI PHY' diet showing wider bones. Keel bone width also showed a trend, indicating that 'HI PHY' keel bones were wider (p = 0.064). Table 3.3 showed that at D21 significant differences between dietary treatments were found in all bone parameters. Overall, the 'HI PHY' diet had a greater effect on bone growth than the 'LO PHY' diet apart from in the width of the right radius, where the 'LO PHY' diet was significantly better than the 'HI PHY' diet. Also, there were orientational differences in sensitivity of the bones indicating the differences of dietary treatments, but it was not consistent for either the left or right orientation. Bone length was significantly longer in the left humerus (p = 0.013), left ulna (p = 0.025), left femur (p = 0.009), left tibia (p = 0.046), right humerus (p = 0.019) and keel bone (p = 0.006) in the 'HI PHY' diet. D21 bone length also showed trends in the right ulna, right radius, and right tibia to be longer in the 'HI PHY' diet than the 'LO PHY' diet (p = 0.063, p = 0.053, p = 0.083 respectively). Bone width was significantly wider in the left ulna (p = 0.031), left radius (p = 0.016), left femur (p = 0.001), right ulna (p = 0.045), right femur (p = 0.001) and keel bone (p = 0.050) of the 'HI PHY' diet than the 'LO PHY' diet. The right radius was significantly wider in the 'LO PHY' diet than the 'HI PHY' diet (p = 0.001). A trend was also observed in the left humerus width, showing wider bones in from the 'HI PHY' group (p = 0.065). Bone weight showed the left humerus (p = 0.015), left ulna (p = 0.025), left radius (p = 0.015), left femur (p = 0.036), right humerus (p = 0.027), right ulna (p = 0.009), right radius (p = 0.008) and the keel bone (p = 0.011) were all significantly heavier in the 'Hi PHY' diet. Table 3.3 also showed that D21 bone strength was significantly stronger in the 'HI PHY' group in the left radius (p = 0.036), left femur (p = 0.028), right humerus (p = 0.004), right radius (p = 0.003) and right tibia (p < 0.001). A trend was shown in the left ulna (p = 0.085) showing stronger bones in the 'HI PHY' diet compared to the 'LO PHY' diet.

Table 3.2 D14 bone parameters for broiler chickens fed 'LO PHY' and 'HI PHY' diets*†

Bone[a]	Length (mm)			Width (mm)			Weight (g)			Strength (N)		
	LO PHY[b] (±SE)	HI PHY (±SE)	*p* value	LO PHY (±SE)	HI PHY (±SE)	*p* value	LO PHY (±SE)	HI PHY (±SE)	*p* value	LO PHY (±SE)	HI PHY (±SE)	*p* value
LH	37.05 (0.560)	37.62 (0.514)	0.467	3.15 (0.136)	3.09 (0.090)	0.713	1.42 (0.091)	1.42 (0.059)	0.976	61.09 (6.143)	57.50 (4.880)	0.655
LU	34.61 (0.561)	35.30 (0.203)	0.278	2.82 (0.088)	2.78 (0.046)	0.712	0.54 (0.037)	0.52 (0.021)	0.569	24.73 (1.444)	25.70 (1.717)	0.672
LR	32.04 (0.477)	32.39 (0.341)	0.557	1.54 (0.051)	1.50 (0.039)	0.580	0.19 (0.017)	0.20 (0.012)	0.491	7.77 (0.515)	7.00 (0.337)	0.232
LF	40.32 (0.624)	40.93 (0.522)	0.470	3.91 (0.106)	3.83 (0.142)	0.644	1.61 (0.104)	1.67 (0.098)	0.691	62.61 (5.667)	63.57 (3.544)	0.887
LT	52.56 (0.911)	50.28 (2.534)	0.412	3.19 (0.109)	3.20 (0.094)	0.939	2.10 (0.141)	2.09 (0.151)	0.914	39.41 (3.449)	38.27 (3.058)	0.807
RH	37.12 (0.660)	37.78 (0.383)	0.401	3.01 (0.089)	2.98 (0.080)	0.806	1.44 (0.898)	1.38 (0.046)	0.586	57.50 (5.574)	59.05 (3.191)	0.813
RU	35.20 (0.546)	35.15 (0.381)	0.943	2.69 (0.100)	2.71 (0.041)	0.830	0.53 (0.037)	0.52 (0.020)	0.791	26.95 (1.406)	28.94 (1.748)	0.391
RR	32.18 (0.427)	32.01 (0.384)	0.776	1.48 (0.059)	1.50 (0.042)	0.851	0.20 (0.016)	0.20 (0.008)	0.655	11.14 (0.858)	10.94 (0.698)	0.858
RF	39.06 (0.670)	40.38 (0.869)	0.250	3.04 (0.184)	4.01 (0.125)	0.001	1.60 (0.103)	1.64 (0.104)	0.809	68.56 (5.606)	66.99 (4.292)	0.827
RT	52.43 (0.963)	51.88 (0.970)	0.696	3.21 (0.131)	3.21 (0.085)	0.981	2.14 (0.156)	2.03 (0.147)	0.612	39.36 (3.932)	38.19 (2.923)	0.814
KB*	51.18 (1.328)	51.51 (1.221)	0.858	15.94 (0.595)	17.47 (0.477)	0.064	1.96 (0.207)	1.87 (0.131)	0.711			

†Keel bone strength was not measured as the bones were not fully calcified

[a] LH = left humerus, LU = left ulna, LR = left radius, LF = left femur, LT = left tibia, RH = right humerus, RU = right ulna, RR = right radius, RF = right femur, RT = right tibia, KB = keel bone

[b] LO PHY = 500FTU phytase inclusion, HI PHY = 1500FTU phytase inclusion

*Figures highlighted in grey show a significant difference

Table 3.3 D21 bone parameters for broiler chickens fed 'LO PHY' and 'HI PHY' diets*†

Bone[a]	Length (mm)			Width (mm)			Weight (g)			Strength (N)		
	LO PHY[b] (±SE)	HI PHY (±SE)	*p* value	LO PHY (±SE)	HI PHY (±SE)	*p* value	LO PHY (±SE)	HI PHY (±SE)	*p* value	LO PHY (±SE)	HI PHY (±SE)	*p* value
LH	45.79 (0.903)	48.77 (0.529)	0.013	3.89 (0.120)	4.17 (0.072)	0.065	2.64 (0.167)	3.22 (0.130)	0.015	129.28 (11.624)	137.81 (9.224)	0.574
LU	43.73 (0.815)	46.11 (0.491)	0.025	3.44 (0.104)	3.72 (0.052)	0.031	1.05 (0.069)	1.27 (0.057)	0.025	54.49 (4.219)	65.37 (4.087)	0.085
LR	40.93 (0.884)	42.48 (0.427)	0.137	1.91 (0.047)	2.07 (0.040)	0.016	0.39 (0.031)	0.49 (0.017)	0.015	16.43 (1.236)	20.85 (1.449)	0.036
LF	47.94 (0.614)	50.44 (0.553)	0.009	4.80 (0.136)	5.63 (0.137)	0.001	2.97 (0.145)	3.47 (0160)	0.036	108.81 (9.645)	139.10 (7.669)	0.028
LT	64.22 (1.222)	67.38 (0.763)	0.046	4.17 (0.119)	4.36 (0.103)	0.253	4.31 (0.312)	5.00 (0.278)	0.120	68.15 (4.620)	87.78 (6.176)	0.230
RH	45.92 (0.974)	48.75 (0.448)	0.019	3.93 (0.109)	4.10 (0.054)	0.202	2.61 (0.164)	3.09 (0.105)	0.027	124.23 (10.388)	169.23 (7.811)	0.004
RU	44.48 (0.883)	46.56 (0.524)	0.063	3.40 (0.110)	3.72 (0.093)	0.045	1.04 (0.070)	1.30 (0.046)	0.009	62.33 (5.083)	69.94 (4.033)	0.260
RR	40.32 (0.899)	42.47 (0.480)	0.053	1.88 (0.058)	1.50 (0.042)	< 0.001	0.40 (0.023)	0.49 (0.018)	0.008	23.41 (1.688)	33.32 (2.119)	0.003
RF	48.58 (1.242)	50.74 (0.440)	0.136	4.79 (0.165)	5.68 (0.133)	0.001	3.10 (0.251)	3.58 (0.114)	0.100	114.75 (9.940)	139.42 (8.135)	0.750
RT	64.26 (1.364)	67.14 (0.588)	0.083	4.21 (0.130)	4.42 (0.098)	0.206	4.35 (0.317)	4.81 (0.230)	0.260	58.53 (4.256)	122.04 (7.604)	< 0.001
KB*	63.99 (1.832)	71.14 (1.209)	0.006	21.71 (0.818)	23.94 (0.640)	0.050	3.90 (0.308)	5.11 (0.277)	0.011			

†Keel bone strength was not measured as the bones were not fully calcified

[a] LH = left humerus, LU = left ulna, LR = left radius, LF = left femur, LT = left tibia, RH = right humerus, RU = right ulna, RR = right radius, RF = right femur, RT = right tibia, KB = keel bone

[b] LO PHY = 500FTU phytase inclusion, HI PHY = 1500FTU phytase inclusion

*Figures highlighted in grey show a significant difference

3.2.5 Discussion

3.2.5.1 Validity of results

Body weight was not recorded in this pilot for the sample birds taken, therefore the bone measurements are not relative to bird weight or size. If bird weight was recorded, perhaps more differences would have been found between dietary treatments at D14 compared to what was observed. Conversely, it could also reduce the number of differences seen at D21. There is some uncertainty to whether the results by the right radius at D21 are correct as it is the only bone that shows the 'LO PHY' diet is significantly wider than the 'HI PHY' diet. As the radius is such as small and thin bone at this age, the differences could be a result of some human error when measuring radius width. Furthermore, all bones at D14 and D21 are still cartilaginous and therefore not mineralised fully. Through dissections when de-fleshing the bones it could be possible to remove some of the cartilage caps or cartilaginous areas from bones as they are delicate. Particularly, the keel bone in broilers at these ages were mostly cartilage with somewhat little mineralised structure. It may be that during dissection, some parts of the keel could have been removed in error and made the measure more variable. As the keel was so undeveloped in terms of mineralisation, no strength values were taken as there was insufficient area of the bone to get a representative breaking value.

3.2.5.2 D14 bone parameters

Table 3.2 shows that only right femur width showed an effect of dietary treatments ($p = 0.001$) at D14 with keel bone width also showing a trend ($p = 0.064$), with bone bones showing greater widths from the 'HI PHY' diets. No other differences could be elucidated at D14, though at D21 many more differences were shown in multiple bones and measurements. The lack of significant differences between diets at D14 compared to D21 may be due to the diet type used at each age group. At D14 onwards, the birds were switched to a grower pellet which could have increased food intake and mineral supply prompting more growth; hence the increased need for phytase to help make more phosphorus available.

Previous research found that the inclusion of phytase to a low calcium and phosphorus diet (LCaP) or a control diet showed lower bone breaking strength than without phytase inclusion

in the respective diets at the starter phase (D0-D14) though results were not significant (p = 0.14) (Powell *et al*., 2008). There were some effects of inclusion of non-phytate phosphorus (nPP) and phytase at the grower stage (D14 to D32) (p = 0.01), with bone breaking strength decreasing in broilers fed the low calcium and phosphorus diet with the nPP and phytase (Breaking strengths: Control = 33.39kg, LCaP = 34.14kg, Control + Phy = 34.14kg, LCaP + Phy = 31.49kg). The previous research somewhat supports the results from the present study, as there was a no significant differences in bone strength at D14 from differing phytase inclusions in both studies. Furthermore, in the Powell *et al*., (2008) phosphorus levels in the diets provided to birds until around D14 may have been sufficient (ranging from 5.6g/kg – 6.8g/kg depending on diet) as the growth rate may not be as high at this stage and may not require the same usage of phytase to increase available phosphorus for skeletal development. Though between 4.8g/kg and 5.4g/kg ('LO PHY' and 'HI PHY' respectively) were used in the present study indicating the phytase product used may differ in effectiveness between each study. Less phosphorus is needed in the present study than Powell *et al*., (2008) but ultimately the same effect was shown at D14.

3.2.5.3 D21 bone parameters

At D21 the 'HI PHY' diet had a significant effect on the length of left humerus, left ulna, left femur, left tibia, right humerus and keel bone – all longer than the 'LO PHY' diet (Table 3.3). A recent study found that phytase inclusion did not affect relative length of the left tibia (p = 0.881) at 500 FTU compared to 0 FTU phytase in 24-day old broilers (Akter *et al*., 2016). Although only tibia length was measured in the previous study, the results do not support the current study as an effect of phytase was found in multiple bone samples including the left tibia. Though the difference between inclusions used in this study and the previous study differ as 500 and 1500FTU were used in the present study compared to 0 and 500 FTU in Akter *et al*., (2016). Tibia length in Akter *et al*., (2016) was also relative to body weight, whereas values in the present study did were not relative to body weight perhaps causing different results. Another study also found that femur length significantly increases with increased inclusions of phytase ($p < 0.05$) (0 to 2000FTU) (Fernandes *et al*., 2019). The results from the previous study do support results from the present study, however only the left femur showed significant increase in the 'HI PHY' diet and not the right femur. Width of the left ulna,

left radius, left femur, right ulna, and keel were all significantly improved by the 'HI PHY' diet (p = 0.050 or less) and the right radius was significantly wider in the 'LO PHY' diet, though is suggested to be an anomalous result as it is the only bone to show a greater effect in the 'LO PHY' diet. Tibia width results from Akter *et al*., (2016) also do not support the results from the present study as phytase does not have a significant effect on tibia width (p = 0.469) whereas multiple bones in the present study do. Though neither of the tibias in the present study showed a significant effect of phytase inclusion, somewhat also agreeing with the previous study. Again, the differences in results could be due to the difference in phytase inclusions or because results in Akter *et al*., (2016) are relative to body weight which may conceal some effect of dietary treatments due to bird size. The study by Fernandes *et al*., (2019) also found that femur and tibia widths were significantly affected by phytase inclusion at different levels (p < 0.05), with the femur showing a quadratic effect between inclusion of phytase and bone width and tibia showing a linear effect between inclusion of phytase and tibia width. Fernandes *et al*., (2019) supports results from the present study that femur widths are affected by increase in phytase inclusion but do not support results on tibia width as in the present study no effect on either left or right tibia were present.

The 'HI PHY' diet had a significant effect on bone weight of all bones except the left tibia, right femur, and right tibia (Table 3.3). A previous study found that left tibia bone weight was increased with the supplementation of phytase at 500FTU (p < 0.001) (Powell *et al*., 2011). The results from Powell *et al*., (2011) both support and disagree with results from the present study. One the one hand, both studies confirm that phytase can increase bone weight, however in the present study both tibias did not show a significant effect of phytase inclusion as in Powell *et al*., (2011). A difference in the results for the tibias between the two studies may be due to the difference in dietary treatments, Powell *et al*., (2011) used more diets with differing levels of calcium as well as the inclusion or exclusion of phytase. Surprisingly the effects of tibia weight in the previous study were shown at only 500FTU which was comparable to the 'LO PHY' diet in the present study, suggesting that phytase efficacy is determined by dietary level of calcium as opposed to quantity of phytase (Driver *et al*., 2005). Another previous study also found that right tibia weight was significantly improved by phytase inclusion at 500FTU (p = 0.002). However, when calculated relative to 100g of body

weight the phytase inclusion did not have a significant effect on tibia weight ($p > 0.05$) (Viveros *et al*., 2002). These results do not support the present study as there was no effect of phytase on either tibia weight in the present study, however other bones do show an effect of phytase inclusion. If results in the present study were calculated as relative to body weight the effect of phytase may have been diminished and therefore the results of Viveros *et al*., (2002) would have been supported. Calculating relative to body weight would have negated the effects of bird size in the results and possibly given more accurate results.

Table 3.3 shows that the HI PHY diet improved bone breaking strength in the left radius, left femur, right humerus, right radius and right tibia ($p = 0.036$ or less). Previous studies from Akter *et al*., (2016) and Powell *et al*., 2011 both found phytase supplementation to improve bone breaking strength in the tibia bone ($p = 0.002$ and $p < 0.001$ respectively). Both studies used the left tibia bone though in the present study no effect was seen between the LO PHY and HI PHY diet in the left tibia ($p = 0.230$), but a difference was found in the right tibia ($p < 0.001$) among other bones not used in the previous studies. Furthermore, another previous study found that there was no effect of phytase inclusion at 1500FTU compared to a control diet (0 FTU) in D21 broilers ($p = 0.322$) on right tibia strength (Lee *et al*., 2017). Lee *et al*., (2017) does not support results from the present study as the right tibia showed an effect of 'HI PHY' diet yet left did not. It is uncertain as to why the different studies show the tibia bone to differ in effect of phytase depending on orientation but perhaps bone development during the growing phase effects individual bones differently on a bird-to-bird basis.

3.2.6 Conclusion

It is clear from previous research that the leg bones of broilers are more focussed on in similar studies, however the present study does show that other bones such as the wing bones and keel bones can show differences and be of some use in determining dietary effects on broiler growth. It would be suggested that taking multiple bones, or a particular bone from a particular area of the body would allow for more comprehensive results in future work as different bones have different forms and functions which may cause changes in development. Though some bones such as the ulna, radius and keel at a young age can be difficult to dissect and remove adhering flesh. Furthermore, it is clear from the results that the orientation of

the bone samples used can influence the results. Some bones showed a significant effect on bone parameters in the left side and some in the right only. It is unclear as to why this is occurring, though it is advised to take both orientations of a bone particularly in young birds like broilers as bone development is still occurring and growth of a particular bone may be affected differently between birds depending on factors such as nutrition, behaviours, or husbandry.

3.3 Pilot Study 2: Identifying which are the optimal bones to assess skeletal health in end of lay hens

3.3.1 Introduction

As previously mentioned, skeletal problems not only occur in broiler chickens but also laying hens. Osteoporosis and Keel Bone Damage (KBD) can affect large percentages of flocks during the egg laying period (Casey-Trott *et al.*, 2015). It is therefore important that skeletal health in layers can be assessed in a precise and consistent way. In this pilot study, sample hens at the end lay and housed in either a free-range system or caged system were used. Sample bones collected were the same in pilot study 1 and the factors assessed were the effect of housing system and the effect of orientation of the bone. As the birds collected were from a commercial producer some factors could not be controlled such as age, diet, and bird management.

3.3.2 Aim

The aim of this pilot study was to confirm if the bones collected previously in broilers were also appropriate for assessing skeletal health in laying hens by assessing the results of effects from housing system and orientation.

3.3.3 Methods

Fifteen Lohmann Brown free-range hens aged 76 weeks, and fifteen Lohmann Brown colony caged hens aged 79 weeks were collected from a processing plant of a large UK egg producer. Birds were culled before collection by cervical dislocation. Birds were weighed individually, and the sample bones were dissected out as previously described in section 2.7.1 and 2.7.2. Bones were stored at -20°C until all birds had been dissected. Bones were then de-fleshed, and length, width, weight, and breaking strength were recorded as in section 2.7.3. Univariate analyses (ANCOVA) (IBM SPSS 26) with body weight as a covariate were used to compare the effects housing system and orientation separately for each bone type and bone parameter. The free-range and caged results for each bone were averaged together when evaluating the effect of orientation. The significance threshold was set at $p < 0.05$.

3.3.4 Results

Table 3.4 showed that housing system had an effect on hen body weight, showing significantly heavier birds in the free-range system compared to the caged system ($p < 0.001$). Hence body weight was used as a covariate in further analysis. Table 3.5 showed no effect of orientation on any bone or bone parameter in end of lay hens ($p > 0.05$).

Table 3.6 showed that housing system had little effect on bone length when using body weight as a covariate. Only the keel bone ($p < 0.001$) showed an effect with free-range bones being longer. The right humerus did show a trend from the effect of housing system ($p = 0.051$), showing free-range bones were fractionally longer but not significant. Housing system did have an effect on bone width (Table 3.6). The left humerus ($p = 0.07$), left tibia ($p = 0.013$), right humerus ($p = 0.002$) and keel bone ($p = 0.001$) were significantly wider in free-range hens than caged hens. No other bone showed a significant effect of housing system on bone width. Bone weight was significantly affected by housing system effect in the left ulna ($p = 0.029$) and left radius ($p = 0.024$), showing free-range systems to have heavier respective bones. The right tibia also showed a trend in the free-range bones being heavier but was not significantly different ($p = 0.071$). Lastly, housing system also showed a significant effect on bone strength. The left femur, right humerus, right radius, right femur, right tibia and keel bone were all shown to be stronger in free-range compared to caged ($p = 0.027$ or less). The left tibia also showed a trend indicating that bones from free-range were stronger than caged, though this was not significant ($p = 0.054$).

Table 3.4 Body weights of free-range and caged hens aged 76 and 79 weeks respectively

System	Body weight (g) (±SE)
Free-range	2249.6 (79.97)
Caged	1672.1 (56.34)
***p* value**	< 0.001

Table 3.5 Bone parameters of hens at the end of lay from different orientations†

	Length (mm)			Width (mm)			Weight (g)			Strength (N)		
Bone[a]	Left (±SE)	Right (±SE)	*p* value	Left (±SE)	Right (±SE)	*p* value	Left (±SE)	Right (±SE)	*p* value	Left (±SE)	Right (±SE)	*p* value
H	80.56 (0.378)	79.93 (0.722)	0.395	8.27 (0.097)	8.19 (0.098)	0.341	5.77 (0.175)	5.76 (0.193)	0.970	239.19 (8.290)	237.02 (11.899)	0.838
U	79.47 (0.419)	79.39 (0.419)	0.857	6.59 (0.086)	6.44 (0.062)	0.141	3.72 (0.096)	3.71 (0.098)	0.923	194.28 (8.928)	192.95 (8.172)	0.869
R	72.10 (0.389)	71.90 (0.485)	0.686	3.49 (0.038)	3.57 (0.034)	0.103	1.21 (0.036)	1.22 (0.035)	0.985	66.37 (2.318)	67.86 (2.629)	0.615
F	88.39 (0.676)	88.30 (0.707)	0.898	8.44 (0.087)	8.46 (0.073)	0.847	11.00 (0.318)	11.10 (0.326)	0.684	348.43 (27.187)	336.92 (25.585)	0.599
T	123.43 (0.821)	123.52 (0.800)	0.900	8.00 (0.082)	8.09 (0.081)	0.348	12.90 (0.354)	12.86 (0.346)	0.850	283.65 (21.832)	296.22 (22.547)	0.474

† Means and standard error not adjusted for covariate (body weight)

[a] H = humerus, U = ulna, R = radius, F = femur, T = tibia

Table 3.6 Bone parameters of hens at the end of lay housed in free range and caged systems*†

	Length (mm)			Width (mm)			Weight (g)			Strength (N)		
Bone[a]	Free Range (±SE)	Caged (±SE)	*p* value	Free Range (±SE)	Caged (±SE)	*p* value	Free Range (±SE)	Caged (±SE)	*p* value	Free Range (±SE)	Caged (±SE)	*p* value
LH	81.17 (0.450)	79.95 (0.580)	0.346	8.66 (0.112)	7.89 (0.075)	0.007	6.10 (0.152)	5.44 (0.296)	0.560	261.99 (10.713)	216.39 (9.781)	0.301
LU	80.28 (0.574)	98.66 (0.552)	0.529	6.62 (0.167)	6.57 (0.053)	0.204	4.11 (0.110)	3.32 (0.059)	0.029	222.76 (13.973)	165.80 (4.366)	0.496
LR	72.62 (0.448)	71.59 (0.623)	0.423	3.54 (0.055)	3.44 (0.052)	0.354	1.36 (0.040)	1.07 (0.029)	0.024	73.69 (3.214)	59.05 (2.067)	0.135
LF	90.26 (0.802)	86.52 (0.865)	0.795	8.66 (0.091)	8.22 (0.127)	0.915	12.03 (0.431)	9.97 (0.287)	0.683	463.01 (31.849)	233.86 (13.134)	0.008
LT	126.36 (0.834)	120.50 (0.934)	0.415	8.02 (0.112)	7.99 (0.123)	0.013	14.03 (0.465)	11.78 (0.348)	0.180	371.37 (25.525)	195.93 (14.957)	0.054
RH	79.94 (1.350)	79.91 (0.582)	0.051	8.60 (0.106)	7.78 (0.070)	0.002	6.12 (0.175)	5.40 (0.325)	0.778	285.75 (12.123)	188.28 (10.016)	0.013
RU	80.23 (0.555)	78.54 (0.565)	0.397	6.33 (0.112)	6.55 (0.043)	0.299	4.07 (0.127)	3.35 (0.067)	0.244	223.72 (10.714)	162.18 (5.155)	0.145
RR	72.83 (0.462)	70.90 (0.810)	0.867	3.61 (0.055)	3.53 (0.040)	0.441	1.34 (0.036)	1.09 (0.035)	0.163	79.20 (2.532)	55.71 (1.222)	< 0.001
RF	89.63 (1.136)	86.96 (0.723)	0.194	8.58 (0.092)	8.33 (0.105)	0.501	12.18 (0.440)	10.02 (0.282)	0.647	439.12 (31.920)	234.73 (14.180)	0.027
RT	126.37 (0.820)	120.66 (0.903)	0.421	8.20 (0.135)	7.99 (0.086)	0.101	13.89 (0.482)	11.84 (0.336)	0.071	385.00 (27.885)	207.45 (14.234)	0.090
KB	136.72 (1.608)	120.88 (1.454)	0.003	30.59 (0.521)	26.35 (0.320)	0.001	12.23 (0.562)	9.69 (0.226)	0.231	269.15 (19.699)	150.47 (9.402)	0.002

† Means and standard error not adjusted for covariate (body weight)

*Figures highlighted in grey show a significant difference

[a] LH = left humerus, LU = left ulna, LR = left radius, LF = left femur, LT = left tibia, RH = right humerus, RU = right ulna, RR = right radius, RF = right femur, RT = right tibia, KB = keel bone

3.3.5 Discussion

3.3.5.1 Validity of results

As birds were not selected by the researchers, it could be possible that birds culled were of poorer condition than might have been expected if the birds were randomly selected by the researchers. Though as the birds were collected from a commercial processing site access was not possible. Bird choice or selection from the processing site may have either exacerbated results between the free-range and caged groups or even concealed effects, as poorer conditioned birds in both groups may have similar bone parameters. In addition, this was the first-time bone dissections had been performed by the researchers in laying hens. It could have been possible that lack of practice may have influenced the succession of dissections or reduced the accuracy of the measurements taken, possibly increasing the uncertainty of the results. As shown in table 3.4, body weight was drastically different between caged and free-range birds. Therefore, correcting for body weight by using it as a covariate within bone analysis data was thought to improve the accuracy of the results between housing systems, improving the ability to develop explanations of the data.

3.3.5.1 Body weight

Table 3.4 showed that free-range hens sampled (2249.6g) had significantly heavier body weight compared to caged hens collected (1672.1g) ($p < 0.001$). A previous study found that caged hens were significantly heavier than caged-free hens at 78 weeks of age (2.11kg v 1.97kg, $p < 0.05$)), and free-range hens (2.01kg) were intermediate and not significantly different to either cage or caged-free hens (Regmi *et al*., 2016). The results from the previous study do not support the present study as the free-range hens were not significantly heavier than caged hens and interestingly, caged hens were the heaviest – contradicting results from this study. A difference in the results could have been caused by a difference in diets used and exercise performed by individual birds. It could be suggested that a more active housing system which encourages more exercise could have lighter birds as more energy is being used, therefore increasing metabolism (Regmi *et al*., 2017). As previously mentioned, birds were not selected by the researchers at the processing facility. Bird selection may have also

affected the outcome of bird weights as worse birds could have been picked in the free-range system compared to the caged system and cause anomalous results.

3.3.5.2 Effect of orientation

Table 3.5 showed no effect of orientation on any bone in end of lay hens. In contrast, pilot study 1 showed some left and right bones differed in results from the effect of dietary treatments. Although the studies are not totally comparable it could be suggested that the difference in age of the birds used in both studies influenced the results. For example, it could be suggested that orientational differences in bone parameters are more noticeable before sexual maturity as bone development is still occurring and external factors such as exercise, diet and bird behaviour could affect bone growth differently in different bones. Therefore, within this study orientation showed no effect as all hens were past sexual maturity and bone development did not influence the results. Little research is available on the differences between bone orientation. However, previous research stated that both left and right bones should be take where possible when assessing skeletal health in laying hens as bone growth and development could have impacted each bone differently up to sexual maturity which may have amplified any differences later in life (Martínez-Cummer *et al*., 2006). Exercise is known to effect bone health in laying hens (Leyendecker *et al*., 2005, Regmi *et al*., 2016). Therefore, if bird husbandry routines effect bird behaviours, or are repetitive, it could cause some bones to be exercised more than others and create a difference between bones of difference types or orientations.

3.3.5.3 Effect of housing system

Length and width are not commonly measured as a way of assessing skeletal health in laying hens. However, within this study differences between housing systems were found in multiple bones (Table 3.6). Bone length was only significantly higher in the keel bone of free-range birds, compared to cage birds. Bone width was also significantly improved in the free-range system in all bone types which showed housing system effect (LH, LT, RH, KB). As bone development stops around 20 weeks of age when the hens reach sexual maturity (Hester, 2017), it is not likely that the effects housing system have occurred at end of lay. It could be suggested that any effect of housing system is likely to have influenced bone growth or

development before the point of lay, within the rearing systems for the prospective housing systems and results of these differences have persisted up until sampling at end of lay. More recently, rearing systems tend to use the same configuration as the system the hens are destined for, as matching the rearing system to laying system has found to be beneficial in terms of welfare, development, and natural behaviours (Casey-Trott *et al*., 2017c). A previous study found right tibia and humerus cortex were thicker in aviary pullets compared to caged pullets ($p < 0.05$) (Regmi *et al*., 2015). A difference between free-range and caged systems is the ability to exercise, therefore it could be supported that exercise increases bone development of which the effects maintain throughout lay. If parameters such as cortex thickness is increased in pullets, length and width of bones could also be affected by the change exercise through the difference in housing system. Ultimately, this could be the reason as to why differences in bone geometry were seen in end of lay hens, even though sexual maturity has passed, and bone growth is thought to have stopped by this age. In terms of keel bone measurements, the results could have also been influenced by the abstract shape of the bone and ability to measure correctly, in this case keel width. Furthermore, KBD may have also impacted keel length, as a severely damaged keel bone in either system is likely to influence bone geometry of which some damage in samples were noted.

Housing system had a significant effect on the left ulna and left radius bone weights ($p = 0.029$ and $p = 0.024$ respectively), with the right tibia showing a trend ($p = 0.071$). The free-range bones were found to be heavier than cage bones. It was expected that more differences in bone weight would have been shown between housing systems compared to length or width parameters, as weight was measured using electronic scales and thus reduced human error. Though as body weight was used as a covariate the correlation between a heavier bird and heavier bones may have been negated through the analysis. Previous research found that dry bone weight of the right tibia and right femur were not significantly affected by housing system ($p = 0.19$ and $p = 0.13$) when comparing furnished cage, conventional caged and free-range systems (Regmi *et al*., 2016). Though keel bone did show a significant effect ($p < 0.01$) of housing system, showing furnished cage (7.94g) and free-range (8.02g) keels were significantly heavier than in conventional caged birds (6.12g) but were not significantly different from one and other. The results from Regmi *et al*., (2016) disagree with results from

this study, as the keel bone was not affected by housing system. The results for the tibia and femur weight do concur with the current study, as neither result showed tibia or femur weight to be affected by housing system. However, the right tibia in the present study did show a trend. It could be that the number of samples used per group may have caused a difference in results, as the current study only used 15 hens per housing system whereas the previous study used 60 hens per group. The increase in number of samples could have increased the statistical power of the Regmi *et al*., (2016) study compared to the present study, which may likely show more reliable results. In relation to system effect, Regmi *et al*., (2016) supports the results that free-range systems produce significantly heavier bones than caged systems, however the previous study used conventional cages opposed to colony cages (present study). A direct comparison may not be possible but results from both studies do support each other and show similar effects of housing system.

