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Effects of soy supplementation on bone characteristics and egg quality in peak production laying hens

Kealani Izquierdo
Clemson University, kizquie@clemson.edu

EFFECTS OF SOY SUPPLEMENTATION ON BONE CHARACTERISTICS AND EGG QUALITY IN PEAK PRODUCTION LAYING HENS

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Kealani Joy Izquierdo
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Dr. Julie K. Northcutt, Committee Chair
Dr. Paul Dawson
Dr. Vivian Haley-Zitin

ABSTRACT

In the last few decades equol, a metabolite of the isoflavone daidzein, has become the target of various animal and human studies. Coined the key to health benefits associated with soy foods, equol formation been implicated in the healthy profiles associated with Asian populations. As a metabolite of daidzein, equol must be synthesized from its precursor by intestinal bacteria; however, clinical and epidemiological studies indicate that only about a third of the general human population possess the microflora necessary to metabolize equol. Research indicates that by supplementing laying hens with soybean meal, equol deposition into table eggs can be stimulated, thereby creating a functional direct source of this estrogenic compound, independent of gut microflora. Though equol enhanced eggs may be created through soy supplementation, isoflavones are bioactive and have various physiological effects when consumed. Thus, the purpose of this research project was to examine the impact of various levels of soy supplementation (soy-free-SF, standard soy-SS, soy enhanced-SE) on bone characteristics and egg quality in young battery-cage (BC) and free-range (FR) laying hens. The research was conducted concurrently with a study that determined the level and rate of equol deposition into table eggs from the same flock.

Findings of the current research showed that SE combined with physical activity had a significant impact on bone health as evidenced by increased femoral weight ($P=0.005$), length ($P=0.0066$), bone strength ($P=0.016$) and compression energy ($P=0.013$). Additionally, higher levels of phosphorous ($P=0.012$), magnesium ($P<0.0001$), zinc ($P=0.0002$) and the calcium/ phosphorous ratio ($P=0.001$) were observed in femurs originating from FR layers given SE feed. Quality analysis showed

that eggs collected from both soy treatments (SS and SE) were larger and heavier ($P<0.05$) than eggs collected from SF layers. Moreover, eggs collected from SF hens had thinner eggshells ($P=0.0435$) than those from soy treated (SE and SS) layers. Furthermore, data analysis also showed that soy treatments can negatively affect yolk quality as evidenced by decreased vitelline membrane strength ($P=0.034$) and less red yolks ($P<0.0001$) in hens fed SE and SS diets.

Overall, the effects associated with dietary isoflavones via soy supplementation beneficially altered various quality characteristics that affect both animal welfare and poultry farmers. Decreased bone fractures associated with avian osteoporosis could improve animal welfare as well as curb economical losses associated with layer mortality. Furthermore, stronger eggshells could reduce the percentage of cracked and downgraded eggs.

DEDICATION

This thesis is dedicated to my amazing family for encouraging me to pursue my passions no matter how impossible they seem. To Dr. Manuel and Luz Izquierdo, my caring parents and first nutrition advisors, your unrelenting love and support has taught me to fearlessly follow the path less traveled. I am indescribably thankful you chose to embrace holistic nutrition and instill those values in us Mom, years of sneaking tofu into my sandwiches has finally paid off. Rebeca, my sweet sister, you are one of only people that can handle me when I get anxious and start pacing…it must be your laid back demeanor. Thanks for believing enough for the both us. Manuel, your dedication inspires and drives me and I have not doubt you will be a brilliant doctor. Breanna and Mariah, you little ladies are two of the greatest things in my life and I am so blessed by you both.

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LITERATURE REVIEW

1.1 Introduction

Epidemiological studies have consistently revealed that Asians have lower incidences of several diseases that plague the Western world in almost pandemic proportions. Osteoporosis, cardiovascular disease, breast and prostate cancer, as well as vasomotor symptoms related to menopause are markedly less in Asian countries (Messina, 2000; Friedman et al., 2001; Ogbewu et al., 2010). Though genetics and racial factors have been cited as a source for the discrepancies in epidemiological data, dietary factors, specifically soy isoflavones, have been scrutinized as a major cause for the geographical variation in data (Morton et al., 2002). The evidence further suggests that soy isoflavones may act as phytoestrogens causing various beneficial estrogenic effects when consumed over time in large quantities (Morito et al., 2001; Vaya et al., 2004). However, soy supplementation in clinical trials has yielded inconclusive results leading researchers to question the safety and efficacy of isoflavone treatments (Sakai, 2008; Ogbuewu, et al., 2010; Andres et al., 2011).

Since soy challenges have produced varied results, researchers have formulated the equol hypothesis, suggesting that the clinical effectiveness of soy protein can be attributed to an individual's ability to produce the metabolite, equol (Setchell, 2002). Equol is a non-steroidal estrogenic compound that does not exist naturally, but rather is metabolized by colonic bacteria from its precursor daidzein, a soy isoflavone (Magee, 2011). Detailed in various extensive reviews, equol's estrogenic activity is due to its structure, bioavailability and affinity for estrogen receptors (Yuan et al., 2007; Setchell

and Clerici, 2010a; 2010b; Magee 2011;). Though equol is more bioactive than its precursor, its classification as a metabolite indicates that its formation is entirely dependent on an individual's intestinal microflora (Setchell et al., 2005). An intestinal tract lacking the necessary bacteria to synthesize equol will lead to formation of O-desmethylangolensin (O-DMA), a relatively less bioactive and estrogenic metabolite of daidzein (Lampe, 2009). About 80-90% of the general human population produces O-DMA when challenged with soy while only ~25-30% produces equol (Lampe, 2009). In comparison, in Asian countries where soy intake is high, 60% of adults are classified as equol producers (Setchell and Cole, 2006).

Since only about a third of the general population can produce equol via bacterial metabolism, alternative methods of equol supplementation have been examined. Functional foods in the form of probiotics and prebiotics have been designed to alter colonic bacteria and stimulate equol production, but have yielded varying results (Decroos et al., 2005; 2006; Larkin et al, 2007). Furthermore, though chemical synthesis of an equol supplement has been successful, it has produced a relatively less bioactive (±) racemic mixture, rather than the natural S (-) form (Shinkaruk et al., 2010). Finally, collected research indicates that equol deposition into table eggs can be stimulated by supplementing laying hens with soy and soy isoflavones (Saitoh et al., 2004).

Stimulating equol deposition into table eggs would allow consumers access to a direct source of the metabolite via a functional food, thereby addressing the problems created by inter-individual variability in gut bacteria. Defined as a food or food ingredient that may enhance health through their provision of a physiological benefit beyond its traditional nutrients, functional foods are a vastly growing category of food

products (Stadelman, 1999). Though functional foods encompass a broad spectrum of food products, designer or specialty eggs have been a rapidly growing sector and represent up to 3-5% of retail eggs in the country (Patterson et al., 2001). Reports indicate that consumers are willing to pay premium prices for specialty eggs and according to USDA, organic eggs, a subtype of specialty eggs, retailed for $2.75 - $3.10 per dozen compared to conventional eggs with a national average of $1.39 (Blank, 1997; USDA, 2012). According to Patterson, functional foods and designer eggs fulfill one or more specific needs such as quality, emotional, or health benefits that lead the consumer to pay premium prices (Patterson et al., 2001).

Research indicates that equol-enhanced eggs can be created by diet manipulation; however, an extensive review of literature indicates that little data exists on the effects high levels of soy supplementation have on the biomechanisms that control avian egg formation (Saitoh et al., 2004). According to Ahmadi and Rahimi (2011), egg formation is a delicate physiological process dependent on various factors, specifically diet and stress. Therefore, the research described in this thesis was conducted to determine the effects soy supplementation would have on bone metabolism and egg quality. Isoflavones have been cited to have estrogenic effects and influence bone metabolism when ingested (Wu et al., 2003; Liu et al., 2007; Weaver and Legette, 2010). Since avian osteoporosis is a disease that affects a large percentage of the laying hen population (Gregory and Wilkins 1989; 1990), isoflavones could significantly impact the mechanisms that contribute to the development of the disease. Furthermore, eggshell formation is highly dependent on the mobilization of skeletal calcium and other minerals,

thus any shift in this equilibrium could affect both internal and external egg quality (Whitehead and Fleming, 2000; Chukuwuka, 2000; Ahmadi and Rahimi, 2011)

1.2 Avian Bone Metabolism and Osteoporosis

Osteoporosis in laying hens is defined as a progressive decrease in the amount of mineralized structural bone over time that eventually leads to bone fragility and an increased likelihood of fractures either during the production period or during depopulation (Whitehead and Fleming, 2000). This condition was first described in caged layers and was characterized by bone brittleness, vertebral weakness, and paralysis (Couch, 1955). Production losses also rise with the development of avian osteoporosis since bone breakage is associated with low egg productivity and mortality (Rath et al., 2000). High fracture incidences during processing of spent layers can lead to bone fragments in carcasses and discoloration of meat, both undesirable to consumers and further increasing economic losses (Whitehead and Fleming, 2000).

The development and incidence of osteoporosis in laying hens is partially attributed to the high demands for skeletal calcium necessary for eggshell formation (Whitehead and Fleming, 2000). During formation, each egg requires 2.0-2.2g of calcium (Ca) (Newberry et al., 1999), 30-40% of which is provided by skeletal reserves (Mueller, 1964). However, osteoporosis in laying hens is the consequence of several nutritional, environmental and genetic components. Combined utilization of battery cage production systems and prolonged periods of production has heightened the incidence of the disease in caged layers (Webster, 2004; Clark et al., 2008). Furthermore, since osteoporosis is a multi-factorial disease, it can result as a loss of mineralized bone, deterioration of micro-architecture or reduction of total bone mass (Ilich, 2000).

Similar to humans, laying hens have two types of bone composed of hydroxyapatite crystals of calcium phosphate: cortical and cancellous (Whitehead and Fleming, 2000). Cortical bone is more prevalent in the long bones such as the femur and humerus while cancellous bone is found in flat bones such as the vertebral and skull bones (Rath et al., 2000). Cancellous bone is also found in the metaphyseal ends of long bones and is less calcificied, playing a more prominent role in bone remodeling (Siefert and Watkins, 1997). Additionally, these forms of bone are slowly synthesized and have well organized arrangements of collagen (Whitehead and Fleming, 2000). Collagen is not only the major constituent of bone, it also provides intrinsic strength and support for the rest of the mineral matrix (Riggs, et al., 1993; Whitehead and Fleming, 2000).

Unlike other animals, female avian species develop a third type of bone upon maturation, known as medullary bone. Medullary bone (MB) is a nonstructural, highly labile bone that is formed in response to high circulating levels of estrogen (Beck, 2004). As a hen's ovarian follicles mature, dramatic hormonal changes also occur preparing the hen to enter lay. Growth plates at the end of cortical bone are mineralized causing them to cease lengthening (Whitehead, 2004), and simultaneously causing them to increase in diameter by about 20% prior to lay (Riddel, 1992). These changes allow for an increased surface area for osteoblasts (bone forming cells) to deposit MB on the endosteal surfaces of long bones, specifically on the tibia and femur (Whitehead, 2004). Once synthesized, MB serves as a source of Ca and can be resorbed 10-15 times faster than structural bone due to its high level of osteoclasts (Dacke, 1993). Additionally, MB has a higher mineral content than structural bone, and lacks much of the collagen found in cortical and trabecular bone (Rath et al, 2000).

Though MB provides up to 30-40% of Ca necessary for eggshell formation (Mueller et al., 1965), its unorganized arrangement of collagen fibers can make the bone substantially weaker than structural bone (Fleming et al., 2008). MB is also created at the expense of structural bone and an imbalance between these processes can contribute to the development of osteoporosis (Whitehead and Fleming, 2000).

Although medullary bone lacks the intrinsic strength of structural bone, it may be able to contribute to the mechanical strength of cortical bone (Fleming et al., 1998). Previously, MB had been thought to contribute little to bone integrity; however, recent studies have found that in large quantities MB may actually increase fracture resistance (Fleming et al., 1998; 2006; Whitehead, 2000). In a series of studies, Fleming et al. (1998) found that the presence of MB in large amounts in the pneumatized area of long bones, such as the humerus and femur, could actually increase breaking strength in end-of lay chickens. In a subsequent study, these same researchers found that accumulation and large amounts of MB in genetically selected laying hens had a greater tibiotarsal strength than those with less accumulated MB (Fleming et al., 2006). While MB can contribute to fracture resistance, in general, losing structural bone can weaken overall skeletal strength and integrity (Whitehead, 2004).

1.3 Factors Affecting Bone Metabolism

Though laying hens are inherently predisposed to osteoporosis due to large amounts of skeletal Ca utilized for eggshell formation, several other factors can influence and exacerbate the development of the disease (Whitehead and Fleming, 2000; Beck and Hansen, 2004; Silversides et al., 2012). Of the factors, estrogenic effects, production system and nutrition are of vital importance.

1.3.1 Estrogen

In laying hens, estrogen has direct and indirect effects that influence the microstructural architecture of the skeleton (Beck and Hansen, 2004). By affecting skeletal bone composition and intestinal Ca absorption, estrogen is a vital factor to consider in osteoporotic development.

Briefly highlighted above, loss of structural bone begins when hens reach sexual maturity and occurs in response to elevated levels of circulating estrogen (Wilson et al., 1992). The high levels of estrogen cause a bone formation shift, as osteoblasts begin to synthesize MB and simultaneously, cease synthesizing structural bone (Whitehead and Fleming, 2000). This process is reversed when a hen goes out of lay causing estrogen levels to decline and eggshell formation to stop (Whitehead, 2000). Laying in clutches followed by an incubation period allows for structural bone to recommence and be deposited on MB, permitting the hen to maintain bone integrity over its lifetime (Whitehead, 2000). However, since modern laying hens are selected for prolonged lay, their structural bone content continuously declines causing increased incidences of osteoporosis (Wilson, 1992; Knowles and Wilkins, 1998).

This biomechanism is largely controlled by levels of circulating estrogen (Whitehead, 2004). Endogenous estrogen stimulates osteoblast function and has an inhibitory effect on osteoclast function. Therefore, the estrogenic surge that occurs at maturity causes hens to rapidly produce bone, but rather than structural bone, MB (Whitehead and Fleming 2000). Since the loss of structural bone is progressive, coinciding with levels of estrogen, osteoporosis is most severe in hens at the end of lay (Whitehead and Fleming, 2000).

Additionally, estrogen has several indirect effects on laying hens dependent on its influence on other biomechanisms, including Vitamin D3, or 1,25-D_3 and Calbindin D28K (CaBP-D28K) (Beck and Hansen, 2004). Vitamin D3 is a vital component of bone health due to its role in duodenal Ca absorption. However, in order to be effective, Vitamin D3 must be synthesized from its precursor 25-hydroxycholecaliferol by 25(OH)2-1α-hydroxylase, an enzyme activated by estrogen (Soares, 1984). This activated form of Vitamin D3, or 1,25-D_3, mediates the transport of Ca across the intestinal brush border (Newbrey, 1992). Furthermore, estrogen in laying hens up-regulates gut mucosal 1,25 D_3 receptors (Wu et al., 1996), and together with estrogen synthesizes CaBPD28K, a protein vital to initiating Ca binding and absorption in the avian duodenum (Nys et al., 1992).

1.3.2 Production System

While estrogenic effects, specifically the shift from structural bone to MB formation, can contribute to the development of osteoporosis, the role of exercise and production systems have been examined in various studies and have been found to further influence metabolism as measured by bone mass, strength and mineralization. Furthermore, free-range production systems that allow access to outdoors may stimulate greater vitamin D3 synthesis, further affecting bone metabolism (Fleming, 2007)

Though laying hens are inherently predisposed to osteoporotic conditions, it was not until the utilization of battery cages in the mid 20th century that cage layer fatigue became an issue of industrial proportions (Webster, 2004). As the name implies, cage layer fatigue occurs predominately in hens housed in battery cages (Couch, 1955). These hens have considerably weaker bones than those raised in high-level aviaries and free-

range production systems where they have more opportunities for flight, flapping and perching (Whitehead, 2004). A study conducted by Gregory and Wilkins (1989) found that at the end-of-lay, 29% of hens housed in battery cages had at least one bone fracture during their lifetime. These same researchers also found 24% of hens from battery cages had freshly broken bones, compared to 10% in an aviary system (Gregory and Wilkins, 1990). Therefore, confinement of birds in cages with limited mobility and negligible physical activity has contributed to the manifestation of disuse atrophy and osteoporosis (Whitehead and Fleming, 2000).

According to Lanyon (1992), housing systems allowing the greatest physical activity produce hens with stronger bones while those housed in restricted environments (battery or furnished cages) have weaker and more brittle bones. Additionally, a recent study conducted in the United Kingdom, reported that of 67 flocks housed in four primary housing systems, those housed in furnished cages had the lowest peak bone strength, primarily weaker humerus bones (Wilkins et al., 2011). Conversely, hens housed in organic systems, free-range equipped with a variety of perches and next boxes, tended to have higher peak strengths (Wilkins et al., 2011). These studies serve to illustrate that the improvement of bone integrity is related to the biomechanical load experienced by the bone; therefore, exercise can have effects on bone mass, bone strength and bone mineralization (New, 2001).

Bone Mass

Exercise and mechanical loading have been cited as a source of bone accretion, increasing bone size by inducing skeletal development, bone growth and bone turnover in laying hens (Lanyon, 1998; Fleming et al., 2006; Jendral et al., 2008, Silversides et al.,

2012). Conversely, inactivity is linked to bone fragility, largely due to the loss of functional loading (Fleming et al., 2006). A study conducted by Silversides et al., (2012), found that hens housed in floor pens where they were able to stretch their wings and fly had heavier radius bones than their caged counterparts (1.178 g versus 1.063 g; $P<0.001$). Jendral et al., (2008) conducted a similar study comparing bone quality of laying hens housed in conventional battery cages, modified cages with a nest box and perch and furnished colony cages with or without a dust bath. The hens housed in the conventional battery cages had the lowest cortical bone area in the femur, humerus and tibia ($P=0.0001$).