Another previous study found that left humerus weight was significantly heavier in flattened flooring (similar to barn systems) compared to conventional cages (p = 0.009), though left femur and tibia weight were not significantly different (Qiaoxian *et al*., 2020). The previous study can somewhat support results in the present study as left femur and tibia were also not significant. Though as a different system was compared to the caged system a direct comparison cannot be made, though a flattened floor system is thought to be similar to a free-range system without outdoor access. Humerus results from Qiaoxian *et al*., (2020) do not support the results of which bones showed a significantly different bone weight. In the present study, humerus bone weight (left and right) did not show an effect of housing system. A difference in results could have been caused by the difference in age of the birds used in each study. Birds used in Qiaoxian *et al*., (2020) were aged between 32 and 57 weeks of age whereas birds in this study were around 20 weeks older. The effects of exercise and bone resorption in the different aged birds in each study could have affected the results, as younger birds have undergone less egg production, thus possibly less bone resorption causing a difference in the amount of mineral content present in the bones between ages. The strain of bird used by Qiaoxian *et al*., (2020) could have also caused some differences as it is an uncommon strain that would rarely found in the UK, and most likely not used commercially.

Table 3.6 showed that there was an effect of housing system on bone strength of the left femur, right humerus, right radius, right femur and keel bone (p = 0.027 or less), with the left and right tibia showing a trend (p = 0.054 and p = 0.090 respectively). All effects and trending results showed free-range birds had a higher bone strength than caged birds. A previous study investigating the influence of housing system on bone strength and keel bone fractures in multiple laying hen flocks found that humerus, tibia and keel strength showed a significant effect of housing system ($p < 0.05$) (Wilkins *et al*., 2011).

In the previous study humerus strength was found to be significantly stronger in the free-range (18.7kg) (FR), free-range with A-frames (FRAA) (19.1kg) and free-range with suspended perches (FRAS) (21.9kg) systems compared to furnished cage (FC) humerus strength (14.3kg). Free-range and free-range with A-frames were not significantly different from one and other and free-range with suspended perches showed the highest humerus strength of the free-range systems. Although different free-range systems were not recorded in the present study, the results from Wilkins *et al*., (2011) support results showing humerus strength was significantly stronger in the free-range systems compared to caged, though only the right humerus showed an effect in the present study, where Wilkins *et al*., (2011) measured both humerus collectively.

Tibia strength from the previous study was also shown to be significantly higher in FR (22.9kg), and FRAS (23.6kg) compared to FC (19.1kg), though FRAA (20.8kg) was not significantly different to FC and significant weaker than FRAS. Tibia strength results from Wilkins *et al*., (2011) somewhat support tibia strength results from this study that free-range are stronger than caged bones, though results were only trends in the present study. It was suggested by Wilkins *et al*., (2011) that the type of apparatus within subcategories of free-range systems could also affect tibia strength differently. Another previous study also found that humerus and tibia strengths from an aviary system (similar to free-range) humerus = 247N, tibia = 175N) were significantly stronger than furnished ($p < 0.001$, humerus = 130N, tibia = 122N) and conventional caged systems ($p < 0.001$, humerus = 105N, tibia = 117N) (Leyendecker *et al*., 2005). The results from Leyendecker *et al*., (2005) supports results from the present study

and Wilkins *et al*., (2011) as it confirms free-range systems provide greater bone strength than caged systems.

As previously mentioned, keel bone strength was significantly higher in free-range compared to caged within the present study. Wilkins *et al*., (2011) also showed that housing system significantly influenced results ($p < 0.05$) (tested at the same point as methods in this study). The authors found FR keel strength was significantly stronger than FC, FRAA and FRAS. FRAA, FRAS and FC keel strength were not significantly different from one other. Results for keel bone strength from Wilkins *et al*., (2011) agree with the present study, however it is surprising that the FRAA and FRAS were not significantly stronger to the FC system. It is possible that although the additional apparatus may increase exercise which reduces bone resorption rates (Shipov *et al*., 2010), the additional apparatus could cause a higher prevalence of collisions and KBD (Sandilands *et al*., 2009). More collisions or KBD could therefore lower bone strength compared to a free-range system without additional apparatus.

3.3.6 Conclusion

Unlike young broiler chickens, there was no effect of orientation in bone parameters of end of lay laying hens. This thought to be because bone growth and development should have ceased many weeks prior to the end of lay. It could therefore be concluded that orientation is not a key factor in assessing skeletal health in end of lay hens. Though it is worth noting that behaviours and exercise at the rearing stage may influence differences in orientational bone growth before sexual maturity and the effects could remain until the end of lay. It may also be beneficial to still collect both left and right of a bone and calculate an average per bone type in any future work to provide a more accurate representation. Additionally, it was found that keel, humerus and tibia seemed to be the most assessed bones when measuring skeletal health in layers. It is still advised as in pilot study 1 that multiple bones be taken to assess skeletal health as form and function vary. However, as dissections were laborious it would be advised that only these bones be used in future work instead of all the bone within this study. This will not only reduce workload but align future work with previous research. Furthermore, it could be suggested that the credibility of length and width showing any effect of housing system is ambiguous. This is because hens at the end of lay have long ago surpassed sexual maturity, which is known as that point that growth and development stops. It could therefore be concluded that bone strength and weight are more reliable measures for revealing housing system effects as bone resorption and calcium metabolism still occur throughout lay and do not stop at the point of sexual maturity.

3.4 Pilot study 3: Identifying which bones are optimal bones to assess skeletal hens at the beginning of lay

3.4.1 Introduction

Skeletal health and development are known to change as hens age, with bone metabolism altering over the course of a hen's life (Fleming *et al*., 2006; Hester 2017). Therefore, this pilot study was created to confirm if the same bones taken in pilot studies 1 and 2 (Keel, humeri, radii, ulnae, femurs, and tibiae) were also optimal at assessing skeletal health in hens at the beginning of lay. Similar to pilot study 2, the birds collected were from commercial producers.

3.4.2 Aim

The aim of this pilot study was to confirm if bones from hens at the beginning of lay show the same pattern in determining differences between housing system or orientation of bone as those at the end of lay (pilot study 2).

3.4.3 Methods

Twelve Lohmann Brown hens (6 free-range, 6 colony caged) aged 24 weeks were collected from a collaborating farm and culled via cervical dislocation. Individual body weight was recorded and then the birds were dissected as in section 2.7.1. Information on the dietary treatments for these hens can be found in Appendix 1. The bones that were taken were identical to those used in the previous pilot studies, keel, humeri, radii, ulnae, femurs, and tibiae from each bird. Bones were de-fleshed and measured as described in section 2.7.2 and 2.7.3. The data was analysed using independent T-tests (IBM SPSS 26) comparing housing systems and the orientations of bone independently. The free-range and caged results for each bone were averaged together when evaluating the effect of orientation. The significance level was set at $p < 0.05$.

3.4.4 Results

Table 3.7 showed that there was no significant difference between body weights of the free-range and caged hens at 24 weeks of age ($p = 0.447$). Table 3.8 showed that bone length was

not affected by orientation. There was an effect of orientation on the radius bone (p = 0.044), showing that the right bones were significantly wider than the left bones. Bone weight did not show any effect of orientation in any bone (p = 0.587 or higher). Only bone strength of the ulna showed an effect of orientation (p = 0.050), with the right bone having greater strength compared to the left bone.

Table 3.9 shows that housing system had no effect on any bone length measurement in hens aged 24 weeks (p = 0.113 or higher). There was also no effect of housing system on any bone width measurement in this study (p = 0.213 or higher). Furthermore, no bone weight measurement was significantly affected by housing system, though the left humerus did show a trend being stronger in free-range than caged hens (p = 0.073). Bone strength was the only parameter to show an effect of housing system. The left humerus (p = 0.011), left ulna (p = 0.030), left femur (p = 0.008), left tibia (p = 0.036), right humerus (p = 0.001), right radius (p = 0.030) and right femur (p = 0.029) all showed a significant effect of housing system, all showing greater breaking strengths in the free-range system over the caged system.

Table 3.7 Body weights of free-range and caged hens aged 24 weeks

System	Body weight (g) (±SE)
Free-range	1719.8 (53.26)
Caged	1630.9 (118.91)
***p* value**	0.447

Table 3.8 Bone parameters of hens at the beginning of lay from different orientations*

	Length (mm)			Width (mm)			Weight (g)			Strength (N)		
Bone[a]	**Left (±SE)**	**Right (±SE)**	***p* value**	**Left (±SE)**	**Right (±SE)**	***p* value**	**Left (±SE)**	**Right (±SE)**	***p* value**	**Left (±SE)**	**Right (±SE)**	***p* value**
H	79.20 (0.426)	78.93 (0.424)	0.652	8.12 (0.116)	8.20 (0.110)	0.611	5.49 (0.148)	5.64 (0.219)	0.587	215.02 (13.017)	210.61 (11.994)	0.805
U	77.70 (0.611)	77.74 (0.624)	0.966	6.58 (0.115)	6.59 (0.086)	0.973	3.61 (0.085)	3.58 (0.101)	0.831	162.08 (8.912)	188.48 (9.048)	0.050
R	70.91 (0.590)	70.96 (0.604)	0.952	3.21 (0.078)	3.39 (0.021)	0.044	1.20 (0.029)	1.21 (0.027)	0.779	62.16 (2.305)	62.06 (1.806)	0.974
F	86.57 (0.564)	86.78 (0.587)	0.804	8.11 (0.125)	8.11 (0.134)	1.000	9.90 (0.250)	9.93 (0.235)	0.931	227.66 (15.473)	251.20 (14.450)	0.278
T	119.29 (1.240)	120.28 (1.108)	0.561	6.70 (0.093)	6.71 (0.079)	0.989	11.29 (0.359)	11.50 (0.341)	0.673	274.32 (12.259)	276.28 (11.121)	0.907

[a] H = humerus, U = ulna, R = radius, F = femur, T = tibia

*Figures highlighted in grey show a significant difference

Table 3.9 Bone parameters of hens at the beginning of lay housed in free range and caged systems*

	Length (mm)			Width (mm)			Weight (g)			Strength (N)		
Bone	**Free Range (±SE)**	**Caged (±SE)**	***p* value**	**Free Range (±SE)**	**Caged (±SE)**	***p* value**	**Free Range (±SE)**	**Caged (±SE)**	***p* value**	**Free Range (±SE)**	**Caged (±SE)**	***p* value**
LH	78.75 (0.590)	79.66 (0605)	0.304	8.04 (0.130)	8.20 (0.200)	0.531	5.76 (0.160)	5.23 (0.209)	0.073	245.32 (18.176)	184.72 (6.928)	0.011
LU	77.01 (0.825)	78.39 (0.877)	0.276	6.44 (0.155)	6.73 (0.160)	0.213	3.57 (0.112)	3.66 (0.137)	0.637	180.57 (9.845)	143.58 (10.755)	0.030
LR	70.37 (0.830)	71.44 (0.852)	0.389	3.26 (0.092)	3.16 (0.131)	0.533	1.19 (0.035)	1.21 (0.050)	0.759	65.96 (1.671)	58.35 (3.847)	0.113
LF	85.76 (0.777)	87.38 (0.732)	0.160	7.97 (0.141)	7.96 (0.202)	0.272	9.59 (0.309)	10.22 (0.376)	0.227	264.81 (16.553)	190.51 (15.078)	0.008
LT	117.24 (2.097)	121.35 (0.819)	0.113	6.74 (0.123)	6.73 (0.152)	0.835	11.08 (0.512)	11.49 (0.536)	0.594	299.04 (17.014)	249.60 (11.286)	0.036
RH	78.47 (0.512)	79.39 (0.668)	0.303	8.08 (0.125)	8.32 (0.177)	0.283	5.91 (0.360)	5.37 (0.229)	0.231	243.78 (11.898)	177.43 (7.162)	0.001
RU	77.00 (0.817)	78.48 (0.911)	0.255	6.52 (0.136)	6.65 (0.109)	0.474	3.53 (0.123)	3.64 (0.168)	0.627	202.73 (13.634)	174.23 (9.648)	0.119
RR	70.36 (0.852)	71.56 (0.857)	0.343	3.39 (0.026)	3.39 (0.037)	0.971	1.20 (0.030)	1.22 (0.047)	0.802	66.07 (0.807)	58.05 (2.696)	0.030
RF	85.86 (0.790)	87.69 (0.745)	0.122	8.11 (0.132)	8.11 (0.248)	0.991	9.66 (0.242)	10.21 (0.392)	0.259	281.23 (16.850)	221.18 (16.556)	0.029
RT	118.92 (1.983)	121.64 (0.855)	0.249	6.69 (0.098)	6.73 (0.133)	0.807	11.19 (0.477)	11.82 (0.493)	0.380	294.35 (15.766)	258.22 (12.850)	0.106
KB	119.73 (3.370)	122.46 (1.478)	0.460	31.49 (1.066)	33.37 (1.100)	0.248	9.57 (0.788)	9.20 (0.579)	0.712	92.28 (8.742)	78.99 (6.434)	0.249

[a] LH = left humerus, LU = left ulna, LR = left radius, LF = left femur, LT = left tibia, RH = right humerus, RU = right ulna, RR = right radius, RF = right femur, RT = right tibia, KB = keel bone

*Figures highlighted in grey show a significant difference

3.4.5 Discussion

3.4.5.1 Validity of results

As there were only six birds per housing system group within this study, it could be suggested that the low sample size used might not have been representative of the flock and given inaccurate results. Also, some bird weights were not recorded from the caged group, and this could have resulted in further errors within the body weight results and the effect of housing system between freer-range and caged systems. Particularly within the orientation results, there is some uncertainty to how accurate the measurements of the radius width and ulna strength are, as they were the only bones to show a significant difference (p = 0.044 and p = 0.050 respectively) while all other bones and parameters showed non-significant differences. It could be possible that as the radius a small bone, errors may have been made when measuring radius width and caused incorrect results. As for the ulna strength, outliers may have caused a false result as no outliers were checked or removed in the results before statistical analysis. As only 6 birds per housing system was used, this could have also affected the accuracy of the results as it is thought not be representative the flock. In addition, calculating the results of the bone parameters relative to body size (body weight), could have also improved the reliability of the results in either the orientation or housing system factor as bird size is likely to influence bone geometry.

3.4.5.2 Body weight

Table 3.7 showed that at 24 weeks of age, no difference in body weight was caused by the effects of housing system (p = 0.447). Previous research found that body weight in young laying hens (aged 16 weeks) was not affected by housing systems when using aviary (1204.6g) or conventional caged (1202.1g) rearing systems (p = 0.875) (Casey-Trott *et al*., 2017b). Results from Casey-Trott *et al*., (2017b) supports results found in this study that housing system had no effect on body weight of young hens, though different systems were used in the previous study aviary and conventional cages are similar to free-range and colony cages used within the present study.

3.4.5.3 Effect of orientation

The effect of bone orientation is not commonly reported when assessing skeletal health of laying hens. This study showed few differences between orientations of the bones with only radius width and ulna strength showing a difference ($p = 0.044$ and $p = 0.050$ respectively). As previously stated, these results could be due to errors within measurements or outliers. However, it is also possible that as the hens are only just past the point of sexual maturity at ~20 weeks (Hester, 2017), some bones may still be going through latter stages of development and bone growth may not have completely stopped at 24 weeks of age suggested that individual bones develop at different rates (Kim *et al*., 2012). Although the system types of pilot study 2 and pilot study 3 were the same, the differences within individual houses on the different farms may have also influenced the results as not all commercial housing systems within the same type are identical.

3.4.5.4 Effect of housing system

As previously mentioned in section 3.5 bone length and width are not commonly used in assessing skeletal health in laying hens. Within this study, housing system had no effect on bone length or bone width at this age. However, in pilot study 2 housing system did show significant effects on bone length and width of multiple bones. Bone length showed the left femur ($p = 0.004$), left tibia ($p < 0.001$), right ulna ($p = 0.043$), right tibia ($p < 0.001$), and keel bone ($p < 0.001$) were all longer from the free-range system. Bone widths showed the left humerus ($p < 0.001$), left femur ($p = 0.009$), right humerus ($p < 0.001$) and keel bone ($p < 0.001$) were significantly wider in the free-range system. The results from pilot study 2 do not support results from this study as no effect of housing system was found in bone length and width of hens ages 24 weeks. Although the ages of the different between the two studies, the previous assumption that bone growth does not occur after sexual maturity or point of lay may be incorrect. It is possible that the differences in bone parameters at end of lay were caused by the effects of housing system and is newly suggested that the difference of opportunity for exercise between the housing systems does influence bone morphometry even passed the point of lay. It is possible that as the hens will have only recently moved into the respective laying systems at 24 weeks, the effect of housing system has not influenced bone length or width yet. Birds may have become stressed during transportation (Hedlund *et*

al., 2019), or may still be acclimatising to their new environmental and thus performing less exercise.

Table 3.9 showed that bone weights in laying hens at the beginning of lay were not significantly affected by housing system and only a trend was shown in the left humerus being heavier in free-range hens compared to caged hens ($p = 0.073$). A previous study showed that the right tibia and right femur were not significantly affected by housing system ($p = 0.19$ and $p = 0.13$) between conventional cages, furnished cages and free-range (Regmi *et al*., 2016). Keel on the other hand did show a significant difference between housing systems ($p < 0.01$), showing a free-range and furnished cage keel weights were significantly heavier than conventional cage keel weights. The results from Regmi *et al*., (2016) support results from the right tibia and right femur as in both studies neither bone showed an effect of housing system. However, results for the keel weight in the previous study do not support this study as keel weight was not affected by housing system in the current study. Although Regmi *et al*., (2016) support the results of tibia and femur weights and disagrees with results on keel bone weight, the differences in age between the two studies could have had an effect on the results as one study uses hens at beginning of lay (this study) and Regmi *et al*., (2016) uses end of lay. Another recent study found that left femur and left tibia weight were not affected by housing system, though left humerus was significantly heavier in a flattened floor system compared to conventional cages ($p = 0.009$) (Qiaoxian *et al*., 2020) Although Qiaoxian *et al*., (2020) compared a flattened floor system against conventional cages, results could support the present study as left humerus weight was trending in this study ($p = 0.073$) with both studies also showing no effect on left and right tibia. A reason why housing system was more likely to effect humerus in these studies than femur and tibia could be due to the difference in opportunities for vertical exercises which utilise the wing bone and muscles more in free-range or flattened floor systems compared to conventional or furnished cages (Campbell *et al*., 2019).

In addition, all bones apart from the left and right humerus showed a significant effect of housing system on bone weight ($p = 0.002$ or less) in pilot study 2. The difference between the results of this study compared to pilot study 2 could be the effect of age and bone

resorption. For example, it is known that as birds age bone resorption increases (Lay Jr *et al*., 2011) and the effect of exercise on supressing bone resorption diminishes (Hester, 2017). Therefore, bone resorption may be exacerbated in later ages as between different housing systems differing levels of exercise can occur – causing a difference in results seen in pilot study 2 and the present study. At a younger age, mineral deposits within a bone are thought be sufficient to produce and eggshell without high levels of bone resorption (Dacke *et al*., 2015).

Bone strength was the only bone parameter to show an effect of housing system (Table 3.9), with all bones, left humerus (p = 0.011), left ulna (p = 0.030), left femur (p = 0.008), left tibia (p = 0.036), right humerus (p = 0.001), right radius (p = 0.030) and right femur (p = 0.029) showing greater breaking strengths from the free-range system. Results from a previous showed that the failure moment of the left tibia (free-range = 5.08Nm v Caged = 4.53Nm) and left humerus (free-range = 3.62Nm v 2.51Nm) were significantly greater in an aviary system than a conventional caged system (p < 0.001). It is implied that breaking strength (N) and failure moment (Nm) are similar measures and therefore the results from Regmi *et al*., (2015) support this study as both humerus bones and the left tibia within the present study also showed an effect of housing system, though the systems used in the previous study are not identical but similar. Another study also found that tibia breaking strength (left and right averaged) of hens aged 67 weeks were affected by housing system, with a floor-based system (156.6N) providing higher breaking strength than bones from conventional (92.7N) or furnished cages 131.4N) (p < 0.005) (Lichovníková and Zeman, 2008). Although a free-range system was not used, this study (Lichovníková and Zeman, 2008) supports results from the present study that housing systems with more exercise opportunities improve bone strength as both studies showed an increase the tibia bone strength. A study by Casey-Trott *et al*., (2017c) found that bone breaking strength of the humerii in birds 73 weeks of age was significantly increased by the type of rearing system used (p < 0.001), with humerus strength (kg) coming from a conventional rearing system (9.2kg) found to be stronger than those in an aviary rearing system (6.3kg). Although a direct comparison cannot be made because rearing system was not reported in the present study, Casey-Trott *et al*., (2017c) highlights the impact

rearing systems can have in later life, albeit not in support of this study as humerus breaking strength was significantly greater in free-range than cages in the present study.

Compared to pilot study 2, less bones were found to be affected by housing system in this study. All bones in pilot study 2 showed a significant increase of bone strength in the free-range system compared to the caged system. Whereas in this study, bones except the left radius, right ulna, right tibia, and keel bone were affected by housing system (p = 0.106 or higher). The difference in number of bones showing an effect of housing system shown between the two studies could have been influenced by the differences in age (24 weeks compared to 76 and 79 weeks). Younger hens have been in the egg laying cycle less, therefore less bone resorption has occurred from the demand of eggshell production and had less of an impact on the structural integrity of the bones which can influence bone strength. It is known that bone resorption removes structural bone and replaces it with medullary bone (Whitehead, 2004). Although this may cause bone weights to be similar, medullary bone does not provide the mechanical support that structural bone does. Hence, the more structural bone lost through bone resorption, the weaker bones will be with age (Whitehead and Fleming, 2000).

3.4.6 Conclusion

There was little effect of orientation on any bone or bone parameter in this study with only two results showing effect of orientation being radius width and ulna strength (p = 0.044 and p = 0.050 respectively). Though the credibility of these results is uncertain as all other results showed no effect of orientation, it may still be advised to assess left and right bones in young laying hens. This is because laying hens aged around the point of lay or sexual maturity may have not totally stopped bone development at this stage and require more time to fully mature, although the majority of bone growth is known to have stopped by this point it could be possible. Moreover, as in pilot study 2 it would still be recommended to take multiple bone types as the form and function of bones differ which could affect bone parameters even at a younger age, particularly as the effect of rearing system is known to effect bone development (Casey-Trott *et al*., 2017a). In this study length, width and weight showed no significant effects of housing system on any parameter yet strength did. It could be proposed that strength is

the most sensitive bone parameter for measuring skeletal health in laying hens at the beginning of lay and should be used in future work as is most common in previous research. However, as length, width and weight showed many effects of housing system in pilot study 2 in end of lay hens, it could be suggested that these parameters become more or less effective at highlighting any differences in the factors being assessed depending on the age of the bird.

3.5 Pilot studies' conclusion and outcomes

As mentioned, pilot study 1 was carried out opportunistically in an ongoing broiler trial. Although not laying hens, some key outcomes and conclusions were gained from the study. Pilot study 1 showed the basis of taking multiple bones which cover different areas of the body, as different bones have different forms and functions which may also be affected differently by growth and development. The usefulness of sampling different orientations of bone is also something that should be considered in future studies. Studies that focus on younger poultry should monitor bone orientation as bone development was suggested to be inconsistent between orientation of the same bone. However, the cause of different orientations of a bone developing at different rates is still unclear.

Unlike pilot study 1, there was no effect of orientation in bone parameters of end of lay hens in pilot study 2. This was thought to be because sexual maturity is reached before the birds are introduced to the laying system and skeletal growth should have stopped at the beginning of lay. It was therefore concluded that orientation was not a key factor in end of lay hens when assessing skeletal health. Though it is possible that factors effecting growth and development during the rearing stage of the hen's life could carry through to the end of lay and show historic differences. An outcome of this result was that although orientation showed no effect, both bones could still be taken to provide a more accurate dataset by averaging data when assessing skeletal health. As in pilot study 1, data from laying hens in pilot study 2 also confirmed that sampling multiple bones to cover different bones' form and functions was beneficial, though not all the bones taken in this study were required. The bones most assessed in previous work seemed to be the keel, humerus and tibia. Therefore,

another outcome of this pilot study was that these bones should be used in future work to cover locomotory activity of different parts of the hens' body and the effects on skeletal health. Within pilot study 2, the credibility of length and width parameters in assessing skeletal health were considered ambiguous. This was because significant effects in length and width data were present although growth of the bone should have ceased once sexual maturity was passed. It was considered that perhaps bone breaking strength and bone weight are more reliable bone parameters for examining the effects of housing system on skeletal health, as bone metabolism still occurs throughout lay help to produce eggshell. As such an outcome of this pilot study is that bone breaking strength and bone weight should be considered more suitable parameters of assessing skeletal health and applied into subsequent work.

Pilot study 3 was conducted similarly to pilot study 2, to investigate if similar effects of housing systems and orientation were present in hens at the beginning of lay opposed to the end of lay. Pilot study 3 showed little effect of orientation on any bone or bone parameter. Radius width and ulna strength did show an effect of orientation, though the reliability of these results is uncertain. An outcome of this study was to still assess left and right bones in young laying hens as skeletal development may not have stopped fully after sexual maturity, causing some minor changes between orientations of the same bone. This would also help achieve a more accurate dataset as stated previously. Again, as in pilot studies 1 and 2, results from pilot study 3 also concluded that multiple bones are recommended to assess skeletal health as results showed different bones react differently depending on form and function. The need to use multiple bones in younger birds may be more crucial. As already indicated, growth may not have fully stopped in birds just reaching the point of lay and the effects of the rearing system, which is known to influence bone development may have yet to be seen at this age. In this pilot study, the only bone parameter to show any significant effect of housing system was bone breaking strength. The results could confirm suggestions that bone strength is a reliable measure of skeletal health, supported by pilot study 2 which also showed similar results. However, bone weight in pilot study 3 showed no effect of housing system. It could be possible that different bone parameters, length, width, weight, or strength be more or less effective depending on the age of the birds being sampled. Still, an outcome of this study was

that bone strength could be considered the most sensitive bone parameter when assessing skeletal health and should be a key focus in subsequent work.

Chapter 4: How housing systems effect skeletal integrity

4.1 Introduction

Over the past 20 years, consumer perceptions of how eggs should be produced has changed significantly, thus causing the industry to adapt to the needs of its consumers (Fröhlich *et al*., 2012). From 2012, the EU Council Directive 1994/74/EC banned the use of conventional cages forcing the EU to only use enriched caged systems or alternative systems to produce eggs. Though the move was accepted by producers as the correct way to proceed in-regards to hen welfare, challenges in the egg industry have arisen since the directive. Such as how to maintain skeletal integrity throughout varying systems to achieve the best welfare possible whilst still achieving a similar output (Chambers *et al*., 2017). It is common knowledge within the poultry sector that the type of housing system and availability to perform exercise has a large part to play in maintaining skeletal integrity in laying hens (Leyendecker *et al*., 2005; Wilkins *et al*., 2011; Regmi *et al*., 2016). Exercise or weight bearing activities, such as climbing on a perch, can improve bone mass in pullets (Regmi *et al*., 2015) and decrease bone resorption in hens due to the reduction of osteoclast activity (Fleming *et al*., 2006; Shipov *et al*., 2010). On the other-hand, alternative systems with additional apparatus may also cause more skeletal fractures and KBD (Wilkins *et al*., 2004). Though there is an abundance of research comparing the effects of housing system on skeletal integrity at specific time intervals, there is a lack of research investigating the effect of housing systems over longitudinal studies. The general aim of this study was to investigate the effects of housing system on skeletal integrity throughout the laying period and then to begin to predict skeletal integrity measures using the data collected.

Aims

- To investigate how bone strength as a measure of skeletal integrity is affected by housing system and age throughout the laying period
- To investigate how bone ash content as a measure of skeletal integrity is affected by housing system and age throughout the laying period
- To model predicted bone strength for each system throughout the laying period

4.2 Methods

4.2.1 Trial period

Six laying hens of various breeds (Lohmann Brown Classics, Lohmann Brown Lite, Hy-line Brown or Bovan Brown depending on farm) were collected from 14 farms every six weeks from age 18 weeks until 72 weeks (n = 840). The participating farms were sourced by Noble Foods Ltd, and birds were housed in various housing systems including free-range flat deck, free-range multi-tier, colony cage, barn or organic with each system group comprising of 3 farms (2 for barn). All birds were provided with a customised commercial diet, specific to farm requirements and egg production weights. The majority of farms used diets produced by Noble Foods Ltd, with some farms using their own feed producers or another external feed producer. Dietary information for all participating farms can be found in Appendix 1. Any additional supplements or medication were recorded at the time of sample collection. Water was provided *ad libitum* in all farms via line drinkers, nipple drinkers or floor drinkers. National guidelines for bird husbandry were followed throughout the study.

Upon arrival to the farms, hens were selected by the farmer then euthanised via cervical dislocation and were stored in a cool box ready to be transported back the Poultry Research Unit. A collection sheet was filled in by the farmer detailing, the farm name, date of collection, housing system, breed, age of the bird (weeks), mortality, diet, feeding regime, health or environmental concerns and medication or supplements provided (Appendix 2).

4.2.2 Bird and bone samples

On return to the labs, birds were weighed individually on a 1.d.p top pan balance (Mettler Toledo International). The birds were then dissected to remove the keel bone, left humerus, right humerus, left tibia and right tibia, put in labelled bag per bird and farm and stored at -20°C if not defleshed immediately (section 2.7.1). The sample bones were defleshed of muscle and connective tissues using a scalpel and scissors ensuring to keep any cartilage intact following the methods described in section 2.7.2. For each bone, bone weight was recorded using a 4.d.p analytical balance (Satorius, UK). Bone breaking strength was measured using a texture analyser machine (TA.XT 100; Stable Micro Systems, Guildford) with a 3-point bend

attachment (HDP/3PB; Stable Micro Systems, Guildford) and a 100KG loadcell. Bones were placed horizontally across the attachment and then broken using the methods and settings outlined in section 2.7.3. Once broken, bones were placed in individual foil pie trays and dried at 105°C for 3 days and then weighed to determine dry weight on a 4.d.p analytical balance (Satorius, UK). Next the bones were placed individually in pre-labelled and pre-weighed crucibles and burnt in a furnace at 650°C for 12 hours and recorded for ash weight afterwards as explained in section 2.7.3. Bone ash content was then calculated using the formula:

$$Bone\ ash\ content\ (\%) = \left(\frac{Bone\ ash\ weight\ (g)}{Bone\ dry\ weight\ (g)}\right) \times 100$$

4.2.4 Data analysis

Data from all samples were stored in a database created in Microsoft Excel. A two-way ANOVA (R version 4.1.0 and RStudio version 1.1.463) was used to investigate the effect of housing system and age on body weight and bone strength and ash content for each bone. Outliers were removed using 2 standard deviations from the mean and any effects or interactions were considered significant using $p < 0.05$ as the significance threshold. In addition, a Gaussian Linear Mixed Model (LMM) (R version 4.1.0 and RStudio version 1.1.463) was used to model bone strength over the period of lay for each bone and housing system. The formulation for the model of bone strength was as follows:

$Strength_{ij} \sim Gaussian(\mu_{ijk}, \sigma^2)$

$E(Strength_{ijk}) = \mu_{ijk}$ and $\text{var}(Strength_{ijk}) = \sigma^2$

$\mu_{ijk} = Intercept + Weight_{ijk} + Age_{ijk} \times Housing_{ijk} + Age_{ijk} \times Bone_{ijk} +$

$Bird_j + Farm_k$

$Bird_j \sim Gaussian\ (0, \sigma^2_{Bird})$

$Farm_k \sim Gaussian\ (0, \sigma^2_{Farm})$

Where $Strength_{ij}$ is the breaking strength (N) of bone *i* from *Bird j* reared on *Farm k* assuming a normal distribution with mean μ_{ijk} and variance σ^2. $Weight_{ijk}$ is a continuous covariate indicating the weight of bone *i* (g), Age_{ijk} is a continuous covariate indicating the age of bird (weeks) from which bone *i* was obtained, $Housing_{ijk}$ is a categorical covariate indicating the housing systems from which bone *i* was obtained, and $Bone_{ijk}$ is a categorical covariate indicating bone type. The random intercepts $Bird_j$ and $Farm_k$ are included in the model to introduce a correlation structure between observations for bones obtained from the same bird nested within the same farm, each with variance σ^2_{Bird} and σ^2_{Farm} which is assumed to be distributed normally and equal to 0.