The benefit of exercise in increasing bone mass is important in curbing the development of osteoporosis. Attaining and maintaining a peak bone mass (PBM) has been found to help attenuate the development of this disease (Matcovik, 1979; Ilich, 2000). While a low PBM has been linked to higher risks of bone fragility, a high PBM achieved in early adulthood can reduce the risk of osteoporosis (Matcovik, 1979). Furthermore, immobility has been linked to a reduction in skeletal and bone mass, and data shows that even a 5-10% decrease in PBM can potentially contribute to a 50% increase in fracture rates (Matkovic, 1979; 1995). Additionally, bone mass is vital as it is directly proportional to bone strength (Frost, 1997).

Bone Strength

Bone strength is the toughness and ability to endure stress, or the stress applied at which the bone will break (Rath et al., 2000). Though bone strength is related to its physical, architectural, and material properties, (Rath et al., 2000) limited activity or mechanical loading can reduce bone strength (Knowles and Broom, 1990). Hence, the

degree of bone strength is related to the degree of the biomechanical load experienced by the bone (Whitehead, 2004).

The correlation between exercise and bone strength was illustrated in a study conducted by Newman and Leeson (1998). Tibial strengths were measured in laying hens raised in both conventional battery cages and in aviary production systems and measured at 193.4 versus 257.1 Newtons (N), respectively. A randomized sample was moved from the battery cages to the aviary system and within 20 days, their breaking strength was similar to that of indigenous population 259.3 N versus 262.1, both significantly greater than bone strength observed for the battery cage population ($P<0.05$) .

Similar data has been found in subsequent studies, further illustrating the effects of exercise on bone strength (Leyendecker et al., 2005; Fleming et al., 2006; Wilkins, 2011). Data collect by Leyendecker et al. (2005) showed that bone breaking strength was consistently higher in laying hens housed in aviaries than those in caged systems. Furthermore, tibial and humeral breaking strengths were significantly higher in hens housed in aviaries when compared to those in conventional and furnished cages ($P<0.001$). Similar results were collected by Fleming et al., (2006) when he compared bone breaking strengths of aviary and caged laying hens. Aviary birds had tibial and humeral strengths 32-35% higher, respectively than their caged counterparts. Finally, data collected by Wilkins et al., (2011) found that laying hens housed in alternative organic systems where they were free to exercise had significantly greater peak humeral ($P<0.001$), tibial ($P<0.001$) and keel ($P<0.001$) breaking strengths than their caged counterparts.

Bone Mineralization

Apart from increasing skeletal mass and strength, exercise can also contribute to bone quality as measured by bone mineral content (BMC) and bone mineral density (BMD) (Choi et al,, 2005). Though these factors are largely influenced by diet, physical activity has been cited as an important contributor to mineral deposition, as it can increase bone turnover (Lanyon, 1986; Ilich, 2000; Welch, 2005). Maintaining BMC and BMD is extremely important to skeletal health and can dictate the quality of bone in laying hens (Rath et al., 2000). Therefore, changes in the inorganic or organic materials of the bone can alter the quality of the bone, contributing to the development of osteoporosis (Schrweiwis et al., 2003).

Phosphorous (P) and Ca are the predominant inorganic materials in the mineral matrix and contribute 60-70% of bone weight, providing both compressional strength and stiffness (Rath et al., 2000). Furthermore, these minerals present in the form of hydroxyapatite provide a framework by binding to adjacent collagen fibers (Rath et al., 2000). The Ca/P ratio is extremely important in bone health since data indicates that deviating from the ideal 2:1 ratio can have negative effects on skeletal development (Clark et al., 1969).

While Ca and P are the primary inorganic nutrients present in the mineral matrices, several other inorganic elements are present in the bone and necessary for skeletal growth (Rath et al., 2000). Magnesium (Mg) plays an important role in Ca and bone metabolism, and a Mg deficiency can contribute to hypocalcemia (Ilich, 2000). Zinc (Zn) and copper (Cu) are essential cofactors for several enzymes such as collagenase, essential for the development and mechanical strength of collagen (Ilich,

2000). Manganese (Mn) is necessary for the synthesis of mucopolysaccharides, as well as other structural proteins in bone (Watts, 1990).

Maintaining optimum levels of these inorganic nutrients as measured by BMD and BMC, is of utmost importance to maintain skeletal health and support bone metabolism (Choi et al., 2005). Since exercise is linked to bone turnover and deposition, it can also coincide with the maintenance of BMC and BMD. In the study conducted by Jendral et al., (2008) the laying hens in battery cages had a significantly lower total BMD when compared to alternative production systems ($P=0.0304$). Similarly, the results reported by Silversides et al., (2012) found that the hens housed in floor pens had significantly greater radial trabecular, cortical and total mineral content ($P<0.001$) as well as greater tibial cortical and total mineral content ($P<0.001$).

Vitamin D

As mentioned above, Vitamin D is necessary for skeletal development and subsequent eggshell formation and functions by enabling Ca absorption (Frost et al, 1990). Evidence also suggests that maximizing vitamin D status in chicks can alleviate bone disorders in both laying hens and young pullets (Newbrey, 1992; Whitehead, 2004). Though it can be supplemented through feed (Mattila et al., 2004), it has been hypothesized that alternative production systems are linked to higher conversion and synthesis of Vitamin D3 due to layer access to outdoors and natural sunlight (Fleming, 2008). Vitamin D3 is synthesized through photochemical reactions in response to exposure to sunlight and its ultraviolet-β photons (Bar et al., 1999). A study conducted on chicks found that simulation of daylight via UVB lamps stimulated Vitamin D3 synthesis and resulted in tibia strength >30% higher than the control groups (Fleming,

2008) These UV-treated birds had higher mineralization as determined by radiographic density and tibia ash values ($P<0.001$).

1.3.3 Nutritional Factors

According to Rath and Hansen (2000), nutritional factors are probably the most relevant to bone strength, and accordingly several dietary factors have been examined to determine their effects on bone integrity. As noted above, maintaining sufficient levels of organic and inorganic compounds is of vital importance to bone health. Nutritional deficiencies of Ca, P, Cu and Vitamin D have all been linked to bone loss and bone fragility (Wilson and Duff, 1991). Furthermore, supplementation with boron (Wilson and Ruzler, 1998), fluoride and carbonated water (Koelbeck et al., 1993) have been beneficial in remedying bone fragility. Conversely, excess aluminum (Huff et al., 1996), and protein (Heany, 1998) have been linked to having negative effects on bone characteristics.

Soy Isoflavones and Metabolites

While feed enhancement has varied results on avian bone metabolism, recent studies have begun to focus on soy supplementation and the effects it could have on ameliorating osteoporotic conditions by influencing bone homeostatic mechanisms (Zhao et al., 2004, 2005: Ni et al, 2007; Sahin et al, 2007). Soy isoflavones, specifically genistein and daidzein, can exert estrogenic activity due to their structure similar to 17β – estradiol, the most potent form of endogenous estrogen (Figure 2) (Ogbuewu, 2010). Due to their structure, isoflavones can bind to estrogen receptors α and β (ER α and ER β), thus having potential protective and therapeutic effects in a range of estrogen-dependent diseases including osteoporosis (Ishimi, 2010; Weaver and Legette, 2010),

breast and prostate cancers (Fuhrman et al., 2004; Lund, 2011), and atherosclerosis (Cheng, 2010). Moreover, isoflavones are classified as selective estrogen receptor modulators (SERM's), a class of compounds that can act as ER agonists in some tissues, or ER antagonists in other tissues (Cassidy, 2003). SERM's are chemically diverse molecules that possess a tertiary structure that allow them to bind to ER's. Due to their ability to selectively bind to ER's, SERM's can up-regulate ER's in target tissues causing an amplified estrogenic effect, or conversely, competitively bind to ER's inhibiting estrogen's total effects (Gennari et al., 2007).

While daidzein possesses estrogenic activity that is about 10^{-3}-10^{-5} times that of 17-β estradiol (Kuiper et al., 1998), the isoflavone can be metabolized to the more endogenously potent compound, equol (Figure 3). Equol is synthesized exclusively by intestinal bacteria, after its precursor undergoes a series of biotransformations (Figure 4)(Setchell et al., 2002). Once ingested, daidzein is converted to the intermediate dihydrodaidzein, and concurrently either O-desmethylangolensin (O-DMA) or 4-hydroxy-equol which is further converted to equol (Bolca, 2007). Like its precursor, equol has a structure very similar to 17-β estradiol allowing it to bind competitively to ER's, specifically having an affinity for ER-β 20% higher than 17- β estradiol (Figure 5) (Setchell et al., 2005). Additionally, approximately 50% of equol circulates endogenously in free form, while only 18.7% of daidzein and less than 5% of 17-β estradiol circulate unbound to serum proteins such as albumin, globulin and α-fetoprotein (Nagel et al., 1998). Hence, of the isoflavone metabolites, equol is the most bioactive, and due to its high bioactivity has been targeted as the key to the effectiveness of soy and isoflavone consumption (Setchell, 2002).

Though only 30-40% of the adult human population can produce equol when challenged with soy, animals inherently possess intestinal bacteria necessary to metabolize equol (Lampe, 2008). Subsequently, equol has been isolated in urine and plasma samples from horses (Marrian, 1935), chimpanzees (Adelcrutz, 1986), rats (Uehara, 2001) and mice (Ishimi, 2010) when fed soy supplements. Additionally, after laying hens were supplemented with dietary isoflavones, researchers isolated serum equol and, more notably, found that daidzein supplementation stimulated equol deposition in eggs (Saitoh et al., 2004).

Agonistic Effects

As noted, soy isoflavones may have beneficial effects on bone metabolism due to their ability to behave as SERM's. Because they have the ability to preferentially bind to various target tissues, they can be effective in treating specific conditions like osteoporosis without having the total systemic effects of estrogen (Gennari et al., 2007). Consequently, researchers have begun to study the impact of isoflavone supplementation as a potential preventative treatment in avian osteoporosis (Ni et al., 2007; Sahin et al., 2007). Since the risk for osteoporosis increases as laying hens age and levels of circulating estrogen decreases, most studies have focused on the agonistic role of isoflavones, specifically daidzein and equol. According to Setchell and Cassidy (1999), when endogenous estrogen levels are low, isoflavones can bind to vacant estrogen-binding sites amplifying the estrogenic effects.

These findings have been supported in avian studies highlighting bone integrity in end-of-lay hens supplemented with soy isoflavones (Ni et al., 2007; Sahin et al.,2007). Ni and associates conducted a feeding study in 1000 post-peak laying hens for 445 days,

representing 60-70% of the egg laying rate, in which half of the hens were supplemented with 10 mg of daidzein (Ni et al., 2007). At the end of the 9-week feeding period, hens supplemented with daidzein had tibial BMD significantly higher than the tibias recovered from their control counterparts ((P<0.05). Similar results were found when researchers supplemented end-of-lay Japanese quail with isoflavones (Sahin et al., 2007). Half the quail were subjected to heat stress to induce impairment of nutrient utilization available for bone and eggshell formation. Within these temperature-controlled and heat-stressed treatment groups, the quail were further split into diet treatments and either given a basal control diet, or the basal diet supplemented with either 400 mg or 800 mg of isoflavones, primarily daidzein. At the end of the 80 day study, soy isoflavone supplementation had significantly increased BMD in both heat-stressed and temperature-controlled laying hens (P<0.05). Temperature-controlled laying hens had mean BMD 0.58, 0.65 and 0.70 g/cm^2 in the basal diet, 400 mg and 800 mg supplementation respectively. Similar isoflavone effects were observed in the heat-stressed hens with mean BMD of 0.40, 0.49 and 0.54 g/cm^2 in the basal diet, 400 mg and 800 mg supplementation respectively. Furthermore, serum Vitamin D, Ca, and P were all significantly (P<0.05) higher in supplemented quail (Sahin et al., 2007).

Antagonistic Effects

The data collected from the aforementioned studies are similar to several animal and clinical studies that have solidified the agonist effects of isoflavone supplementation and the resultant beneficial effects on bone metabolism (Lydeking and Olsen, 2004; Uegesi, 2002; Wu et al, 2003). However, little data exists on the role of isoflavones and their metabolites on bone homeostasis when endogenous estrogen levels are moderate to

high. In laying hens, estrogen acts as the initiating factor in structural bone loss and eventual osteoporosis development (Whitehead, 2004). Due to layers' prolonged production period, eggshell formation remains fairly constant with prolonged elevated levels of estrogen preventing regeneration of structural bone (Beck and Hansen, 2004). It has been suggested that equol may actually function to slow the rate of turnover from structural to medullary bone, ultimately resulting in beneficial effects on bone health but with a possible negative impact on egg production or egg shell quality (Chilibeck, 2004).

Though little data exists on the antagonistic effects of equol related to bone metabolism, an *in-vivo* study conducted by Hwang et al., (2006) suggested that equol may serve as a competitive inhibitor for estrogen when endogenous estrogen levels are high. These researchers found that equol had the highest binding affinity for ER-β of any of the soy isoflavones and thus, could serve to decrease the cell proliferative effect of estrogen, specifically inhibiting osteoclast formation (Hwang et al., 2006).

Combined Effects of Isoflavone Supplementation and Exercise

While the combined effects of isoflavones, primarily genistein and daidzein, and exercise have not been examined in avian species, other animal studies have found that the combined effects could have positive effects on skeletal integrity and bone metabolism. Data collected by Wu et al. (2003), found that BMD in rats was significantly affected by isoflavone administration (0.4 mg/day) combined with exercise (running) ($P<0.05$). However, the data also indicated that BMD was not affected by either intervention alone (Wu et al, 2003). A subsequent study (Liu et al., 2007), found similar cooperative effects of exercise and isoflavone supplementation on bone loss prevention. Ovariectomized rats treated with isoflavones (50 mg/kg body weight) and

forced to exercise, experienced less bone loss as evidenced by improved serum bone metabolism markers. Alkaline phosphate, a marker of osteogenesis, was significantly increased ($P<0.05$) and tartare-resistant phosphatase (TRAP), a marker of bone resorption, significantly declined ($P<0.05$).

Overall, avian osteoporosis occurs as an aggregation of factors that can affect skeletal health and bone turnover. Battery cages that inhibit inherent behaviors combined with prolonged egg production have led to increased incidences of the disease causing monetary losses for the poultry industry and animal welfare concerns. As previously discussed, various studies have noted the positive effects related to free-range production systems and soy supplementation in end-of-lay hens. However, to date no data exists on high soy supplementation in young laying hens and the effects isoflavones and equol could have on estrogen. Furthermore, the combined effects of isoflavones and exercise have not been examined in young laying hens.

1.4 Egg Quality

As the aforementioned research indicates, isoflavone supplementation, primarily genistein and daidzein, may have beneficial effects on bone health by impacting the role of endogenous estrogen. Though isoflavone supplementation can serve to enhance skeletal integrity, it is also vital to consider the effects that diet manipulation could have on egg quality. The process of egg formation and subsequent egg quality is dependent on several biomechanisms that can be altered by various environmental, genetic and nutritional components (Chukuwuka et al, 2011; Ahmadi and Rahimi, 2011). Thus, the next portion of the literature review will focus on egg quality and examining relevant factors that can alter the quality parameters.

Egg quality is associated with a variety of parameters, both internal and external. According to Jones et al. (2002), 3.3 billion cartons of a dozen eggs were produced in 2000, resulting in a per capita consumption of 258 eggs and millions of dollars of revenue for the poultry industry. Therefore, maintaining egg quality is of utmost importance since it affects the economical welfare of the poultry industry as well as consumer acceptance and safety.

1.4.1 External Quality

External egg quality is related to an egg's physical characteristics, and more importantly, shell quality (Ingram et al, 2008). Physical characteristics are related to shape, quantified by shape index, weight, and specific gravity (Ingram et al., 2008). These properties dictate the distribution of shell material over the egg surface, thus as hens age, egg size may increase while shell quality decreases (Chukuwuka et al, 2011).

The term shell quality is often used synonymously for shell strength and implies the ability of eggshells to withstand externally applied forces without cracking or breaking (De Ketelaere et al, 2002). Poor eggshell quality leading to an increased risk of cracks, can affect both producers as well as consumers (Rodriguez-Navarro et al., 2002). Increased egg breakage linked to poor shell integrity account for 6-10% of all produced eggs (Roland, 1988). According to Hunton (1995), every increase of 1% in cracked eggshells leads to an income reduction of $10,000 by poultry farmers. Additionally, an increase in cracks can compromise the safety of the eggs, leading to contamination of the internal contents by Salmonella or other pathogens (Holt et al, 2011). Furthermore, Ledvinka et al. (2000) concluded that the shell quality was one of the most important parameters for the technology of further egg manipulation.

Shell quality or shell strength, is dictated by both structural and material properties (Bain, 2005). Though the physical characteristics mentioned above can influence structural properties, shell thickness is the most influential structural property as it is indicative of the amount of deposited organic and inorganic material (Bain, 2005). Furthermore, a linear relationship has been seen between shell thickness and shell strength (Cooke, 1979).

Though shell thickness is an important parameter in defining strength, a thicker shell does not necessarily mean a stiffer or stronger shell (Bain, 2005). Changes in shell ultrastructure or crystallization account for about 40% of cited variances in shell strength (Rodriguez Navarro et al, 2002). The eggshell is a complex composite composed of about 95% calcium carbonite in the form of calcite, and about 1-3.5% of organic material, consisting mainly of proteins with disulphide cross links, collagen, proteoglycans, and glycoproteins (Arias et al., 1993). Composed of six layers and about 3 grams of calcium, the eggshell is a sophisticated structure formed as the egg passes through distinct regions of the oviduct (Nys et al., 2004).