4.3 Results

4.3.1 Body weight

Table 4.1 and Fig. 4.1 shows that the body weight of the hens was significantly affected by both age and housing system ($p < 0.001$) but there was no interaction between these factors ($p = 0.252$). As age increases bodyweight in all systems increases. Generally, the organic systems have the highest weight, followed by the free-range systems, caged and barn shows the lowest body weight. The lack of any interaction indicates that the effect of housing system on body weight does not depend on age.

Table 4.1 A comparison of hen body weights between different housing systems throughout the laying period

Age (weeks)	Housing System	Body weight (g) (±SE)
18	**MT**	1562.1 (50.01)^{ab}
	C	1486.9 (46.00)^{a}
	FD	1616.8 (55.29)^{ab}
	O	1655.0 (41.47)^{b}
	B	1487.1 (67.71)^{a}
24	**MT**	1711.4 (39.09)^{a}
	C	1744.7 (41.47)^{a}
	FD	1716.9 (39.09)^{a}
	O	1780.1 (40.23)^{a}
	B	1754.5 (47.88)^{a}
30	**MT**	1717.6 (39.09)^{a}
	C	1740.7 (39.09)^{a}
	FD	1882.3 (47.88)^{b}
	O	1921.3 (39.09)^{b}
	B	1740.7 (47.88)^{a}
36	**MT**	1724.8 (40.23)^{a}
	C	1769.3 (39.09)^{ab}
	FD	1780.1 (39.09)^{ab}
	O	1863.2 (39.09)^{b}
	B	1718.3 (47.88)^{a}
42	**MT**	1736.8 (39.09)^{a}
	C	1796.8 (41.47)^{ab}
	FD	1867.2 (39.09)^{b}
	O	1864.7 (40.23)^{b}
	B	1681.8 (50.01)^{a}
48	**MT**	1961.2 (40.23)^{a}
	C	1837.4 (40.23)^{b}
	FD	1899.5 (39.09)^{ab}
	O	1959.2 (40.23)^{a}
	B	1827.5 (47.88)^{b}

54	**MT**	1919.1 (58.64)[a]
	C	1832.5 (40.23)[ab]
	FD	1839.0 (47.88)[ab]
	O	1921.4 (41.47)[a]
	B	1751.9 (47.88)[b]
60	**MT**	1876.8 (39.09)[ab]
	C	1897.8 (39.09)[a]
	FD	1908.2 (39.09)[a]
	O	1913.1 (44.33)[a]
	B	1760.7 (50.01)[b]
66	**MT**	1882.1 (40.23)[a]
	C	1901.3 (41.47)[a]
	FD	1952.7 (47.88)[a]
	O	1903.3 (39.09)[a]
	B	1753.7 (47.88)[b]
72	**MT**	1956.5 (39.09)[a]
	C	1839.0 (40.23)[bc]
	FD	1949.1 (47.88)[ab]
	O	1931.2 (39.09)[ab]
	B	1771.2 (52.45)[c]
***p* value**		
Age		< 0.001
System		< 0.001
Age*System		0.252

Means within an age group with different letters are significantly different ($p = 0.05$)
MT = multi-tier free-range, C = colony cage, FD = flat deck (one-tier) free-range, O = organic, B = Barn

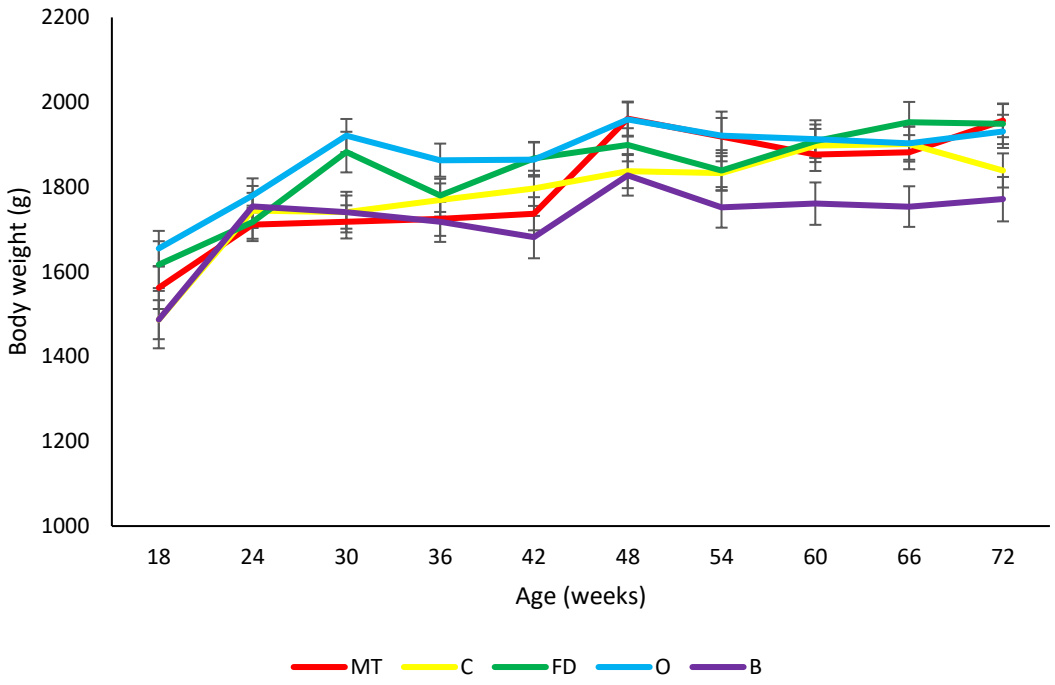

Fig. 4.1 Comparison of body weight at different ages in different systems

4.3.2 Bone strength

Table 4.2 and Fig. 4.2 – 4.4 show that the strength of keel, humerus and tibia were significantly affected by age, housing system, with an interaction effect between age and housing system ($p < 0.001$). The interaction results indicate that the effect of housing system on bone strength depends on the age of the bird. For example, keel bone strength at age 18 weeks is significantly different between the multi-tier and caged systems but are not significantly different at 24 weeks of age. Humerus strength was not significantly different in barn or caged systems at 18 weeks or 24 weeks but was significantly different at 30 weeks. In tibia strength, multi-tier and flat deck systems were significantly different at 18 weeks but were not at 24 weeks.

Table 4.2 Summary of effect of housing system over time on bone strength of keel, humerus and tibia

Age (weeks)	Housing System	Bone strength (N) (±SE)		
		Keel	**Humerus**	**Tibia**
18	**MT**	173.13 (13.542)^{a}	251.65 (12.197)^{a}	242.37 (10.975)^{a}
	C	105.38 (13.542)^{b}	190.04 (12.197)^{b}	230.76 (10.666)^{a}
	FD	191.53 (16.585)^{a}	271.28 (14.939)^{a}	278.65 (13.644)^{b}
	O	123.90 (14.363)^{bc}	282.58 (12.937)^{a}	278.08 (11.313)^{b}
	B	154.58 (16.585)^{ac}	260.53 (14.939)^{a}	248.95 (13.063)^{ab}
24	**MT**	179.63 (13.542)^{a}	285.95 (12.197)^{a}	303.74 (10.666)^{a}
	C	156.57 (13.542)^{ab}	203.26 (12.197)^{b}	280.16 (10.666)^{a}
	FD	120.59 (13.542)^{bc}	241.32 (12.197)^{c}	290.99 (10.666)^{a}
	O	120.05 (13.542)^{bc}	273.90 (12.197)^{ac}	308.23 (10.666)^{a}
	B	94.46 (16.585)^{c}	239.24 (14.939)^{bc}	288.60 (13.063)^{a}
30	**MT**	141.68 (13.542)^{ab}	256.42 (12.197)^{a}	288.27 (11.313)^{a}
	C	85.05 (13.542)^{c}	163.12 (12.197)^{b}	219.37 (10.666)^{b}
	FD	150.34 (16.585)^{ab}	268.55 (14.939)^{a}	327.32 (13.644)^{c}
	O	169.19 (13.542)^{a}	267.06 (12.197)^{a}	304.36 (10.975)^{ac}
	B	127.12 (16.585)^{b}	270.81 (14.939)^{a}	284.40 (13.063)^{a}
36	**MT**	130.32 (13.542)^{ab}	249.05 (12.197)^{ab}	228.73 (10.666)^{a}
	C	107.16 (13.542)^{a}	189.42 (12.551)^{c}	238.93 (10.975)^{a}
	FD	163.51 (13.542)^{b}	241.48 (12.197)^{ad}	270.74 (10.666)^{b}
	O	236.83 (13.542)^{c}	280.41 (12.197)^{b}	326.57 (11.684)^{c}
	B	101.89 (16.585)^{a}	204.00 (14.939)^{cd}	248.39 (13.644)^{ab}
42	**MT**	108.96 (13.542)^{a}	230.46 (12.197)^{a}	267.21 (10.666)^{a}
	C	129.38 (13.542)^{ab}	182.02 (12.197)^{b}	235.53 (11.313)^{b}
	FD	155.81 (13.542)^{b}	210.09 (12.197)^{ab}	239.35 (10.666)^{ab}
	O	142.59 (13.542)^{ab}	235.22 (12.197)^{a}	297.95 (10.975)^{c}
	B	113.05 (16.585)^{a}	174.65 (14.939)^{b}	257.99 (13.063)^{ab}
48	**MT**	146.04 (13.542)^{ab}	208.99 (12.197)^{ab}	242.50 (10.666)^{a}
	C	169.09 (13.542)^{a}	182.88 (12.197)^{ac}	225.53 (10.666)^{a}
	FD	113.58 (13.542)^{bc}	209.92 (12.197)^{ab}	248.49 (10.666)^{a}
	O	119.69 (13.542)^{bc}	230.06 (12.551)^{b}	285.30 (10.975)^{b}
	B	92.79 (16.585)^{c}	164.82 (14.939)^{c}	251.70 (13.063)^{a}

54	**MT**	209.09 (16.585)^{a}	199.73 (14.939)^{ab}	256.40 (13.063)^{ab}
	C	116.55 (13.542)^{b}	185.65 (12.197)^{ab}	215.67 (10.666)^{c}
	FD	112.37 (16.585)^{b}	180.21 (14.939)^{a}	265.65 (13.063)^{ab}
	O	113.86 (13.542)^{b}	218.79 (12.197)^{b}	290.32 (11.684)^{a}
	B	121.84 (16.585)^{b}	172.35 (14.939)^{a}	245.50 (13.063)^{bc}
60	**MT**	126.77 (13.542)^{a}	197.49 (12.197)^{a}	255.64 (10.666)^{ab}
	C	79.65 (13.542)^{b}	168.89 (12.551)^{a}	207.68 (10.666)^{c}
	FD	85.53 (13.934)^{b}	169.75 (12.197)^{a}	234.41 (10.666)^{ac}
	O	132.14 (13.542)^{a}	233.66 (12.197)^{b}	271.18 (12.094)^{b}
	B	96.87 (16.585)^{ab}	160.50 (14.939)^{a}	227.51 (13.063)^{ac}
66	**MT**	131.16 (13.542)^{a}	192.98 (12.197)^{ab}	270.22 (11.313)^{ab}
	C	78.90 (13.934)^{b}	145.73 (12.197)^{c}	207.46 (10.666)^{c}
	FD	96.82 (16.585)^{ab}	189.50 (14.939)^{ab}	243.08 (13.063)^{ad}
	O	113.92 (13.542)^{ab}	218.97 (12.197)^{a}	287.69 (10.666)^{b}
	B	110.46 (16.585)^{ab}	180.73 (14.939)^{bc}	232.29 (13.063)^{cd}
72	**MT**	130.80 (13.542)^{a}	196.42 (12.197)^{ab}	270.08 (10.975)^{a}
	C	81.74 (14.363)^{b}	155.00 (12.937)^{c}	205.94 (11.313)^{b}
	FD	116.34 (23.455)^{ab}	199.09 (21.126)^{abc}	301.38 (20.237)^{a}
	O	109.20 (13.542)^{ab}	215.62 (12.197)^{a}	268.11 (10.975)^{a}
	B	109.14 (16.585)^{ab}	164.11 (14.939)^{bc}	218.73 (13.644)^{b}
***p* value**				
Age		< 0.001	< 0.001	< 0.001
System		< 0.001	< 0.001	< 0.001
Age*System		< 0.001	< 0.001	< 0.001

Means within a column within an age group with different letters are significantly different (p = 0.05)

MT = multi-tier free-range, C = colony cage, FD = flat deck (one-tier) free-range, O = organic, B = Barn

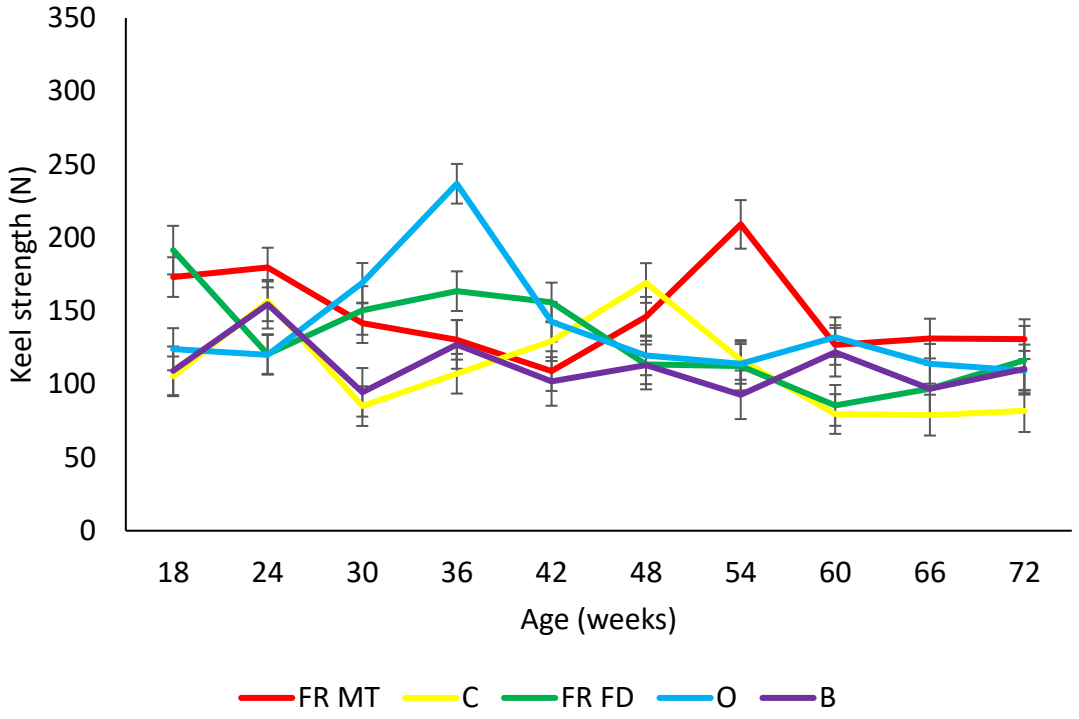

Fig. 4.2 Effect of age and housing system on keel bone strength

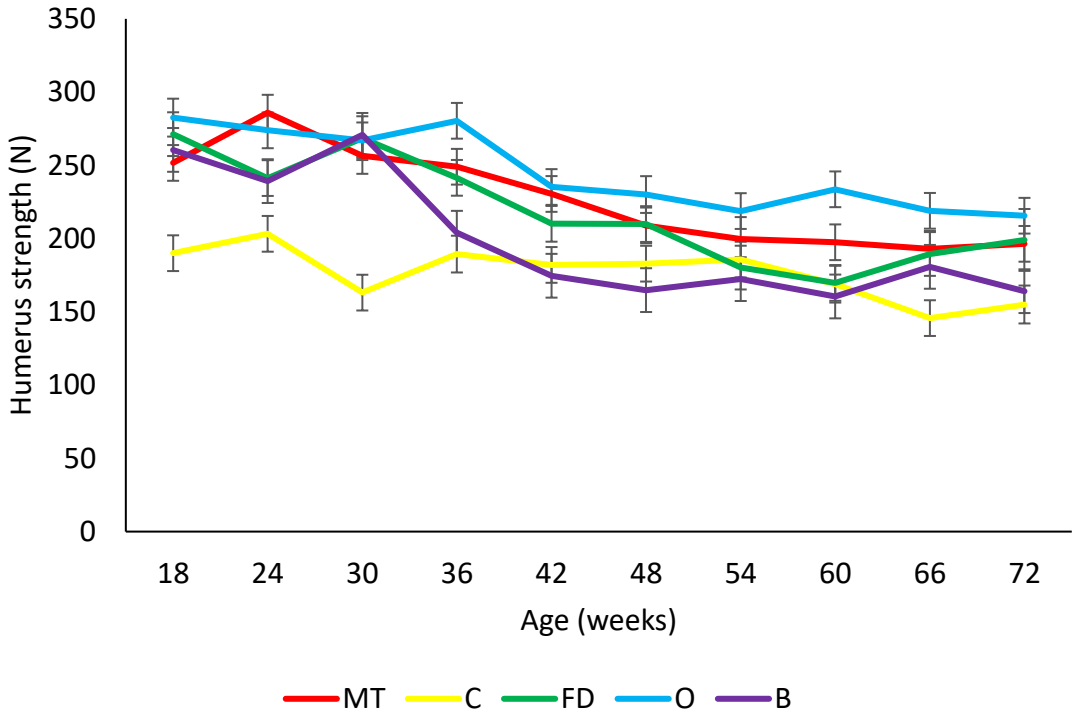

Fig. 4.3 Effect of age and housing on humerus strength

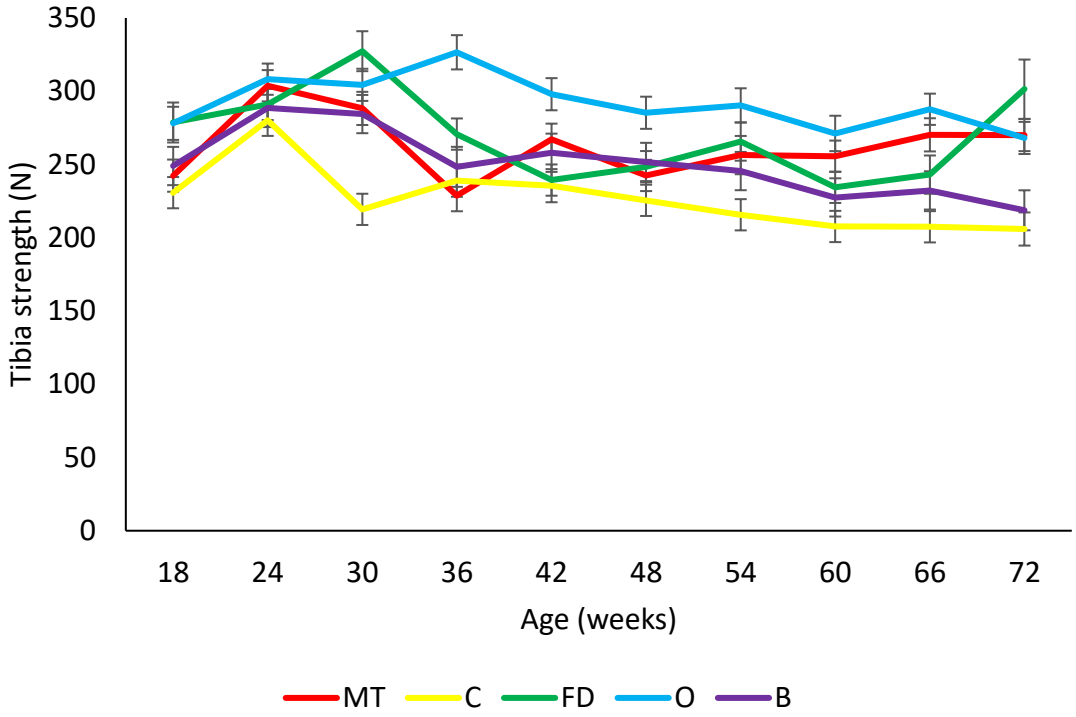

Fig. 4.4 Effect of age and housing system on tibia strength

4.3.3 Bone ash content

Table 4.3 and Fig. 4.5 – 4.7 show bone ash content of the keel, humerus and tibia were also significantly affected by age and housing system, and an interaction effect was present ($p < 0.001$). The interaction effect indicates that the effect of housing system on bone ash content of each bone is dependent on the age of the bird. For example, ash content of the keel bone in multi-tier and caged birds at age 60 weeks are not significantly different from one and other, yet at 66 weeks there is a significant difference. The humerus ash content in organic and multi-tier are not significantly different at 24 weeks but are significantly different at 30 weeks. In tibia ash content, multi-tier and flat deck show a significant difference at 60 weeks but no significant difference at 66 weeks.

Table 4.3 Summary of effect of housing system over age on bone ash content of keel, humerus and tibia

Age (weeks)	Housing System	Ash content (%) (±SE)		
		Keel	**Humerus**	**Tibia**
18	**MT**	42.25 (0.866)^{a}	54.25 (0.611)^{a}	38.77 (0.768)^{ab}
	C	40.84 (0.866)^{a}	49.47 (0.648)^{b}	35.91 (0.867)^{c}
	FD	42.83 (1.060)^{a}	53.81 (0.748)^{a}	40.05 (0.909)^{a}
	O	42.70 (0.918)^{a}	52.79 (0.648)^{ac}	38.03 (0.797)^{abc}
	B	43.06 (1.060)^{a}	51.21 (0.748)^{bc}	36.80 (1.016)^{bc}
24	**MT**	45.79 (0.866)^{ab}	54.35 (0.611)^{ab}	42.33 (0.677)^{a}
	C	44.08 (0.866)^{a}	52.79 (0.629)^{a}	41.91 (0.677)^{a}
	FD	47.12 (1.060)^{bc}	54.82 (0.782)^{b}	42.94 (0.830)^{ab}
	O	48.43 (0.866)^{c}	54.55 (0.611)^{b}	44.36 (0.677)^{b}
	B	48.37 (1.107)^{bc}	53.71 (0.748)^{ab}	43.84 (0.830)^{ab}
30	**MT**	47.81 (0.866)^{a}	56.29 (0.611)^{a}	43.87 (0.697)^{ab}
	C	42.72 (0.866)^{b}	50.30 (0.748)^{b}	40.08 (0.677)^{c}
	FD	45.35 (1.060)^{ab}	52.23 (0.748)^{b}	42.10 (0.867)^{ac}
	O	46.79 (0.918)^{a}	54.29 (0.611)^{c}	44.72 (0.677)^{b}
	B	47.59 (1.107)^{a}	54.38 (0.748)^{c}	43.69 (0.830)^{ab}
36	**MT**	47.03 (0.866)^{a}	55.76 (0.611)^{a}	43.10 (0.677)^{ab}
	C	44.83 (0.866)^{a}	51.46 (0.629)^{b}	41.43 (0.697)^{a}
	FD	46.10 (0.891)^{a}	53.87 (0.611)^{c}	43.34 (0.677)^{ab}
	O	42.03 (0.866)^{b}	53.32 (0.629)^{c}	44.46 (0.677)^{b}
	B	44.96 (1.060)^{a}	52.48 (0.782)^{bc}	42.95 (0.867)^{ab}
42	**MT**	43.95 (0.866)^{ab}	53.30 (0.611)^{a}	43.04 (0.677)^{abc}
	C	44.37 (0.866)^{ab}	50.97 (0.611)^{b}	41.21 (0.677)^{a}
	FD	42.31 (0.866)^{a}	51.32 (0.693)^{b}	42.63 (0.677)^{ab}
	O	46.89 (0.866)^{c}	53.68 (0.611)^{a}	44.68 (0.718)^{c}
	B	45.77 (1.060)^{bc}	54.09 (0.82)^{a}	43.74 (0.867)^{bc}
48	**MT**	44.61 (0.866)^{ab}	54.10 (0.611)^{a}	42.72 (0.677)^{a}
	C	42.19 (0.918)^{a}	50.92 (0.611)^{b}	40.44 (0.677)^{b}
	FD	46.14 (0.866)^{b}	53.73 (0.648)^{a}	42.76 (0.677)^{a}
	O	45.11 (0.866)^{b}	53.62 (0.629)^{a}	44.08 (0.677)^{a}
	B	43.81 (1.107)^{ab}	53.37 (0.748)^{a}	43.60 (0.867)^{a}

54	**MT**	42.33 (1.060)[a]	51.80 (0.748)[a]	41.38 (0.830)[a]
	C	45.36 (0.918)[b]	52.04 (0.648)[a]	42.08 (0.677)[ab]
	FD	46.50 (1.060)[bc]	53.36 (0.748)[ab]	44.50 (0.830)[c]
	O	47.45 (0.866)[bc]	54.19 (0.611)[b]	44.89 (0.742)[c]
	B	49.57 (1.298)[c]	56.83 (0.916[)c]	43.98 (0.958)[bc]
60	**MT**	45.74 (0.866)[a]	53.36 (0.611)[a]	43.41 (0.677)[a]
	C	45.16 (0.866)[a]	52.84 (0.611)[a]	42.73 (0.697)[ab]
	FD	42.67 (0.866)[b]	52.24 (0.611)[a]	41.33 (0.677)[b]
	O	45.40 (0.891)[a]	53.42 (0.648)[a]	44.28 (0.742)[a]
	B	45.90 (1.060)[a]	51.97 (0.748)[a]	43.75 (0.830)[a]
66	**MT**	46.00 (0.866)[a]	53.94 (0.611)[a]	44.18 (0.718)[a]
	C	37.74 (1.060)[b]	47.39 (0.748)[b]	38.95 (0.867)[b]
	FD	45.88 (1.060)[a]	52.26 (0.748)[ac]	43.78 (0.830)[ac]
	O	46.34 (0.866)[a]	53.68 (0.611)[a]	44.80 (0.677)[a]
	B	44.81 (1.060)[a]	51.12 (0.748)[c]	41.90 (0.830)[c]
72	**MT**	45.64 (0.866)[a]	53.35 (0.611)[ab]	43.87 (0.742)[ab]
	C	42.73 (0.891)[b]	51.82 (0.629)[a]	41.35 (0.697)[c]
	FD	46.43 (1.060)[a]	53.84 (0.748)[b]	45.62 (0.909)[a]
	O	45.49 (0.866)[a]	52.80 (0.611)[ab]	43.66 (0.697)[ab]
	B	46.26 (1.060)[a]	53.25 (0.748)[ab]	42.55 (0.830)[bc]
***p* value**				
Age		< 0.001	< 0.001	< 0.001
System		< 0.001	< 0.001	< 0.001
Age*System		< 0.001	< 0.001	< 0.001

Means within a column within an age group with different letters are significantly different (p = 0.05)

MT = multi-tier free-range, C = colony cage, FD = flat deck (one-tier) free-range, O = organic, B = Barn

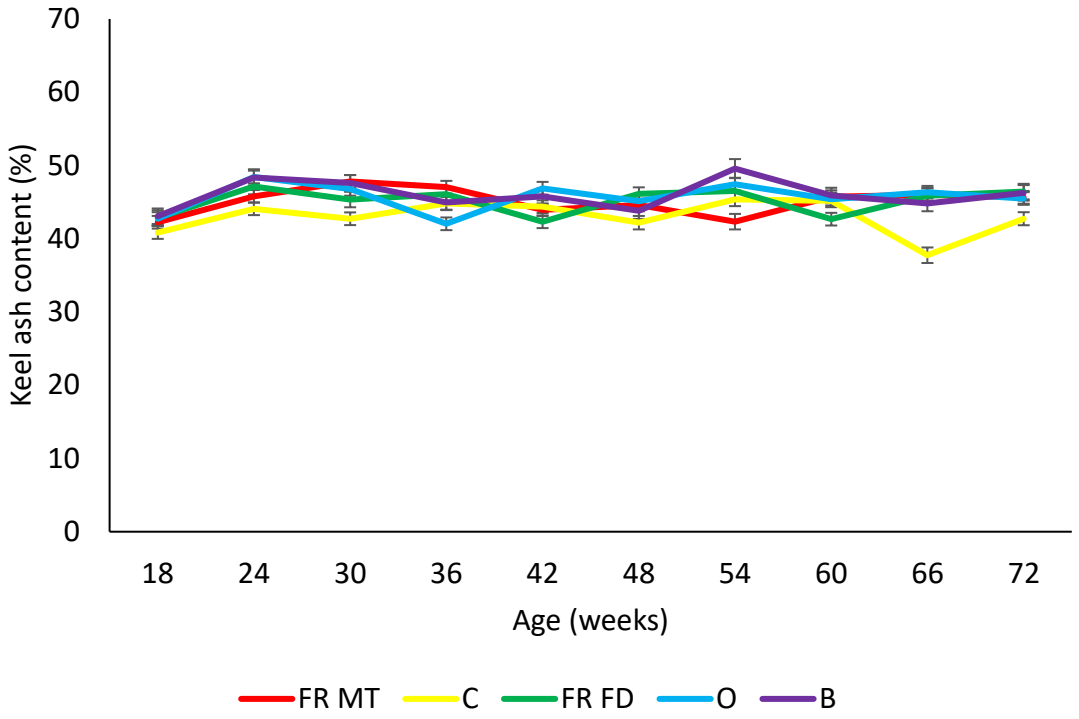

Fig. 4.5 Effect of age and housing system on keel ash content

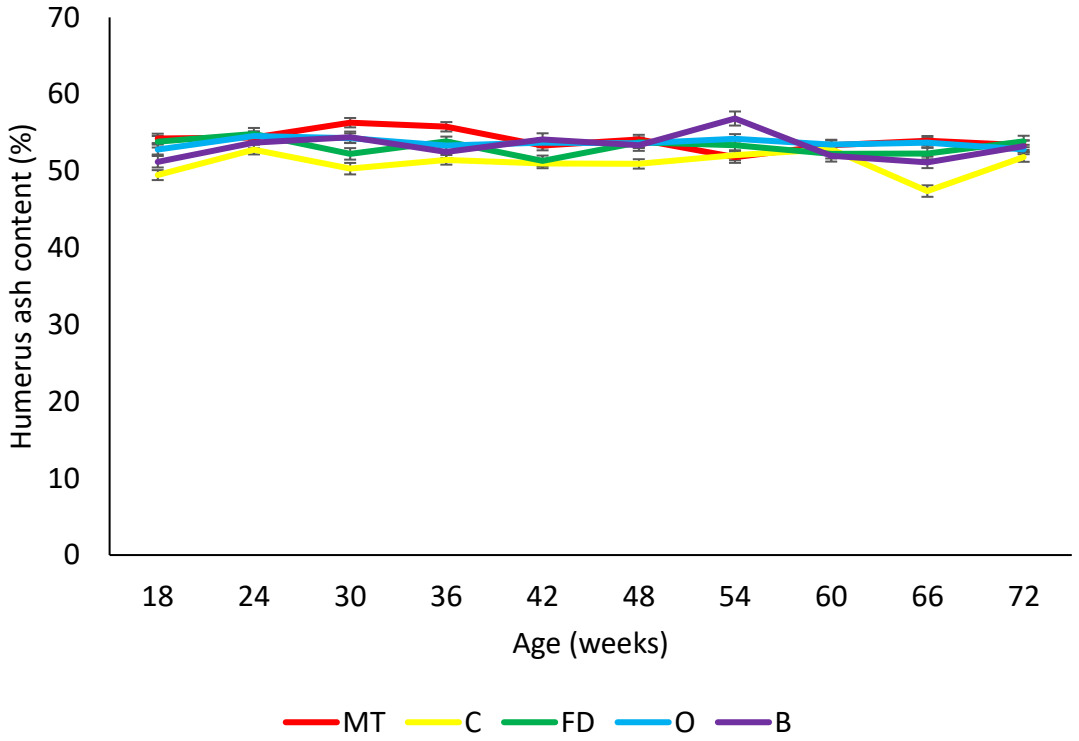

Fig. 4.6 Effect of age and housing system on humerus ash content

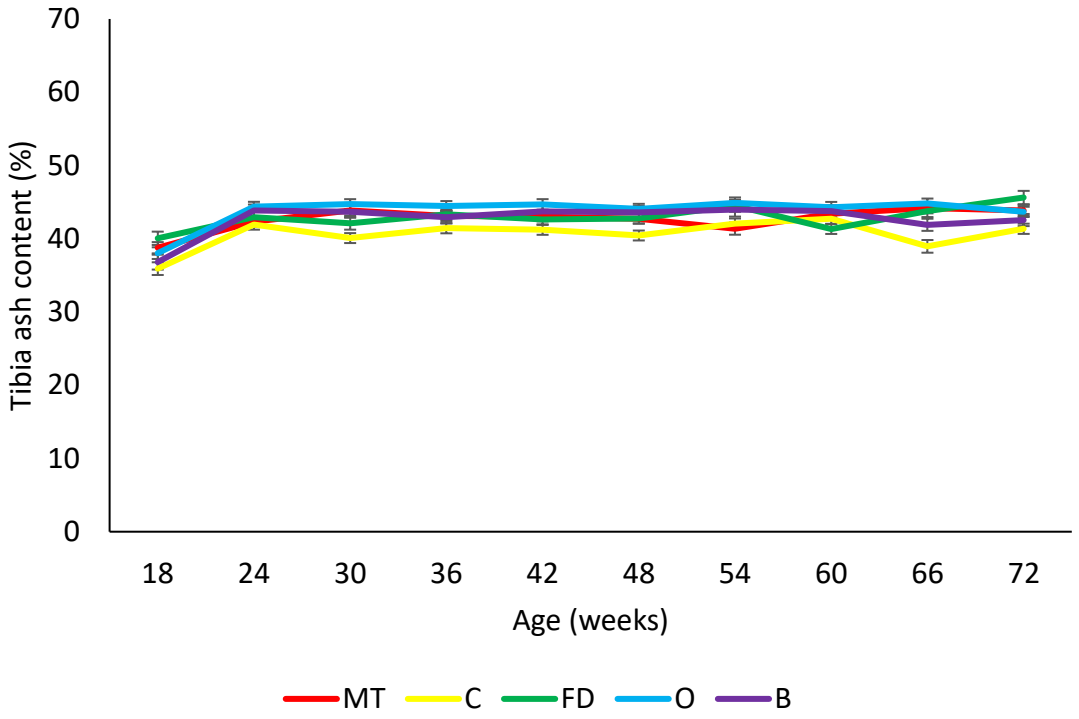

Fig. 4.7 Effect of age and housing system on tibia ash content

4.3.4 Predicting bone strength

A Gaussian Linear Mixed Model was fitted to the bone data to predict bone breaking strength over the laying period of hens from UK farms in multiple housing systems. Individual farm and bird were fitted as random terms and bone weight as a covariate. Conditional R^2 was 0.703 and marginal R^2 was 0.492. Model parameter estimates are shown in Table 4.4 and Fig. 4.8.

Table 4.4 shows the intercept (Caged tibia strength) differs significantly from 0 ($p < 0.001$). Bone weight is a significant predictor of bone strength ($p < 0.001$), with a parameter estimate of 23.60, indicating that as bone weight increases, bone strength increases at this rate. As a main effect, age was not significantly different from 0 ($p = 0.072$; estimate = -5.75), though as a trend as age increased, caged tibia strength decreased. Free-range flat deck bone strength was significantly greater than caged tibia strength ($p = 0.020$; estimate = 27.37). Free-range multi-tier bone strength is significantly stronger than caged tibia strength ($p < 0.001$; estimate = 38.30). Organic bone strength was also significantly stronger than caged tibia strength ($p < 0.001$; estimate = 56.78). The keel bone was significantly weaker than caged tibia ($p < 0.001$; estimate = -127.17). Barn bone strength and free-range flat deck bone strength both decline significantly faster than caged bone strength ($p = 0.024$; estimate = -10.64 and $p = 0.025$; estimate = -10.92 respectively). Humerus bone strength declines significantly faster than tibia bone strength ($p < 0.001$; estimate = -12.04). Keel bone strength also declines significantly faster than tibia bone strength ($p < 0.001$; estimate = -8.74).

Table 4.4 Summary of Gaussian LMM to model bone strength over laying period of UK laying hens with farm fitted as a random term. The estimated value for σ^2 is 1969.71, N_{farms} = 14, N_{birds} = 781, N_{obs} = 3869. The caged housing system was set as the baseline coefficient

		Gaussian model	
Coefficient	**Estimates**	**CI (95%)**	***p***
(Intercept)	214.44	197.88 – 231.01	**< 0.001**
Bone weight	23.60	19.05 – 28.15	**< 0.001**
Age	-5.75	-12.01 – 0.51	0.072
House [B]	16.31	-9.17 – 41.78	0.210
House [FR FD]	27.37	4.30 – 50.43	**0.020**
House [FR MT]	38.30	15.47 – 61.14	**0.001**
House [O]	56.78	33.96 – 79.59	**< 0.001**
Bone [Humerus]	-4.49	-13.93 – 4.95	0.351
Bone [Keel]	-127.17	-131.31 – -123.02	**< 0.001**
Age * House [B]	-10.64	-19.91 – -1.37	**0.024**
Age * House [FR FD]	-10.92	-20.43 – -1.40	**0.025**
Age * House [FR MT]	-2.35	-10.71 – 6.01	0.581
Age * House [O]	-0.61	-9.00 – 7.78	0.886
Age * Bone [Humerus]	-12.04	-15.19 – -8.89	**< 0.001**
Age * Bone [Keel]	-8.72	-12.65 – -4.78	**< 0.001**
Random Effects			
σ^2	1969.71		
$\tau_{00\ Bird}$	1218.19		
$\tau_{00\ Farm}$	175.11		
ICC	0.41		
$N_{\ Bird}$	781		
$N_{\ Farm}$	14		
Observations	3869		
Marginal R^2 / Conditional R^2	0.492 / 0.703		

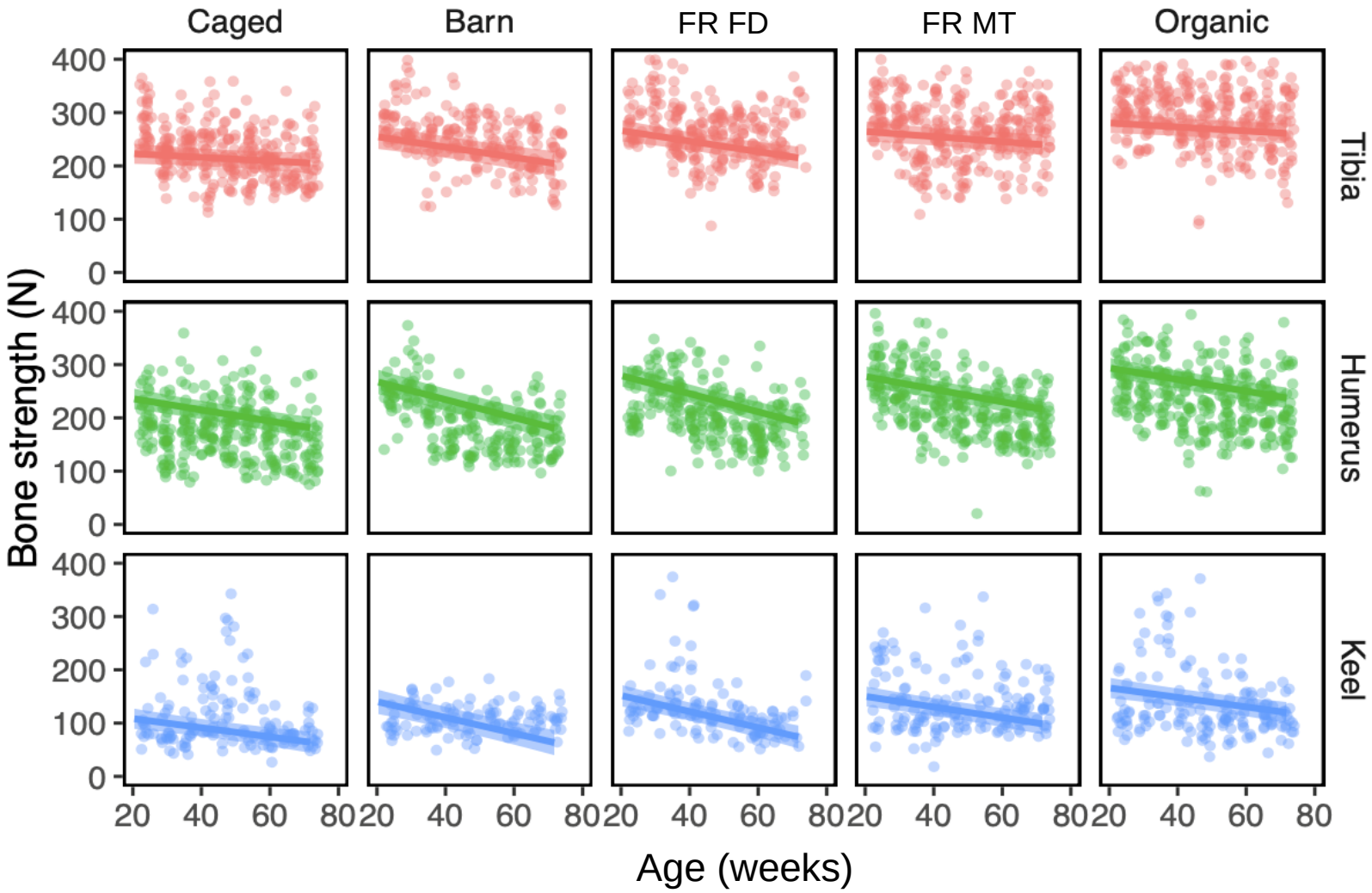

Fig. 4.8 Mean fitted bone strength of UK laying hens (solid line) and 95% confidence intervals (shaded area) over age (weeks) modelled with a Gaussian LMM, with farm and individual bird fitted as random terms in the model. Data is split by housing system and bone type

4.4 Discussion

4.4.1 Validity of results

Whilst this study has gone some way in trying to determine the effect of housing system on skeletal integrity over the laying period, there are many variables which should have been considered and possibly investigated in the future. This data may not be representative of UK egg production as only three farms (two for barn) per system were used due to time constraints on the project. Furthermore, the number of barn systems currently used in the UK is low, yet production may move this direction to maintain supply after the movement away from caged systems by 2025. This lack of farms using barn systems impacts the validity of the results of this study and causes less data to be generally available to create an informed evaluation on the effects of the barn system on skeletal integrity in egg production. In relation to this, some farms were hesitant to contribute to this study due to the cost incurred to the farmer by having healthy livestock culled for research, with some farms declining to participate. The selected or volunteered farms for this study were decided by the collaborating egg producer (Noble Foods Ltd), therefore any farm selected will have been selecting from their best performing farms to show their business in the best light. The farm selection may have created bias results as sampling from well managed farms could lead to unrepresentative selection of birds in the study, as these farms may not be a comprehensive example of the average farms in the UK for that specific system.

Variability between the farms selected may have also impacted the data collected. For instance, depending on farm, sample birds were either collected by the farmer or by the researcher. Bias sample collection could have occurred if the farmer chose any lame or weak birds that would not have impacted profits as much as a healthy bird. On the other hand, selection by the researcher could also create bias as birds could have been selected based on how healthy the birds appeared to be. In any instance the use of a randomised collection design based on different areas of the housing system may have reduced the bias in human selection. Farm to farm variability may also cause uncertainty in the results due to the different management or routines within farms and how a producer performs daily husbandry checks. For example, a farmer actively checking for behaviours such as feather pecking could

reduce the incidence of this occurring and maintain higher egg productivity within the flock by incorporating additional management changes such as foraging enrichment or changes to diet (Lambton *et al*., 2013). The majority of farms that contributed were provided with diet specific to Noble Foods Ltd dietary plans, however some farms were externally sourced and asked to join through Noble Foods which could have also caused variation in results due to different dietary specifications for the external farms to use throughout the period of lay. Specifications for additives or supplements for internal and external farms may also have been controlled differently. It is also to note that breed used by each farm was dependent on the management of that farm which could have led to breed variation within results of this study. Further examination of the effects of housing system on skeletal integrity over the period of lay, with controls for breed selection a standardised dietary plan within the study may allow for a more accurate representation in future research. A key factor within egg production is the rearing stage of a hen's life. As such, it is more recently common practice align rearing systems with the laying systems the hens are going to move into at point of lay (Janczak and Riber, 2015). For a more representative outcome in the results of this study, the rearing systems should have been standardised throughout the trial period. However, as it is common practice to align rearing systems and laying systems it could be seen as a negative effect on welfare to rear birds in the same system as hens will be moved on to alternative laying systems.

Generally, no matter which bone is selected, bone strength changes per housing system depending on age. Therefore, from these results it is difficult to provide a recommendation as to which housing system is most advantageous for the industry. The factors within a housing system will also affect each bone differently, making it more difficult to evaluate the effect of housing system and age on skeletal health. In keel bone strength, week-to-week variability particularly noted in multi-tier, caged and organic systems may be caused by bird-to-bird variation, whether that be from space utilisation, varying levels of KBD, differing genetic lines between farms used in one system, and possibly differences in nutrition due to farms changing diets at different times depending on egg weights. The differences and fluctuations within keel bone strength across housing systems and age could be due to multiple different factors, some of which may also have affected tibia and humerus bone

strengths over the course of the study. One factor that may have affected keel bone strength over the longitudinal study could be the prevalence of KBD, as the more damage to a keel as either deviations or fractures is often suggested as deleterious to the strength of the bone (Casey-Trott *et al*., 2015). Housing systems which provide more apparatus for their birds often have an increased prevalence of KBD (Wilkins *et al*., 2011). Furthermore, the prevalence of KBD is further increased with age. A previous study found that both keel bone fractures increased with age when palpating hens at 30, 50 and 70 weeks of age with prevalence % in order of age 35.2 (2.5), 55.2 (2.8) and 62.4 (2.6) ($p < 0.001$) Deviations also increased with age, 28.1 (2.6), 40.0 (2.6) and 51.6 (2.6) respectively ($p < 0.001$) (Casey-Trott *et al*., 2017a). In addition, deviations and fractures were also strongly associated with one and other across all age groups. As over 50% of hens in the previous study were found to have either a fracture or deviation by 70 weeks of age, this could explain the larger drop in keel strength in most systems, seen after 60 weeks of age within the present study. The usefulness of keel bone strength as a measure of skeletal integrity could also be examined. No previous studies have utilised keel bone strength as a value, with many opting to use bone mineral density (Hester *et al*., 2013), keel bone damage (Casey-Trott *et al*., 2017a) or ash weight (Neijat *et al*., 2019) when using the keel as a sample bone.

For all the bones measured in this study, exercise is another factor which is likely to affect bone strength (Whitehead, 2000). An assumption as to why alternative systems often have greater bones strengths over caged systems is because of an increased availability to exercise within the system (Shipov *et al*., 2010). As the keel bone acts as an anchor for the *pectoralis major* and *pectoralis minor* muscles used in wing movements (Lilburn *et al*., 2019), systems with more vertical apparatus such as multi-tier or may increase or maintain keel bone strengths better than those with less vertical apparatus. The same can be said for humerus and tibia strength, though the manner in which they are used for exercise varies. For instance, the tibias are used more often in walking, perching, and jumping whereas the humerus bones are commonly used in jumping or wing-assisted movements when moving between different tiers in a system. The rate at which they are used most likely depends on how active the bird is. Like the present study, Leyendecker *et al*., (2005) found that housing system had a significant effect on bone breaking strength, with humerus strength of the aviary system more

than double that of a the conventional caged and almost double of the furnished caged system. Tibia strength in the aviary systems were approximately 50% greater than conventional cage and 44% greater than furnished cages, compared to the present study where multi-tier (closest to aviary set up) was 16% greater than colony caged birds. Keel strength was not measured in the study. Leyendecker *et al*., (2005) also summarised birds kept in caged systems performed less exercise compared to aviary systems and that the exercise taken by caged hens was insufficient to prevent bone resorption. Increased amounts of exercise are known to maintain higher levels of osteoblastic activity may maintain bone strength (Petrik *et al*., 2015). Although, with age the effectiveness of exercise is reduced as osteoblast receptors begin to degrade (Hester, 2017).

4.4.2 Body weight

Age and housing system showed significant main effects on body weight, but no interaction effect was present. This means that the two factors were not dependent on the other. The differences in age and housing system could be influenced by some factors not considered within this study. For example, the strain of birds used within the study varied between housing system and ultimately farm to farm, due to strain selection being decided by the farmer for the best commercial output. Although all commercial strains will be somewhat alike growth performance will still differ. Regmi *et al*., (2016) found that genetic strain had no effect on body weight, but housing system did, with conventional caged birds (2.11kg) being heavier than caged free birds (1.97kg); free range was intermediate (2.01kg). The lack of effect of genetic strain are contradictory to the assumptions made in this study, that genetic strain may influence body weight. Cage free from the study by Regmi *et al*., (2016) could be considered similar to the barn system in this study, they found cage free birds were significantly lighter than conventional caged birds. Within the later part of this study barn body weight was significantly lighter than all other systems (54 to 72 weeks). Although housing system in both these studies showed an effect on body weight, it is important to note that in this study many secondary factors within a housing system may have influenced the overall outcome. Farm to farm variations in management practices, dietary schedules and farm environment may all have contributed to the overall effect of housing system.

Fig. 4.1 shows that though not always significantly different, barn often provides the lightest birds among the different systems. Barn also shows the steepest increase in body weight at an early age compared to other systems. It could be suggested that the steep increase in body weight at early stage of life in the barn system could be due to the compatibility of the rearing system and the laying system used. However, when rearing in aviary systems (1213.2g), birds had a lower body weight than when reared in a caged system (1240.7g) (p = 0.042), suggesting that systems that encourage more exercise will have lighter birds as more energy is being expended (Regmi *et al*., 2017). Within this study it could be that the rearing system used for barn hens were closely matched to the specifications of the laying system for barn hens, therefore increasing the confidence of birds, allowing them to perform more natural behaviours and increasing the amount of exercise that, occurs therefore increasing growth rate (Cambell *et al*., 2019). It is also possible that hens destined for a barn system may have been reared in rearing systems used for free-range hens and thus the reduced exercise when moved to the barn laying system – due to lack of outdoor access, reduced energy expenditure and caused birds to gain more weight.

Rearing systems are now thought to be beneficial to the long-term effects of skeletal growth in laying hens, and better bone growth may increase body weight (Regmi *et al*., 2015). The free-range flat deck system and organic had the most prolonged increase in body weight at early stages of lay and maintained some of the heaviest birds throughout the study. The availability of an outdoor space or lower stocking density within these systems may have increased the amount of natural foraging behaviours exhibited by hens in these systems and influenced body weight. It has been shown in 36-week-old ISA brown hens that there is a positive trend (p = 0.07) between body weight and hours spent outside at 2000 hens per hectare stocking densities. It was also shown that there was a negative correlation with body weight and time outside in stocking densities of 10000 hens per hectare at 26 weeks of age. (Campbell *et al*., 2016a). These results would somewhat agree with the present study as the organic systems used in this study are known to have lower stocking densities and show the highest body weight throughout the period of lay. Furthermore, the lack of space in barn systems without outdoor space shows the lowest body weight, supporting results from Campbell *et al*., (2016a) that low or no outdoor access and a high stocking density negatively

effects body weight. However, the multi-tier system had showed a much lower body weight until mid-lay. The reason why barn birds may be the lightest could be down to the least amount of stimulation in a system compared to free-range systems, so hens' behaviours increased amount of exercise and energy expenditure it creates. This cannot be confirmed within this study however as feed intake or feed conversion ratio was not measured. Furthermore, hens kept within a barn often crowd up more than other systems and can sometimes cause smothering or piling (Campbell *et al*., 2016b). It was suggested that piling therefore reduced activity within a barn could be caused by hen behaviour. For example, if there is something attractive to peck with the barn, hens are drawn to one area and will often overpopulate certain areas without moving for long periods (Gebhardt-Henrich *et al*., 2016). The theory of lower activity in barn due to crowding could be a reason why bird weight was lower in barn systems throughout the present study. It is also worth highlighting that due to the lack of barn systems currently used in the UK, only two farms were used in the barn housing system group, a third farm may have caused a different outcome. It is surprising that only two farm systems were able to be used within this study as the barn system is thought likely to replace the majority of caged systems within the UK leading up the cage-free by 2025 movement. Overall, it is difficult to provide an accurate conclusion of the effects of the interaction effect as there is much variation between similar housing systems possibly due to farm-to-farm variation within factors such as farm management, rearing system of the hens, dietary schedule, and strain of birds, as previously mentioned.

4.4.3 Bone strength

From the results (Table 4.3), age, housing system and an interaction effect was shown for bone strength in all bones collected ($p < 0.001$). Generally, the keel bone displayed the weakest bone strength followed by the humerus and with the tibia recording the highest breaking strengths. These results support previous studies such as Wilkins *et al*., (2011), where tibia also showed to be the strongest bone, followed by humerus, and then keel (when broken at the same point in this study). Overall, in this study, keel was the most variable in terms of measured bone strength (Fig. 4.2). There are several factors which can impact keel strength, and all contribute to variability within the results. The most predominant factors being prevalence and severity of KBD. It is possible that due to the prevalence of KBD and the

location of the damage, weak spots may have skewed keel strength between samples considerably.

In this study caged bone strength was the weakest for all bones, whilst organic generally the strongest - supporting results from Wilkins *et al.,* (2011) investigating the effect of housing systems on bone strength and KBD in end of lay flocks. Though in contrast to our findings, they found that subcategories of free-range systems with additional perches or A-frames were not significantly different in bone strength (apart from the humerus bone) to furnished caged systems. Whereas in this study, bones were generally significantly stronger in the multi-tier and flat-deck free range systems compared to colony cage systems. When comparing bones at the end of lay, a system similar to the subcategories in Wilkins *et al*., (2011) could be the multi-tier system in this study, which at 72 weeks was found to be significantly different to the caged system in all bones measured ($p < 0.001$).

Other previous trials have also found that caged bone strength is the weakest compared to other non-caged systems (Table 4.5). Keel bone strength in the caged system of this study was not much different to barn, whereas in Wilkins *et al*., (2011) it was stronger and more similar to the keel strengths of free range or organic. The differences between humerus strength in free-range and caged systems in Leyendecker *et al*., (2005) were much greater than the differences in free-range and caged systems in the current study as well as the study by Wilkins *et al*., (2011). Furthermore, the differences in humerus strength of barn and caged systems from Qiaoxian *et al*., (2020) were larger than the differences within the current study. In tibia strength results, caged was shown to be the weakest in across all previous studies. However, the difference between free-range and caged tibia strength was larger in Newman and Leeson, (1998) and Leyendecker *et al*., (2005) than the current study as well as Wilkins *et al*., (2011). The current study and the study by Wilkins *et al*., (2011) also showed the same trend in tibia bone strength across housing systems. Organic showed the highest, followed by free-range barn and then caged. Compared to the current study, Qiaoxian *et al.*, (2020) showed roughly the same difference between barn and caged tibia weight. Overall, there are some factors which will have influenced the results between different studies, these include

what strain of birds were used, the methods used to measure bone strength, what diets were used and even the differences in housing system within the same categories between studies.

Notably, the differing effect of exercise available per housing system may affect bone strength differently depending on bone strength. Wilkins *et al*., (2011) also suggested that the reduced humerus strength in caged may be caused by the reduced capabilities of flapping or other wing-assisted movements. The study by Leyendecker *et al*., (2005), also supports the theory that configuration of housing systems may prevent or increase occurrence of different types of exercise. On the other hand, housing systems that encourage exercise to increase bone strength may also increase the prevalence of skeletal damage, as the likelihood of collisions is known to be increased, causing a paradox when deciding which system to rear or keep hens in throughout lay (Sandilands *et al*., 2009). Hypothetically, the modern-day layer was originally developed for primary use within caged systems, as 20-30 years ago intensive farming was more acceptable. Therefore, the effects of caged systems on any bone parameter may have been reduced through high genetic selection over time, showing caged systems have better skeletal health than is true (Wilkins *et al*., 2011). In addition, as caged systems provide worse skeletal strength throughout age, the effects of the caged systems do not decline bone strength faster than other systems as the bones are already in a worse state. It could be suggested that there should be a limit at which bone strength cannot go below otherwise it could be seen to compromise hen welfare and the birds should be culled to prevent suffering.

Table 4.5 Bone strength values from previous studies compared with the on-farm collection project

	Housing System strengths (N)*			
	FR	**FC**	**B**	**O**
Keel				
Current study	139	111	112	138
Wilkins *et al*., (2011)	136	95	134	130
Humerus				
Current study	223	177	199	246
Leyendecker *et al.,* (2005)	247	130		
Wilkins *et al.,* (2011)	183	140	182	209
Qiaoxian *et al.,* (2020)		155	87	
Tibia				
Current study	266	226	250	291
Newman and Leeson (1998)	257	193		
Leyendecker *et al.,* (2005)	175	121		
Wilkins *et al.,* (2011)	225	187	214	282
Qiaoxian *et al.,* (2020)		134	164	

*MT and FD were averaged for this study = FR. FR and OM were used from Wilkins *et al*., 2011 for free range and organic categories. Some results were converted to newtons

Age had a significant effect on the bone strength of the keel, humerus and tibia ($p < 0.001$). A slight decline in humerus and tibia strength can be observed in Fig. 4.3 and 4.4 with humerus strength decline observed to be slightly steeper. Keel strength is affected by age but the decline over time is more unclear than for the humerus and tibia. A study by Regmi *et al*., (2017a) studied the effect of age and housing system on the mechanical properties of tibia and humerus bones through young's modulus attributes. Although not measuring bone strength with the same methodology, the study showed properties such as the energy to failure (J) in the humerus was significantly different between Lohmann white hens aged 26, 56 and 72 weeks (564J, 439J, 397J respectively). The current study mirrors the findings of this paper in that the bones became weaker with age (Regmi *et al*., 2017a). Although the effect of age was not assessed, Newman and Leeson (1998) there was a noticeable difference in strength of tibia from 10 days-in-trial to 20 days-in-trial for both caged and aviary systems. A recent study by Qiaoxian *et al*., (2020) found that age had a significant effect on femur breaking strength ($p = 0.008$), but not on tibia or humerus in Taihang chickens aged 32 to 57 weeks of age. Though femur showed a significant effect of age in the previous study, the data

from tibia and humerus strength in the current study contrasts with these findings as both bones showed a significant reduction with age.

The effects of age and housing system are dependent on each other. As the main differences between housing systems are often either stocking density or the opportunity for the birds to exercise, these beneficial effects may diminish with age. Although exercise is known to reduce the effect onset of osteoporosis through bone resorption, there becomes a point in lay where the effect of exercise is not sufficient to prevent the increasing bone resorption demands (Whitehead and Fleming, 2000; Whitehead, 2004; Fleming *et al*., 2004). Nevertheless, there are other factors within a farm setting which could also affect the interaction between house and age on bones strength, including genetic strain, diet, and bird husbandry as previously mentioned. It is therefore likely that the effect results of the age*housing interaction within this study is more multifactorial than just age and housing alone. The study by Qiaoxian *et al*., (2020) found no age*housing interaction effect on femur, humerus or tibia breaking strength, contradicting the results of this study. Conversely, the study by Regmi *et al*., (2017a) showed an interaction effect of age and housing on tibia ($p = 0.04$) and humerus ($p = 0.01$) properties when analysing young's modulus, indicating as age increases depending on housing system bones become more brittle. Although a direct comparison cannot be made with breaking bone strength from the present study, as different variables for assessing strength were used, the results of Regmi *et al*., (2017a) theoretically support the results from this study that as age increases bone strength decreases but the rate changes between housing system.

4.4.4 Bone ash content

All bone ash contents measured within this study were significantly affected by age, housing system and the interaction effect ($p < 0.001$) (Table 4.3). Bone ash content shows a similar pattern in all three sample bones and for all housing systems over the period of lay. Caged ash content was the lowest numerically and organic recorded the highest values with flat deck and multi-tier just below organic. Interestingly, barn ash content throughout the study in all bones was also high, showing similar values to flat deck, multi-tier, and organic systems (Fig. 4.5 – 4.7). This contrasts with the findings of bone strength where there was a more marked difference between free-range and organic systems compared to barn.

It could be suggested that ash content was less variable since medullary bone is being produced from the point of lay, whilst structural bones is being resorbed, therefore dulling

the effect of housing system and age on ash content (Whitehead, 2004). Medullary bone is spongy and non-uniform in structure and provides less mechanical support than structural bone (White and Fleming, 2000). The loss of structural bone would therefore suggest that bone strength would be affected to greater extent, and that ash content and mineral composition would show smaller variations. Returning to the barn ash results, an explanation for these results could be caused by the lack of access to an outdoor range. Outdoor access is known to increase the rate of exercise and natural behaviours hens exhibit compared to no outdoor access (Knierim, 2006). Hence, an improved bone strength within a free range of organic systems compared to barn. In terms of ash content, as previously stated the effect of housing system is not as prominent mainly because of medullary bone production during lay. Consequently, if only ash content was used to assess skeletal health the barn system would not appear to be as detrimental as when bone strength is also considered.

Overall, the humerus ash content seemed to be higher than either the keel or tibia ash content. Humerus bones are known to be pneumatic, meaning they are hollow inside, developed for flight in most other avian species (Whitehead and Fleming, 2000; Neijat *et al*., 2019). It could be possible that due to the extra space within a humerus bone, more medullary bone can be produced or stored during lay compared to the keel and tibia bones. It is noted that keels can sometimes be pneumatised, but this does not occur frequently (Whitehead, 2004), hence this study keel showed no overall difference in ash content compared to tibia. Furthermore, even though the patterns between bones are similar for ash content, the keel bone ash content values again fluctuate more than the humerus and tibia values. This could be down to the effects that KBD is having on keel integrity (Casey-Trott *et al*., 2015). For instance, a keel fracture will require repairs and increase the ash content via excess mineral deposition to fix the damage, leading to more frequent fluctuations in data. It should be considered that when investigating skeletal health in laying hens across the laying period in different housing systems, that bone strength shows more meaningful differences compared to ash content, primarily due to the production of medullary bone and resorption of structural bone throughout lay.

A study by Regmi *et al*., (2016) found no differences in ash content of the keel, femur, and tibia bones between conventional cage birds, furnished cages and free-range did not differ (p = 0.42, p = 0.55 and p = 0.23 respectively). Although femur was not used in the present study, results from the tibia and keel do not agree with the results from Regmi *et al*., (2016). Bones within this study showed lower ash values than that found in the previous study. In the present study, bones were not fat extracted before being ashed, whereas in Regmi *et al*., (2016) bones were extracted via the Soxhlet method for fat extraction therefore providing higher values. A previous study from Nottingham Trent University has shown substantial effects of methodology of bone preparation on ash content in broiler chickens. It was found that fat extraction increased ash weight by 22% (Sanni and Burton, 2016). In future work, it is suggested that for better comparability, bones be extracted. However, the number of bones generated within this study would consume high amounts of petroleum ether if de-fatted. The large quantity of petroleum ether used would increase the cost significantly whilst also having negative environmental effects when disposing of the chemical. Therefore, it was decided that bones were not defatted on this basis.

Strain of the birds used in each study may have also caused a disparity within results. Regmi *et al*., (2016) found that there was an effect of strain within all bones measured (p = 0.04 or less), using Hy-line Brown, Hy-Line Silver Brown, and Plymouth Rock hens. Strain as a factor was recorded in the present study but not assessed, it is perhaps possible that strain selections of different farms caused an underlying effect in difference between housing systems on ash content. A study by Regmi *et al*., (2015) investigating the effect of housing system at the rearing stage (16 weeks) found that humerus bone ash content was significantly higher in aviary housed hens than conventional caged hens (~57.5% compared to ~55.5%, p < 0.005). Although rearing stage was not covered within the present study, the effects multi-tier (closest related to aviary) compared to caged on humerus bone as at 18 weeks was also significantly different 54.25% vs 49.47% respectively. Furthermore, a study by Newman and Leeson, (1998) found no difference in tibia bone ash content from 69-week-old hens at 0 and 10 days into the trial but did find a difference at day 20, showing a greater ash percentage from the aviary system over caged (58.5% vs 56.7%; p < 0.05).