As the egg is formed, it is the organic matrix that determines the rate of formation, physical characteristics and orientation of inorganic calcite crystals (Nys et al, 2001). Robinson and King (1970), attributed the incidence of weak eggshells to the presence of abnormal mammillary cores, specific sites on the outer shell surface that attract crystallized calcium salts and initiate eggshell formation. Moreover, a structurally imperfect mammillary layer and subsequent abnormal mineralization has been implicated in poorly constructed and weak eggshells (Nys et al., 2004; Bain, 2004).

Structural analysis by scanning electron microscopy (SEM) further suggests that it is the microstructure of the palisade layer that dictates the overall shell integrity (Meyer et al., 1973). Comprised of columns that grow from the mammillary knobs and eventually fuse together during calcification, the palisade layer is the thickest portion of the shell at about 200 micrograms (Solomon, 2010). Researchers have found that the earlier column fusion occurs, the thicker and stronger the shell will be (Bain, 1992; Solomon, 1999).

1.4.2 Internal Quality

The interior of the egg consists of the albumen and the yolk and each has their own parameters associated with them. The albumen quality is largely dictated by albumen height (AH) and Haugh Units (HU), while yolk quality is determined by vitelline membrane strength (VMS), yolk index and color

Albumen Quality

The albumen, or egg white, is composed of water and protein specifically, ovalbumin, ovotransferrin, ovomucoid, ovomucin and lysozyme (Ahmadi and Rahimi, 2011). Thus, albumen quality is related to the changes that occur within these protein interactions, primarily the lysozyme-ovomucin complex that gives the albumen its viscous properties (Li-Chan and Nakai, 1989). Changes in albumen quality begin as soon as the egg is laid as carbon dioxide escapes through the egg shell and pH becomes more alkaline (Benton, 2000). Therefore, internal quality begins to decline as thick albumen, or the gelatinous area directly surrounding the yolk, loses viscosity and transforms to thin albumen (Aboonajami et al., 2010).

Since albumen quality is dictated by the thick albumen, albumen height (AH) has largely defined the quality of sound eggs by indicating freshness (Silversides and Budgell 2004). Furthermore, AH can be utilized to calculate the Haugh Unit (Haugh, 1937). Developed in 1937 by Raymond Haugh, this measurement is a correlation between egg weight and AH, and has been coined the gold standard of interior egg quality (Jones, 2005). Though researchers have argued this destructive tests produces a skewed measurement (Silversides, 1994) , the USDA has accepted the HU as a valid and reliable method for determining the interior egg quality (Jones, 2009).

Yolk Quality

There are two main components to quantifying yolk quality and those are associated with yolk color as well as vitelline membrane strength (VMS) (Ahmadi and Rahimi, 2011). Additionally, the yolk index, usually associated with an egg's freshness, can be calculated as well.

The vitelline membrane is the membrane which separates the yolk from the albumen, and gives it the round appearance associated with egg quality (Kirunda and McKee, 2000). Furthermore, vitelline membrane strength is also associated with the functionality and safety of eggs. Only slight contamination of the albumen with yolk can alter the protein functionality and reduce the foaming properties of egg albumen important in baking products (St John and Flor, 1931). Apart from its contribution to functionality, the vitelline membrane acts as a line of defense against contamination of microbes such as *Salmonella enteriditis* (Gast end Beard, 1992). Though the albumen has antimicrobial properties, the yolk is nutrient dense and allows bacteria to multiply rapidly once they infiltrate the vitelline membrane (Gast et al, 2002). Therefore, the

vitelline membrane can act as a mechanical barrier to block entry of microbes into the yolk (Gast et al, 1992).

Similar to albumen quality, VMS begins to decline as soon as the egg is laid (Ahmadi and Rahmidi, 2011). According to Kido (1976), membrane weakening occurs due to the degradation of one of its major structural glycoproteins (glycoprotein II). This process allows water to be displaced from the albumen to yolk by the process of osmosis. As water enters over time across the vitelline membrane, VMS will weaken and the egg yolk will become enlarged and flattened (Aboonajami et al, 2010). Therefore, yolk width and height are inversely correlated and can be utilized to calculate the yolk index (Aboonjami et al., 2010).

Finally, egg yolk color is a quality indicator that can be altered by manipulating a hen's diet. The color of the yolk is dependent on the amount of xanthophylls, fat soluble pigments, in a hen's diet (Hasin et al, 2006). Typically hens fed diets rich in yellow corn and greens produce dark-yellow to orange colored yolk (Hasin et al., 2006.) Conversely, hens fed diets with white corn, sorgum, millet or wheat will have a lighter yellow yolk (Chukuwuka etal., 2011). Additionally, if the hen has access to green grasses, silage or cottonseed meal, the yolks may acquire a reddish or olive color (USDA, 2011). Feed can also be supplemented to stimulate higher xanthophyll deposition by the addition of marigold petals and other natural or synthetic xanthophylls (Chukwua et al, 2011).

1.5 Factors Influencing Egg Quality

Avian egg formation is a delicate process consisting of various interdependent biomechanisms; therefore, several factors can influence egg quality including stress, genetics, aging, nutrition, disease and contaminants (Ahmadi and Rahimi, 2011).

Although various factors can induce change in quality, only factors relevant to this study will be highlighted including aging, housing and isoflavone supplementation.

1.5.1 Aging Effects

Bird age is the most important factor affecting the egg quality of freshly laid eggs (Jin et al., 2011). Aging causes an increase in egg weight and size in order to facilitate embryonic development (Finkler, et al., 1998). The eggs increase in size as the yolk: albumen ratio is altered and effectively impacts internal quality characteristics (Yasmeen et al., 2008). These variations are also dependent on genetic strain (Scott and Silversides, 2000).

Several studies conducted by Silversides et al., (2001; 2006a; 2006b) found that hen age significantly impacts egg quality. Data collection indicated that internal quality as measured by AH and albumen percentage declined ($P<0.01$). Similar results were noted in a study conducted by Tumova and Ledvinka (2009). The researchers found that egg weight increased from 54.9 grams to 65.0 grams over the course of 40 weeks. A similar trend was noted in yolk weight that increased from 13.4 to 17.1 grams; however, albumen weight decreased significantly ($P<0.001$).

According to Safaa et al. (2008), most egg losses are related to poor shell quality produced at the end of the production cycle. Due to their finite capacity to deposit Ca, as egg size increases, shell quality decreases (Safaa et al., 2008). Data collected by Van Den Brand (2004) showed that as hens aged, shell thickness increased from .354 mm to .372 mm, and strength decreased from 4793 g/cm3 to 4539 g/cm3. He also noted that an increase in egg weight and size directly correlated with aging and inversely correlated with eggshell percentage and layer age ($P<0.001$). Furthermore, data collected by De

Ketaelere et al., (2002) and Suk and Park (2001) confirm that shell thickness decreases as layers age.

1.5.2 Housing Effects

Changes in egg quality can further be associated with production systems (Silversides and Scott, 2001). With welfare concerns of battery cage production systems, alternative housing systems and their effects on egg quality has become the target of several studies (Van Der Brand et al., 2004; Pištěková et al., 2006; Wang et al., 2009; Sekeroglu et al., 2010). Though avaries and free-range systems permit laying hens to perform natural behaviors, the consequences of these systems on egg quality are still unclear (Holt et al, 2011).

According to Van Den Brand et al. (2004), hens raised in an outdoor system produced eggs with inconsistent quality characteristics, exhibiting greater fluctuation than battery cages. Of the characteristics measured, shell index was significantly affected by housing systems with FR hens producing smaller eggs ($P<0.001$). Yolk color was also darker from the hens with outdoor access ($P<0.001$). Furthermore, they found that shell thickness decreased as hens aged in the BC system but remained consistent in eggs from FR systems suggesting that activities like walking can be effective in mineral metabolism (Van Der Brand et al, 2004). Similar results were noted in data collected by Mungai et al., (2009) and Küçükyilmaz et al., (2012) with little effects on egg weight or albumen quality.

Pištěková et al. (2006), also found that egg quality was altered in hens raised in a deep litter floor system when compared to those from BC. However, unlike the studies above, egg weight was statistically higher in the deep litter system ($P<0.01$) and also

corresponded with statistically higher yolk weight ($P<0.01$). Similar to the aforementioned studies, yolk color was darker in eggs gathered from the deep litter system. Additionally, shell strength was not affected by either housing system. Data collected by Sekeroglu et al., (2010) further indicates that housing does affect egg size, with a notable absence of differences in albumen and yolk quality. Hens reared in deep litter and FR systems produced smaller eggs than those gathered from BC ($P<0.01$). Differences were not noted in egg weight, AH, HU, yolk index or yolk color.

A market study conducted by Hidalgo et al., (2008), found the greatest variability between cage and free-range egg quality. The researchers concluded that weight, size and shell thickness were all significantly greater in eggs from FR chickens ($P<0.001$). However, eggs collected from BC hens exhibited greater breaking strength, compressional energy and shell index ($P<0.001$).

Additional studies have concluded that production system does not influence egg quality. Wang et al., (2009), noted that caged and FR systems did not influence egg weight, eggshell thickness, shell index AH, HU, yolk index or yolk color. Moreover, a series of studies (Abrahammson and Tausson 1995: 1998), also noted an absence of differences in both shell and internal quality.

1.5.3 Isoflavone Effects

Since eggshells contain about 3 grams of Ca (Ahmadi and Rahmini, 2011), and various other minerals, the provision of adequate nutrients specifically Ca, P, and Mg is essential for good eggshell quality (Cusack et al, 2003). Although the effects of isoflavones have not been researched for their direct effects on egg quality, they have been utilized to enhance bone metabolism in laying hens. As previously discussed, there

is a vital symbiotic relationship between bone metabolism and eggshell formation (Whitehead, 2004); therefore, supplements that could trigger changes in bone metabolism could subsequently impact eggshell synthesis. By affecting the biomechanisms that control Ca homeostasis, data indicates that isoflavone supplementation has effects on eggshell formation and quality (Zhao et al., 2004; 2005, Ni et al., 2007; Sahin et al., 2007).

Sahin et al., 2007, examined the effects of isoflavone supplementation in end-of-lay Japanese quail populations. At the end of the 80 day experiment, the researchers found that the supplemented groups had a higher BMD ($P<0.05$), as well as increased shell quality as measured by egg production ($P<0.05$), egg weight ($P<0.01$) eggshell thickness ($P<0.01$) and eggshell weight ($P<0.01$). Ni et al., 2007, found similar results after supplementing 445-day old laying hens with daidzein supplements for a 9 week experimental period. Daidzein supplementation increased the egg laying rate ($P<0.05$), increased shell thickness ($P<0.05$) and decreased the percentage of cracked eggs ($P<0.05$) without affecting the average egg weight or egg size. There was also a simultaneous increase in serum Ca levels BMD ($P<0.05$).

A series of studies conducted by Zhao et al. (2004; 2005), also noted positive effects of daidzein supplementation on end-of-lay egg production. After 415 day old Shaoxing ducks were treated with daidzein supplements for 9 weeks, egg production and egg mass increased by 7.70% and 6.44%, respectively (Zhao et al., 2004). A subsequent study was conducted by the same researchers to further how daidzein supplementation would affect the ducks in different stages of the egg production cycle (Zhao et al., 2005). Similar to the first study, the supplementation in post-peak ducks (415 days old) caused

an increase in egg-laying rate ($P<0.05$) and egg mass ($P<0.05$). However, in young ducks (100 days old) daidzein supplementation decreased egg production and quality when compared to the control group. Adverse reactions were seen as evidenced by a significantly lower egg laying rate ($P<0.01$) and decreased egg mass from 22.4g in the control group to 16.61 g in the supplemented group (Zhao et al, 2005).

Though limited data exists on the role that isoflavones play in avian bone metabolism and subsequent eggshell formation, data suggests that when estrogen levels are low they can behave as agonists (Zhao et al, 2004; 2005; Sahin et al., 2007, Ni et al, 2007). By amplifying the estrogen effects in end-of-lay hens, positive results have been noted in both bone and egg quality. However, data also suggests that when endogenous estrogen levels are already high, isoflavones can behave as competitive inhibitors, creating a different cascade of events in bone metabolism and subsequently affecting eggshell formation (Zhao et al., 2005).

The purpose of the following research was to determine the effects that high isoflavone supplementation would have on young or peak-production laying hens. The data collected focused on the effects isoflavone supplementation would have on bone characteristics and the role of endogenous estrogen as measured by both mechanical and chemical parameters. Furthermore, egg quality analysis was conducted on both internal and external parameters to examine if dietary isoflavones and subsequent metabolites would affect the biomechanisms controlling egg formation. Additionally, since data suggests that exercise facilitated by FR production systems may also play a role in both bone and egg quality, housing effects were also examined.

FIGURES 1.6

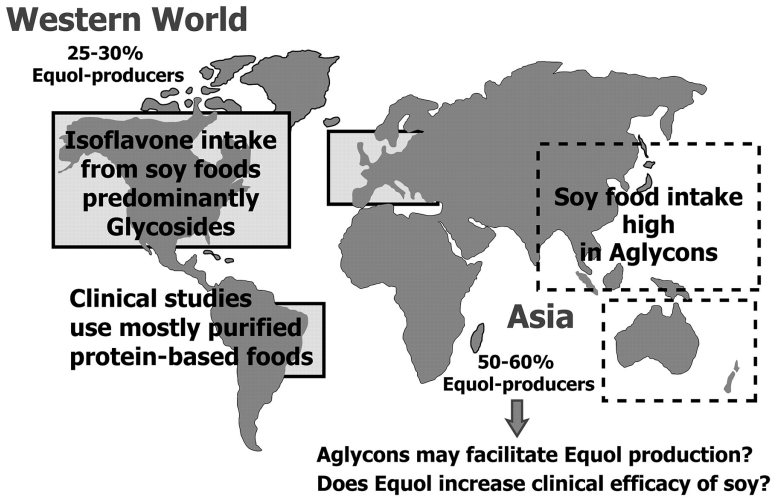

Figure 1. Equol producers in Asian versus Western populations (Setchell and Clerici, 2010).

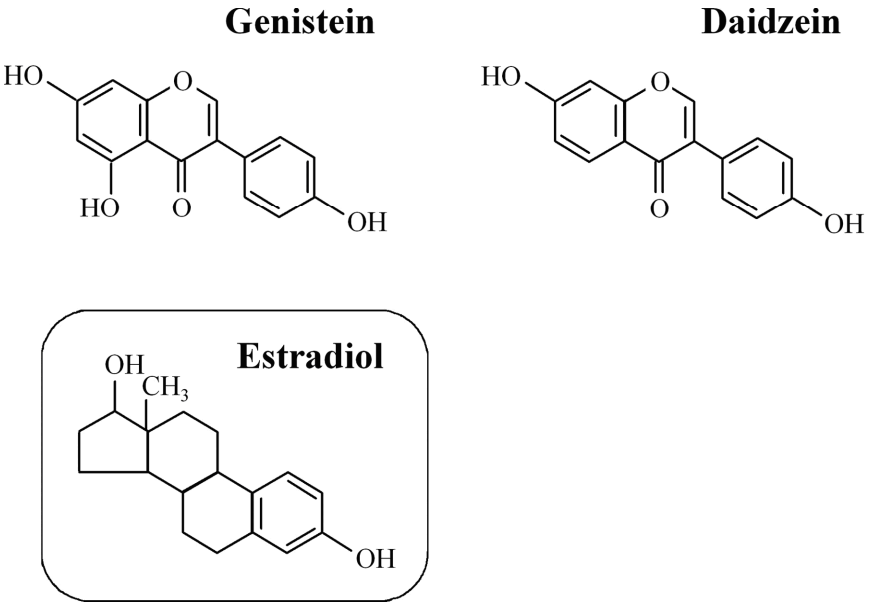

Figure 2. Chemical structure of genistein, daidzein and 17-β estradiol (Wood et al., 2006).

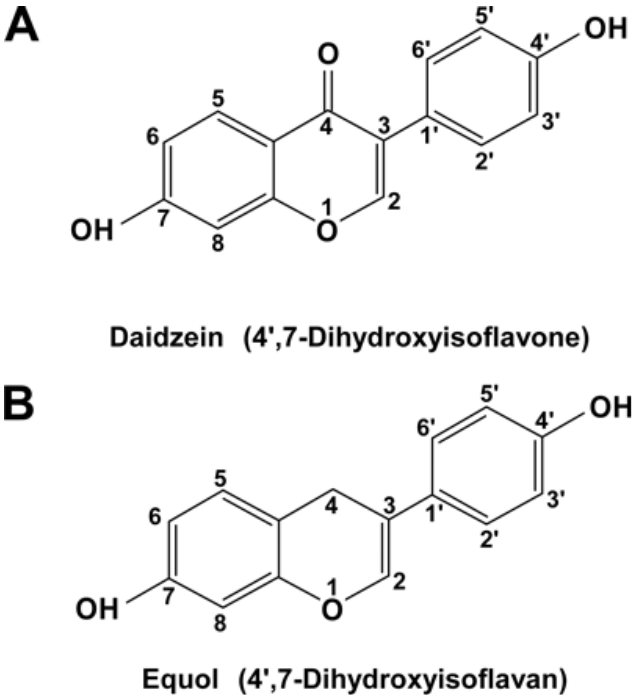

Figure 3. Chemical structures of of equol and its precursor daidzein (Kang et al., 2007).