4.4.5 Modelling data

Previous studies modelling multiple bones strengths across a range of UK based housing systems over a period of lay are scarce. The model parameters shown in Table 4.4 showed that bone weight is a significant predictor of bone strength in UK based farms. Bone weight has used as a covariate to outline the size of an individual bone though this is not commonly used in previous studies. Though a study by Neijat *et al*., (2019) found that regardless of housing system, bone breaking strength was correlated with amount of ash and total bone weight ($r = 0.60$; $p < 0.01$). In more technology focussed studies, many researchers opt to use methods such as QCT for measuring bone characteristics. A study by Casey-Trott *et al*., (2017c) found that a higher bone breaking strength was found in tibiae with more positive QCT values for bone characteristics. Although a more basic measurement, the similar conclusions are drawn from either more positive QCT values, or higher bone weight meaning better bone strength. Ash content and bone mineral density has also correlated to stronger bone strength. The more ash content or BMD subsequently better mineral composition, the greater the strength (Regmi *et al*., 2015; Vaughan *et al*., 2016).

In the model parameters there was no main effect of age when controlling for farm and bird as random factors. The previous work within this chapter disagrees with this model outcome as age has a significant main effect on all parameters measured, however the two-way ANOVAs used in the previous work did not control for farm and bird as a random factor, which will have most likely caused the difference in outcomes between the two tests. Overall, it is accepted that increasing age decreases bone strength as osteoporosis can begin to set in (Whitehead and Fleming, 2000; Whitehead, 2004; Fleming *et al*., 2006). Although not a main effect in the model, the effect of age did show a trend, indicating that caged tibia strength would decline with age but not significantly.

Model parameters also show caged bone strength was significantly lower than flat deck, multi-tier, and organic bone strength. These results support studies previously investigating the effect of housing system on bone strength. Most similarly, a study by Wilkins *et al*., (2011) found that caged tibia strength was significantly weaker, than barn, multiple organic systems, and multiple free-range systems apart from those with additional A-frames. The study also

found that caged humerus were significantly weaker than all other systems. Keel bones were also found to be significantly weaker in caged flocks compared to other flocks, apart from free-range with addition A-frames or free-range with addition suspended perches. The addition apparatus was thought to have increases KBD and thus lowered keel strength (Wilkins *et al*., 2011). From the model results, the keel bone was shown to be weaker than caged tibia. As previously mentioned, KBD not only effects hen welfare, but can also reduce keel bone integrity and therefore strength compared to other bones which are less likely to become damaged in everyday life (Casey-Trott *et al*., 2017c). As humerus strength does not show any significant difference to the caged tibia strength (p = 0.351), the suggestion of KBD drastically effecting the strength of the keel can be supported.

Surprisingly, the model for bone strength showed that with age, the barn and flat deck systems declined significantly faster than the caged system. It is unclear as to why this is occurring and is most likely caused by a multitude or factors previously discussed. Housing system configuration as a factor could be a causing the difference in decline, for example the barn and free-range flat deck systems may not have been equipped with adequate equipment or apparatus to encourage the hens to exercise or show natural behaviours as much as those used in multi-tier or organic systems. The stocking density or usage of an area by the hens in these systems could also have been worse, causing less movement and ultimately not offsetting the effects of osteoporosis or bone resorption through the form of adequate exercise (Leyendecker *et al*., 2005). In organic systems the stocking density is usually low and therefore increasing the opportunities to exhibit natural behaviours such as foraging. Bird husbandry or farm routine could also play a role in effecting the results shown within this model. A more active farmer who may encourage birds to move around indoor or outdoor may see greater bone strength values than a farmer that does not (Gerbhardt-Henrich *et al*., 2014). Conversely, could it be possible that the configuration of caged systems may reduce bone metabolism by not providing as much exercise opportunities than other systems. Therefore, possibly forcing the skeletal system to become more efficient in bone metabolism due to the pressures of being reared and housed in a caged system, like a stress response found in a biological mechanism. Another possibility is that as caged bone health is lower from the start to the end of lay compared to other systems. Therefore, there may be less of a

decline before reaching a minimum value which is considered poor health due to osteoporosis, before being culled on welfare grounds minimising the actual effects of the caged system. These theories link to suggestions previously proposed by Wilkins *et al*., (2011) suggesting that modern-day layer could have originally been developed for primary use within caged systems as 20-30 years ago intensive farming was more acceptable. Therefore, the effects of caged systems currently seen in recent research is supressed by generations of genetic selection within the industry.

Bone strength in the model also declined significantly faster in the keel and humerus bones compared to the tibia. As different bones have different functions within the body, it is clear that they will be subject to different amounts and types of exercise. For example, the tibia is used the most in all systems as the birds move around and is used in walking, running, or jumping. The humerus bones and keel will receive less exercise in systems with less vertical apparatus or environmental enrichment as there may be less need for wing-assisted movements (Shipov *et al*., 2010). Consequently, the tibia is exposed to prolonged amounts of exercise compared to the keel or humerus and therefore the rate of decline of bone strength is reduced. In addition, the humerus is a pneumatic bone and contains no or very little trabecular bone compared to tibia (Hester, 2017). This could therefore imply that the trabecular bone contained within a tibia bone helps to reduce the rate of decline of bone strength and provides some additional structural support compared to the humerus. Keel bone damage is known to be highly prevalent in laying hens and varies between system, ranging from 5% to 97% in some flocks (Gregory *et al*., 1990; Rodenburg *et al*., 2008b; Casey-Trott *et al*., 2017a). The difference in the rate of decline between the keel and tibia could be caused by the prevalence of KBD. Any damage caused to a bone will require bone remodelling, consequently, weak spots may develop where the bone has been damaged and therefore influence the rate of decline of keel bone strength.

4.5 Conclusion

Overall, from this study it could be concluded that bone breaking strength is more informative when assessing skeletal health in layers compared to using bone ash content. This is most likely since medullary bone is produced during lay and replaces some structural bone in terms of mineral content but not the structural support, therefore could provide a false evaluation on skeletal health. The move away from caged systems within the industry is also an incentive that could be supported by the results from this study. Regardless of whether measuring ash content or bone strength, caged systems displayed the weakest results in all analysis and other non-caged systems ultimately showed better skeletal health through the analysis of bone strength and ash content over time. However, although alternative systems increase bone strength and ash content, they can increase the change of collisions and the prevalence of KBD, creating a paradox for farmers when deciding what system to use – better bone health or less KBD (Sandilands *et al*., 2009). A balance between housing system configuration to improve bone health while providing enough space to reduce KBD is therefore recommended when configuring housing systems.

There is also still some uncertainty to the true effects of housing system in this study as farm-to-farm variation within a multitude of factors that could not be controlled for, such as management style, environmental conditions (outside of the housing system), dietary schedules and bird strains used. In future research it would be recommended to assess some of these factors to better understand the multifactorial effects that individual farms present when trying to assess skeletal health.

In modelling bone data, it was concluded that using a range of bones to assess skeletal health is essential as different bone types perform different locomotory functions thus bone parameters are affected differently. In addition, it would be advised in future work to include additional factors within the model for bone strength such as diet and breed to increase the accuracy of the predictions. Ultimately, modelling bone health data could act as an early warning system for skeletal health in UK based farms so better longevity and production is achieved whilst not compromising welfare of the hens.

Chapter 5: Effect of housing system on egg quality

5.1 Introduction

As a source of protein, eggs now contribute to a large proportion of animal proteins within the human diet as a relatively low-cost food source with an easy production chain (Elson and Tauson, 2011). In the last 10-20 years, the methods by which eggs are produced, especially in the EU and UK have gathered interest from consumers. Hen welfare and healthier eggs are now a key influence of consumer perception, with healthier eggs thought to come from a free-range system rather than an enriched caged system (Dikmen *et al*., 2016). Due to consumer perception and evolving legislation, the EU banned the use of non-enriched caged systems in 2012, with an aim to increase hen welfare (EU Directive 1999/74/EC), though there is an aim by most large UK retailers to be cage free by 2025. The question now is whether barn, organic, or free range will take up the majority of supply currently provided by cage (Williams, 2018). In 2018, 30,943 cases of eggs were produced in the UK (360 eggs per case). Of this total, 51.75% were produced by free-range, 44.33% by enriched cages, 2.49% by organic and 1.43% by barn. By 2019, more eggs were produced in total (31,633 cases) but less by enriched cages (42.33%) and more in free-range (52.62%), organic (3.14%) and barn (1.90%) (DEFRA, 2020). These results may suggest that the supply can be provided by alternative systems and not caged systems, but there is still some way to go. As such, more research continues to be published on the effect of housing system on egg quality to help improve the supply of alternative systems into the egg market. In addition to bird related differences such as behaviour and skeletal health, research has also found that egg quality traits can differ depending on system (Lichovníková and Zeman, 2008; Lewko and Gornowicz, 2011; Jones *et al*., 2014). A high standard of egg quality coupled with a high standard of skeletal health may be able to determine the overall usefulness of different systems in the future and help determine a balance between meeting high hen welfare whilst maintaining a suitable egg production rate, at a pivotal time for the egg production industry. This study

begins to investigate the effect of housing systems in UK farms on some egg quality traits throughout the period of lay.

Aims

- To investigate the effect of housing system on egg quality traits
- To model what is normal eggshell strength for UK systems
- Provisionally determine if there is a relationship between egg and bone strength

5.2 Methods

5.2.1 Trial period

Thirty eggs from various breeds (Lohmann Brown Classics, Lohmann Brown Lite, Hy-line Brown or Bovan Brown) were collected from 14 farms every 12 weeks from 24 weeks of age to 72 weeks of age, in conjunction with bird samples collected in chapter 5 (n = 2100). The participating farms were also the same farms used in chapter 5 and were sourced by Noble Foods Ltd. The housing systems collected from were free-range flat deck, free-range multi-tier, colony cage, barn and organic. Each system group had 3 farms (except barn, n = 2). Egg collections were originally planned for collection every 6 weeks to match the bird collection in chapter 5, however due to the number of eggs needed and turn around for egg data (eggs needed to be processed within 3 days for accurate results), egg collections were changed to every 12 weeks per farm. In addition, only 2 farms were able to be used for the barn system group as there is a lack of producers currently using barn as a system that were available for this study. All birds were provided with a customised commercial diet, specific to farm requirements and egg production weight by age. Farms either used a commercial diet produced by Noble Foods Ltd, or by an external feed producer. Dietary information for all participating farms can be found in Appendix 1. Water was provided *ad libitum* in all farms via line drinkers, nipple drinkers or floor drinkers. If a farm used any additional supplements or medication, these were recorded on a collection sheet (Appendix 2). National guidelines for bird husbandry were followed throughout the study. On arrival at a farm, the eggs were collected from the egg belt by a farm operator and packed into an egg tray ready for transportation. All eggs used within the study were categorised as firsts, according to the British Egg Industry Council.

5.2.2 Egg samples

Eggs were weighed individually using a 4.d.p analytical balance (Sartorius, UK). Egg height was recorded using a 2.d.p digital calliper at the longest points of the eggs as described in section 2.7.4. Next, the circumference of the egg was identified by halving the measured egg height and 4 points were marked on the egg, roughly one per quarter. Eggs were then broken to test shell strength using a texture analyser (TA.XT 100; Stable Micro Systems, Guildford) with a

cylindrical probe (P36/R; Stable Micro Systems, Guildford) placed horizontally across the texture analyser. Once the eggs were broken, the yolks and albumen were discarded, and the shell was wiped out. Once cleaned, eggshell thickness was measured using 2.d.p digital callipers at the 4 points previously marked, taking care not to measure fragmented shell doubled up behind the 4 points. An average eggshell thickness was then calculated from the four measurements. Next, the eggshells were then wiped out again and left to dry for 24 hours. After 24 hours the eggshells were placed in individual pre-weighed, pre-labelled crucibles and then placed in a muffle furnace (SNOL 22/1100 LHM01) at 650°C for 12 hours. Ash weights were then recorded using a 4.d.p analytical balance (Satorius, UK) as stated in section 2.7.4, so eggshell ash content (as % of egg weight) could be calculated using the equation below:

$$Eggshell\ ash\ content\ (\%) = \left(\frac{Eggshell\ ash\ weight\ (g)}{Egg\ weight\ (g)}\right) \times 100$$

5.2.3 Data Analysis

Egg sample data was stored in a database created in Microsoft Excel. Multiple two-way ANOVAs (R (version 4.1.0) and RStudio (version 1.1.463)) were used to determine the effect of age, housing system and whether an interaction effect was present in all egg sample data. Outliers were removed at 2 standard deviations from the mean and any effect was considered significant at a threshold level of $p < 0.05$. A Gaussian Linear Mixed Model (LMM) (R version 4.1.0) was also used to model eggshell strength over period of lay for each individual system. In addition, residuals of variance of bone strength and eggshell strength were plotted against each other to see if a relationship between the two parameters was present. The formulation for the model of eggshell strength was as follows:

$Strength_{ij} \sim Gaussian(\mu_{ij}, \sigma^2)$

$E(Strength_{ij}) = \mu_{ij}$ and $\text{var}(Strength_{ij}) = \sigma^2$

$\mu_{ij} = Intercept + Weight_{ij} + Age_{ij} \times Housing_{ij} + Farm_j$

$Farm_j \sim Gaussian\ (0, \sigma^2_{Farm})$

Where $Strength_{ij}$ is the breaking strength (N) of egg *i* from *Farm j* assuming a normal distribution with mean μ_{ij} and variance σ^2. $Weight_{ij}$ is a continuous covariate indicating the total weight of egg *i* (g), Age_{ij} is a continuous covariate indicating the age of bird that laid egg *i* (weeks), and $Housing_{ij}$ is a categorical covariate indicating the housing systems in which egg *i* was produced. The random intercept $Farm_j$ is included in the model to introduce a correlation structure between observations for eggs from the same farm, with variance σ^2_{Farm} distributed normally and equal to 0.

5.3 Results

5.3.1 Effect of housing system and age on egg quality

Table 5.1 and Fig. 5.1 – 5.5 show that egg weight, egg height, eggshell strength, ash content and eggshell thickness were significantly affected by age ($p < 0.001$). Housing system also had a significant effect on egg weight, egg height, eggshell strength, ash content and eggshell thickness and housing system ($p = 0.007$). An interaction effect between age and housing system was also present and significant in all egg quality traits ($p < 0.001$). The interaction therefore indicates that the effect of housing system on these parameters are dependent on age of the bird. For example, egg weight in multi-tier and cage systems were not significantly different at 24 weeks but at 36 weeks, caged eggs weighed significantly less than multi-tier eggs. In egg height, multi-tier and organic eggs were not significantly different at 24 weeks, whereas at 72 weeks, organic eggs were significantly longer than multi-tier eggs. An example of the interaction effect in eggshell strength can be shown by the lack of differences in caged and barn eggshell strength at 24, 36 and 42 weeks but a significant difference shown at 60 and 72 weeks. Table 5.1 shows that ash content is not significantly different between any housing system at 24 weeks but there are differences at 72 weeks of age, with barn showing significantly higher eggshell thickness compared to all other systems, whilst organic was significantly thinner. Furthermore, eggshell thickness is not significantly different between barn and flat deck systems at 36 weeks but shows a significant difference at 72 weeks.

Table 5.1 Summary of effects of housing system and age and their interaction on egg quality traits (±SE)

Age (weeks)	Housing System	Egg weight (g)	Egg height (mm)	Eggshell strength (N)	Ash content (%)	Eggshell thickness (mm)
24						
	MT	56.13 (1.528)^{a}	53.23 (0.644)^{a}	56.63 (2.374)^{a}	9.58 (0.349)^{a}	0.37 (0.015)^{a}
	C	55.93 (1.528)^{a}	54.25 (0.644)^{ab}	51.38 (2.480)^{ab}	8.90 (0.349)^{a}	0.33 (0.015)^{b}
	FD	56.18 (1.248)^{a}	53.84 (0.525)^{ab}	54.14 (1.938)^{ab}	9.39 (0.285)^{a}	0.36 (0.012)^{ab}
	O	56.24 (0.817)^{a}	54.59 (0.344)^{ab}	51.09 (1.300)^{b}	9.14 (0.187)^{a}	0.39 (0.008)^{a}
	B	57.00 (0.882)^{a}	54.84 (0.372)^{b}	50.80 (1.390)^{b}	9.40 (0.201)^{a}	0.44 (0.009)^{c}
36						
	MT	64.41 (0.817)^{a}	56.78 (0.344)^{ab}	45.35 (1.284)^{a}	8.76 (0.187)^{a}	0.35 (0.008)^{a}
	C	61.56 (0.817)^{b}	55.99 (0.344)^{a}	49.16 (1.269)^{b}	9.02 (0.187)^{a}	0.39 (0.008)^{b}
	FD	62.32 (0.652)^{b}	57.82 (0.274)^{c}	44.43 (1.028)^{a}	8.09 (0.149)^{b}	0.37 (0.007)^{c}
	O	65.65 (0.817)^{a}	57.31 (0.344)^{bc}	52.51 (1.300)^{b}	8.96 (0.187)^{a}	0.34 (0.008)^{a}
	B	63.53 (0.882)^{ab}	56.93 (0.372)^{abc}	50.56 (1.410)^{b}	9.24 (0.201)^{a}	0.38 (0.009)^{bc}
42						
	MT	64.63 (0.558)^{a}	57.49 (0.235)^{a}	47.26 (0.882)^{a}	9.09 (0.127)^{ab}	0.36 (0.006)^{a}
	C	64.42 (0.558)^{a}	57.37 (0.235)^{ab}	46.44 (0.877)^{ab}	9.17 (0.127)^{ab}	0.39 (0.006)^{b}
	FD	61.72 (0.558)^{b}	56.54 (0.235)^{c}	45.39 (0.872)^{ab}	9.02 (0.127)^{a}	0.34 (0.006)^{c}
	O	63.13 (0.558)^{ab}	56.83 (0.235)^{bc}	47.13 (0.908)^{a}	9.39 (0.127)^{b}	0.39 (0.006)^{b}
	B	62.26 (0.684)^{b}	57.21 (0.288)^{abc}	44.23 (1.062)^{b}	9.12 (0.156)^{ab}	0.36 (0.007)^{a}

60						
	MT	62.27 (0.558)^{a}	57.31 (0.235)^{a}	45.88 (0.892)^{ab}	9.14 (0.128)^{ab}	0.38 (0.006)^{a}
	C	63.68 (0.558)^{ab}	57.79 (0.235)^{ab}	47.92 (0.877)^{a}	9.07 (0.127)^{ab}	0.39 (0.006)^{ab}
	FD	62.49 (0.558)^{a}	57.93 (0.235)^{ab}	45.87 (1.099)^{ab}	9.39 (0.127)^{a}	0.42 (0.006)^{c}
	O	64.58 (0.558)^{b}	58.02 (0.235)^{b}	47.64 (0.903)^{a}	8.92 (0.127)^{b}	0.40 (0.006)^{bc}
	B	63.46 (0.684)^{ab}	58.11 (0.288)^{b}	44.47 (1.119)^{b}	9.00 (0.156)^{ab}	0.39 (0.007)^{ab}
72						
	MT	61.96 (0.558)^{a}	57.42 (0.235)^{a}	48.68 (1.080)^{a}	9.42 (0.127)^{a}	0.39 (0.006)^{a}
	C	63.09 (0.558)^{ab}	57.98 (0.236)^{ab}	45.54 (0.908)^{b}	9.44 (0.127)^{a}	0.38 (0.006)^{ab}
	FD	63.41 (0.684)^{ab}	58.75 (0.288)^{c}	46.50 (1.501)^{ab}	9.22 (0.156)^{ab}	0.37 (0.007)^{c}
	O	66.08 (0.558)^{c}	59.48 (0.235)^{d}	46.85 (1.140)^{ab}	8.93 (0.127)^{b}	0.38 (0.006)^{bc}
	B	63.82 (0.689)^{b}	58.37 (0.407)^{bc}	42.05 (1.089)^{c}	10.10 (0.157)^{c}	0.33 (0.007)^{d}
***p* value**						
Age		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
System		< 0.001	< 0.001	< 0.001	0.007	0.001
Age*System		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Means within a column within an age group with different letters are significantly different (p = 0.05)

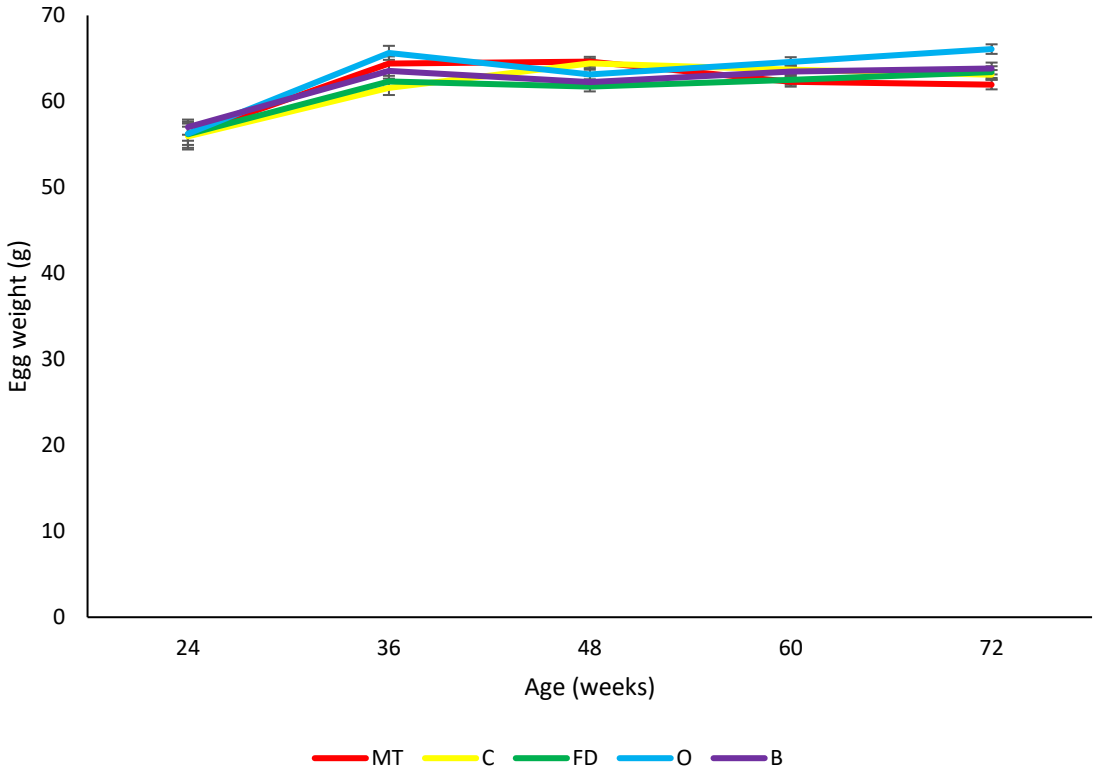

Fig. 5.1 Effect of age and housing system on egg weight

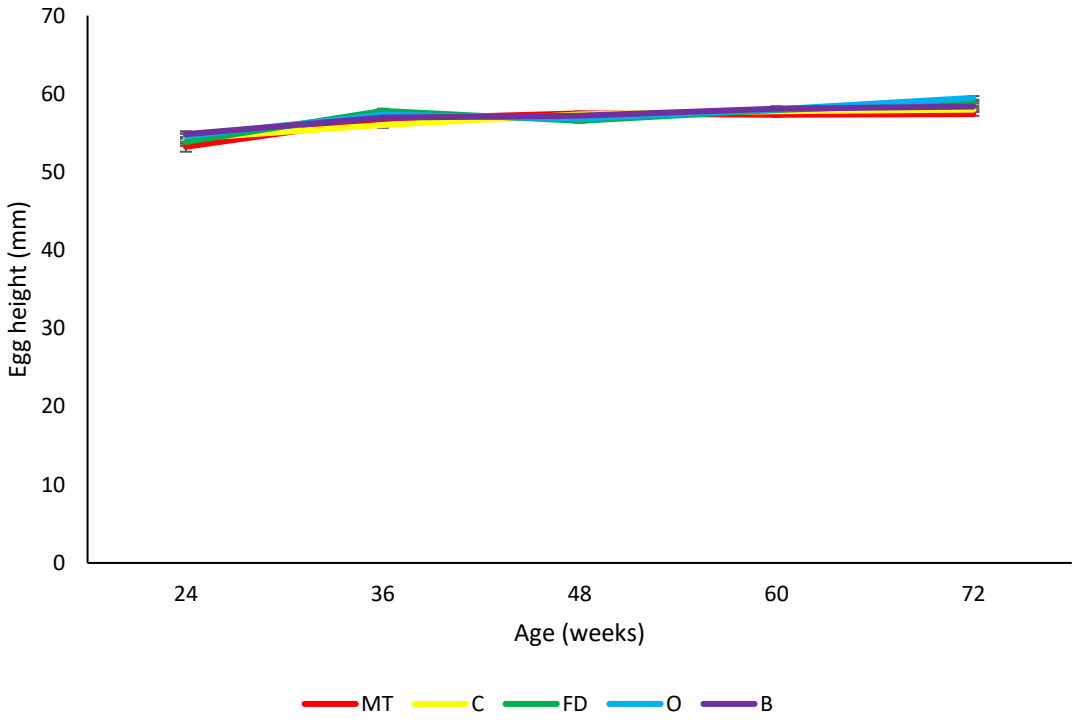

Fig. 5.2 Effect of age and housing system on egg height

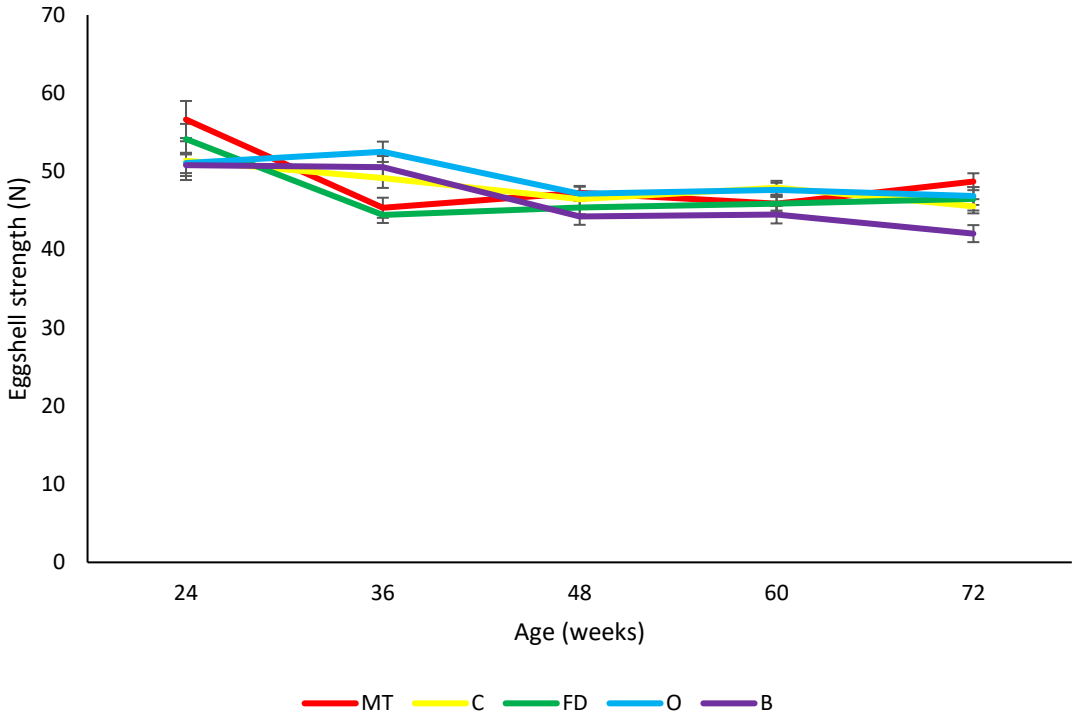

Fig. 5.3 Effect of age and housing system on eggshell strength

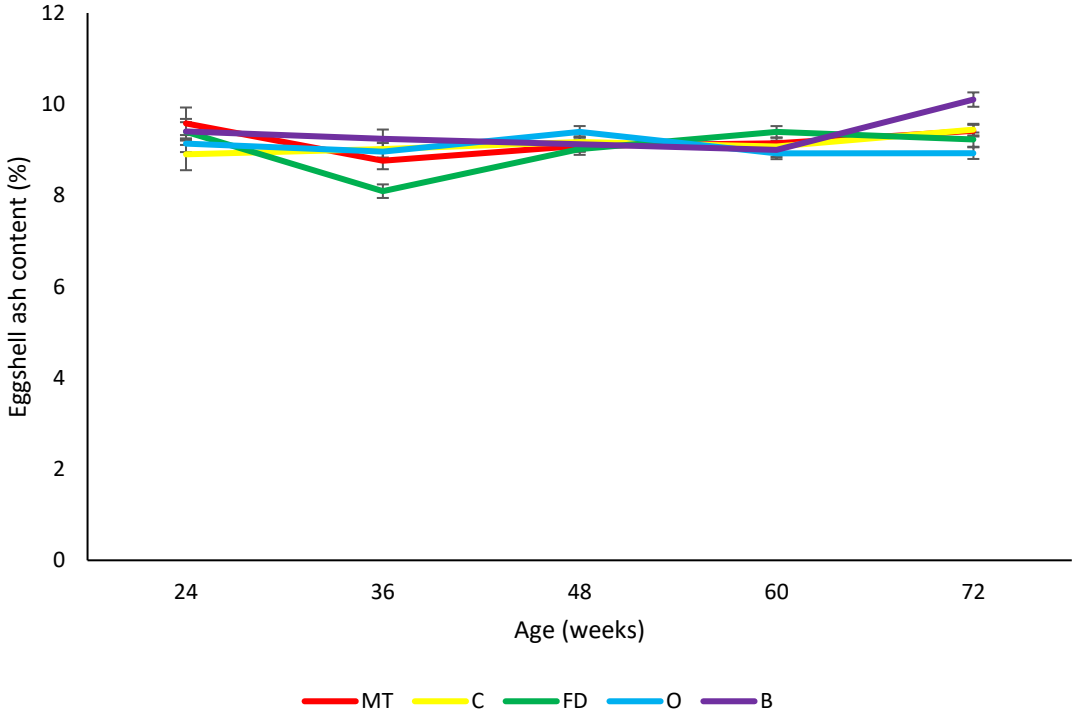

Fig. 5.4 Effect of age and housing system on eggshell ash content

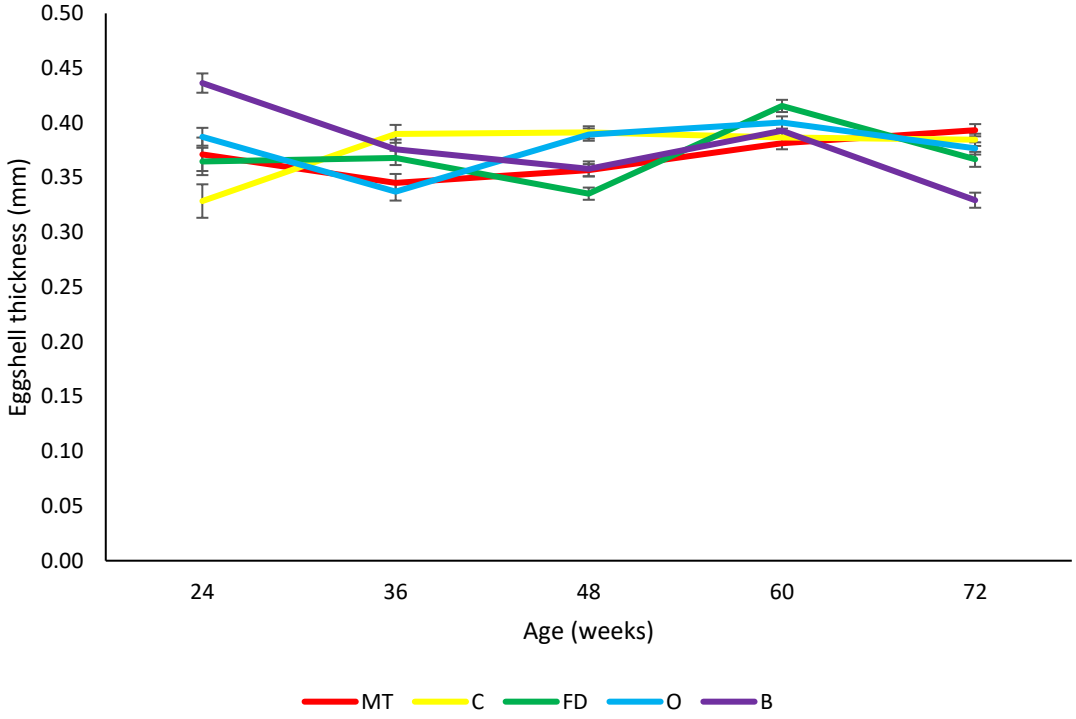

Fig. 5.5 Effect of age and housing system on eggshell thickness

5.3.2 Predicting eggshell strength

A Gaussian Linear Mixed Model was fitted to the egg data to predict egg breaking strength over the laying period of hens from UK farms in multiple housing systems. Individual farm was fitted as a random term and eggshell ash weight as a covariate. Conditional R^2 was 0.191 and marginal R^2 was 0.136. Model parameter estimates are shown in Table 5.3 and Fig. 5.6.