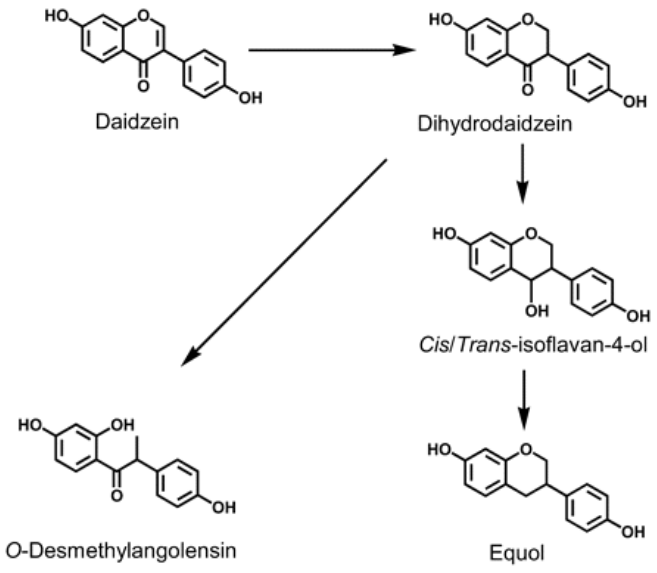

Figure 4. Metabolic conversion of daidzein to O-DMA or equol (Lampe, 2009).

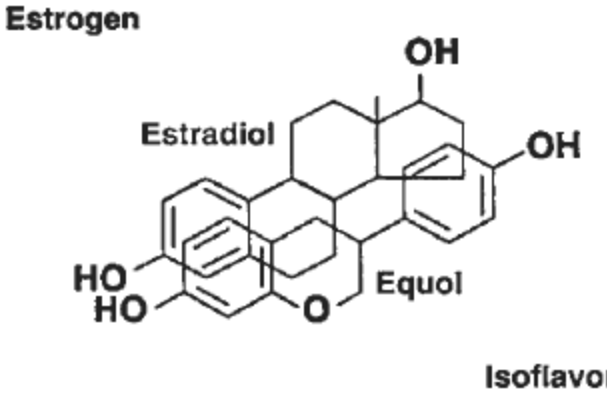

Figure 5. Comparison of equol and 17 β-estradiol (Setchell and Cassidy, 1999)

1.7 Works Cited

1. Abrahamsson, P., and R. Tauson. 1995. Aviary systems and conventional cages for laying hens. Acta. Acrig. Scand. Anim. Sci. 45: 191-203.

2. Abrahammson, P. and R. Tauson. 1998. Performance and egg quality of laying hens in an aviary system. J. Appl. Poult. Res. 7: 225-232.

3. Aboonajami M., A. Akram, T.Nishizu, N. Kondo, S.K. Setarehdan, and A. Rajabipour. 2010. An ultrasound based technique for the determination of poultry egg quality. 56: 26-32.

4. Adlercreutz, H., P.I. Musey, T. Fostis, C. Bannwart, K. Wahala, T. Makela, G. Brunow, and T. Hase. 1986. Identification of lignans and phytoestrogens in urine of chimpanzees. Clin. Chim. Acta. 158: 147-154.

5. Ahmadi, F., and F. Rahimi. 2011. Factors affecting quality and quantity of egg production in laying hens: A review. World Appl. Sci. J. 12: 372-384

6. Arias, J.L., S. Xia, A.H. Heuer, and A.I. Caplan. 1993. Biomineralization and eggshells-cell mediated acellular compartments of extracellular matrix. Int. Rev. Cytol. 145: 217-250.

7. Atkinson C., H.E. Skor, E.D. Fitzgibbons, D. Scholes, C. Chen. K. Wähälä, S.M. Schwartz, and J.W. Lampe. 2003 Urinary equol excretion in relation to 2 hydroxysterone and 16α-hydroxyestrone concentrations: an observational study of young to middle-aged women. J. Steroid Biochem. 86: 71-77.

8. Bain, M.M. 1992. Eggshell strength: a relationship between the mechanism of failure and the ultrastructural organization of the mammilary layer. Brit. Poult. Sci. 33: 303-319.

9. Bain, M.M. 2005. Recent advances in the assessment of eggshell quality and their future application. World Poultry Sci. J. 61: 268-277.

10. Baird, H.T., D.L. Eggett, and S. Fulmer. 2008. Varying ratios of omega-6: omega-3 fatty acids on the pre- and postmortem bone mineral density, bone ash, and bone breaking strength of laying chickens. Poult. Sci. 87: 323-328.

11. Bar, A., E.Vax. S. Striem. 1999. Relationships among age, eggshell thickness and vitamin D metabolism in its expression in the laying hen. Comp. Bioch. Physiol. 123: 147-154.

12. Beck, M.M., and K.K Hansen. 2004. Role of estrogen in avian osteoporosis. Poult. Sci. 83: 200-206.

13. Benton, C.E. and J. Brake. 2000. Effects of atmospheric ammonia on albumen height and pH of fresh boiler eggs. Poult. Sci. 79: 1562-1569.

14. Blank, C. 1997. Demand for organic eggs soars. Egg Industry. 100: 3-7.

15. Bolca S., S. Possemiers, A. Herregat, I. Huybrechts, A. Heyerik, S. De Vriese, M. Verbruggen, H. Depypere, D. De Keukeleire, M. Bracke, S. De Henauw, W. Verstrate, T. Van de Wiele. 2007. Microbial and dietary factors are associated with the equol producers phenotype in healthy postmenopausal women. J. Nutr. 137: 2242-2246.

16. Cassidy A. 2003. Potential risks and benefits of phytoestrogen-rich diets. Int. J Vitam. Nutr. Res. 73: 120-126.

17. Cheng S., S. Sipila, D.R..Taffe, J. Puokala, and H. Suominen. 2002. Change in bone mass distribution induced by hormone replacement therapy and high impact physical exercise in post-menopausal women. Bone. 31: 126-151.

18. Cheng, C., X. Wang, S.M. Wakley, P. Kougias, P.H. Lin, Y. Qizhi, and C. Chen, 2010. Soybean isoflavonoid equol blocks ritonavir-Induced endothelial dysfunction in porcine pulmonary arteries and human pulmonary artery endothelial cells. J. Nutr. 140: 12-17.

19. Chilibeck, P. 2004. Exercise and estrogen or estrogen alternatives (Phytoestrogens, bisphosphates) for preservation of bone mineral in preservation of bone mineral in postmenopausal women. Can. J. Physiol. 29: 59-75.

20. Choi, M. 2005. Effects of exercise on bone mineral density and bone mineral content in postmenopausal women. J. Comm. Nutr. 7: 93-99.

21. Chukwuka, O.K., I.C. Okoli, N.J. Okeudu, A.B.I Udedibie, I.P. Ogbuewu, N.O. Aladi, O.O.M Ihesiulor, and A.A. Omede. 2011. Egg quality defects in poultry management and food safety. Asian Journal of Agricultural Research 5: 1-16.

22. Clark, I. 1969. Importance of dietary Ca: PO4 rations on skeletal, Ca, Mg, and PO4 metabolism. Am. J. Physiol. 217: 865-870.

23. Clark, W.D. W.R. Cox, and F.G. Silversides. 2008. Bone fracture incidence in end-of-lay high-producing noncommercial laying hens identified using radiographs. Poult. Sci. 87: 1964-1970.

24. Cooke, A.S. 1973. Shell thinning in avian eggs by environmental pollutants. Environ. Pollut. 4: 85-152.

25. Couch, J.R. 1955. Cage layer fatigue. Feed Age. 5:55-57.

26. Cusack, M., A.C. Fraser and T. Fraser. 2003. Magnesium and phosphorous distribution in the avian eggshell. Comp. Biochem. Phys. B. 134: 63-69.

27. Dacke, C.G., S.Arkle, D.J. Cook, I.M., Wormstone, S. Jones, M. Zaidi, and Z.A Bascal. 1993. Medullary bone and avian calcium regulation. J. Exp. Biolo.184:63-66.

28. De Ketelaere, B., T. Govaerts, P. Coucke, E. Dewil, J. Visscher, E. Decuypere, and J. De Baerdemaeker. 2002. Measuring the eggshell strength of 6 different genetic strains of laying hens: Techniques and comparisons. Brit. Poult. Sci. 43: 238-244.

29. Decroos K., S., Cattoir, S., Boon, N. Verstraete, and W. Verstraete. 2005. Isolation and characterization of an equol-producing mixed microbial culture from a human fecal sample and its activity under gastrointestinal conditions. Arch. Microbiol. 183, 45-55.

30. Decroos K., E. Eekhaut, S. Possemiers, and W. Verstraete. 2006. Administration of equol-producing bacteria alters the equol production status in the simulator of the gastrointestinal microbial ecosystem (SHIME). J. Nutr. 136: 946-952.

31. Fleming R.H., H.A. McCormack, L. McTeir, and C.C. Whitehead. 1998. Medullary bone and humeral breaking strength in laying hens. Res. Vet. Sci. 64: 63-67.

32. Fleming, R.H., H.A. McCormack, L.Mcteir, and C.C. Whitehead. 2006. Relationship between genetic environmental and nutritional factors influencing osteoporosis in laying hens. Brit. Poult. Sci. 47: 742-755.

33. Fleming, R.H. 2008. Nutritional factors affecting poultry bone health. P. Nutr. Soc. 67: 177-183.

34. Friedman, M., and D.L Brandon. 2001. Review: Nutritional and health benefits of soy proteins. J. Agric. Food Chem. 1069-1086.

35. Frost, H.M. 1997. Obesity and bone strength, and mass: a tutorial based on insight from new paradigm. Bone. 21: 211-214.

36. Fuhrman BJ., B.E. Teter, M. Barbara, C. Byrne. A. Cavalleri, B.J. Grant, P.J. Hovarth, D. Morelli, E. Venturelli, and P.C. Muti. Equol status modifies the association of soy intake and mammographic density in a sample of postmenopausal women. Cancer Epidemiol. Biomarkers Prev. 17: 33-42.

37. Gennari, L. D. Merlotti, F. Vallegi, G. Martini, and R. Nuti. 2007. Selective estrogen receptor modulators for postmenopausal osteoporosis. Drugs Aging. 24: 361-379.

38. Gast R.K. and C.W. Beard. 1992. Detection and enumeration of Salmonella enteriditis in fresh and stored eggs laid by experimentally infected hens. J. Food Prot. 55: 152-156.

39. Gast, R.K. and P.S. Holt. 2001. Assessing the frequency and consequences of Salmonella enteriditis deposition on the egg yolk membrane. Poult. Sci. 80: 997-2002.

40. Gregory, N.G., and L.J. Wilkins. 1989. Broken bones in domestic fowl: Handling and processing damage in end-of-lay battery hens. Br. Poult. Sci. 30:555-562.

41. Gregory, N.G. and L.J Wilkins. 1990. Broken bones in chickens: Effects of stunning and processing in broilers. Br. Poult. Sci. 31:53-58.

42. Haugh R.R. The haugh unit for measuring egg quality. US Egg Poult. Mag. 1937. 43: 522-525.

43. Heany, R.P. 1998. Excess dietary protein may not adversely affect bone. J. Nutr. 128: 1054-1057.

44. Hidalgo, A., M. Rossi, F. Clerici, and S. Ratti. 2008. A market study on the quality characteristics of eggs from different housing systems. Food Chem. 106: 1031-1038.

45. Holt, P.S., R.H. Davies, J. Dewulf, R.K. Gast, J.K. Huwe, D.R. Jones, D. Waltman, and K.R. William. 2011. The impact of different housing systems on egg safety and quality. 90: 251-262.

46. Huff, W.E., P.A. Moore, J. M. Balog, G.R. Bayyari, and N.C. Rath. 1996. Evaluation of toxicity of alum (aluminum sulfate) in young boiler chickens. Poult. Sci. 75: 1359-1364.

47. Hunton, P. 1995. Understanding the architecture of the eggshell. World Poult. Sci. J. 51: 140-147.

48. Hwang, C.S., H.S. Kwak, H.J. Limb, S.H. Lee, Y.S. Kanga, T.B. CHoec. H.G. Hurd, and K.O. Hana. 2006. Isoflavone metabolites and their in vitro dual functions: They can act as an estrogenic agonist or antagonist depending on the estrogen concentration. J. Ster. Biochem. 101: 246-353.

49. Ilich , J.Z. and J.E. Kerstetter. 2000. Nutrition in bone health revisited: A story beyond calcium. J. Am. Coll. Nutr. 19: 715-737.

50. Ingram, D.R., L.F. Hatten, and K.D. Homan. 2008. A study on the relationship between eggshell color and eggshell quality in commercial broiler breeders. Int. J. Poult. Sci. 7: 700-703.

51. Ishimi Y. 2010. Dietary equol and bone metabolism in postmenopausal Japanese women and osteoporotic mice. J. Nutr. 140: 1373-1376.

52. Jendral M.J., D.R. Korver, J.S. Church, and J.J.R. Feddes. 2008. Bone mineral density and breaking strength of white leghorns housed in conventional, modified, and commercially available colony battery cages. Poult. Sci. 87:828–837.

53. Jin. Y.H., K.T. Lee, and Y.K. Han. 2011. Effects of storage temperature and time on the quality of eggs from laying hens at peak production. Asian-Aust J. Anim Sci. 24: 279-284.

54. Jones D.R. and M.T. Musgrove. 2005. Effects of extended storage on egg quality factors. Poult. Sci. 84: 1774-1777.

55. Jones. D.R. 2009. Determining Haugh Units. National Egg Quality School Proceedings. Section IV, p 83-84.

56. Kang, N.M., K.W. Lee, E.A. Rogozin, Y.Y. Cho, A.M. Bode, H.Y. Lee, and Z. Dong. 2007. Equol, a Metabolite of the Soybean Isoflavone Daidzein, Inhibits Neoplastic Cell Transformation by Targeting the MEK/ERK/p90RSK/ Activator Protein-1 Pathway. J. Biol. Chem. 282: 32856-32866.

57. Kato, A., K. Ongino, Y. Kuramto, and K. Kobayashi. 1979. Degradation of the o-glycasically linked carbohydrate units of ovomucin during egg white thinning. J. Food Sci. 44:1341-1344.

58. Kido, S. M. Janado, and H Nunoura. 1976. Macromolecular components of the vitelline membrane of hens eggs. Membrane structure and deterioration with age. J. Biochm. 79: 1351-1356.

59. Kuipuer, G.G., J.G. Lemmen, B.Carlsson, J.C. Corton, S.H. Safe, P.T. van der Saag. B. van der Burg, J.A. Gustafsson. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology. 139: 4252-4263.

60. Koelkebeck, K.W., P.C. Harrison, and T. Madindou. 1993. Effect of carbonated drinking water on production performance of laying hens exposed to high environmental temperatures. Poult. Sci. 72: 1800-1803.

61. Knowles, T.G., and D.G. Broom. 1990. Limb bone strength and movement in laying hens from different housing systems. Vet. Rec. 126: 354-356.

62. Knowles, T.G. and L.J. Wilkins, 1998. The problem of broken bones during the handling of laying hens-A review. Poult. Sci. 77: 1798-1802.

63. Küçükyilmaz, K.,B. Mehmet, E.N. Herken, M. Çinar, A. U. Çath, E. Bintaş, and F. Çöven. 2012. Effects of rearing systems on performance, egg characteristics and immune response in two layer hen genotype. Asian-Aust. J. Anim. Sci. 25: 559-568.

64. Lampe J.W. 2008. Is equol the key to the efficacy of soy foods? Am. J. Clin. Nutr. 89: 1664-1667

65. Lanyon, L.E. C.T. Rubin, and G. Baust. 1986. Modulation of bone loss during calcium insufficiency by controlled dynamic loading. Calcif. Tissue Int. 38: 209-216.

66. Lanyon, L.E. 1992. Functional load-bearing as a controlling influence for fracture resistance in the skeleton. Pages 61-66 in: Bone biology and skeletal disorders in poultry. Carfax Publishing CO., Abingdon, UK.

67. Larkin T.A., W.E. Price, and L.B. Astheimer. 2007. Increased probiotic yogurt or resistant starch intake does not affect isoflavone bioavailability. Nutr. 23: 709-718.

68. Ledvinka Z., E. Tumova, E. Arent, J. Holoubek, and L. Klesalova. (2000): Egg shell quality in some white-egged and brown-egged cross combinations of dominant hens. Czech J. Anim. Sci. 45: 285–288.

69. Leyendecker, M., H. Hamann, J.Haturng, J. Kamphues, U. Neumann, C. Suire, and O. Distl. 2005. Keeping laying hens in furnished cages and an aviary housing system enhances their bone stability. Brit. Poult. Sci. 46: 536-544.

70. Liu, K., G. Ma, G. Lu, Y. Zhoue, W. Wang, L. Liu, P. Yan, L. Jiang, Y. Liu. And Z. Liu. 2007. Effects of soybean isoflavone dosage and exercise on the serum markers of bone metabolism in ovariectomized rats. Asia Pac. J. Clin. Nutr. 16: 193-195.

71. Lund TD., C. Blake, A.N. Hamaker, and E.D Lephart. 2011. Equol an isoflavonoid: potential for improved prostate health, in vitro and in vivo evidence. Reprod. Biol. Endocrin. 9: 4-13.

72. Lydeking-Olsen, E. J.E. Jensen, K.D.R. Setchell, and T. Holm Jensen. 2004. Soymilk or progesterone for prevention of bone loss-a 2 year randomized placebo-controlled trial. Euro.J. Nutr. 43: 246-257

73. Magee, P.J. 2011. Influences of food constituents on gut health: Is equol production beneficial to health? Proc. Nutr. Soc. 70: 10-18.

74. Marrian, G.F., and G.A.D. Haslewood. 1932. Equol, a new inactive phenol isolated from the ketohydroxyoestrin fraction of mare's urine. J. Biochem. 26:1227–32.

75. Matkovic, V., K. Kostial, I. Simonovic, R. Buzina, A. Brocarec, and B.E. Nordin. 1979. Bone status and fracture rates in two regions of Yugoslovia. Am. J. Clin. Nutr. 58: 219-227.

76. Matcovich V., Z. Crncevic-Orlic, and J.D. Landoll. 1995. Identifying fracture risk and preventing osteoporosis in children: Diminished bone mass and reduced bone mineral density are key factors. J. Muscoskelet. Med. 24: 380-384

77. Mattila, P., J. Vajala, L. Rossow, E. Venäläinen and T. Tupasela. 2004. Effect of vitamin D2 and D3 enriched diets on egg vitamin D content, production and bird condition during an entire production period. Poult. Sci. 83: 433-440.

78. Messina M. 2000. Soyfoods and soybean phyto-oestrogens (isoflavones) as possible alternatives to hormone replacement therapy (HRT). Eur. J. Cancer. 36: 71-77.

79. Meyer, R., R.C. Baker, and M.L. Scott. 1973. Effects of hen egg-shell and other calcium sources upon egg-shell strength and ultrastructure. Poult Sci. 52: 949-955.

80. Morito, K., T. Hirose, J. Kinjo, T. Hirakawa, M. Okawa. T. Nohara. S. Ogawa, S. Inque, M. Muramatsu, and Y. Masamune. 2001. Interactions of phytoestrogens with estrogen receptors α and β. Bio. Pharm. Bull. 24: 351-356.

81. Morton, M.S., O. Arisaka, N. Miyake, L.D. Morgan, B.A.J. Evans. 2002. Phytoestrogen concentrations in serum form Japanese men and women over forty years of age. J. Nutr. 132: 3168-3171.

82. Mueller W. J., Schraer R. And H. Schraer. 1965. Calcium metabolism and skeletal dynamics of laying pullets. J. Nutr. 84:20-26.

83. Mugnai, C. A. Dal Bosco, and C. Castellini. 2009. Effects of rearing system and season on the performance and egg characteristics of Ancona laying hens. Ital. J. Anim. Sci. 8: 175-188.

84. Nagel, FS, vom Saal FS, and Welshons WV. 1994. The effective free fraction of estradiol and xenoestrogens in human serum measured by whole cell uptake assays; physiology of delivery modifies estrogenic activity. Proc. Soc. Exp. Biol. Med. 217: 300-309.

85. Ni, Y., Q. Zhu, Z. Zhou, R. Grossman, J. Chen, and R. Zhao. 2007. Effect of dietary daidzein on egg production, shell quality, and gene expression of ER-α, GH-R and IGF-IR in shell glands of laying hens. J. Agric. Food Chem. 55: 6997-7001.

86. New, S.A. 2001. Clinical metabolism and nutrition group symposium on nutritional aspects of bone metabolism from molecules to organisms. Proc. Nutr. Soc. 60: 265-274.

87. Newberry, R.S., A.B. Webster, N.J. Lewis and C.Van Arr. 1999. Management of spent hens. J. Appl. Welfare Sci. 2:13-29.

88. Newbrey, J.W., S.T. Truitt, T. Truitt, D.A. Roland, T.J. Frost, and G.G. Untawale. 1992. Bone histomorphometry in 1,25 (OH)2D3 and vitamin D3-treated aged laying hens. Avian Dis. 36: 700-706.

89. Nys, Y., J. Gaturon, J.M. Garcia-Ruiz, and M.T. Hincke. 2004. Avian eggshell mineralization: biochemical and functional characterization of matrix proteins. C.R. Pale. 3549-562.

90. Nys, Y., K. Baker, R. Bouillon, H.Van Baelen, and D.E.M. Lawson. 1992. Regulation of calbindin D 28 K and mRNA in the intestine of the domestic hen. Gen. Comp. Endocrinol. 86: 460-468.

91. Nys, Y., J. Gautron, M.D. McKee, J.M. Garcia-Ruiz, and M.T. Hincke. 2001. Biochemical and functional characterization of egg shell matrix proteins in hen. World Poultry Sci. J. 57: 401-413.

92. Ogbuewu IP., MC Uchegbu, IC Okoli, MU Iloegje. 2010. Overview of the chemistry of soy isoflavones, potential threats and potential therapeutic benefits. EJEAFChe. 9: 682-695.

93. Orban, J.I., D.A. Roland, K. Cummins, and R.T. Lovell. 1993. Influence of large doses of ascorbic acid on performance plasma calcium, bone characteristics and eggshell quality in broilers and leghorn hens. Poult. Sci. 72: 691-700.

94. Patterson, P.H., K.W. Koelkbeck, D.D. Bell, J.B. Carey, K.E. Anderson, and M.J. Darre. 2001. Egg marketing in national supermarkets- Specialty eggs- Part 2. Poult. Sci. 80: 390-395.

95. Pištěková V., M. Hovorka, V. Večerek, E. Straková, and P. Suchý. 2006. The quality comparison of eggs laid by laying hens kept in battery cages and in a deep litter system. Czech. J. Anim. Sci. 51: 318-325.

96. Rath, N.C., G.R. Huff, W.E. Huff, and J.M Balog. 2000. Factors regulating bone maturity and strength in poultry. Poult. Sci. 79: 1024-1032.

97. Riddel, C. 1992. Non-infections skeletal disorders of poultry: an overview. Bone biology and skeletal disorders in poultry. Poultry Science Symposium v.23 Carfax Publishing Company. Abingdon, UK.

98. Riggs C.M.L., L.C. Vaghan,, G.P. Evans, L.E. Lanyon, and A. Boyde. 1993. Mechanical implications of collagen fibre orientation in cortical bone of equine radius. Anat. Embryol. 187: 239-248.

99. Robinson, D.S.and N.R. King. 1970. The structure of the organic mammillary cores in some weak egg shells. Brit. Poult. Sci. 11: 39-44.

100. Rodriguez-Navarro, A., O. Kalin, Y. Nys, and J.M. Garcia-Ruiz. 2002. Influence of the microstructure on the shell strength of eggs laid by hens of different ages. Brit. Poult. Sci. 9: 395-403.

101. Roland, D.R. 1988. Research note: egg shell problems: estimates of incidence and economic impact. Poult. Sci. 676: 1801-1803.

102. Safaa, H.M., M.P. Serrano, D.G. Valencia, M. Frihka, E. Jimenez-Moreno, and G.G. Mateos. 2008. Productive Performance and Egg Quality of Brown Egg-Laying Hens in the Late Phase of Production as Influenced by Level and Source of Calcium in the Diet Poult. Sci. 87: 2043-2051.

103. Sahin N., M. Onderci, T.A. Balci, G. Cikim, K. Sahin, and O. Kucuk. 2007. The effect of soy isoflavones on egg quality and bone mineralization during the lay laying period of quail. 48: 363-369.

104. Saitoh S., T. Sato, H, Haranda, and T. Matsuda. 2004. Biotransformation of soy isoflavone-glycosides in laying hens: intestinal absorption and preferential accumulation into egg yolk of equol, a more estrogenic metabolite of daidzein. Biochimica. et Biophysica Acta. 1674: 122-130.

105. Sakai T., and M. Kogiso. Soy isoflavones and immunity. 2008. J. Med. Invest. 55: 167-173.

106. Schreiweis, M.A., J.I. Orban, M.C. Ledur, and P. Y. Hester. 2003. The use of densitometry to detect differences in bone mineral density and content of live white leghorns fed varying levels of dietary calcium. Poult. Sci. 82: 1292-1301.

107. Scott, T.A., and F.G. Silversides. 2000. The effect of storage and strain of hen on egg quality. Poult. Sci. 79:1725-1729.

108. Setchell K.D.R., and A. Cassidy. 1999. Dietary isoflavones: biological effects and relevance to human health. J. Nutr. 129: 758-767.

109. Setchell KDR., N.B. Brown, and E. Lydeking-Olsen. 2002. The clinical importance of the metabolite equol- a clue to the effectiveness of soy and its isoflavones. J. Nutr. 132: 3577-3584.

110. Setchell, KDR, C. Clerici, ED Lephart, SJ Cole, C. Heenan, D. Castellani, BE Wolfe, LN-Zimmer, NM Brown TD Lund, RJ Handa, and JE Heubi. 2005. S-Equol, a potent ligand for estrogen receptor β, is the exclusive enantiomeric form of soy isoflavone metabolite produced by human intestinal bacterial flora. Am. J. Clin. Nutr. 81: 1072-1079.

111. Setchell KDR., SJ. Cole. 2006. Method of defining equol-producer status and its frequency among vegetarians. J. Nutr. 136: 2188-2193.

112. Setchell KDR., C. Clerici. 2010. Equol: pharmacokinetics and biological actions. J. Nutr. 140L 1363-1368.

113. Setchell KDR., C. Clerici. 2010. Equol: History, chemistry, and formation. J. Nutr. 140: 1355-1362.

114. Shinkaruk S., C. Carreau, G. Flouroit, C. Bennetau-Pelissero, M. Potier. 2010. Comparative effects of R- and S- equol and implication of transactivation functions (AF-1 and AF-2) in estrogen receptor induced transcriptional activity. Nutr. 2: 340-354.

115. Sekeroglu, A., M. Sarica, E. Demir, Z. Ulutas, M. Tilki, M. Saatci, and H. Omed. 2010. Effects of different housing systems on some performance traits and egg qualities of laying hens. J. Anim. Vet. Adv. 9: 1739-1744.

116. Siefert, M.F., and B.A. Watkins. 1997. Role of dietary lipid and antioxidants in bone metabolism. Nutr. Res. 17: 1209-1228.

117. Silversides F.G. 1994. The Haugh unit correction for egg weight is not adequate for comparing eggs from chickens of different lines and ages. J. Appl. Poult. Res. 3: 120-126.

118. Silversides, F.G. and T.A. Scott. 2001. Effect of storage and layer age on quality of eggs from two lines of hens. Poult. Sci. 80: 1240-1245.

119. Silversides F.G., and K. Budgell. 2004. The relationship among measurements of egg albumen, pH, and whipping volume. Poult .Sci. 83: 1619-1623.

120. Silversides, F.G., T.A. Scott, D.R. Korver, M. Afsharmanesh, and M. Hruby. 2006. A study on the interaction of xylanase and phytase enzmes in wheat based diets fed to commercial white and brown egg laying hens. Poult. Sci. 85: 297-305.

121. Silversides, F.G., D.R. Korver, and K.L. Budgell. 2006. Effect of strain of layer and age at photostimulation on egg production, egg quality and bone strength. Poult. Sci. 85: 1136-1144.

122. Silversides, F.G., R. Singh, K.M. Singh, K.M Cheng, D.R. Korver. 2012. Comparison of bones of 4 strains of laying hens kept in conventional cages and floor pens. Poult. Sci. 91: 1-7.

123. Soares, J.H. 1984. Calcium metabolism and its control-A review. Poult. Sci. 63: 2075-2083.

124. St. John, J., and I.H. Flor. 1931. A study of whipping and coagulation of eggs of varying quality. Poult. Sci. 10: 71-74.

125. Stadelman, W.J. 1999. The Incredibly Functional Egg. Poult. Sci. 78: 807-811.

126. Suk, Y and C. Park. 2001. Effects of broiler breeder age and length of egg storage on albumen characteristics and hatchability. Poult. Sci. 80: 855- 858

127. Tumova, E., and Z. Ledvinka. 2009. The effect of time of oviposition and age on egg weight, egg components and eggshell quality. Arch. Geflügelk. 73: 110-115.

128. Uehara A., K Ohta, K. Sakai, K, Suzuki, S .Watanabe, and H. Adlercretuz. 2001. Dietary fructopolysaccharides modify intestinal bioavailability of a single does of genistein and dadizein and affect their urinary excretion and kinetics in blood of rats. J. Nutr. 131: 787-795.

129. Uesugi, T., Y. Fukui, and Y. Yamori. 2002. Beneficial effects of soybean isoflavone supplementation on bone metabolism and serum lipids in postmenopausal Japanese women: a four week study. J. Am. Coll. Nutr. 21: 97-102

130. USDA (United States Department of Agriculture). 2011. Shell eggs from farm to table. Washington, D.C. USA.

131. USDA. 2012. Egg Market News Report. Agricultural Marketing Service, USDA, Washington, D.C.

132. Van Den Brand, H.K. Parmentier, and B .Kemp. 2004. Effects of housing system (outdoor vs. cages) and age of laying hens on egg characteristics. Brit. Poult. Sci. 45: 745-752.

133. Vaya J., S. Tamir. 2004. The relation between the chemical structure of flavonoids and their estrogen-like activities Curr. Med. Chem. 11:1333-43

134. Wang, X.L., J.X. Zheng, Z.H. Ning, L.J. Qu, G.Y. Xu, and N. Yang. 2009. Laying performance and egg quality of blue-shelled layers as affected by different housing systems. Poult. Sci. 88: 1485-1492.

135. Watts, D. 1990. The nutritional relationship of manganese. J. Orthomolecular Med. 5:219-222.

136. Weaver CM., and LC. Legette. 2010. Equol, via dietary sources or intestinal production may ameliorate estrogen deficiency-induced bone loss. J. Nutr. 140: 1377-1379.

137. Webster, A.B. 2004. Welfare implications of avian osteoporosis. Poult. Sci. 83:184-192.

138. Welch, J.M. and C.M. Weaver. 2005. Calcium and exercise affect the growing skeleton. Nutr. Rev. 63: 361-372.

139. Whitehead, C.C. Wilson, and S. Wilson. 1992. Characteristics of osteopenia in hens. In bone biology and skeletal disorders in poultry. Ed. C.C. Whitehead. Abingdon, Carfax Publishing Co. pp. 265-280.

140. Whitehead, C.C, and R.H. Fleming. 2000. Osteoporosis in cage layers. Poult. Sci. 79: 1033-1041.

141. Whitehead, C.C. 2004. Overview of bone biology in the egg-laying hen. Poult. Sci. 83: 193-199.

142. Wilkins L.J., J.L. McKinstry, N.C. Avery, T.G. Knowles, S.N. Brown, J. Tarlton, and C.J. Nicol. 2011. Influence of housing system and design on bone strength and keel bone fractures in laying hens. Vet. Rec. 169: 414-420.

143. Wilson, S. and S.R.I Duff. 1991. Effects of vitamin or mineral deficiency on the morphology of medullary bone in laying hens. Res. Vet. Sci. 50: 216-221.

144. Wilson, S., and S.R.I Duff. 1992. Effects of vitamin or mineral deficiency on the morphology trabecular bone of laying genetic group domestic fowl. Res. Vet. Sci. 50: 52-58.

145. Wilson, S., H. James and P.L. Ruszler. 1998. Effects of boron on growing pullets. Biol. Trace Elem. Res. 56: 287 – 294.

146. Wood, C.E., S.E. Appt, T.B. Clarkson, A.A. Franke, C. J. Lees, D.R. Doerge, and J.M. Cline. 2006. Effects of High-Dose Soy Isoflavones and Equol on Reproductive Tissues in Female Cynomolgus Monkeys Biol. Reprod. 75: 477-486.

147. Wu, W.X., J. Owiny, Q. Zhang, X.H. Ma, and P.W. Nathaniels. 1996. Regulation of the estrogen receptor and its messenger ribonucleic acid in the ovariectomized sheep myometrium and the endometrium: The role of estradiol and progesterone. Biol. Reprod. 55: 762-768.

148. Wu, J., X.X. Wang, H., Chiba, M. Higuchi, M. Takasaki, A. Ohta, and Y. Ishimi. 2003. Combined intervention of exercise and genistein prevented androgen deficiency-induced bone loss in mice. J. Appl. Physiol. 94: 335-342.

149. Yasmeen F., S. Mahmood, M. Hassan, N. Akhtar, and M. Yaseen. 2008. Comparative productive performance and egg characteristics of pullets and spent layers. Pakistan. Vet. J. 28: 5-8.

150. Zhao R., Y. Wang, Y. Zhou, Y. Ni, L. Lu, R. Grossman, and J. Chen. 2004. Dietary daidzein influences laying performance of ducks (Anas platyrhynchos) and early post-hatch growth of their hatchlings by modulating gene expression. Comp. Biochem. Physiol. 138: 459-466.

151. Zhao, R.Q., Y.C. Zhou, Y.D. Ni, L.Z. Lu, Z.R. Tao, W.H. Chen and J.Chen. 2005. Effect of daidzein on egg-laying performance in Shaoxing duck breeders during different stages of the egg production cycle. Brit. Poult. Sci. 175-181.

Influence of production system and level of dietary soybean meal on bone composition and bone strength in commercial laying hens

K. J. Izquierdo, M. A. Parisi, J. K. Northcutt[1] and P. L. Dawson

Department of Food, Nutrition and Packaging Sciences, Clemson University, Box 340316, Clemson, SC 29634.

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[1] To whom correspondence should be addressed:jknorth@clemson.edu

SUMMARY

Bone fragility in laying hens leading to fractures and osteoporosis has become an economic issue and welfare concern for the poultry industry. Laying hens are inherently predisposed to bone fragility due to high amounts of skeletal calcium (Ca) necessary for egg formation. However, prolonged laying cycles and utilization of battery cages have increased the incidence of osteoporotic conditions in commercial layers. Alternative production systems that allow exercise have been found to produce hens with stronger and more resilient skeletons when compared to hens grown in battery cages that inhibit behaviors like flapping and flying. Furthermore, common poultry feed ingredients such as soybean meal and its subsequent isoflavones and metabolites have been found to have positive effects on bone health in estrogen deficient animals; however, the exact role these compounds play when estrogen levels are sufficient has not been established. A study was conducted to determine the effects of varying levels of dietary soy on bone growth and strength parameters in free-range (FR) and battery caged (BC) layers. Prior to data collection, 16 wk old pullets were split into two production systems, battery and free-range, with subgroups in each system given feed treatments with high or moderate (traditional) levels of soy. Control groups of free-range and battery caged pullets received feed which did not contain soy. At the end of an 8-week feeding trial, hens were euthanized and femoral bones removed to determine physical (mass and strength) and chemical characteristics (bone mineral content or BMC). Overall, the results revealed that hens from the FR production system receiving high soy supplementation had larger and stronger bones with a higher BMC than those given no soy or standard levels of soy

(P<0.05). In BC hens, soy supplementation did not provide protective effects on any of the bone parameters studied (P<0.05).