Table 5.3 shows the intercept (Caged eggshell strength) differs significantly from 0 ($p < 0.001$). Ash weight is also significantly different from 0 ($p < 0.001$), with a parameter estimate of 3.73, indicating that as ash weight increases, eggshell strength increases. Overall, as age increased, caged eggshell strength decreased ($p < 0.001$; estimate = -0.16). Barn eggshell strength declines significantly faster than caged eggshell strength ($p = 0.036$; estimate = -0.29) (Fig. 5.6).

Table 5.3 Summary of Gaussian LMM to model eggshell strength over laying period of UK laying hens with farm fitted as a random term. The estimated value for σ^2 is 87.24, N_{farms} = 14, N_{eggs} = 1408. The caged housing system was set as the baseline coefficient

	Gaussian model		
Coefficient	**Estimates**	**CI (95%)**	***p***
(Intercept)	33.43	27.28 – 39.59	**< 0.001**
Ash weight	3.73	3.10 – 4.37	**< 0.001**
House [B]	4.47	-3.22 – 12.17	0.255
House [FR FD]	1.46	-6.12 – 9.04	0.705
House [FR MT]	-3.40	-11.02 – 4.22	0.382
House [O]	2.06	-5.04 – 9.16	0.570
Age	-0.16	-0.24 – -0.08	**< 0.001**
Age * House [B]	-0.13	-0.25 – -0.01	**0.036**
Age * House [FR FD]	-0.05	-0.18 – 0.07	0.387
Age * House [FR MT]	0.06	-0.06 – 0.18	0.319
Age * House [O]	-0.05	-0.15 – 0.06	0.403
Random Effects			
σ^2		87.24	
$\tau_{00\ Farm}$		5.84	
ICC		0.06	
$N_{\ Farm}$		14	
Observations		1408	
Marginal R^2 / Conditional R^2		0.136 / 0.191	

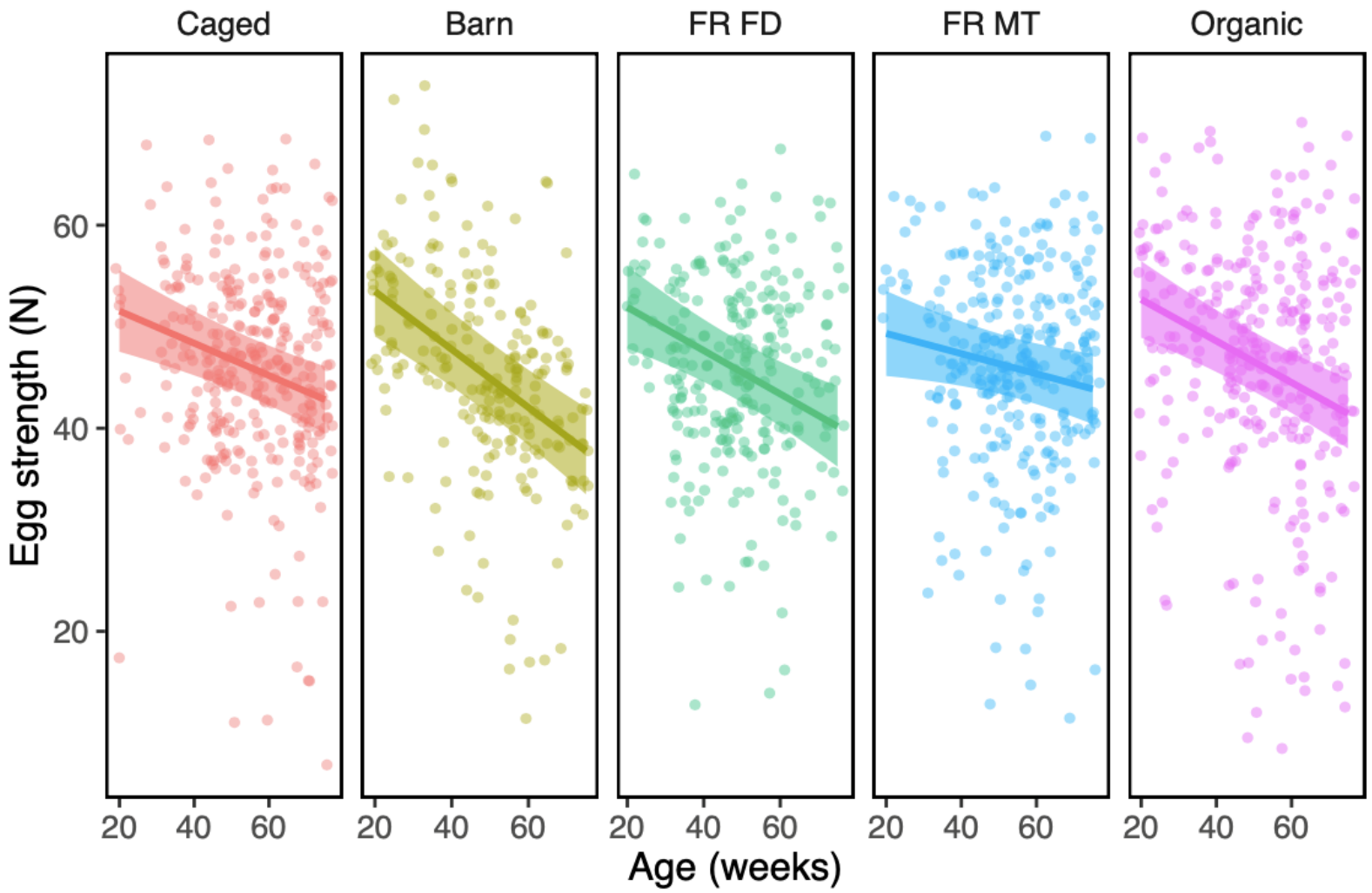

Fig. 5.6 Mean fitted eggshell strength of UK laying hens (solid line) and 95% confidence intervals (shaded area) over age (weeks) modelled with a Gaussian LMM, with farm fitted as a random term in the model. Data is split by housing system

5.3.3 Relationship between bone and eggshell strength

Fig. 5.7 shows that there was no relationship between bone strength and the egg residual variance for eggshell strength. The egg and bone data were collapsed at a farm level and a new model was fitted for eggshell strength, as in the previous section. Strength residuals were collapsed to match bone data on a farm level and were fitted into another linear model. This was to predict the unexplained variance in eggshell strength based on keel, humerus and tibia strength. Fig 5.7 indicated that there was no relationship between bone strength and the eggshell strength residuals.

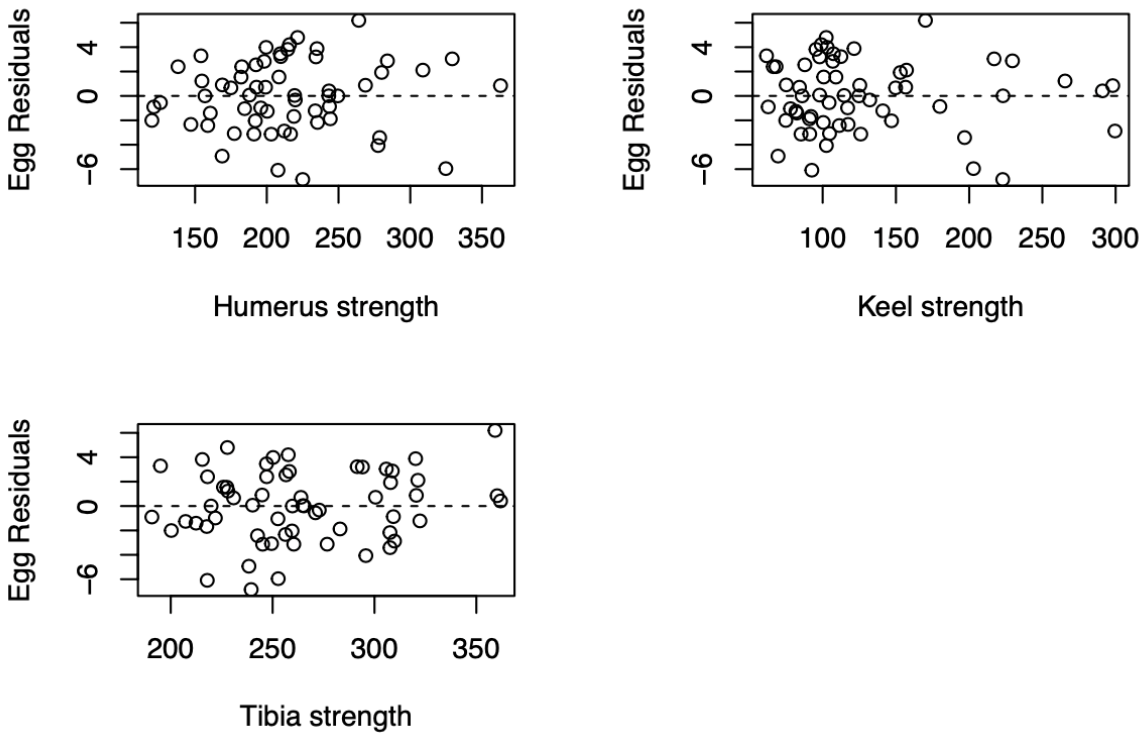

Fig. 5.7 Egg residuals plotted against keel, humerus and tibia strength, after data has been collapsed to a farm level. A model for eggshell strength with egg ash weight, housing and age as predictors was fitted. Farm was used as a random term. The residuals from the model were then fitted to a linear model to predict unexplained variance based on various bone strengths

5.4 Discussion

5.4.1 Egg quality traits

All egg quality traits measured within this study were significantly affected by age, housing system and the interaction between age and housing system (Table 5.1, Fig. 5.1 – 5.5) (p = 0.007 or less). The results of egg weight showed lower egg weight at 24 weeks, an increase at 36 weeks which then stabilised until the end of the trial. The noticeable difference in egg weight from 24 weeks to 36 weeks is caused by the hens having only started their laying cycles a few weeks prior to this sample point, at the point of sexual maturity (18-20 weeks). It is known at the start of lay egg sizes often start smaller and are more variable (Hester, 2017). There was also a slight increase in egg weight over time across all housing systems. Throughout the period of lay, organic eggs were some of the heaviest eggs compared to other systems. A study by Ketelaere *et al*., (2002) found that through a Pearson's correlation analysis, egg weight had a positive correlation with week, indicating that as hens age, they lay heavier eggs (coefficient = 0.415).

Another study by Van Den Brand *et al*., (2004) also found that age influenced egg weight (p < 0.001) as well as the interaction between age and house (p = 0.002), but housing did not (p = 0.920), therefore both previous studies agree with the present study that egg weight increases throughout the laying period. Interestingly, past research showed that housing system did not support the results of the present study (Van Den Brand *et al*., 2004). It could be suggested that as the Van Den Brand *et al*., (2004) study used less types of housing systems, the effects of housing systems on egg weight were less pronounced, as only battery cage and outdoor systems were investigated, compared to the 5 systems used in this study. On the other hand, results of egg weights from Van Den Brand *et al*., (2004) and Wang *et al*., (2009) do support that there is an age*housing system interaction indicating that the effect of housing on egg weight depends on age.

This study measured egg height as a way of estimating egg size. Age, housing system and the interaction between the two showed a significant effect on egg height. Few studies have been found that measure solely egg height as a measure of egg size with most using shape index (a value based on egg length (height) and width (Van Der Brand *et al*., 2004; Hidalgo *et al*., 2008; Kraus *et al*., 2021). As seen for egg weight there is a noticeable difference in egg height from 24 to 36 weeks of age, with very slight increase until the end of the laying period. As previously mentioned, younger birds generally lay smaller eggs as they have only recently passed the point of sexual maturity and egg development can cause fluctuations in size, normally settling further into the laying period (Hester, 2017).

A study by Hidalgo *et al*., (2008) found that egg height differed significantly between housing systems ($p < 0.05$). Free-range (6.05cm) and organic eggs (5.92cm) were significantly longer than eggs from caged (5.74cm) or barn (5.89cm) systems. Organic eggs were shown to be longer within the current study but only towards the end of lay (Fig. 5.2). It could be possible that a multitude of factors such as stocking density and ability to exercise cause less competition within the system. This then would allow the hen to produce larger sized eggs as they are able to perform more natural behaviours and have reduced stress levels compared to other systems. Other factors not considered within the present study could have also affected egg size, primarily the strain of birds and diets used. As previously mentioned, the use of egg height to assess egg quality is not commonly used, often studies use shape index which combines length and width of the egg to give an indication of size. A study by Van Den Brand *et al*., (2004) also found there was an effect of age and housing on egg shape index but no effect of an interaction ($p < 0.001$, $p < 0.001$ and $p = 0.61$ respectively). Results showed increasing age decreases shape index % meaning eggs became smaller. The effect of housing system also showed that the outdoor eggs had a higher shape index % than caged eggs (75.44% to 74.70% respectively). Although a direct comparison between those results and egg height results from the present study cannot be made, perhaps results would differ and support Van Den Brand *et al*., (2004) if shape index was used. In addition, a more recent study by Kraus *et al*., (2021) also found that age influenced egg shape index % ($p = 0.028$), though housing system had no effect ($p = 0.710$), and the interaction had no effect ($p = 0.872$). It

would be advantageous for future work studying egg quality to use egg shape index over egg height as it seems to be a more commonly acceptable measurement of egg size.

In previous studies, eggshell breaking strength has been a common measurement used to assess egg quality (Guinette and Nys, 1991; Wang *et al*., 2009; Hidalgo *et al*., 2008; Englmaierová and Tumová, 2014). Eggshell strength was affected by age, housing system and the interaction of age and housing system in this study. The eggshell strength of the multi-tier and flat deck systems were highest at the beginning of lay, though declined quicker than all other systems from 24 weeks to 36 weeks (Fig. 5.3). Conversely the egg height and weight (possibly overall size) increase at 36 weeks, therefore the drop in eggshell strength at this age could be due to the crystalline structure of eggshell being less organised as there is more surface area to cover (Rodriguez-Navarro *et al*., 2002). From 36 weeks the organic systems showed greater eggshell strength, though at 72 weeks multi-tier is higher. After 36 weeks, the barn system also showed the lowest eggshell strength until the end of lay. Interestingly, Vits *et al*., (2005) described a lower eggshell strength was found in higher density systems. This could explain why organic systems showed a high eggshell strength throughout while barn showed the lowest. Caged systems showed a higher eggshell strength after 36 weeks of age than barn, but this could be because the caged systems used were colony cages and stocking density comparatively low per cage. Furthermore, previous studies have found systems with outdoor access increases eggshell strength, supporting results from the present study although, not always significantly different. It is known that outdoor access can provide more vitamin D and therefore this could have also caused an increase in eggshell strength. Research showed that an increase in vitamin D_3 increased eggshell strength at 8,348 IU D_3/kg diet, 35,014 IU D_3/kg diet and 68,348 IU D_3/kg diet compared to a control diet (1,681 IU D_3/kg diet) by approximately 0.20kg of force ($p < 0.001$) (Wen *et al*., 2019). A study by Wang *et al*., (2009) investigating the effects of housing systems and age on blue shelled layers found age did have a significant effect on eggshell strength ($p < 0.001$) but housing system and the interaction between age and housing system did not ($p = 0.50$ and $p = 0.91$ respectively). Wang *et al*., (2009) sampled 60 eggs per collection over 4 age groups and systems whereas this study used 30 eggs, 5 age groups and 5 systems. The difference in methodologies could have caused the differences in results. The effect of age from this previous study supports data from the

present study, however, a housing system and interaction effect was also shown. Other studies such as Habig and Distl, (2013) found as age increases eggshell strength decreases (p = 0.010; 3 months = 44.31N, 9 months = 39.57N, 12 months = 36.91N). It is suggested that the effect of age and the onset of osteoporosis can negatively affect eggshell strength as the demand for skeletal calcium increases thus increasing overall demand and subsequently effecting eggshell deposition (Rodriguez-Navarro *et al*., 2002). A more recent multifactorial study by Kraus *et al*., (2021) found that age did not influence eggshell strength, though housing system and the interaction of housing system and age did (p = 0.368, p = 0.005 and p = 0.022). These results are opposite to what was found by Wang *et al*., (2009) but do support the present study as all main effects and the interaction were significantly affecting eggshell strength. As previously mentioned, method design such as what housing systems and age groups were used in a study can cause differences between comparable studies (Kraus *et al*., 2021). As the present study was a commercial trial, some factors such as diet and genetic strain could not be controlled, and these could have altered results without being assessed. Furthermore, eggshell strength is likely to be heavily linked to other egg qualities such as egg size, and eggshell thickness (Sapkota *et al*., 2017). A study by Rodriguez-Navarro et al., (2002) showed that better organisation of calcite crystals provided greater eggshell strength and an improved eggshell ultrastructure. Furthermore, it was shown that as crystal size increases, eggshell thickness increases and ultimately leads to an increase in eggshell strength. The difference in eggshell strength per housing system over age in this present study could have therefore been influenced by the level of organisation at the time of shell deposition.

Interestingly some studies modelling eggshell strength have found that caged systems have thinner eggshells but higher eggshell strength due to differences in shell ultrastructure compared to aviary systems (Karcher *et al*., 2015). Calcium intake between in caged birds was also found to be higher in caged systems compared to a floored system, ranging from an increase of 0.2 to 1g a day (Lichovníková and Leman, 2008). Caged eggshell strength was higher than barn towards the end of lay in this study. It could be suggested that the caged birds within this study may have also had a higher intake of calcium than the barn system (similar to a floor system) and therefore improved eggshell deposition and subsequently eggshell strength. However, as feed intake was not measured in this study this theory cannot

be confirmed. Furthermore, the changes in eggshell strength could be influenced by dietary differences between farms. Though the dietary schedules were provided, the diets used could not be controlled as this study was run in a large commercial setting. As such, additives and supplements could have been provided such as extra calcium in the form of oyster shell.

Age, housing system and the interaction all had a significant effect on eggshell ash content (p = 0.001 or less). This study found that housing system effects on eggshell ash content depend on the age of the bird. The differences between housing system over age could have been caused by varying calcium intake or more direct dietary differences including feed additives or supplements. Fig. 5.4 shows multi-tier and flat-deck systems decline in ash content at 36 weeks before then increasing at 48 weeks. The results also show that barn is significantly higher in ash content at 72 weeks compared to all other systems, though this is not mirrored in other egg quality measurements. It would be advised to reconsider the usefulness of eggshell ash content when assessing effects on egg quality. However, it may be useful when comparing bone qualities to egg qualities as differences in calcium mobilisation may be better interpreted. Eggshell ash content is not a commonly used measurement of eggshell quality. A similar but more commonly used measurement is shell percentage. Van Den Brand *et al*., (2004) found an interaction of age and housing system effected shell percentage showing shell weight started high, decreased around mid-lay and then increased towards the end of lay, though housing system results were comparable throughout. Another study by Kraus *et al*., (2021) found that housing system (p = 0.046) had a main effect on shell percentage, but age (p = 0.473) and the interaction did not (p = 0.330). Although a direct comparison cannot be made, it is assumed that shell percentage and ash content would be strongly correlated with one and other as a greater amount shell would contain more mineral content (Hester, 2017). It would be advised in future work to measure shell weight and assess shell percentage rather than ash content as more previous studies have used this measurement when assessing egg quality (as seen in chapter 1, Table 1.12).

Although age, housing system and their interaction showed a significant effect on eggshell thickness, it is difficult to draw any conclusions on actual effect of housing system and age as there is much variation within the results (Fig. 5.5). As previously stated, if eggshell thickness

is increased, eggshell strength is thought to increase also (Rodriguez-Navarro *et al*., 2002; Lichovníková and Zeman, 2008; Karcher *et al*., 2015). However, the trends in eggshell thickness and eggshell strength do not match within this study, indicating the accuracy of the eggshell thickness results could be poor. For example, the barn system shows the greatest eggshell thickness at 24 weeks, however eggshell strength for barn at 24 weeks shows one of the lowest. It is possible that after an eggshell had been tested for strength, fragments of shell may have stuck together on the points at which eggshell thickness was measured. Any fragments that stuck together may have caused eggshell thickness values to be greater, causing errors in the results. Furthermore, if eggs were not emptied and cleaned correctly, broken shell fragments would often stick to the digital callipers with residual albumen or yolk and could cause additional errors. Diets used in the study could have also had an effect on the variation with these results, but the effect of diet was not assessed. The study by Kraus *et al*., (2021) found that age ($p < 0.001$) and housing system ($p = 0.022$) as main effects influenced eggshell thickness, but the interaction effect did not, though there was a trend indicating the effect of housing system on eggshell thickness depended on age ($p = 0.080$). This study supports the results for age and housing system for the present study but contradicts the results from the interaction effect. Kraus et al., (2021) found that the lowest values of eggshell thickness was found in the oldest hens. The variation within eggshell thickness results in the previous study was much less than the current study ranging from 0.289 – 0.338mm compared to 0.329 – 0.436mm. Another study by Dikmen *et al*., (2017) found that housing system and age interaction did significantly affect eggshell thickness, thus supporting this current study but contradicting the study by Kraus *et al*., (2021). The current study and Dikmen *et al*., (2017) therefore indicate that housing system effects eggshell thickness differently depending on the age of the bird. Though in the current study when considering the relationship between eggshell strength and thickness that is usually shown (increase in eggshell thickness = greater eggshell strength) (Rodriquez-Navarro *et al*., 2002), care should be taking in summarising the interaction effect as there are no clear trends in eggshell thickness data.

5.4.2 Modelling data

As was the case for bone strength models (chapter 4), studies modelling eggshell strength have been scarce. The model parameters in Table 5.3 showed that ash weight of the eggshell was a significant predictor of eggshell strength within this data. This indicates that a higher ash weight will provide a higher eggshell strength value. Eggshell ash weight is not a commonly used measurement. The study by Alfonso-Carillo *et al*., (2021) found that there was a significant positive relationship between shell weight and shell breaking strength when analysing via Pearson's correlation (0.652; $p < 0.05$). Shell weight was not collected in this dataset to reduce sampling times. Though as the previous study shows a relationship between shell weight and breaking strength (Alfonso-Carillo *et al*., 2021), it could be theorised that ash weight would also have a similar effect as a heavier shell weight would likely indicate higher mineral contents. Therefore, modelling these parameters appears to agree with other reported literature.

Table 5.3 also showed an overall effect of age on caged eggshell strength (estimate = -0.16; $p < 0.001$). It is suggested that as birds age, the organic matrix content which provides a precursor for egg development declines in older hens, therefore altering the structure of the egg, increasing the likelihood of weaknesses and poorer shell strength (Rodriguez-Navarro *et al*., 2002). The study by Rodriguez-Navarro *et al*., (2002) supports the results from this study as they also found eggshell strength to be weaker in older hens compared with younger hens ($p < 0.001$). Furthermore, they also showed that eggshell strength as a function of eggshell thickness was higher in younger birds than older birds. Another study by Ketelaere *et al*., 2002 also supports the results of this study. Dynamic static stiffness and breaking force were found to have a significant correlation with age (weeks) (-0.218 and -0.080 respectively). A more recent study by Kraus *et al*., (2021) found no main effect of age on eggshell strength when determining egg parameters in Czech and Slovak native hens ($p = 0.368$), thus contradicting results from the present study. One difference causing a lack of main effect of age could have been the trial length. Kraus *et al*., (2021) only studied the birds at 34, 42 and 50 weeks whereas the present study used more time points (5 age groups) over a longer period (24 to 72 weeks). It could be possible that the increase in age groups used, and the extended trial period exacerbated the effects of age on eggshell strength compared to the previous study.

Table 5.2 and Fig. 5.6 show that eggshell strength declines quicker in the barn system than the caged system (estimate = -0.13; p = 0.036). It is unclear why the barn system declines faster than the caged system. It could be possible that the calcium utilisation is greater in barn than caged as a higher percentage of dietary calcium must go towards repairing bone damages as barn birds are more often more active than a caged bird (Leyendecker *et al*., 2005). The results of this part of the study could also have been influenced by the management on the farms which provided the eggs. In both barn farms, eggs and bird samples were picked by workers and not the researchers involved with this study, therefore possibly allowing for some biased selections. Furthermore, due to the lack of farms currently using barn systems to keep hens, only two barn systems were monitored compared to three farms used for other systems. Future work to add more data to this model would be beneficial in creating a more robust model of why eggshell strength declines quicker in some systems compared to others.

Results shown in Fig. 5.7 show that there is no correlation between bone and eggshell strength residuals meaning that there is no clear relationship between the two variables. This suggests that bone strength is not compromised by egg quality (based on eggshell strength). These results could be caused by the fact that after collapsing all data to the farm level, there was substantially reduced data available to be able to determine a relationship between egg and bone strength. To investigate the relationship more accurately, bone and egg data need to be collected for an individual bird. The bone and egg samples would then be linked and a more accurate analysis of the relationship between bone and eggshell strength would be possible. It is unlikely that collections of individual eggs from a commercial system would be a plausible method of research as it would require large amounts of planning, system modifications, costs, and labour when collecting the data. Attempting to collect eggs from individual hens might mean keeping hens in individual cages or similar, therefore making the housing systems irrelevant and therefore impossible to assess.

A study by Jansen *et al*., (2020) investigating bone traits against eggshell production in multiple different strains, also found no strong association with eggshell production and bone

characteristics (BMD in particular) overall. Though results did show that the R11 laying strain in both tibia and humerus did show a significant regression coefficient between BMD and eggshell production, but no other strains showed a significant regression. Furthermore, the same figure did indicate that there was a trend showing as eggshell production increased, BMD decreased. Another study by Alfonso-Carillo *et al*., (2021) found that when analysing bone and egg properties using a Pearson's correlation, that the relationship between bone and egg production is weak and not obvious. The previous study showed a significant negative correlation between uterus weight and mineral content in cortical bone (-0.237) but a positive correlation between egg weight and tibia diameter (0.221). Though egg and bone strength were analysed in the study, they did not show a significant correlation between each other and showed an extremely weak relationship (0.024) not dissimilar to the lack of relationship shown in the present study. It could therefore be advised that additional work is needed on determining the relationship between bone and eggshell strength or in general between egg and bone characteristics, when determining the effect of egg production on bone health.

5.5 Conclusion

Overall, it could be suggested that measuring the effect of housing system and age on egg qualities is not informative on skeletal health or welfare. It is thought indicatively that egg production will take precedent over skeletal maintenance due to large amounts of genetic selection to control for mineral mobilisation over time. Although egg quality traits are of high importance to the egg industry, the results from this chapter add little to the understanding of the consequences of egg production on skeletal health. It is suggested that there is some correlation between bone health and egg production, but the relationship is highly unclear (Alfonso-Carillo *et al*., 2021). It would be advised that future research is needed in order to better understand if there is a relationship, though it seems that ultimately hens are now bred to maintain egg production at the cost of skeletal health to meet demands of the industry.

Chapter 6: Validity of methods for assessing skeletal integrity

6.1 Introduction

Assessing skeletal integrity is an important aspect of maintaining hen welfare throughout the laying period. As previously mentioned in section 1.7, there are many approaches to assess skeletal integrity, including invasive or non-invasive methods each with their own advantages and disadvantages (Regmi, *et al*., 2016). This study begins to validate whether the methods, samples and parameters developed in chapter 3 and used in chapter 4 are beneficial to assessing skeletal health in laying hens. This study uses end of lay hens moved to a pen-based floor system fed a commercial diet with or without a silicon supplement over the course of 7 weeks, to see if the nutritional intervention (silicon supplement) to improve bone health can be identified using the methods from the previous chapters.

Aims

- To determine if methods from previous chapters can identify the effects of a nutritional intervention on bone health in laying hens
- To conclude if egg parameters could be useful in assessing skeletal health

6.2 Trial methods

6.2.1 Bird husbandry

Lohmann Brown Classic birds were sourced from Longbelt Farm, Newark (Noble Foods Ltd) from a flock aged 72 weeks and had previously been housed in a multi-level colony cage system. Birds were weighed on arrival and any birds showing signs of ill health were culled and health status of the culled hens were recorded. Birds were also ringed with blue, yellow, and pink bands per pen for identification purposes. Seventy-eight hens were weighed individually before being allocated to twenty-six 0.64m^2 purpose built wooden, mesh sided pens (13 per diet, 2 of which were spare pens) bedded on 3cm of clean wood shavings with two nest boxes available, stacked on top of one and other (nest box volume = 0.027m^3). 38mm wooden dowel perches were provided 20cm above the litter in all pens. Temperature was controlled using thermostat and set at 21°C throughout the trial period. Lighting was set as 16 hours light and 8 hours darkness, with a 15-minute twilight period each side of darkness. The light intensity of the room was set to measure 15 lux at feed trough height. Bird health checks and environmental checks were undertaken twice daily. Food and water were provided *ad libitum* and husbandry guidelines were followed as stated in section 2.2.2. The room layout and pen layout can be seen below (Fig. 6.1 & Fig. 6.2).

					Door
19		13	12		1
20		14	11		2
21		15	10		3
22		16	9		4
23		17	8		5
24		18	7		6
		spare	spare		

Figure 6.1 Layout of the trial room

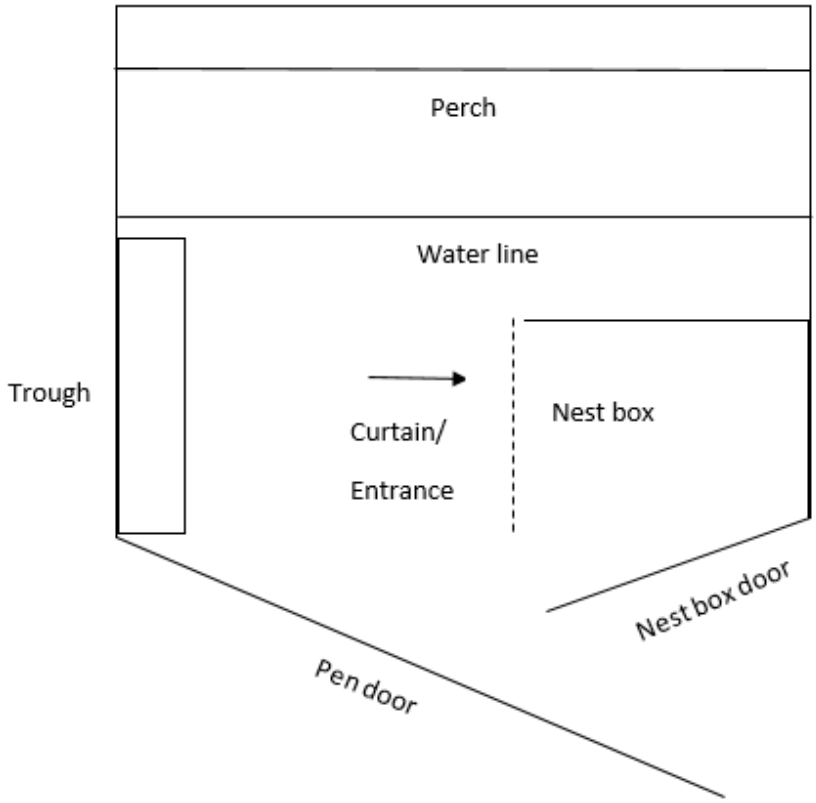

Figure 6.2 Top-down layout of an individual pen

6.2.2 Diet formulation

Birds were fed a generic commercial layer mash (GoldNLay, GLW; Shepshed, UK) throughout the 7-week trial period, with or without the inclusion of a novel silicon supplement. Dietary treatments can be seen below (Table 6.1).