DESCRIPTION OF PROBLEM

Bone fragility and osteoporosis have become major problems in the poultry industry leading to economic losses and animal welfare concerns. Osteoporosis in laying hens has been characterized by the decrease in mineralized structural bone over time. The condition eventually leads to bone fragility and an increased likelihood of fractures during either the production period or depopulation [1]. While laying hens are predisposed to osteoporosis as a result of prolonged periods of egg production, battery cage systems have exacerbated the disease, causing incidence of fractures to increase [2]. In advanced stages, osteoporosis can manifest itself in the form of cage-layer fatigue characterized by spontaneous bone fractures, vertebral weakness and in extreme cases even paralysis [3]. Osteoporosis in laying hens may be the result of several nutritional, environmental and genetic factors; however, physical activity and endogenous estrogen levels are two notable factors that influence the incidence of osteoporosis in commercial hens [1][4[5].

It was not until the utilization of battery cages in the mid 20th century that osteoporosis and cage layer fatigue became issues of industrial proportions [6][7]. Cage layer fatigue occurs almost exclusively in hens housed in battery cage [8]. These hens have considerably weaker bones than hens grown in high-level aviaries or free-range production systems where they have more opportunities for flight, flapping and perching [6]. A study conducted by Gregory and Wilkins [2] examined hens over the course of their entire production period and found that 29% of the hens housed in battery cages had

at least one bone fracture during their lifetime. A subsequent survey found that 24% of hens from battery cages had freshly broken bones compared to broken bones in 10% of the hens in an aviary system [9]. Confinement of birds in cages, where they have limited mobility and negligible physical activity, contributes to the manifestation of disuse atrophy and osteoporosis [1].

Another factor that affects the incidence of osteoporosis in laying hens is endogenous estrogen which largely controls the formation of medullary bone [4]. Medullary bone (MB) is a non-structural type of woven bone exclusive to female egg-laying birds that can be resorbed 10-15 times faster than structural bone and provides the necessary calcium (Ca) for egg shell formation [10]. Shortly before hens enter lay, circulating plasma 17-β estradiol increases triggering follicular maturation and medullary bone formation [1]. During the laying period, structural bone formation ceases and is continuously resorbed to supply Ca for medullary bone synthesis causing skeleton fragility and increasing the chance of fractures [6]. Decreased 17- β estradiol circulation signals a decline in egg production and causes medullary bone formation to cease [1]. As estradiol levels decline, formation of structural bone recommences, and is evident by the appearance of a layer of structural bone deposited on top of the existing medullary bone [11]. This homeostatic mechanism is normal in all laying hens enabling them to lay in clutches with the follow-up incubation giving them time to regenerate structural bone [1]. However, commercial laying hens are typically selected for production capacity, and prolonged lay leads to bone fragility and eventual osteoporosis [4].

Whitehead and Fleming [1] reported that nutrition during rearing plays a critical role in reducing but not preventing osteoporosis in laying hens by maximizing deposition

of structural bone prior to sexual maturation. Traditionally, Ca, P and Vitamin D supplements have been examined for their role in maintaining skeletal quality and integrity in chickens [12]; however, recent research has begun to investigate the effects of soybean isoflavones on avian bone formation, development and maintenance [13][14][15][16]. Structurally similar to estrogen, isoflavones and their subsequent metabolites, particularly equol, have been found to exhibit estrogenic behavior by binding to estrogen receptors (ER's) [17]. Based on levels of endogenous estrogen, the effects of isoflavones can be classified as agonistic, amplifying the effects of estrogen, or antagonist, suppressing and down-regulating estrogen [18].

Isoflavones and their agonist role in estrogenic Ca regulation and bone homeostasis has been well documented in various avian studies with most notable effects stemming from daidzein, equol's precursor [15][16]. Both Ni et al., (2007) and Sahin et al., (2007) studies found that isoflavone supplementation in aged avian populations had beneficial effects on bone metabolism, enhancing BMD and levels of serum Ca, P and Vitamin D. Where estrogen deficiency in end-of lay hens could stimulate bone loss, the presence of circulating isoflavones helped regulate estrogenic Ca homeostasis and induce a state of equilibrium by binding to ER's [18].

Furthermore, a series of studies conducted by Zhao et al. [13][14] focused on the effects isoflavone supplementation would have on shell production and quality. By altering bone metabolism, isoflavones could have a concurrent effect on the biomechanisms that control shell synthesis. In both studies egg production and shell quality was increased in end-of lay Shaoxing ducks when they were supplemented with daidzein [13][14]. However, data collected in the latter study found that younger ducks

supplemented with daidzein had lower egg production, weight and mass [14]. These results illustrate that supplementing avian species when circulating estrogen levels are high will result in a different sequence of events, and may suggest its ability to act as an ER competitive inhibitor.

Though isoflavone supplementation has been widely studied in estrogen deficient models, its effects on bone metabolism and subsequent shell formation when estrogen levels are sufficient are yet to be fully elucidated. Furthermore, an extensive review of the existing literature has shown no data on the impact of isoflavone supplementation from dietary soy on laying hens grown in different production systems. Therefore, the purpose of this study was to examine the role housing, isoflavone supplementation and their combined effects have on bone strength parameters and bone deposition in young laying hens. The study focused on two production systems, battery cage (BC) and free-range (FR), to compare the effects constricted movement versus mechanical loading have on bone growth and strength. Within each production system, hens were further subdivided into soy supplementation groups to determine the effect that soy and subsequent isoflavone ingestion would have on bone parameters including mass, strength and mineralization.

MATERIALS AND METHODS

The study described in this manuscript details the effects of soy supplementation and production system on the egg quality of peak production laying hens. This study is a complement to a series of concurrent studies conducted by Parisi [19] where she focused on the effects of hen production system, soy supplementation of feed and subsequent equol deposition in table eggs.

Briefly, 84 two-day old Bovan Brown chicks were purchased and transferred to the Clemson University Morgan Poultry Center in Clemson, South Carolina. Chicks were housed in six indoor floor pens with *ad libitum* access to feed and water. At 16 weeks of age, pullets from three of the pens were moved into a free-range housing system (FR) and further divided into three 5 x 10 feet pens, each with access to a separate 25 x 45 foot outdoor range and separate nest boxes, feeders and waterers. The remaining pullets were further split into three groups and were housed in conventional cages measuring 24 x 24 x 16 inches and maintained in an indoor poultry house.

During the grow-out period (2 days to 16 weeks of age) hens were given starter feed [20] with FR hens receiving plant based diets and BC hens receiving animal sources of fat and protein (Table 1). At 16 weeks, hens were given layer feeds. One FR pen and 1 BC pen were assigned as control pens and were fed soy-free feed (SF) throughout the entire experiment [21-22]. A second pen in each production system was assigned a diet containing the standard 25% soybean meal typical of commercial layer formulations (SS) [23-24]. The third FR and BC pens were assigned to standard soybean meal feed with added soy germ in the amount of 5 grams soy-germ/100 grams feed (SE) to enhance isoflavone content. Soy germ [25] was obtained in 1 bulk shipment and mixed with the 25% SS FR and BC feed at University of Georgia's (UGA) Poultry Research Facility in Athens, Georgia.

The feeding study designed by Parisi [19] consisted of 2 periods, each beginning with a washout week followed by a four week feeding trial (Table 2). Data collection occurred over an 8 week period when hens were 20, 24, 25 and 28 weeks old. Each period began with a washout or control treatment where hens were given SF diets (20 and

25 weeks). At week 21, hens in each production system were assigned to SS, SE, and SF feeds. After the washout period at week 26, hens previously on the SS diet were assigned the SE feed, and hens on the SE diet were given the SS feed. Hens assigned SF were maintained on these diets for the duration of the feeding study to act as a control group. Parisi's feeding design allowed each group of hens to serve as their own controls when evaluating the effects of diet and housing system on performance [19].

Bone Parameters

At 29 weeks of age and after the completion of the 8 week cross-over feeding study, 18 hens (9 from each production system) were euthanized by cervical dislocation. Within the production BC and FR pens, hens were chosen at random to include 3 from each soy treatment (SE, SS, SF).

Both femurs were removed from each carcass, cleaned of adhering tissue and stored at 40° F prior to data collection. Femur length was measured from medial condyle to the greater trochanter using digital calipers and recorded to the nearest 100^{th} decimal place. Similarly, width measurements were taken at the calculated midpoint or 50% of length [26][27][28]. Bone volume was determined by utilizing the water displacement method described by Cheng and Coon [29]. Briefly, this method measures bone volume by placing femurs in a 100 mL graduated cylinder filled with 70 mL with distilled water. Femur volume can be determined from the change in volume after the femur is fully submerged. Wet densities of the femurs were calculated by dividing the mass in g by the volume (mL) of the bone.

Once initial measurements were recorded, bone strength parameters were determined utilizing a TA-XT Plus Texture Analyzer [30] equipped with a 50-kg load

cell and programmed to conduct a 3-point bend test using a 1-mm blade. While loaded at the midpoint of the shaft, fulcrum points were set to 62.33 mm apart, and the femur was subject to shear test to failure at the constant loading rate of 20 mm/s [31].

Bone strength parameters were automatically calculated using a Dell® computer interfaced with the TA-XT machine and included force (N), stress (MPa), strain (%),Young's Modulus (elastic modulus) using the stress-to-strain curve and compressional energy (Table 3). After mechanical testing was conducted and data was recorded, each broken femoral bone was crushed and pulverized in a Waring® blender. The pulverized particles were placed in labeled 7 oz. Nasco® Whirl-pak bags and sent to Clemson Agricultural Service Laboratory for standard mineral analysis to determine phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), zinc (Zn), copper (Cu), manganese (Mn), iron(Fe), sulfur (S) and Ca/P amounts [32]. Mineral analysis was performed using the inductively coupled plasma-mass spectrometry method recommended by the US Environmental Protection Agency with detection limits between 1 and 100 ng/L [[33].

Statistical Analysis

Data was analyzed by the General Linear Model procedure of the SAS/STAT program [34] using feed formulation, production system (caged or free-range) and their interactions as the main effects for the model. All first order interactions were tested for statistical significance ($P < 0.05$) using the residual error mean squares. Means were separated using the least-squares means option and reported along with the standard error [34].

RESULTS

Data analysis showed that femoral mass, strength, and mineral deposition were significantly affected by production systems ($P<0.05$). Furthermore, within the free-range (FR) production system where bone metabolism is enhanced by exercise, soy isoflavone supplementation influenced specific growth and strength parameters (femoral length, weight, breaking force and compressional energy).

Bone Mass

Significant differences in bone measurements were evident when comparing production systems (Table 4). Although femoral width, volume, density and weight were not significantly different, femoral length among FR laying hens was greater than the femoral length of BC hens with a mean of 87.8 mm vs. 85.2 mm, respectively ($P=0.0015$). Furthermore, within the FR production system, high levels of soy had a positive effect on bone parameters. Hens treated with SE diets had a greater femoral weight ($P=0.0018$), length ($P=0.0396$) and density ($P=0.0495$) than SS and SF; however, high soy had minimal impact on these same parameters in BC hens ($P<0.05$). Femurs from FR hens fed a SE diet weighed 12-13% more and were 3-4% longer than femurs from layers fed a SS and SF diets.

Bone Strength

Statistical analysis on strength parameters showed that they were not altered by either production system or diet (Table 5; ($P<0.05$). However, a positive interaction effect was noted in FR laying hens given high soy supplementation ($P<0.05$). Bone strength in FR laying hens given SE diets had a significantly higher breaking strength with a force 26% and 29% greater than femurs from SS and SF diets, respectively

(P=0.016). Compressional energy was also significantly higher in the same group of laying hens with values of 2.81±0.69 MPa, as compared to 1.75± 0.56 and 2.70 ± 0.80 in SS and SF, respectively (P=0.013)

Bone Mineral Content

Bone mineral analysis showed that femurs extracted from FR chickens had greater deposition of several minerals than the BC hens (Table 6.) Minerals included Ca (P=0.014), Mg (P=0.009), Cu(<0.0001),Mn (P=0.002) Ca/P(P<0.0001) in amounts 6.8%, 10.5%, 74.3% and 6.5% greater, respectively than the bone mineral levels from hens in battery cages. Within the FR femoral bones, laying hens supplemented with soy enhanced feed had higher amounts of P (P=0.012), Mg (P <0.001) and Zn (0.0002)

DISCUSSION

Based on the overall data collected, FR laying hens had superior bone characteristics, further solidifying the positive effects exercise and mechanical loading have on skeletal integrity. These findings are consistent with previous data that indicate bone parameters are improved when laying hens are permitted to perform innate behaviors such as flapping, flying, perching and running [3][9]. Similarly, laying hens raised in caged production systems that restrict movement have decreased bone mass, as well as weaker and more brittle bones [35].

Free-Range Production System

Data collected in this study indicated that bone mass in FR laying hens was higher than their BC counterparts. Silversides et al. [5], noted similar results when he compared radial bones from caged and floor pen hens. The hens kept in floor pens allowed to stretch their wings and fly had radial bones 9.76% higher than those raised in BC [5].

Similarly, Jendral et al. [3], found significantly reduced femoral, humeral and tibial cortical bone area in conventional BC laying hens when compared to hens from systems equipped with a next box, perch and dust bath (P=0.0001).

Increased bone mass and accretion is also directly proportional to bone strength [36], and as the collected data in this study indicates, higher bone strength was noted in the larger bones from FR hens. According to Whitehead [11], the degree of bone strength is related to the degree of the biomechanical load experienced by the bone, thus, similar to bone mass, there is a direct correlation between exercise and bone strength [37]. Previous studies have consistently found that tibial and humeral breaking strengths are negatively affected by limited mobility and conversely significantly improved in alternative production systems (P<0.001)[38][39]. Additionally, Fleming et al. [35], found that tibial and humeral breaking strengths were 32-35% higher in hens raised in avaries than those from caged laying hens .

Femoral bones from FR laying hens were not only larger and stronger, mineral analysis indicated that they had greater deposition of inorganic material as well. Maintaining optimum levels of these inorganic nutrients as measured by bone mineral content (BMC) and bone mineral density (BMD), is of utmost importance to maintain skeletal health and support bone metabolism [40]. Since exercise has been linked to enhanced bone metabolism, specifically accretion, it may also benefit the quality by increasing deposition of individual nutrients in the mineral matrix. [41] Jendral et al. (2008), found that BMD in laying hens raised in alternative production systems was significantly higher than those raised in battery cages (P=0.034) [3]. Additionally, a study by Silversides et al. [5] found greater BMC in both the radial and tibial bones of

laying hens raised in floor pens than those raised in caged production systems ((P<0.001) [5].

As illustrated, exercise directly affects bone quantity and quality by beneficially impacting skeletal turnover; however, it may also have indirect effects by enhancing vitamin D3 synthesis. Vitamin D3 or 1,25-dihydroxycholecalciferol, is synthesized through a photochemical reactions a consequence of greater exposure to sunlight and its ultraviolet β photons [42]. Because vitamin D3 facilitates intestinal Ca absorption by synthesis of Ca binding protein, Calbindin, it has been demonstrated to have beneficial effects on bone density and strength [43]. Evidence suggests that maximizing vitamin D status in young chicks can alleviate bone disorders in both laying hens and young pullets [11][43]. Additionally, Fleming [44] found that simulation of daylight stimulated vitamin D3 synthesis and resulted in tibial strength to be >30% higher, additionally, UV-treated birds had higher mineralization than those in the control group (P<0.001).

Soy Supplementation

Within production systems, there was a notable positive effect of soy supplementation within FR laying hens as indicated by bone size, strength and mineralization; however, within the BC system, femoral characteristics were not impacted by soy treatments.

As discussed above, isoflavones may exert an agonist or antagonistic effect based on the amounts of endogenous estrogen present [45]. The majority of past research conducted on bone metabolism and dietary isoflavones has been conducted when endogenous estrogen levels are low as in cases of ovariectomized animals, postmenopausal women or end-of-lay avian species [15][16][46][47][48][49]. The data

collected in these studies indicate that isoflavones can amplify the estrogenic effect by binding to ER's and exerting positive effects on bone cells. Furthermore, daidzein's metabolite, equol, has the highest binding affinity for ER's, specifically, ER-β further enhancing bone metabolism in estrogen depleted species [50]. Moreover, the therapeutic effects of isoflavones on bone turnover have been found to be higher in ovariectomized rats forced to exercise, rather than isoflavone supplementation alone [46][47].

In this particular study, the femoral samples were obtained from young hens in the early laying period with already high endogenous estrogen levels. Therefore, the isoflavone biomechanisms would differ, perhaps behaving like antagonist and competitively binding to ER's slowing the rate at which bone loss occurs by suppressing osteoclasts [50]. Rather than increased resorption of structural bone linked to high circulating estrogen, dietary isoflavones may regulate and further inhibit the rate of medullary and structural bone resorption in FR laying hens. This data may further suggest that isoflavones can act like an estrogen antagonist by suppressing the rates of bone turnover that occur as a result of high circulating estrogen during the early laying period. Whereas, increased endogenous estrogen stimulates turnover from structural to medullary bone [1][11], equol may actually function to slow this rate sustaining levels of structural and MB and ultimately resulting in favorable bone mass, strength and quality. Though limited data exists on the antagonistic isoflavone effects on bone an in vivo study by Hwang et al. [50], found that high levels of equol can competitively bind to ER's serving to decrease the cell proliferative effect of estrogen, specifically inhibiting osteoclast formation.

The results collected indicate that high levels of soy supplementation can ameliorate bone loss in young laying hens raised in FR production systems. Though the exact biomechanisms by which daidzein and equol function need to be further elucidated, the data suggests that isoflavone supplementation exerts protective effects by decreasing the rate at which bone, specifically structural bone, is resorbed. By acting as a competitive inhibitor, isoflavones can mediate the resorption of structural bone stimulated by high levels of circulating estrogen that accompany maturation. Additional studies need to examine how the shift in bone metabolism caused by circulating isoflavones affects shell formation and eggshell quality to determine if it impairs the amount of Ca available for eggshells. Further studies need to be conducted to determine how isoflavone supplementation continues to affect laying hens as they age and the amount of circulating endogenous estrogen declines.

CONCLUSIONS AND APPLICATIONS

1. These results indicate that laying hens raised in a free range production system had larger and heavier bones that those raised in conventional battery cage systems. Bone mineralization was also greater indicating that exercise and mechanical loading positively affect both physical and chemical bone characteristics.
2. Soy supplementation did not affect any of the bone parameters measured in battery cage laying hens. High soy supplementation combined with exercise did increase bone mass, strength and mineralization illustrating the protective effects of isoflavones are maximized when combined with load-bearing exercise.

3. None of the parameters examined were negatively affected by high levels of soy supplementation or conventional soy feed.

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REFERENCES AND NOTES

1. Whitehead, C.C, and R.H. Fleming. 2000. Osteoporosis in cage layers. Poult. Sci. 79: 1033-1041.

2. Gregory, N.G., and L.J. Wilkins. 1989. Broken bones in domestic fowl: Handling and processing damage in end-of-lay battery hens. Br. Poult. Sci. 30:555-562.

3. Jendral, M.J., D.R. Korver, J.S. Church, and J.J.R. Feddes. 2008. Bone mineral density and breaking strength of white leghorns housed in conventional, modified, and commercially available colony battery cages. Poult. Sci. 87:828–837.

4. Beck, M.M., and K.K Hansen. 2004. Role of estrogen in avian osteoporosis. Poult. Sci. 83: 200-206.

5. Silversides, F.G. R., Singh, K.M. Singh, K.M. Cheng and D.R. Korver. 2012. Comparison of bones of 4 strains of laying hens kept in conventional cages and floor pens. Poult. Sci. 91: 1-7.

6. Webster, A.B. 2004. Welfare implications of avian osteoporosis. Poult. Sci. 83:184-192

7. Couch, J.R. 1955. Cage layer fatigue. Feed Age. 5:55-57.

8. Knowles, T.G., and L.J. Wilkins. 1998. The Problem of broken bones during the handling of laying hens-A review. Poult. Sci. 77: 1798-1802.

9. Gregory, N.G., and L.J Wilkines. 1990. Broken bones in chickens: Effects of stunning and processing in broilers. Br. Poult. Sci. 31:53-58.

10. Dacke, C.G., S. Arkle, D.J. Cook, I.M. Wormstone, S. Jones, M. Zaidi, and Z.A Bascal. 1993. Medullary bone and avian calcium regulation. J. Exp. Biolo.184:63-66.

11. Whitehead, C.C. 2004. Overview of bone biology in the egg-laying hen. Poult. Sci. 83: 193-199.

12. Rath, N.C., G.R. Huff, W.E. Huff, and J.M Balog. 2000. Factors regulating bone maturity and strength in poultry. Poult. Sci. 79: 1024-1032.

13. Zhao, R., Y. Wang, Y. Zhou, Y. Ni, L. Lu, R. Grossman, and J. Chen. 2004. Dietary daidzein influences laying performance of ducks (Anas platyrhynchos) and early post-hatch growth of their hatchlings by modulating gene expression. Comp. Biochem. Physiol. 138: 459-466.

14. Zhao, R.Q., Y.C. Zhou, Y.D. Ni, L.Z. Lu, Z.R. Tao, W.H. Chen and J.Chen. 2005. Effect of daidzein on egg-laying performance in Shaoxing duck breeders during different stages of the egg production cycle. Brit. Poult. Sci. 175-181.

15. Ni, Y., Q. Zhu, Z. Zhou, R. Grossman, J. Chen, and R. Zhao. 2007. Effect of dietary daidzein on egg production, shell quality, and gene expression of ER-α, GH-R and IGF-IR in shell glands of laying hens. J. Agric. Food Chem. 55: 6997-7001.

16. Sahin, N., M. Onderci, T.A. Balci, G. Cikim, K. Sahin, and O. Kucuk. 2007. The effect of soy isoflavones on egg quality and bone mineralization during the lay laying period of quail. 48: 363-369.

17. Cassidy, A. 2003. Potential risks and benefits of phytoestrogen-rich diets. Int. J. Vitam. Nutr. Res. 73: 120-126.

18. Setchell, K.D.R., and A. Cassidy. 1999. Dietary isoflavones: biological effects and relevance to human health. J. Nutr. 129: 758-767.

19. Parisi, M. 2012. The microbiological and nutritional composition of an equol-enhanced egg produced in free-range and conventional production systems. PhD Diss. Clemson Univ., Clemson.

20. Starter feed- 16% Crude Protein (CP) and 1,050 Metabolizable energy/lb

21. Battery Cage- Soy Free 18 CP with 1,300 ME/lb mixed at University of Georgia Poultry Department, 110 Cedar Street Athens, GA 30602

22. Free-Range- Standard Soy Feed-18% CP with 1,250 ME/lb Layena Purina Mills, LLC. PO Box 66812, St. Louis, MO 63166

23. Battery Cage Standard Soy Feed- 19% CP 1,250 ME/lb Newberry Feed and Farm Inc, Carolina Choice Feed. 2431 Vincent St, Newberry, SC 29108

24. Free Range-Soy Free- 19% CP with 1,220 ME/lb Organic Soy Free Layer Feed. 801 Second St., Waynesborough, VA 22980

25. Soy Germ- Frutarom Soylife. Frutarom USA, Inc., 9500 Railroad Ave. North Bergen, NJ, 07407

26. Turner K.A., and M.S. Lilburn. 1992. The effect of early protein restriction (zero to eight weeks) on skeletal development in turkey toms from two to eighteen weeks. Poult. Sci. 71: 1680-1686.

27. Applegate, T.J., and M.S. Lillburn. 2002. Growth of femur and tibia of a commercial broiler line. 81: 1289-1294.

28. Reddish, J.M., and M.S. Lillburn. 2004. A comparison of growth and development patterns in diverse genotypes of broilers and 2. pullet growth. Poult. Sci. 83: 1072-1076.

29. Cheng, T.K., and C.N. Coon. 1990. Sensitivity of various bone parameters of laying hens to different daily calcium intakes. Poult. Sci. 69: 713-720.

30. TA.XT *Plus* Texture Analyzer 123 Stone Mill Lane, Marietta, GA 30064

31. Baird, H.T., D.L. Eggett, and S. Fulmer. 2008. Varying ratios of omega-6: omega-3 fatty acids on the pre- and postmortem bone mineral density, bone ash, and bone breaking strength of laying chickens. Poult. Sci. 87: 323-328.

32. Clemson University Agricultural Service Laboratory 171 Old Cherry Road, Clemson, SC 29634

33. U.S. Environmental Protection Agency. 1994. Determination of trace elements in waters and wastes by inductively coupled plasma-mass spectrometry, Method 200.8 U.S. Environmental Protection Agency, Environmental Monitoring Systems Lab, Cincinnati, Ohio.

34. SAS. 1999. SAS/STAT User's Guide. Release 8.0 Edition. SAS Institute Inc., Cary, NC.

35. Fleming, R.H., H.A. McCormack, L.Mcteir, and C.C. Whitehead. 2006. Relationship between genetic environmental and nutritional factors influencing osteoporosis in laying hens. Brit. Poult. Sci. 47: 742-755.

36. Frost, H.M. 1997. Obesity and bone strength, and mass: a tutorial based on insight from new paradigm. Bone. 21: 211-214.

37. Newman, S., and S. Leeson. 1998. Effect of housing birds in cages or an aviary system on bone characteristics. Poult. Sci. 77: 1492-1496.

38. Leyendecker, M., H. Hamann, J.Haturng, J. Kamphues, U. Neumann, C. Suire, and O. Distl. 2005. Keeping laying hens in furnished cages and an aviary housing system enhances their bone stability. Brit. Poult. Sci. 46: 536-544.

39. Wilkins, L.J., J.L. McKinstry, N.C. Avery, T.G. Knowles, S.N. Brown, J. Tarlton, C.J. Nicol. 2011. Veterinary Record. 169: 414-420.

40. Choi, M. 2005. Effects of exercise on bone mineral density and bone mineral content in postmenopausal women. J. Comm. Nutr. 7: 93-99.

41. Chilibeck, P. 2004. Exercise and estrogen or estrogen alternatives (Phytoestrogens, bisphosphates) for preservation of bone mineral in preservation of bone mineral in postmenopausal women. Can. J. Physiol. 29: 59-75.

42. Newbrey, J.W., S.T. Truitt, T. Truitt, D.A. Roland, T.J. Frost, and G.G. Untawale. 1992. Bone histomorphometry in 1,25 (OH)2D3 and vitamin D3-treated aged laying hens. Avian Dis. 36: 700-706.

43. Bar, A., E.Vax, and S. Striem. 1999. Relationships among age, eggshell thickness and vitamin D metabolism in its expression in the laying hen. Comp. Bioch. Physiol. 123: 147-154.

44. Fleming, R.H. 2008. Nutritional factors affecting poultry bone health. P. of Nutr. Soc. 67: 177-183.

45. Atkinson C., H.E. Skor, E.D. Fitzgibbons, D. Scholes, C. Chen. K. Wähälä, S.M. Schwartz, and J.W. Lampe. Urinary equol excretion in relation to 2 hydroxysterone and 16α-hydroxyestrone concentrations: an observational study of young to middle-aged women. J. Steroid Biochem. 2003. 86: 71-77.

46. Wu, J., X.X. Wang, H., Chiba, M. Higuchi, M. Takasaki, A. Ohta, and Y. Ishimi. 2003. Combined intervention of exercise and genistein prevented androgen deficiency-induced bone lose in mice. J. Appl. Physiol. 94: 335-342.

47. Liu, K., G. Ma, G. Lu, Y. Zhoue, W. Wang, L. Liu, P. Yan, L. Jiang, Y. Liu. And Z. Liu. 2007. Effects of soybean isoflavone dosage and exercise on the serum markers of bone metabolism in ovariectomized rats. Asia Pac. J. Clin. Nutr. 16: 193-195.

48. Ishimi Y. 2010. Dietary equol and bone metabolism in postmenopausal Japanese women and osteoporotic mice. J. Nutr. 140: 1373-1376.

49. Weaver CM., LC. Legette. 2010. Equol, via dietary sources or intestinal production may ameliorate estrogen deficiency-induced bone loss. J. Nutr. 140: 1377-1379.

50. Hwang, C.S., H.S. Kwak, H.J. Limb, S.H. Lee, Y.S. Kanga, T.B. Choec. H.G. Hurd, and K.O. Hana. 2006. Isoflavone metabolites and their in vitro dual functions: They can act as an estrogenic agonist or antagonist depending on the estrogen concentration. J. Ster. Biochem. 101: 246-353.

Table 1: Feed Analysis on Dry Matter Basis[1]

	BATTERY CAGE			**FREE-RANGE**		
Based on 100% dry matter	SS[2]	SE	SF	SS	SE	SF
crude protein %	18.8	19.7	18.4	18.6	19	18.1
fat %	2.5	2	7.7	3.3	3.2	3
P %	0.72	0.77	1.49	0.79	0.83	1.09
K %	0.9	0.9	0.54	1.14	1.13	0.71
Ca %	3.3	3.2	6.44	4.25	3.33	4.9
Mg %	0.19	0.2	0.22	0.35	0.34	0.24
S %	0.24	0.23	0.33	0.28	0.27	0.26
Zn ppm	180	138	136	172	134	194
Cu ppm	19	22	16	20	14	12
Mn ppm	110	124	135	161	152	140
Fe ppm	271	267	162	174	159	615
Moisture %	10.7	10.2	10.1	10.3	9.7	11.2
Ca/P	4.61	4.16	4.32	5.37	4.01	4.48
Dry matter %	89.3	89.8	89.9	89.7	90.3	88.8

[1] Feeding study as designed by Michelle Parisi

[2] SE represents standard soy feed content with added soy germ in the amount of 5 g soy-germ/100 grams feed; SS indicates standard soy diets with 25% soy content; SF represents soy-free diet

Table 2. Eight week feeding study [12]

Housing/Diet Group	TRIAL 1		TRIAL 2	
	20 weeks of age	24 weeks of age	25 weeks of age	28 weeks of age
BATTERY CAGE (BC) BC Group-1	BCSF[1] Diet	BCSS[2] Diet	BCSF Diet	BCSE Diet[3]
BC Group-2	BCSF Diet	BCSE Diet	BCSF Diet	BCSS Diet
BC Group-3	BCSF Diet	BCSF Diet	BCSF Diet	BCSF Diet
FREE-RANGE (FR) FR Group-1	FRSF[4] Diet	FRSS[5]	FRSF Diet	FRSE[6]
FR Group-2	FRSF Diet	FRSE Diet	FRSF Diet	FRSS Diet
FR Group-3	FRSF Diet	FRSF Diet	FRSF Diet	FRSF Diet

1) BCSF refers to battery caged soy free layer feed

2) BCSS refers to battery caged standard soy feed

3) BCSE refers to battery caged soy enhanced feed

4) FRSF refers to free-range soy free feed;

5) FRSS refers to free-range standard soy feed

6) FRSE refers to free-range soy enhanced feed

Table 3. Characteristics of bone strength

Property	**Units**	**Definition**
Force	Newtons (N)	Measures the ultimate load or force required to break the bone [12]
Stress	Megapascals (Mpa)	Measures force per unit area of bone, independent of size or shape [37]
Strain	%	Measures the rigidity of the bone or the point at which the bone is no longer resilient [12]
Young Modulus	Mpa/	Measures in the intrinsic rigidity or stiffness. Calculated from stress-to-strain curve [37]
Energy	Megapascals (Mpa)	Measures compressional strength [37]

Table 4: Effect of production system and diet on laying hen femoral mass post-slaughter[1,2]

PRODUCTION SYSTEM	**BONE PARAMETERS**					
	Diet[3]	Weight (g)	Width (mm)	Length (mm)	Volume(cm^3)	Density(g/cm^3)
Battery Cage	SE	10.6 ± 0.79	8.14 ± 0.11	86.6 ± 1.05	8.08 ± 0.58	1.31 ± 0.05
	SS	9.92 ± 1.11	8.07 ± 0.19	84.39 ± 2.98	7.33 ± 0.75	1.35 ± 0.03
	SF	10.2 ± 0.78	8.21 ± 0.35	84.5 ± 1.96	7.67 ± 0.75	1.34 ± 0.10
Free Range	SE	11.6 ± 0.97	8.06 ± 0.64	89.9 ± 2.99	8.17 ± 0.75	1.42 ± 0.04
	SS	10.2 ± 0.22	8.10 ± 0.39	87.1 ± 1.81	7.67 ± 0.52	1.34 ± 0.06
	SF	10.1 ± 0.55	8.23 ± 0.26	86.3 ± 1.96	7.50 ± 0.71	1.35 ± 0.07
SOURCE OF VARIATION	**PROBABILITY VALUES[3]**					
Production System	NS[4]	NS	0.0015[5]	NS	NS	
Diet	0.005	NS	0.0066	NS	NS	
Production System x Diet	NS	NS	NS	NS	0.0495	

[1] n=6 samples/ group
[2]Laying hens 29 weeks of age
[3]SE represents standard soy feed content with added soy germ in the amount of 5 g soy-germ/100 grams feed; SS indicates standard soy diets with 25% soy content; SF represents soy-free diet
[4]Probability value for General Linear Model of SAS
[5]NS=Not significant at P>.05

Table 4: Effect of production system and diet on laying hen femurs post-slaughter[1,2]

PRODUCTION SYSTEM	**STRENGTH PARAMETERS**					
	Diet[3]	Force (N)	Max Stress (MPa)	Max Strain (%)	Energy (MPa)	Young's Modulus (MPa/)
Battery Cage	SE	217.8 ± 30.1	16.92 ± 3.33	0.286 ± 0.07	2.146 ± 0.60	99.35 ± 31.4
	SS	263.4 ± 59.4	24.57 ± 10.7	0.243 ± 0.06	2.967 ± 0.87	157.1 ± 84.3
	SF	229.1 ± 100.6	18.52 ± 6.89	0.28 ± 0.06	2.451 ± 0.96	161.2 ± 65.2
Free Range	SE	304.7 ± 28.0	20.96 ± 5.71	0.314± 0.06	2.805 ± 0.69	176.0 ± 68.6
	SS	217.4 ± 39.8	15.98 ± 8.12	0.408 ± 0.22	1.752 ± 0.56	102.6 ± 102.8
	SF	282.8 ± 34.1	19.86 ± 4.41	0.278 ± 0.07	2.704 ± 0.80	109.7 ± 73.2
SOURCE OF VARIATION	**PROBABILITY VALUES**					
Production System	NS[4]	NS	NS	NS	NS	
Diet	NS	NS	NS	NS	NS	
Production System x Diet	0.016[5]	NS	NS	0.013	NS	

[1] n=6 samples/ group
[2] Laying hens 29 weeks of age
[3] SE represents standard soy feed content with added soy germ in the amount of 5 g soy-germ/100 grams feed; SS indicates standard soy diets with 25% soy content; SF represents soy-free diet
[4] NS=Not significant at P>.05
[5] Probability value for General Linear Model of SAS

Table 5. Effect of production system and diet on femoral bone mineral content (BMC) of laying hens post-slaughter[1,2]