Table 6.1 Dietary treatments for the Si14 trial

Diet	Treatments
A	Commercial mash only (control)
B	Commercial mash with NTU produced silicon supplement at 600ppm

Diet B was manufactured in house as per the method in section 2.3 and was produced in one batch at the beginning of the trial and topped up when needed throughout the trial period. A novel silica supplement (Scholey *et al*., 2018b) was added to the commercial mash to achieve 600ppm of silica. The study allowed for 12 + 1 spare replicate pens per dietary treatment. Each dietary treatment was analysed for energy content, dry matter, ash content, fats, and protein. The methods by which the diets were analysed are outlined in sections 2.8.1 to 2.8.5 (analysis reported in Table 6.3). Diets were randomised around the room by block to reduce the effect of room placement (Table 6.4).

Table 6.3 Analysed content of dietary treatments

	Diet	
Constituents (g/kg)	**A**	**B**
DM	908.22	901.39
Ash	81.06	59.00
Fat	25.78	24.85
Protein	163.13	170.83
Energy (MJ/kg)	13.62	14.23

Table 6.4 Diet allocation per pen blocked for environmental effects

Pen	Diet	Pen	Diet
1	A	**13**	B
2	B	**14**	A
3	A	**15**	B
4	B	**16**	A
5	A	**17**	B
6	B	**18**	A
7	B	**19**	A
8	A	**20**	B
9	B	**21**	A
10	A	**22**	B
11	B	**23**	A
12	A	**24**	B

6.2.4 Sample collection

6.2.4.1 Bird weights, feed intake and FCR

Individual bird weights were recorded on arrival and then every 7 days from D7 (73 weeks) to D49 (79 weeks) and average weekly bird weights were calculated. Birds were ringed with either a pink, yellow or blue band so they could be identified when taking individual weights. Feed was weighed into 5kg bags and provided individually to each pen. The feed bags were tipped back and weighed weekly to calculate average weekly feed intake per bird and feed conversion ratios (FCR) per bird. Feed bags were topped up and recorded where necessary.

6.2.4.2 Bone samples

One bird at D28 (76 weeks) and two birds at D49 (79 weeks) were culled via cervical dislocation. The keel, humeri and tibiae were dissected from each bird post-mortem as per section 2.4 and stored at -20°C until measured. All bone samples were measured for length, width, weight, strength using the methods described in section 2.5.1. Length and width were measured using a 2-decimal place digital calliper. Weight was measured using a 4.d.p top pan balance (Satorius, UK) and strength was measuring using a TA.XT 100 Plus Texture analyser (Stable Micro Systems, Guildford) and a 3-point bend rig with a 100KG load cell (Stable Micro Systems, Guildford) as outlined in section 2.7.3.

6.2.4.3 Egg samples

Two eggs per pen were collected every 7 days from D13 (74 weeks) to D48 (79 weeks) of the trial. These eggs were then measured for egg weight, albumen height, shell weight, shell percentage shell strength and shell thickness as described in section 2.7.4. Egg weight was recorded to 4.d.p using an analytical balance (Satorius, UK), albumen height was measured using a albumen height gauge (QCH; TSS, York), shell weight was recorded using the analytical balance to 4.d.p (Satorius, UK) shell breaking strength was measured using a TA.XT 100 Plus Texture analyser (Stable Micro Systems, Guildford) and a cylindrical probe (P36/R; Stable Micro Systems, Guildford) and shell thickness was measured using 2.d.p digital callipers at 2 points around the mid circumference of the shell. Shell percentage was calculated used the following equation:

$$Shell\ percentage\ (\%) = \frac{Dry\ eggshell\ weight\ (g)}{Egg\ weight\ (g)} \times 100$$

In addition, Haugh unit was also calculated using albumen height and egg weight to determine egg quality. Equation for Haugh unit below:

$$Haugh\ Unit\ (HU) = 100 \times log\ (h\ - 1.7w^{0.37} + 7.57)$$

6.2.5 Data Analysis

Performance data including average weekly body weights, feed intake and FCR were analysed using IBM SPSS 26 (IBM Statistics). Bone and egg data were also analysed with using R version 4.1.0 and RStudio version 1.1.463. A two-way ANOVA was used to analyse the effect of age and diet and their interaction on bone parameters, and another was used to analyse the same factors and interaction effect on egg quality traits. Data for the left and right humerus and the left and right tibia were averaged to provide one per bone value per bird. At D49 (79 weeks), bone data from the two birds collected were averaged to provide a per bird value. The two eggs collected per pen from D13 (74 weeks) to D48 (79 weeks) were also averaged to give one value per pen per week for all parameters. The two shell thickness measurements taken per egg were averaged to give one value per pen.

6.3 Results

6.3.1 Performance data

Average weekly body weights did not differ significantly per week (p = 0.395) meaning a healthy weight was maintained per pen throughout the trial (Table 6.5; Fig. 6.3). Feed intake and FCR were not summarised as there was high amounts of feed wastage throughout the trial.

Table 6.5 Average weekly body weights over the trial period

Week	Body weight (g) (±SE)
Placement	1871.2 (23.18)
1	1800.0 (23.71)
2	1812.6 (23.52)
3	1830.3 (21.65)
4	1836.1 (20.95)
5	1853.2 (29.19)
6	1836.0 (29.17)
7	1877.0 (33.82)
***p* value**	0.395

Means within a column with different letters are significantly different (p = 0.05)

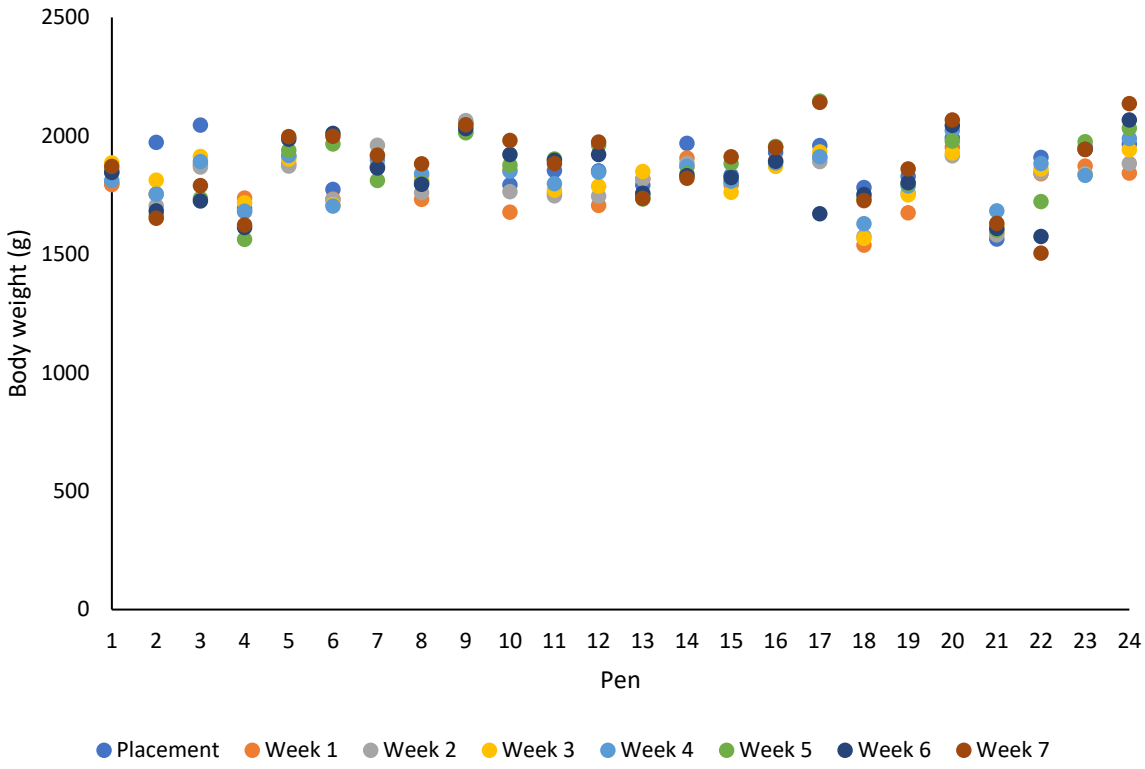

Figure 6.3 Average weekly body weights per pen over the trial period

6.3.2 Body weight data

There was no effect of age, diet, or the age * diet interaction (Table 6.6) on body weight of the hens (p = 0.185, p = 0.844 and p = 0.932 respectively).

Table 6.6 Effect of age, diet, and interaction effect on body weight

	Body weight (kg) (±SE)
76 weeks	
Control	1.80 (0.059)
Si 600 ppm	1.80 (0.059)
79 weeks	
Control	1.87 (0.057)
Si 600 ppm	1.89 (0.057)
p	
Age	0.185
Diet	0.844
Age * Diet	0.932

Means within an age group with different letters are significantly different (p = 0.05)

6.3.3 Bone data

6.3.3.1 Bone length

Table 6.7 showed that age only had a significant effect on tibia length ($p < 0.001$). Diet had no effect on any bone length, but tibia length did show a trend ($p = 0.099$), indicating that the control diet showed longer tibia bones. The interaction of age and diet did have a significant effect on humerus length ($p = 0.018$) but not keel and tibia, indicating that the effect of diet on humerus length is dependent on age. For example, the humerus is longer in the supplemented diet at 79 weeks than the control diet but is significantly smaller at 76 weeks.

Table 6.7 Effect of age, diet, and interaction effect on bone length

	Bone Length (mm) (±SE)		
	Keel	**Humerus**	**Tibia**
76 weeks			
Control	125.09 (1.711)	79.61 (0.604)[a]	116.67 (1.097)[a]
Si 600ppm	123.32 (1.711)	77.58 (0.576)[b]	113.65(1.046)[a]
79 weeks			
Control	120.79 (1.638)	78.83 (0.576)[a]	119.60(1.046)[a]
Si 600ppm	124.38 (1.638)	79.68 (0.576)[a]	118.98(1.046)[a]
p			
Age	0.338	0.233	< 0.001
Diet	0.544	0.344	0.099
Age * Diet	0.117	0.018	0.265

Means within a column within an age group with different letters are significantly different ($p = 0.05$)

6.3.3.2 Bone width

The humerus was the only bone width affected by age ($p < 0.001$), the keel and tibia showed no effect of age ($p = 0.526$ and $p = 0.884$ respectively) (Table 6.8). There was no significant effect of diet on any bone width measurement in the results. The interaction effect influenced keel width ($p = 0.013$) but not humerus width and tibia width. The interaction effect indicated that the effect of diet on keel length was dependent on age – as the keel width of the control is significantly wider than the Si 600ppm group at 76 weeks but not at 79 weeks of age.

Table 6.8 Effect of age, diet, and interaction effect on bone width

	Bone Width (mm) (±SE)		
	Keel	**Humerus**	**Tibia**
76 weeks			
Control	29.01 (0.799)[a]	6.50 (0.105)[a]	7.88 (0.142)
Si 600ppm	26.12 (0.799)[b]	6.37 (0.101)[a]	7.53 (0.136)
79 weeks			
Control	27.47 (0.765)[a]	8.14 (0.101)[a]	7.70 (0.136)
Si 600ppm	28.65 (0.765)[a]	8.31 (0.101)[a]	7.74 (0.136)
p			
Age	0.526	< 0.001	0.884
Diet	0.331	0.842	0.285
Age * Diet	0.013	0.141	0.161

Means within a column within an age group with different letters are significantly different (p = 0.05)

6.3.3.3 Bone weight

Table 6.9 shows that age, diet, and interaction effect had no influence on bone weight from any bone.

Table 6.9 Effect of age, diet, and interaction effect on bone weight

	Bone Weight (g) (±SE)		
	Keel	**Humerus**	**Tibia**
76 weeks			
Control	7.51 (0.447)	3.08 (0.186)	6.90 (0.335)
Si 600ppm	7.19 (0.426)	2.72 (0.168)	6.20 (0.303)
79 weeks			
Control	7.34 (0.408)	2.95 (0.168)	6.51 (0.303)
Si 600ppm	7.20 (0.408)	2.80 (0.168)	6.37 (0.303)
p			
Age	0.866	0.960	0.823
Diet	0.595	0.157	0.202
Age * Diet	0.825	0.570	0.371

Means within a column within an age group with different letters are significantly different (p = 0.05)

6.3.3.4 Bone strength

Table 6.10 shows age had a significant effect on all bone strengths ($p < 0.001$, $p = 0.035$ and $p = 0.030$ for keel, humerus and tibia respectively), with all bones becoming weaker with age. Diet had no effect on strength of any bone, though a trend was shown in keel strength ($p = 0.083$), indicating that the control diet provided stronger keel bones at 76 weeks but not 79 weeks. The interaction effect showed no effect on keel, humerus or tibia strength ($p = 0.188$, $p = 0.828$ and $p = 0.663$ respectively).

Table 6.10 Effect of age, diet, and interaction effect on bone strength

	Bone Strength (N/kg) (±SE)		
	Keel	**Humerus**	**Tibia**
76 weeks			
Control	50.23 (3.193)[a]	87.94 (5.980)[a]	134.94 (9.665)[a]
Si 600ppm	40.57 (3.044)[b]	86.38 (5.409)[a]	120.27 (8.742)[a]
79 weeks			
Control	32.29 (2.915)[a]	74.54 (5.409)[a]	110.08 (8.742)[a]
Si 600ppm	30.70 (2.915)[a]	75.40 (5.409)[a]	103.31 (8.742)[a]
p			
Age	< 0.001	0.035	0.030
Diet	0.083	0.959	0.249
Age * Diet	0.188	0.828	0.663

Means within a column within an age group with different letters are significantly different ($p = 0.05$)

6.3.3 Egg data

Table 6.11 shows that age influenced albumen height ($p < 0.001$), shell weight ($p = 0.003$), shell percentage ($p = 0.038$), shell thickness ($p < 0.001$) and Haugh unit ($p < 0.001$), showing that all these egg parameters fluctuate higher or lower with age. Egg weight ($p = 0.796$) and shell strength ($p = 0.881$) were not affected by age. The table also shows that there was no effect of diet on any egg parameter, though a trend in shell strength was present, indicating the diet containing silicon improved shell strength ($p = 0.094$). There was no significant interaction between age and diet on any egg parameter within this study.

Table 6.11 Effect of age, diet, and interaction effect on egg parameters

	Egg weight (g)	Albumen height (mm)	Shell weight (g)	Shell percentage (%)	Shell strength (N)	Shell thickness (mm)	Haugh unit (HU)
Control							
Week 74	68.45 (1.277)	5.98 (0.306)[a]	6.46 (0.168)[a]	9.49 (0.258)[a]	37.62 (1.821)[a]	0.32 (0.011)[a]	72.67 (2.409)[a]
Week 75	67.21 (1.305)	7.13 (0.313)[a]	6.64 (0.168)[a]	10.10 (0.258)[a]	37.57 (1.783)[a]	0.31 (0.011)[a]	78.82 (2.409)[a]
Week 76	66.68 (1.334)	6.69 (0.327)[a]	6.51 (0.176)[a]	9.79 (0.269)[a]	40.11 (1.862)[a]	0.51 (0.012)[a]	75.55 (2.516)[a]
Week 77	67.02 (1.366)	8.55 (0.335)[a]	6.55 (0.180)[a]	9.82 (0.275)[a]	40.31 (1.906)[a]	0.34 (0.012)[a]	87.55 (2.575)[a]
Week 78	66.59 (1.475)	7.14 (0.353)[a]	6.43 (0.194)[a]	9.69 (0.297)[a]	37.11 (2.118)[a]	0.32 (0.013)[a]	81.40 (2.781)[a]
Week 79	67.80 (1.399)	6.38 (0.335)[a]	6.98 (0.184)[a]	10.31 (0.282)[a]	38.37 (1.953)[a]	0.27 (0.012)[a]	75.74 (2.639)[a]
Si 600 ppm							
Week 74	65.28 (1.277)	5.55 (0.313)[a]	6.52 (0.168)[a]	10.03 (0.258)[a]	40.63 (1.783)[a]	0.32 (0.011)[a]	68.43 (2.409)[a]
Week 75	67.73 (1.277)	7.46 (0.306)[a]	6.96 (0.168)[a]	10.31 (0.258)[a]	42.83 (1.821)[b]	0.32 (0.011)[a]	82.84 (2.409)[a]
Week 76	65.95 (1.277)	6.98 (0.306)[a]	6.40 (0.168)[a]	9.74 (0.258)[a]	39.03 (1.821)[a]	0.50 (0.011)[a]	80.35 (2.409)[a]
Week 77	67.60 (1.366)	9.60 (0.327)[b]	6.33 (0.180)[a]	9.44 (0.275)[a]	37.16 (1.906)[a]	0.33 (0.012)[a]	95.06 (2.575)[b]
Week 78	69.25 (1.436)	7.37 (0.344)[a]	6.87 (0.189)[a]	10.03 (0.289)[a]	39.71 (2.058)[a]	0.34 (0.013)[a]	83.01 (2.575)[a]
Week 79	68.17 (1.334)	6.32 (0.320)[a]	7.11 (0.176)[a]	10.46 (0.269)[a]	42.52 (1.862)[a]	0.29 (0.012)[a]	75.12 (2.516)[a]
P							
Age	0.796	< 0.001	0.003	0.038	0.881	< 0.001	< 0.001
Diet	0.912	0.235	0.349	0.376	0.094	0.346	0.145
Age * Diet	0.406	0.338	0.428	0.621	0.192	0.475	0.213

Means within a column within a diet group with different letters are significantly different (p = 0.05)

6.4 Discussion

6.4.1 Validity of results

The accuracy of all the results of this study could be examined as it is thought that move from a colony caged housing system in a commercial setting to a floor-based pen housing system may have influenced the results. It is known that housing systems impact bone parameters due to the availability to exercise (Wilkins *et al*., 2011; Campbell *et al*., 2019). Moving from a colony caged system to a floor-based pen housing system with extra vertical movements and a much lower stocking density may have allowed for greater exercise and thus impacted skeletal health. It could therefore be suggested that the effect of diet in this study was concealed by the effect of change in housing system. Diet had no effect on bone parameters or egg qualities within this study though there were some trends. This could be because of an incorrectly mixed experimental diet (Si 600 ppm) in-house or that the inclusion of silicon was too low to cause any effect. A study by Faryadi *et al*., (2017) investigating the effect of silicon (nanosilicon dioxide) in laying quail found an improvement in bone ash percentage of around 7.5% in hens fed 4000ppm compared to no supplementation but did not find any difference in birds fed 500ppm. They also found egg weight and shell weight were significantly heavier in hens fed 4000ppm silicon inclusions 12.72g v 13.27g and 1.03g v 1.10g respectively).

Furthermore, the previous study showed that 500ppm silicon inclusion significantly lowered egg weight compared to no inclusion and showed a drop in shell weight but was not significant. The results from this study could support claims that the inclusion of silicon in the present study was too low and maybe had a deleterious effect, as some bone and egg parameters showed lower values from the experimental diet although not significant. A suggestion for future work would be to include multiple inclusions of silicon over the course of the study to gain a more comprehensive overview of the effect of silicon in laying hens. Though the effect of the diets was not the main aim of this study, the aim of the study was to determine how suitable the previously examined bones and measurements were when used to assess skeletal health under trial conditions. Within this study there was a lot of feed wastage in each pen as the hens were seen to perch on the troughs and sometimes tip the

feed out, causing incorrect feed intake and therefore FCR results were not meaningful. It would be suggested to use another method of providing feed if a similar study was repeated.

Additionally, within this study there may have been some user error in measuring certain bones, for example the keel bone width results, which were the only bone to show a significant age*diet interaction effect (p = 0.013) and only humerus width showed an age effect (p < 0.001). The changes in keel width between samples could have occurred when the bone was dissected from the bird. The specific area where keel width is intended to be measured from, can sometimes be removed by user error and cause an incorrect measurement. Additionally, where the keel is measured for width by different operators this may have caused more variation within the results. In terms of the humerus width differing so drastically between age groups, it could suggest that the orientation in which the humerus width was measured was incorrect compared to the methods at 79 weeks of age, as they are consistently wider than at 76 weeks of age. It is possible, that instead of using the flattest part of the humerus to measure width, the humerus was rotated and measured in the wider orientation, giving false results. As data collection in this study was carried out by many researchers, human error was more likely to affect the results. In future research it would be advantageous to have a single researcher carry out the methods for each sample measurement to ensure consistency, though this was not possible on this occasion.

During this study bird selection has also been proposed to have influenced some results, as there were only 3 birds per pen, it was suggested that the length values for tibia (Table 6.7), particularly in the diet effect (p = 0.099) could have been caused by selecting smaller birds at 76 weeks and this has masked some of the effect of diet. For instance, the control and Si 600ppm diets at 76 weeks were significantly smaller than 79 weeks of age and showed a larger difference between the two dietary groups, whereas the dietary groups at 79 weeks were not as different in length. Choice of the birds is likely to have been influenced by bird aggression shown within the pens and therefore could have influenced the results unintentionally.

6.4.2 Body weight

In the present study, age, diet or the age * diet interaction had no effect on body weight of the hens (Table 4.6). Little research has been performed studying the effect of silicon supplementation in laying hens. Although this study found that the diet did not have an effect on body weight, a previous study by Carlisle, (1972) found that body weight gain in broiler chick increased when supplementing with silicon with a 30 – 50% increase in the supplemented groups. The formulated diets in Carlisle, (1972) were deficient in calcium which could have exacerbated the results, perhaps in the present study all mineral requirements of the hen were met by the commercial mash so no difference in treatment effect was seen. As previously mentioned, the change from a colony caged system to a floor-based pen system may have concealed any true effects of diet and age or the interaction effect. Although not significant, both diet groups were heavier at 79 weeks compared to 76 weeks. This could have been influenced the change of housing system more specifically – the increased amount of exercise available which could have increased body weight through increase in muscle mass. On the other hand, assuming the floor-based pens systems are most similar to a barn system, previous research found that body weight is often lower in more active housing systems (Sherwin *et al*., 2010), therefore disagreeing with this statement. It could also be suggested that position in the room may have caused differences in body weight, as birds closer the door may have been disturbed more and more likely their eating patterns disrupted. Additionally, room position could have also affected light/lux levels as lights were dimmed via makeshift covers as the facility is primarily for use in broiler chickens. Pens receiving more light could have showed increased aggression causing less exercise or food consumption. It is known that higher light levels can increase stress and therefore aggression in laying hens, decreasing performance (Parvin *et al*., 2014; Barros *et al*., 2020). Furthermore, as hens were moved out of a familiar setting to a new system, new hierarchies would need to be established and this could have affected body weight performance – as some birds may have been more aggressive than others effecting food consumption and thus body weight. The nonsignificant increase in body weight at 79 weeks compared to 76 weeks could also have been caused by bird selection at 76 weeks. For example, if an aggressive bird was removed earlier on in the study, the more submissive hens may have been able to consume more food after this point and may have influenced results.

6.4.3 Bone data

Humerus was the only bone to have length affected by the age * diet interaction effect (p = 0.018) and age only had an effect on tibia length (p < 0.001), though there was also a slight trend in diet effect on tibia length (p = 0.099). As previously stated, it could be possible that smaller birds were selected at 76 weeks of age compared to 79 weeks as tibia length was highly significantly shorter at 76 weeks of age, than 79 weeks of age. Also, the humerus length in the Si 600ppm group at 76 weeks was significantly shorter than the control group but the effect disappeared at 79 weeks of age. It is also possible that the change in housing systems may have masked many true effects of diet or age within this study and the effect of housing system is stronger than either of the other factors. The additional exercise available in the floor-based pen system compared to a colony cage system may have physiologically stimulated some changes in bone structure to accommodate for the increased exercise, as it is well known exercise can increase bone development in pullets (Regmi *et al*., 2015). Though changing housing system at the end of lay has never been assessed. The interaction effect in humerus length could have been influenced by the change in housing system and confounded by exercise more than the keel and tibia bones as there is more vertical space enabling more wing movements, therefore increasing humerus usage, though after sexual maturity bone growth/development are commonly thought to stop (Whitehead, 2004). It could be theorised that exercise as a factor could be powerful enough to alter bone structure at any point in lay if additional exercise is facilitated.

Though tibia length was affected by age and considered that bird selection and change housing system may have influenced the outcome, diet also showed a trend in tibia length showing that a difference between control and Si 600ppm diminished over time. Although humerus also showed the same pattern, it could be suggested that the tibia bone is more sensitive to diet as it showed a trend (p = 0.099) whereas humerus did not.

Table 6.8 shows keel width was affected by the age * diet interaction effect but humerus and tibia were not. There is some uncertainty whether keel width was measured incorrectly during data collection and that is the cause of this difference, as the keel has not shown any other significant interaction effect in any other parameter. Furthermore, humerus width also

shows a significant effect of age, though it could be possible that errors occurred when measuring the width at 79 weeks due to the bones being consistently wider. As the tibia was not affected by any factor or interaction when measuring width, the results support the theory that humerus width was measured incorrectly. Interestingly the significance value for the effect of diet on tibia width is much less than humerus width (p = 0.285 and p = 0.842). As previously mentioned, the change in housing is likely to have concealed some true effects in the results and without changing housing systems, further diet differences may have been elucidated. Many previous studies use either the humerus or tibia when evaluating skeletal health, with the latter being more commonly assessed (Leyendecker *et al*., 2005; Regmi *et al*., 2015; Neijat *et al*., 2019).

No effect of age, diet or interaction effect was present in any bone weight measurement (Table 6.9). A previous study found that supplementing *Buttiauxella sp.* phytase to Lohmann LSL hens throughout the period of lay increased bone mineral content by approximately 10 and 9% at ages 48 and 70 weeks compared to those without any supplementation, though the results were not significant (p = 0.650) (Bello and Korver, 2019). Though this is not weight, an increase in bone mineral content could also suggest an increase in bone weight. Though two different supplements, phytase and silicon are used with the aim to improve skeletal health by increasing phosphorus utilisation (Hughes *et al*., 2009) or increasing a collagen biosynthesis (Carlisle, 1981). Therefore, it could be insinuated that both supplements may theoretically improve bone weight indirectly by either increasing mineral content (phytase) or increasing the space/structure minerals can bind to in bone development/maintenance (silicon).

Surprisingly, there is no effect of age on any bone weight measurement, yet one would be expected because as age increases, the rate of bone resorption also increases and leads to osteoporosis (Whitehead and Fleming, 2000). However, in this study it is likely that the effect of changing housing system at the end of lay increased the potential for exercise, reducing the rate of bone resorption and therefore reducing the effect of age on bone weight and showing not significant difference within this study. The results of age are effectively confounded by exercise as the ability to exercise in a colony caged system compared to the

system used in this study are not equal and less exercise would take place in a colony caged system, meaning the effect of age would not be miscalculated.

Unlike bone weight, age had a significant effect on all bone strength (keel, humerus and tibia $p < 0.001$; $p = 0.035$ and $p = 0.030$). It could be suggested that the effect of age more strongly effects bone strength than other parameters measured in this study or that bone strength as a measure is more sensitive at indicating differences in factors. The effect of changing housing system at the start of this trial does not seem to have impacted the results of bone as it may have done length, width, or weight. Although medullary bone is produced during the laying stage, it lacks the mechanical support that structural bone provides. Therefore, even if the change in housing system increases exercise and reduces bone resorption, replacement bone will provide less strength that the original structural bone. A previous study found that the energy to failure (J) was significantly different in Lohmann white hens aged 26, 56 and 72 weeks of age (564J, 439J, 397J respectively) (Regmi *et al*., 2017a), supporting results in this study that as age increases bone strength decreases. The results from chapter 4 also show age had an effect on bone strength in the keel, humerus and tibia ($p < 0.001$) showing a negative decline in strength (though less clear in the keel bone). Another study in Taihang chickens (aged 32 to 57 weeks) found that age had a significant effect on femur strength ($p = 0.008$) but not on tibia and humerus strength (Qiaoxian *et al*., 2020). The results from Qiaoxian *et al*., (2020) contradict the results of the present study but the birds used in the present study are ~20 weeks older and therefore a direct comparison cannot be made. The keel showed the most significant effect of age on breaking strength compared to the humerus and tibia, this could imply that as a bone the keel is the most sensitive and can indicate more accurate effects of the examined factors within a study. The keel bone is known to be a calcium reservoir and a highly active bone in terms of calcium metabolism (Prondvai and Stein, 2014) therefore, it would make sense that the keel may be the most sensitive bone. Furthermore, the keel was the only bone to show a trend of diet in bone strength ($p = 0.083$) indicating that the control diet provided better bone strength at 76 weeks of age. Interestingly, the lack of difference at 79 weeks of age, could indicate that the silicon supplement improved bone strength as there is no difference between the control and silicon diet at this age and reduced a deterioration of bone strength over time. Other research

supplementing vitamin D_3 and various intervals found no effect of supplementation on tibia bone strength, though there was a trend high vitamin D_3 levels might improve tibia bone strength (p = 0.09). Both the previous study and the present study showed no effect of diet supplementation on tibia strength, albeit different supplements however the study by Wen *et al*., (2019) did show a trend. It could be plausible to assume that if more dietary treatments were used in the present study an effect of diet may have been revealed. A previous study supplementing omega-3 polyunsaturated fatty acids to commercial laying hens at 30, 50 and 70 weeks found that the breaking strength of keel bone at the manubial spine (point A) and the lateral surface) (B) (same position broken as present study) was significantly greater in omega-3 fatty acids supplemented birds than birds fed the standard diet (overall treatment effect = A: $p < 0.001$; B: $p < 0.05$) (Tarlton *et al*., 2013). The study also found that the mechanical properties of the tibia were improved with supplementation of omega-3 fatty acids, with improvements compared to the standard diets in ultimate stress (MPa) values at 30, 50 and 70 weeks of age with an overall treatment effect of p = 0.002. Results for supplementation on tibia strength contradict the present study as no difference in diet was found (p = 0.249), although the supplements most likely effect bone development in different ways. Results from the keel bone strength in Tartlon *et al*., (2013) also does not support results from the present study as omega-3 fatty acids showed an increase in breaking strength of the keel, whereas supplementing of silicon in the present study did not, though a trend was shown indicating that diet may have had some influence (p = 0.083). The difference in supplements is most likely why the results from both studies differ but, it is clear to the see that the use of keel strength as a parameter to measure skeletal health may be valuable.

6.4.4 Egg data

Egg weight showed no effect of age, diet, or age * diet in this study. Previous research revealed that egg weight was affected by age, showing an increase as age increases ($p < 0.001$) (Van Den Brand *et al*., 2004). The previous study does not support results from the present study as age was shown to have no effect on egg weight, though the difference in length of study could have affected the results. Egg weight in Van Den Brand *et al*., (2004) was observed over a 40-week period compared to 6 weeks in the present study. A study in laying quail found that silicon supplementation of different inclusions can improve egg weight ($p < 0.001$),

4000ppm (13.27g) inclusions were shown to improve egg weight compared to 0 (12.72g), 500 (12.32g), 1000 (12.78g), and 2000ppm (12.85g) (Faryadi *et al*., 2017) However, at 500ppm egg weight was significantly worse than all other inclusions, 2000ppm inclusions showed significantly heavier than 1000ppm and 500ppm but significantly lighter egg weight than 4000ppm but no difference to the standard diet. 1000ppm was significantly better than 500ppm but significantly lighter than 2000 and 4000ppm, and no different to no inclusion of silicon. The study by Faryadi *et al*., (2017) does not align with the current study one reason could be that as the previous study used multiple inclusions of silicon the effect of diet is stronger than only using two diets as in the current study (control and Si 600ppm). Another reason for the lack of effect of diet in the present study could be that the inclusion of silicon is too low, as 500ppm in Faryadi *et al*., (2017) showed a negative effect on egg weight and 600ppm is not much higher and possible ineffective.

Only age had an effect on albumen height ($p < 0.001$) though it could be possible that an error in measurements of albumen height has shown a false result as both 77 weeks in the control and Si 600ppm groups were substantially higher than the rest of the age groups and then lowers again, with those in the Si 600ppm group significantly higher. A study previous study looking at the effect of age on albumen height found that as age increases, albumen height decreases ($p < 0.001$) to around 5.5mm to 6.0mm at 60 weeks of age (Van Den Brand *et al*., 2004). It is therefore unlikely that in the present study, albumen height at 77 weeks is ~25-30% higher (control = 8.55mm, Si 600ppm = 9.60mm) than results found by Van Den Brand at 60 weeks of age. In terms of a diet effect, no difference was found, however a previous study supplementing ginger extract to hens ages 40 weeks of age found that the supplementation of ginger improved albumen height 4 weeks into the trial and 8 weeks into the trial (control = 6.33mm, ginger extract = 7.70mm; $p = 0.044$ and control = 6.32mm, ginger extract = 7.08mm; $p = 0.024$) (Wen *et al*., 2017). Results from Wen *et al*., (2017) do not support the present study and show that diet does effect albumen height when supplements are included. Although the supplements vary, the results from Wen *et al*., (2017) may indicated albumen height can be used to distinguish effects of diet supplementation, though in the present study the supplementation of silicon may have not been effective enough to show any difference.

Table 6.11 shows that only age had an effect on eggshell weight (p = 0.003). A recent study in blue-shelled layers investigated the effect of age and housing system on various egg qualities and found age had a significant effect on shell ratio (%) (p = 0.002), though the effect of age was not linear and varied by age showing a significant difference between caged and outdoor systems at 36 weeks and 50 weeks but not 26 and 42 weeks of age (Wang *et al*., 2009). Although not the same parameter as the present study, shell ratio and shell weight are linked, and it could be assumed one parameter would correlate with the other. As such, the present study also showed that shell weight varied with age in a non-uniform way, therefore the results from Wang *et al*., (2009) somewhat support findings from this study as if eggshell ratio varies so will eggshell weight. In the present study there was also no effect of diet on shell weight, however a recent study looking at silicon supplementation in laying quail found that at high inclusions of silicon (4000ppm) shell weight was increased significantly (4000ppm = 1.10g v 0ppm = 1.03g; p = 0.002) (Faryadi *et al*., 2017) The previous study does not agree with results from the present study that silicon improves shell weight. Although, the inclusion level which caused a significant increase in shell weight in Faryadi *et al*., (2017) is far higher than the silicon supplementation in the present study (4000 v 600ppm), therefore the lack or difference in inclusion levels could have caused the difference in results. Also, the use of quail compared to commercial laying hens may have also influenced results. No effect of age * diet was also seen in shell weight, this could be due to the change in housing system from colony caged to a floor-based pen system, along with a diet supplementation that is relatively low compared to previous results.

Similarly, to shell weight, shell percentage showed an effect of age but no effect of diet and age * diet interaction. Though the significance level of age effect on shell percentage was not as significant as shell weight (p = 0.038 and p = 0.003 respectively). The difference in significance levels could be due to shell percentage accounting for overall egg weight and what fraction of the shell makes up overall egg weight – albumen and yolk weights may vary between egg to egg. As previously mentioned, Wang *et al*., (2009) found that shell ratio was affected by age (p = 0.002), supporting results from the present study, however the ratios from the previous study were on average higher (13-14%) than ratios found in this study (9-11%). Results could differ due to the different breeds used, diets provided or the difference

in age as the birds in this study were 20+ weeks older than the last age group used in Wang *et al*., (2009). Another recent study also found that eggshell percentage was not affected by age ($p = 0.14$), and therefore does not support the current results (Van Den Brand *et al*., 2004). The differences of ages used within each trial could be the cause the of the differences in results. A recent study supplementing boron to hens with a low calcium diet showed no effect on shell percentage ($p > 0.05$) (Olgun *et al*., 2009). Although different supplements – results are similar that the inclusion of each do not increase shell percentage. It could therefore be suggested that the use of egg parameters to evaluate skeletal health may in fact not be useful as the egg production could take precedent over skeletal health.

Eggshell strength is one of the most used egg parameters to assess egg quality (Guinotte and Nys, 1991; Hidalgo *et al*., 2008 Wang *et al*., 2009). Eggshell strength was not affected by age, diet or the age * diet interaction in this study, though there was a trend in diet effecting eggshell strength ($p = 0.94$). Contradictory to this study, a previous study found that as age increases eggshell strength decreases ($p = 0.010$) and does not support results from this study (Habig and Distl, 2013). The differences in results could be due to the fact that Habig and Distl (2013) used age intervals of every 3 months compared to every week within the current study – therefore larger intervals could be suggested to show greater differences. Another study by Kraus *et al*., (2021) showed that age did not have an effect on eggshell strength ($p = 0.368$), though shorter intervals than Habig and Distl (2013), they are still 8-week intervals compared to 1 week. It could be possible that the ages used in Kraus *et al*., (2021), 34 – 50 weeks were not as affected by the effects of bone resorption or osteoporosis at this age range, so eggs were more uniform in terms of breaking strength. Though if this was the case an effect of age at 74-79 weeks of age in the present study would be expected to be shown. A study investigating the supplementation of organic and inorganic trace minerals (Manganese, Zinc and Copper) found that eggshell strength was increased using either organic or inorganic trace mineral supplementations of various levels ($p < 0.005$) (Stefanello *et al*., 2014). Other research has also shown that vitamin D3 supplementation in the diet can increase eggshell strength ($p < 0.001$) (Wen *et al*., 2019). Although not the same supplementations used, the previous studies contradict the results of the current study and indicate that diet can in fact effect eggshell strength. No effect of age * diet on egg strength could be due to the fact that

bone resorption continues throughout lay to provide enough minerals for egg production at the cost of bones (Hester, 2017), therefore making the combined effect of age and diet less clear.

Age had a significant effect on eggshell thickness ($p < 0.001$) though diet and age * diet showed no effect. Results from week 76, indicate that eggshell thickness could be an anomalous result as both control and supplement groups are approximately 0.20mm thicker, almost double any other measurement at other age groups. This could imply that some eggshell was folded on itself when thickness measurements for this week were taken. As in chapter 5 section 5.4.1, eggshell strength results and eggshell thickness results do correspond with each other as age has a significant effect on eggshell thickness but not eggshell strength ($p < 0.001$ and $p = 0.881$ respectively). As stated previously stated, if eggshell thickness is affected one way by a factor, shell strength is likely to correlate as shell thickness through crystal size is likely to effect shell strength results, the bigger the crystals the thicker the shell and ultimately provides greater strength (Rodriguez-Navarro *et al*., 2002). This theory therefore supports the assumption that eggshell thickness may have been incorrectly measured at some time points (76 weeks). A study by Kraus et al., (2021) found that age did influence eggshell thickness significantly ($p < 0.001$), with older hens having thinner eggshells. Diet was not effective at changing eggshell thickness in the current study. A recent research supplementing vitamin D_3 also found that diet did not have an effect on eggshell thickness ($p = 0.28$) (Wen *et al*., 2019). Both studies used commercial basal diets before adding supplements to create each treatment. It could be proposed that as the basal diets were not deficient in any mineral that the hens did not require supplementation as all mineral metabolic needs were fulfilled. If diets supplements were added to diets deficient in minerals such as calcium or phosphorus supplementing either silicon or vitamin D_3 (Wen *et al*., 2019) may have had a different outcome.

Haugh unit was significantly affected by age ($p < 0.001$), though showed no effect from diet or the age * diet interaction. Results from week 77 albumen height, which was suggested to be an error in the results could have subsequently influenced the results for age effect on Haugh unit. This is because week 77 results are also substantially higher than other weeks

and albumen height is used in the equation to calculate Haugh unit. Though there is uncertainty within this result, a previous study also found that age significantly affected Haugh unit (Dikmen *et al*., 2017). The effect of age in the previous study was clearer than in the present study and showed Haugh unit declines somewhat steadily with increasing age. As previously stated, the move from a caged housing system to a more open experimental system could have nullified the effects of the diet slightly. Dikmen *et al*., (2017) also found that housing system did have an effect on Haugh unit and could support this theory. More exercise or movement from a change in housing system could lead to better skeletal health and have a knock-on effect to egg production before the effects of a dietary change can be seen. Another previous study did find a diet effect, showing ginger extract supplementation significantly increases Haugh unit (4 weeks - control = 76.88, ginger extract = 85.37; $p = 0.048$ and 8 weeks - control = 80.20, ginger extract = 84.53; $p = 0.032$) (Wen *et al*., 2017) and therefore does not support the results from the present study. As previously mentioned, if an effect is shown in albumen height, it is probable that an effect will be shown in Haugh unit as the two are interlinked. It may be possible that Haugh unit can show the impact of dietary changes or supplements, though in the present study levels of silicon, or more likely the move from and to different housing systems could have negated or diminished dietary effects in this instance.

6.5 Conclusion

Overall, determining whether the bones chosen in previous work and used in this study were useful at assessing skeletal health is still somewhat unclear. It would be suggested that when using these bones to assess skeletal health, a clearer supplement effect is present to help clarify if the sample bones are useful by possibly showing more differences, if using diet/supplements as a factor. In addition, the decision to use birds from a commercial colony caged system had an impact on the bone data from this study as the change availability in exercise is thought to have skewed the effects of age, diet and the interaction effect. It would be recommended in future work to use a similar housing to the previous commercial setting or to keep hens in the same system to remove the risk of housing system effecting results if it was not intended to be assessed. If housing systems were changed, increasing the acclimatisation period before sampling may also help alleviate the effect of the change in housing system and provide more accurate results. As previously mentioned in the discussion, as the diets before supplementation were commercially made, they are well balanced and should not be deficient in any mineral. On the other hand, if the diets used were deficient in certain minerals, novel supplementations of silicon may have shown more pronounced differences as the diets were deficient, yet the bones will still require a suitable mineral supply. As in chapter 5, it would still be recommended to use multiple types of bones as each bones form, function and usage will differ between housing system and age and be affected by diet differently to one and other, depending on bone metabolism. Furthermore, parameters such as bone length and width are not likely to be affected after sexual maturity as bone growth is known to stop, therefore the usefulness of these measurements is somewhat diminished in laying hens past the rearing stage. Although previous work in end of lay hens (pilot study 2) did show similar results in that length and width were significantly affected by housing system. Strength and weight on the other hand will still be affected throughout the laying as bone resorption continues throughout which is known to negatively impact bone strength and weight. In terms of the effectiveness at assessing the impacts of age and diet on skeletal health, egg quality traits seem to lack any usefulness as per chapter 5 as the modern layer has been developed to prioritise the egg development over bone health

through genetic selection. It would therefore be recommended not to use egg parameters to indicate any effect of a factor on skeletal health.

Chapter 7: Conclusions and Future Work

7.1 Introduction

This chapter is split into three sections to discuss the effectiveness of methods used in the poultry industry to assess skeletal health in laying hens. Firstly, the studies undertaken investigating methods of assessing skeletal health will be discussed alongside key findings from the results. Next, the impacts of the conclusions made from each study of the methods used will be discussed and how these methods may be applied in future. Subsequently, key areas for future work are outlined and any impactful recommendations based on the previous works are specified.

Modern day laying hens are susceptible to skeletal disorders, particularly disorders such as osteoporosis and keel bone damage (KBD) and caged layer fatigue (Whitehead and Fleming, 2000; Casey-Trott *et al*., 2015). The main factors causing these disorders are the demand for egg products, the length of a laying cycle and the systems used to house hens during production (Bain *et al*., 2016; Dikmen *et al*., 2016; Jones *et al*., 2018). The demand for egg products has increased over the past two decades. Consequently, the egg sector has attempted to fulfil the ever-growing demand of egg productions as a source of animal protein. Currently, through genetic selection, a modern-day laying hen can lay an egg roughly every 25 hours for extended periods of time without stopping a laying cycle (Hester, 2017). Recently, there has been an interest in research surrounding extending the laying period closer to 100 weeks whilst maintaining suitable hen welfare – which would be beneficial for producers as a longer cycle would require less repopulation of laying systems and prevent the culling of unnecessary male birds (Bain *et al*., 2016). The knock-on effect of trying to extend the period of lay is that the likelihood of skeletal problems occurring such as osteoporosis, KBD, and generally poor skeletal health will increase as the birds age (Alfonso-Carillo *et al*., 2021). Furthermore, the effects of factors such as housing system, dietary treatments, and differences in genetic strains can all also affect the occurrence of skeletal problems in laying hens through the laying period (Regmi *et al*., 2017a; Wen *et al*., 2019), ultimately negatively effecting hen welfare.

This project used bone measurement methodology from previous research to try and provide an optimal recommended procedure for assessing skeletal health in laying hens throughout the egg-laying cycle. The focus has been on improving the methods used for assessing skeletal integrity to gain a more comprehensive overview of skeletal health in UK flocks, so hen welfare can be maintained whilst still meeting the demand for egg products. Skeletal integrity can be defined as the ability of the skeletal structure of the hen to support its own body weight throughout daily exercises and while exhibiting natural behaviours without pain or discomfort. A similar study investigating methodologies for determining broiler leg health had been performed at NTU (Sanni, 2017), and the original aim of this PhD project was to mirror that study on general skeletal health of laying hens. However, as the project developed, it became clear that additional objectives focussing on the longitudinal effect of egg production on skeletal health in different housing systems and investigation of the methods used was required. The objectives of this project were:

1. Determination of the optimal bones used for assessing skeletal health.
2. Investigation of the effects of different housing systems and age on skeletal integrity throughout the laying period of UK flocks.
3. To model bone parameters from different housing systems and age groups to investigate if predictions can be made.
4. Investigation of the effects of different housing systems and age on egg quality traits throughout the laying period of UK flocks.
5. To model egg quality traits from different housing systems and age groups to investigate if future egg quality can be predicted through modelling procedures.
6. Determination of the relationship between bone parameters and egg quality traits to inform on the impact of housing system and hen age on skeletal health.
7. To identify whether previous methods used to determine the effect of housing system and hen age can be used to assess if a dietary intervention can influence skeletal health.

These objectives were examined progressively on a step-by-step basis from the first objective. Optimal bones were assessed through a series of investigations: utilising a study investigating the effect of phytase inclusions on broiler growth, then the effect of housing system and orientation on skeletal health in end of lay hens collected from a processing facility, and finally using hens at the beginning of lay from collaborating farms to assess the effect of housing system on orientation on skeletal health. Bones were considered useful if differences were found between the factors within the studies, combined with how much labour was required to dissect those specific bones and how common they were used in previous research. These findings were then used to conduct an on-farm study over a full laying cycle for multiple farms across multiple housing system types, with egg traits also assessed. Data was also modelled to determine if skeletal health could be predicted. Following on from this, an in-house trial was undertaken in end of lay hens to investigate whether the methods previously utilised could identify the effects of dietary intervention of supplementation of silicon (developed at NTU) (Scholey *et al*., 2018b).

7.2 Summary of conclusions

Whilst the pilot study chapter went some way in determining which bones to use and which parameters to measure, there are still questions unanswered. The sample birds used in this chapter covered some age groups, and how useful the bones and parameters differ at each age (albeit broiler chickens in pilot study 1). To pinpoint the optimal bones for assessing skeletal health, the number of age groups studied could be increased, so a more comprehensive result can be achieved before using it to inform future studies. It may be likely that certain bones or bone parameters become more or less sensitive at highlighting differences depending on the age they are assessed at. By sampling multiple bones throughout the pilot studies and observing the differing response to sensitivity of effects within each bone, it is indicated that there is a need to evaluate skeletal health in poultry using multiple bones from different skeletal areas. This is suggested because the form and function of bones from different areas of the body perform different physiological functions or exercises. For example, the humerus bone will be more involved in wing exercises than the leg bones and vice versa. These differences in function can be further influenced by the type

of housing system that is used as these are known to affect the availability to exercise (Shipov *et al*., 2010). Results of the pilot studies summarised that orientation effects do differ with age but unexpectedly altered more in older laying hens than younger laying hens, even past the point of sexual maturity when bone development is thought to have stopped. It is unclear as to why more differences in orientation occurred in older laying hens, and more research is needed to confirm these effects. It may be that bones in a particular orientation may have individual growth rates influenced by other external factors within different systems. Similarly, it was also unexpected that bone geometry (length and width) was widely affected by housing system in both laying hen pilot studies as they were post sexual maturity. Greater differences were expected in younger laying hens at the beginning of lay, as bone development may still be occurring, however it is unclear as to why so many differences were seen in end of lay hens. Due to the lack of clarity in the effect of housing system on bone parameters late in lay, it would be advised that more studies need to be carried out in a more highly controlled trial to confirm these results at end of lay. In terms of bone parameters used to assess skeletal health, it could be concluded from the pilot studies that measurements such as bone weight or bone strength were the most sensitive. In particular bone strength, as it was the bone parameter which showed the most differences across all combined studies and was already commonly used within previous research. As bone strength was measured autonomously, it may indicate that bone strength results may be more credible than other parameters and would be advised to be used in future work. The next stage of this project examined the effect of housing system, age, and the interaction effect on skeletal health, in a longitudinal on-farm study over a full laying cycle for multiple farms of different housing systems with laying hens.

The investigation into the longitudinal effect of housing system and age on skeletal health yielded many conclusions. In this study data for bone ash content was calculated in addition to bone strength as it was a common measurement in assessing skeletal health in previous research. The bone data from the on-farm project also suggested that bone strength was more informative than bone ash content, as it was able to highlight more noticeable differences between housing systems and age groups. This study also showed support for the incentive to move away from caged systems within the industry. The caged systems showed

the weakest results in the analysis of both bone strength and ash content throughout the laying period, due to the configuration and the lack of opportunity for exercise (Regmi *et al*., 2017a). This study also confirms that although non-caged systems may increase skeletal health, they may also cause more risk of collisions or prevalence of KBD. This would imply that a decision needs to be made when housing hens, either increase bone health or decrease risk of KBD – a paradox first highlighted by Sandilands *et al*., (2009). A balance between housing system configuration for better bone health whilst reducing high prevalence of KBD would therefore be advised in future housing systems. As in the pilot studies, the on-farm project demonstrated it is essential to use a range of bones when also modelling the effects of housing system and age on bone strength as model outputs varied between bones, especially due to the availability to exercise within different systems. The modelling work initially looks promising as a possible early warning tool for skeletal health for use within the industry, but further research is needed. Adding more data to the model to increase the statistical power and considering factors such as diet or genetic strain not currently in the model parameters would improve precision of the predicted outcomes.

The second part of the on-farm project was to assess the effect of housing system and age on egg quality traits to determine if these provided any indication of skeletal health. Although many egg traits showed a significant effect of housing system, age and the interaction between age and housing effects the reliability of the results to inform on skeletal health. This study indicates that mass genetic selection and laying hen biology leads to egg production taking precedent over skeletal maintenance. Therefore, although highly important within the egg sector, egg traits were assumed to provide little information on skeletal health. This study showed that there was no relationship between egg quality and skeletal health via correlation between residual eggshell strength and bone strength, further implying that egg qualities are not useful to assess skeletal integrity.

The in-house trial demonstrated that the usefulness of the chosen sample bones to assess skeletal health is still unconfirmed. It may be that a clearer supplement effect would be needed in order to clarify the suitability of the sample bones. It seems the move from a colony caged housing system to a pen-based floor system influenced results of this study more than

the supplement, causing some uncertainty as to the specific effects of age and diet investigated. Results from the unexpected effect of change in housing system suggests that a longer acclimatisation period is required in future work to mitigate the effect changing housing system at the end of lay. The fact that diets were commercially made and nutritionally formulated to be specific to the age and role of the hens may suggest that the supplementation of silicon was not required to improve skeletal health. If diets had been lower in mineral content – the effects of inclusion of silicon may have been more marked. It would be suggested that using a low calcium or phosphorus diet may have increased the effectiveness of silicon as seen in previous studies in broilers supplementing phytase (Scholey *et al*., 2018a). As found in the pilot studies and on-farm project, the in-house trial also supports the advice that sampling from multiple bones is beneficial. As seen in pilot study 2, this study also showed that bone geometry (length and width) was somewhat affected by the study factors at the end of lay. It is unclear why this would be the case, as bone geometry is known to stop developing after sexual maturity (or point of lay); however, differences were still being highlighted indicating a possible role of bone geometry in future work. This study also further supports that egg quality traits do not correspond to differences in skeletal integrity, as only a few traits showed differences and these results may be due to errors. In addition, data collection implied that egg production may be prioritised over skeletal maintenance. This study yielded large amounts of data providing the basis for further work in the supplementation of silicon in laying hens. There is scope for deeper investigation into the effect of change in housing system at the end of lay and the usage of multiple inclusions of silicon in dietary treatments in the future.

7.3 Future work

There are many areas of future work which could follow on from this thesis. As all studies within this project were mainly carried out in or relating to commercial settings, some factors could not be controlled for. Greater overlap of results from commercial and experimental settings would help improve and expand on the findings from this project. Some key areas for future work are described below.

7.3.1 Determining the optimal bones to assess skeletal health

The studies looking at which are the optimal bones to assess skeletal health (pilot studies) could be expanded to give further understanding. As it is well documented that age effects bone parameters at multiple ages (Qiaoxian *et al*., 2020), expanding the number of age groups used to determine the optimal bones would give more information on which bones are the most useful. It would also provide a better understanding as to whether different bones are optimal for different points of the laying cycle. In addition, investigating the rearing stage could provide more information as the rearing system effects bone development as reported in previous research (Hester *et al*., 2013), and it may be elucidated whether growth potential at the rearing stage influences the differences seen towards the end of lay. Future pilot studies run in-house or closely mirroring commercial settings would also be advised in future work when confirming useful bones to assess skeletal health. This would allow for the control of dietary treatments and bird husbandry, thus reducing the variability caused by these factors and giving clearer understanding on what may influence the results. Increasing sample numbers would improve statistical power and would make the results are more representative of the flock or farm but requires more hens to be culled. As used in subsequent chapters, bone ash content or parameters such as BMD or BMC could be used when determining optimal bones, as these parameters may help indicate mineral turnover of a bone. Furthermore, the bone parameters which were used in the pilot study may have been more meaningful, if calculated relative to bird weight. This would reduce any effect of bird size and improve the accuracy of the results. The use of a range of different bird strains in determining the optimal bones would also be advised. Like age, the effectiveness of some bones to indicate differences in factors could change depending on the genetic strain of birds

used. Using strains of hens commonly used in UK production would be advised as the this produces more meaningful and relative results for the UK poultry industry. Ultimately, assessing as many factors as possible when determining the best sample bones to evaluate skeletal health will provide the most well-informed decisions going forward.

7.3.2 Effect of housing system on skeletal integrity

Aside from the longitudinal effects of housing system and age on skeletal integrity studied in chapter 5, other factors such as the diet and bird strain could assessed for their impact. As these factors have been previously studied in other research (Tarlton *et al*., 2011, Candelotto *et al*., 2017), these factors could have affected the outcome of skeletal health but were not recorded in this study. In addition, including diet and genetic strain as factors into the bone strength model along with extra farm data from future work could increase the power of the model to accurately predict skeletal health across UK production and therefore increase the potential of it to be used an early warning tool for assessing skeletal health. Further investigation into the longitudinal effect of housing system and age using an increased number of farms per system, increasing the age range to include the rearing stage, increasing the bird samples per collection, and identifying subcategories of different housing systems would all increase the comprehensiveness of the results within future work and improve skeletal assessment. However, as this would increase commercial application it would also highly costly as it would require large commercial participation and increased birds to be taken from production which would be considered disadvantageous to the collaborating farms involved. Also, it is likely that farms willing to collaborate are of a certain standard otherwise they may receive negative attention, implying that the majority of farms able to be used may influence the outcomes of the study and not be inclusive of all UK farms.

In terms of future work surrounding the methods used within this study, it would be advised to randomise selection of birds within different areas of the housing system (by a randomised block design) to prevent bird selection skewing the results from a particular farm. Fat extraction of the bones when calculating bone ash content would also be advised, as it would increase the accuracy of the ash results significantly (Sanni and Burton, 2016). Furthermore, calculating bone strength relative to body weight would have reduced the effect of variability

within bird size as previously mentioned. Due to the large sample size in this study, laboratory work was performed by many different researchers. Having one researcher recording a certain measurement throughout the study could have reduced the amount of possible human error and would be advised in any future work of similar sample size. As exercise has been shown to effect bone parameters in previous research (Casey-Trott *et al*., 2017c), another area of focus for future work could be to record distinct exercise behaviours periodically to gain an understanding of how much exercise is being performed in individual systems. Combined with the numerical data of bone parameters, this would help support or explain conclusions of future work.

7.3.3 Effect of housing system on egg quality traits

Within the egg part of the on-farm study, six eggs were originally collected to analyse, though this was then increased to 30 eggs, so results were more representative of egg production. In future work it would be advised that this number be increased further possibly to a case (360 eggs). This would allow egg data to be more representative of the flock at each age group but also increase labour required for analysis. Aside from increasing the egg numbers per collection, the number of age groups collected from could be increased. Less eggs collected more frequently would provide a more continuous measure of egg quality and be able to highlight differences caused by age or housing system more precisely. As in section 7.3.2, increasing the number of farms participating per system and including the genetic strain and diets of the birds would also help increase the comprehensiveness of the egg quality results further. Although it is thought that egg quality traits are not effective at distinguishing the effects of housing system and age on skeletal health, further development into the modelling of egg and bone residuals could potentially highlight a relationship. It would be suggested that collecting and linking eggs back to individual birds would increase the likelihood of a relationship to be revealed. However, this is highly unlikely to be practical within a commercial setting due the work it would require in order to configure separate pens for individual birds and the husbandry required to run the study. As such, it could be possible that future work in this topic may utilise inert dietary markers which can be taken up in egg production and analysed for when assessing egg quality traits. This could allow for eggs to be traced back to a group of birds or individual bird in order to link egg and bone data. Again,

this would still require costs and modification to the system but would be simpler and less invasive than setting up separate pens within a commercial system.

7.3.4 Confirming validity of methods

There is still much work to be done to ensure the methods used to assess skeletal health in laying hens are fully validified. As the birds were housed in colony caged system for the length of the laying period and then moved to a lab-based pen system (chapter 6), it would be advised that to achieve accurate results a substantial acclimatisation period must be included upon arrival. This would ensure more accurate results by allowing the birds to settle, establish a hierarchy within the pen and begin to show natural behaviours. These behaviours could be monitored to ensure the birds were well settled into their new environment pre-trial. Though exercise could not be controlled for, this acclimatisation period could reduce stress of the birds and prevent it from influencing the results. Furthermore, it is advised that birds used in a future study are from a similar system as the Poultry Unit at NTU, to prevent bird stress and potential skeletal damage caused by birds not being familiar with the configuration of the system. Diet supplementation, in this case silicon inclusion, was found to have little effect on the bone and egg parameters during the study in chapter 6. Increasing the duration of the trial (after the acclimatisation period) and supplementing silicon at multiple inclusion levels is suggested to potentially improve the validation of bone parameters and egg quality traits further. Additionally, it could be proposed that the model parameters of bone strength, egg strength and residual values for bone-egg relationships as in chapters 4 and 5 would be of some usefulness in future work on method validation. As this study was on a smaller scale than the on-farm study, eggs would be easily traceable back to a small group of hens. This would increase the sample size when determining a relationship between eggshell and bone strength and possibly reveal more information about of the relationship. In addition, the effect of silicon supplementation, or another bone supplement (such as Vitamin D) could also be investigated as a factor on this relationship, perhaps further increasing the applicability of bone-egg relationship model for commercial use.

7.4 Recommendations for industry

This project suggests that the use of egg quality traits is not meaningful when attempting to assess skeletal health. Although egg quality traits are of great importance to egg producers, investigating skeletal health is considered important for hen welfare. It is therefore recommended that any studies undertaken do not use egg quality traits to identify any effects relating to skeletal health, as traits are not likely to be affected before the integrity of the skeletal system has been comprised by egg production.

Secondly, when selecting bone samples, it would be recommended to use multiple bone types to ensure all areas of the skeletal system are assessed as the form and function of different bones vary. These bones may also show different effects of the factors being analysed and if all areas of the skeleton are covered, a more comprehensive and detailed assessment of skeletal health would be achieved. In relation to this, the bone parameters used to assess skeletal health are recommended to cover bone geometry as well as measurements such bone weight, ash content and strength which are more commonly used. This is because the project found that bone geometry measurements were affected by the analysed factors, although it is thought that once point of lay is reached, bone development stops. Using these parameters in conjunction commonly used measures will improve the understanding of different factor effects on skeletal health moving forward.

Finally, there is merit for using and further developing the models created within this project. As the data of this project was based on UK egg production – the model parameters could be utilised as an early warning system on skeletal health within the poultry industry, to help maintain high standards of hen welfare and productivity. It would be recommended that this model or variations of this model be improved or added to with data retrospectively, in order to increase the precision of the model parameters and help combat the effects of egg production demands on skeletal health of laying hens proactively.

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Appendices

Appendix 1 – Analysis of diets used in the on-farm project

Table 1 Farm diet codes by age

	FR MT			C			FRFD			O			B	
Age	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1 (own mix)**	**2**
18	210	281	210	110	110	110	281	210		108	108	108	636	117
24	210	281	210	110	110	110	281	210	339	205	205	205	636	110/117
30	210	210	210	110	110	110	281	210		205	205	205	646	110/117
36	209	210	210	110	110	110	210	210	339	205	205	205	646	117
42	209	209	211	110	110	110	220	211	339	235	235	235	646	117
48	209	209	211	110	110	110	220	211	339	235	235	235	646	117
54	210/220	216	211	110	120	120	216	211	339	235	235	235	646	127
60	220	216	211	120	120	110	216	211	339	235	235	235	637	127
66	220	216	211	120	120	130	216	211		235	235	235	637	127
72	220	216	211	120/130	130	130	216	211		235	235	235	638	127

Table 2 Farm reference key

System	Farm number	Farm Name
FRMT	1	Welshs
FRMT	2	Ashlea H3
FRMT	3	Denton Grange H2
C	1	Longbelt 3
C	2	Longbelt 4
C	3	Moores
FRFD	1	Ashlea H1
FRFD	2	Denton Grange H1
FRFD	3	Priory Walcott
O	1	Ings
O	2	Bulbourne
O	3	Gatewood
B	1	Bridgehouse
B	2	Longbelt 1

Table 3 Farm diet analyses (636 - 638 own farm analysis)

Feed code	Unit	110	117	120	127	130	209	210	211	216	220
Calcium	%	3.90	3.80	4.00	3.80	3.90	3.80	3.70	3.70	3.70	3.80
Phosphorus (total)	%	0.38	0.43	0.38	0.41	0.39	0.46	0.46	0.42	0.42	0.44
Ash	%	12.49	12.42	12.67	12.33	12.46	12.55	12.25	12.17	12.17	12.40
Fibre	%	4.48	3.44	4.62	3.75	3.82	4.85	5.56	5.45	4.40	5.68
Oil A-EE	%	2.31	2.85	1.87	2.64	2.45	3.71	3.20	2.80	2.74	2.54
Protein	%	17.07	18.71	15.93	17.93	16.98	17.97	17.09	17.06	18.50	15.68
Dry matter	%	88.66	88.66	88.63	88.64	88.57	88.61	88.89	88.66	88.49	88.87
Feed code	**Unit**	**281**	**339**	**108**	**205**	**235**	**636**	**646**	**637**	**638**	
Calcium	%	3.90	3.80	2.75	4.10	4.20	3.80	3.94	4.10	4.20	
Phosphorus (total)	%	0.48	0.46	0.62	0.52	0.50	0.53	0.49	0.46	0.44	
Ash	%	12.84	12.55	10.45	13.90	14.05	13.00	12.93	13.50	13.50	
Fibre	%	5.94	4.85	5.57	4.80	5.08	2.60	2.91	3.50	3.50	
Oil A-EE	%	2.48	3.71	4.35	4.22	3.97	4.50	4.58	4.30	3.50	
Protein	%	16.71	17.97	18.80	19.74	18.67	18.00	16.49	16.00	15.00	
Dry matter	%	88.76	88.61	88.86	89.41	89.39					

Appendix 2 – Farm collection/health sheet used for on-farm project

Farm Collection Sheet

NOTTINGHAM TRENT UNIVERSITY

Farm Name:			
Date of collection:			
Production details: - **Housing system** - **Breed**			
House:			
Age of birds (weeks):			
Age of parent flock (weeks): (REARING ONLY)			
Mortality in flock: (%)			
Feeding: - **Rations (feed code)** - **Routine**			
Additives in feed (if any):			
Additives in water (if any):			
Health concerns (if any):			
Environmental issues (if any):			
Eggs collected (Y/N)		**Number of eggs:**	