PRODUCTION SYSTEM	CALCULATED NUTRIENT ANALYSIS										
	Diet[3]	P%	K%	Ca%	Mg%	S%	Zn ppm	Cu ppm	Mn ppm	Fe ppm	Ca/P
Battery Cage	SE	7.167 ± 1.12	0.220± 0.02	15.30 ± 2.41	0.227 ± 0.04	0.248 ±0.04	184.7 ± 36.0	1.000 ± 0.00	9.167± 2.79	111.0 ± 31.8	2.135± 0.02
	SS	7.968 ± 0.41	0.233 ± 0.03	16.93± 0.94	0.263 ± 0.02	0.256 ± 0.02	203.3 ± 13.5	1.000 ± 0.00	10.17 ± 0.75	100.3 ± 14.1	2.123 ± 0.01
	SF	7.867 ± 0.55	0.215 ± 0.01	16.42 ± 1.22	0.272 ± 0.20	0.238 ± 0.17	241.2 ± 23.1	1.000 ± 0.00	8.833 ± 1.47	88.33± 13.7	2.087 ± 0.04
Free-Range	SE	8.123 ± 0.34	0.198 ± 0.02	18.04 ± 0.87	0.313± 0.19	0.235 ± 0.01	243.8 ± 43.64	2.667 ± 1.86	9.000 ± 1.67	98.00 ± 24.02	2.221 ± 0.06
	SS	7.557 ± 0.47	0.248 ±0.02	17.16 ± 1.01	0.283 ±0.02	0.261± 0.03	193.7 ± 12.0	4.667± 2.25	7.000 ± 0.00	115.2 ± 7.68	2.272 ± 0.03
	SF	7.401 ± 0.45	0.205 ± 0.026	16.98 ± 1.04	0.256 ± 0.02	0.240 ± 0.02	193.5± 20.1	4.333 ± 2.58	7.000 ± 0.89	92.50± 24.7	2.295 ± 0.02
SOURCE OF VARIATION		**PROBABILITY VALUES**									
Production		NS[4]	NS	0.014[5]	0.009	NS	NS	<.0001	0.002	NS	<.0001
Diet		NS	0.0023	NS	NS	NS	NS	NS	NS	NS	NS
Production x Diet		0.012	NS	NS	<.0001	NS	0.0002	NS	NS	NS	0.001

[1] n=6 samples/ group
[2] Laying hens 29 weeks of age
[3] SE represents standard soy feed content with added soy germ in the amount of 5 g soy-germ/100 grams feed; SS indicates standard soy diets with 25% soy content; SF represents soy-free diet
[4] NS=Not significant at P>.05
[5] Probability value for General Linear Model of SAS

Influence of laying hen production system and level of dietary soybean meal on internal and external egg quality characteristics

K. J. Izquierdo, M. A. Parisi, P. L. Dawson and J. K. Northcutt[11]

Department of Food, Nutrition and Packaging Sciences, Clemson University, Box 340316, Clemson, SC 29634.

Key words: hen production system, soybean meal, egg quality, egg shell

Running head: Diets effects on egg quality

Primary audience: egg producers, researchers, poultry nutritionists, feed mill personnel, ranchers, consumers

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[1 1] To whom correspondence should be addressed:jknorth@clemson.edu

SUMMARY

The effects of production system and dietary soy meal were evaluated in peak production laying hens. The purpose of the present study was to determine how free-range housing systems and soy isoflavones affected both internal and external quality characteristics associated with table eggs. Eighty-four, 2-day old laying hens were obtained and transferred to the Morgan Poultry Farm in Clemson, South Carolina. Once the hens were 16 weeks, they were divided into two housing systems groups: battery-cage (BC) or free-range (FR). Within these systems, the hens were further subdivided into 3 groups and given either a soy-free (SF) diet, standard soy (SS) or soy-enhanced diet (SE). The feeding study began when hens were 20 weeks of age and was comprised of two periods with each period having a washout week (hens fed soy-free diets) followed by a 4-week feeding treatment. Egg quality analyses were conducted after the washout weeks and at the end of the 2 treatment periods.

At the end of the experiment, data analysis showed that eggs from BC were significantly larger ($P=0.0002$), with weaker ($P=0.024$) and thinner eggshells ($P=0.0079$) than those from FR chickens. Egg yolks from FR eggs were more red ($P<0.0001$) and yellow ($P=0.0087$) than the yolks collected from BC laying hens. Soy supplementation also had significant effects on both internal and external qualities. Data collected during both periods, showed that SS and SE treated layers produced heavier ($P<0.0001$ Period 1 and $P=0.037$ Period 2) and larger eggs ($P=0.0002$ Period 1 and $P=0.0049$ Period 2) than the control group. Eggshell thickness was also statistically higher in SE and SS treated layers than in the control group ($P=0.0435$). Yolk quality was adversely affected by SS

and SE treatments and was evident by an increased VMS (P=0.034) and redder yolks (P<0.0001) from eggs in the control group.

DESCRIPTION OF PROBLEM

Functional foods may be defined as foods or food ingredients that may enhance health through their provision of a physiological benefit beyond its traditional nutrients [1]. According to the American Dietetic Association, these foods include 'whole foods and fortified, enriched or enhanced foods, and have a potentially beneficial effect on health when consumed as a part of a varied diet' [2]. Though the term functional food encompasses a large spectrum of foods, recent attention has turned to the egg and its role in nutrition and addressing nutritional deficiencies [3][4] [5].

Eggs may be classified as functional foods since, besides their high macro and micronutrient profiles, they are also sources of compounds like lecithin, carotenoids and choline [6]. Apart from the egg's inherent nutrient profile, modifications to a laying hens diet or altering its breeding system has resulted in specialty or designer eggs [1]. First appearing in the 1950's, designer eggs were marketed by Drew Chemical Company after they developed a feeding program to create an egg with approximately equal parts saturated, monounsaturated and polyunsaturated fatty acids [1]. Since then specialty eggs represent 3-5% of retail carton eggs in the United States and include nutritionally altered eggs, organic eggs, and eggs from hens raised in alternative housing systems [7]. According to Blank [8], specialty egg producers are expanding production and these eggs, specifically organic eggs are being sold at double or triple the price of standard conventional eggs. Additionally in 2012, the USDA found that organic eggs retailed for

$2.75 - $3.10 per dozen compared to conventional eggs with a national average of $1.39[9].

While eggs are naturally nutrient dense, world-wide laying hens diets' are being manipulated to produce nutritionally altered eggs to address micro-nutrient deficiencies. In 2008, Charoensiriwatana et al. [5], found that enriching hen feed with high levels of iodine served as an alternative method of supplying iodine to severely deficient areas of Thailand. Similarly, selenium deficiency was found to be a global problem, thus selenium enriched eggs were formulated and are currently being sold in countries including Ireland, Malaysia, Thailand, Australia, Ukraine, Turkey and the UK [4]. Additionally in the USA, specialty feeding practices have been formulated utilizing both fish and flaxseed oils to produce n-3 enhanced eggs [3][10]. Furthermore, research has also spotlighted soy isoflavones and their ability to be transferred to egg yolks as a potential new product [11][12].

Soy isoflavones have been the subject of both animal and human studies over the past few years, generating mixed results and conclusions regarding the efficacy and safety of soy supplementation [13][14][15][16]. However, more recent research has emphasized the positive impact of the isoflavone equol, a secondary metabolite of daidzein, as being the key to the effectiveness of dietary soy supplementation [17][18]. While *in-vivo* equol formation has been linked to decreased incidences of vasomotor symptoms[19], osteoporosis [15], and cancers [20], only about 25-30% of the adult population has the intestinal bacteria necessary to metabolize its precursor, daidzein, into equol [18][21]. Linked to their high soy diet, about 60% of Asian populations produce equol [22]; however, the majority of Western populations produce O-

desmethyloanglionsen instead, a metabolite with significantly lower estrogenic-effects [17].

While individual equol formation varies greatly, data collected from animal studies indicate that they inherently possess the colonic bacteria to produce equol when challenged with soy feed or other isoflavone sources. Equol was first isolated in equine urine [23], and has since been identified at substantial levels in sheep [13], chimpanzees [24], rats [25], and laying hens after soy consumption [11]. Additionally, a few avian studies have linked daidzein supplementation to increased bone strength and mineral density, further suggesting the protective effects of equol formation [26] [27]. Moreover, Saitoh et al. [11], indicated that laying hens produced equol when challenged with soy isoflavone-enriched feed within just 24 hours of supplementation. Furthermore, within 3 days equol levels were further detected in egg yolks from soy supplemented laying hens . These same authors concluded that soy supplementation in laying hens could be used to create a functional equol-enhanced egg that could be marketed to consumers for its health benefits [11].

Clinical and epidemiological studies show that human health benefits of soy are associated with equol formation [17][19][20][21]; however, the majority of individuals do not naturally harbor the enzymes necessary to produce equol when challenged with soy [17]. Therefore, the creation of an equol-enhanced egg would allow broad spectrum access to a direct source of equol, regardless of inter-individual variation in colonic bacteria.

The impact of equol-deposition via soy supplementation on the quality characteristics of eggs has not been well established and these characteristics are critical

for the production of a marketable specialty egg. According to Ahmadi and Rahimi [28], altering a hen's diet and environment may lead to changes in the eggshell, albumen or yolk. Therefore, several studies were conducted to determine the effect of soy supplementation and production system on egg quality. Prior to data collection, laying hens were split into two production systems, battery cage (BC) and free-range (FR), and subsequently given varied levels of soy over the course of an 8-week period [12]. During this period, several parameters were concurrently measured to ensure that egg quality was not negatively affected. Since soy isoflavones have been documented to have an affect on shell quality by altering calcium homeostatic mechanisms, external quality parameters such as shell strength, thickness and mineral deposition were measured. Other parameters included egg weight, shape index, Haugh units (HU), albumen height (AH), yolk index, vitelline membrane strength (VMS) and yolk color.

MATERIALS AND METHODS

The study described in this manuscript details the effects of soy supplementation and production system on the egg quality of peak production laying hens. This study is a complement to a series of concurrent studies conducted by Parisi [12] where she focused on the effects of hen production system, soy supplementation of feed and subsequent equol deposition in table eggs.

Briefly, 84 two-day old Bovan Brown chicks were purchased and transferred to the Clemson University Morgan Poultry Center in Clemson, South Carolina. Chicks were housed in six indoor floor pens with *ad libitum* access to feed and water. At 16 weeks of age, pullets from three of the pens were moved into a free-range housing system

(FR) and further divided into three 5 x 10 feet pens, each with access to a separate 25 x 45 foot outdoor range and separate nest boxes, feeders and waterers. The remaining pullets further split into three groups and were housed in conventional cages measuring 24 x 24 x 16 inches and maintained in an indoor poultry house.

During the grow-out period (2 days to 16 weeks of age) hens were given starter feed [29] with FR hens receiving plant based diets and BC hens receiving animal sources of fat and protein (Table 1). At 16 weeks, hens were given layer feeds. One FR pen and 1 BC pen were assigned as control pens and were fed soy-free feed (SF) throughout the entire experiment [30-31]. A second pen in each production system was assigned a diet containing the standard 25% soybean meal typical of commercial layer formulations (SS) [32-33]. The third FR and BC pens were assigned to standard soybean meal feed with added soy germ in the amount of 5 grams soy-germ/100 grams feed (SE) to enhance isoflavone content. Soy germ [34] was obtained in 1 bulk shipment and mixed with the 25% SS FR and BC feed at University of Georgia's (UGA) Poultry Research Facility in Athens, Georgia.

The feeding study designed by Parisi [12] consisted of 2 periods, beginning with a washout week followed by a four week feeding trial (Table 2). Data collection occurred over an 8 week period when hens were 20, 24, 25 and 28 weeks old. Each period began with a washout or control treatment where hens were given SF diets (20 and 25 weeks). At week 21, hens in each production system were assigned to SS, SE, and SF feeds. At week 26 after the second washout period, hens previously on the SS diet were assigned the SE feed, and hens on the SE diet were given the SS feed. Hens assigned SF were maintained on these diets for the duration of the feeding study to act as a control group.

Parisi's feeding design allowed each group of hens to serve as their own controls when evaluating the effects of diet and housing system on performance [12].

Egg Quality Analysis

Egg quality analyses were conducted on weeks 20, 24, 25 and 28 of the soy-supplementation challenge. Eggs tested on weeks 20 and 25 represented the wash-out periods where isoflavone levels were at their lowest (control), while samples collected on weeks 24 and 28 representing extended periods of soy supplementation. Eggs were collected from each pen on, both Friday and Saturday morning and held over night at room temperature for 24 hours prior to testing. Data collection occurred over the course of two days and included egg weight, shape index shell strength, Haugh units (HU), albumen height (AH), yolk index, vitelline membrane strength (VMS) and yolk color.

Prior to testing, eggs were weighed on an electronic scale and weights were recorded in grams. The length and width of the eggs were measuring utilizing electronic calipers with a sensitivity of 0.01 mm and, subsequently, shape index was calculated as described by Yannakoupolous and Tserveni-Gousi [35]

Shape index= (short edge/long edge) x 100.

Day 1 Analysis. Shell strength was measured according to the methods of Jones and Musgrove [36], using a Texture Analyzer (TA-XT Plus Texture Analyzer)[37]. The texture analyzer was equipped with a 5 kg load cell, a 75 mm aluminum compression plate, and calibrated with a 2 kg weight. The program was set to test speed of 3.2 mm/s and a 1.0 g trigger force. The egg was placed on an egg holder with the apex facing away from the compression plate and compressed until the shell cracked. Maximum force was recorded in grams.

Albumen height, yolk height and width and HU were determined on a flat surface using a break-out table [38]. The AH was measured with a tripod micrometer, and measurements were taken approximately one centimeter away from the yolk, or about halfway between the yolk and thick albumen [39]. The yolk was then separated from the albumen, with remaining albumen removed by rolling on a damp paper towel [40]. Yolk height was measured with the tripod micrometer, while the yolk width was measured with electronic calipers. Yolk index and HU were calculated utilizing the following formulas:

HU=100 x log (AH+7.57-1.7 x egg weight(g) $^{0.37}$) [41][42]

Yolk Index= (Yolk height/yolk diameter) x 100 [43].

Day 2 Analysis. Vitelline membrane strength (VMS) was measured utilizing the Texture Analyzer equipped with a 5 kg load cell and a 2 mm probe. Test speed was set at 2.00 mm/s with a trigger force of 1.0 gram. At the time of testing, eggs were broken into a shallow 15x100 mm petri dish. Albumen was not separated from the yolk to avoid drying and alteration of data. Care was taken to avoid measuring VMS near the chalazae or germinal disc, since VMS is greatest near the chalazae [44].

Shells of the broken eggs were gently rinsed under flowing tap water to release albumen residues and were then allowed to air dry overnight. Shell thickness was measured with an electronic micrometer and measured at three locations (around the equator of the shell, the blunt region, equatorial region and sharp region) and calculated by utilizing the following formula:

Shell thickness = (pointed edge + equator + blunt end)/3 [35].

Yolk color was determined using a Minolta® colorimeter which was previously standardized using a standard white tile [45]. Color was measured after the yolk was separated from the albumen and placed in a Nasco Whirlpak® bag. Readings were taken from each sample utilizing the CIE colorimetric system L*, a* and b*. Three separate readings on each sample were recorded and averaged to obtain the most accurate calculation.

Mineral Analysis

After data collection on Weeks 24, 25 and 28, a total of 12 eggs were from each treatment were pulverized in a Waring® blender and evenly distributed into 2 labeled 7 oz. Nasco Whirl-pak ®bags. The samples were then sent to Clemson Agricultural and Soil Service Laboratory to undergo standard mineral analysis via inductive coupled plasma method [46][47]. Analysis was done to determine phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), zinc (Zn), copper (Cu), manganese (Mn), iron(Fe), and sulfur (S) and sodium (Na) levels present in the eggshells.

Statistical Analysis

Data was analyzed by the General Linear Model procedure of the SAS/STAT program [48]. Data collected on washout weeks was analyzed to determine the aging and housing effects on the dependent variables. Quality analyses conducted on eggs during the hen feeding trials were analyzed as two separate periods to determine both treatment and housing effects on egg quality. All first order interactions were tested for statistical significance ($P < 0.05$) using the residual error mean squares. Means were separated using the least-squares means option and reported along with the standard error [48].

RESULTS

Aging effects

Data collection during washout periods (SF control), showed that aging effects had a significant effect on egg quality (Table 3). Egg weight significantly increased due to aging ($P<0.0001$) Mean egg weight for BC laying hens increased by 7.69% over the five week period and egg weight for FR laying hens increased by 9.43%. Shell thickness was also significantly affected by hen age ($P<0.0001$). Eggshell thickness decreased over the five week period by 14.8% and 14.6% in BC and FR, respectively. Furthermore, internal quality as measured by HU ($P<0.0001$) and yolk index ($P=0.0002$) declined as the hens aged.

Housing effects

Data collected during washout periods (Table 3) and treatment periods (Table 4 and 5), show that housing systems affected egg quality. During the first treatment period (Table 4), eggs collected from the BC system were significantly larger ($P=0.0002$) and had significantly weaker shells ($P=0.024$) than those from FR layers. During the second treatment period, (Table 5) shell thickness was also greater ($P=0.0079$) in eggs gathered from FR layers than those from BC hens. Though housing system significantly effected VMS during washout weeks (Table 3) ($P=0.0002$), there was a lack of difference in treatment weeks. Yolk color in eggs gathered from the FR production system had a higher redness and yellowness than those collected from BC laying hens (a* $P<0.0001$)(b* $P=0.0087$). Differences in shell mineralization (Table 7), were only noted in zinc levels from BC layers ($P=0.001$), indicating the mineral may have come from the steel cages.

Soy Supplementation effects

Analysis from both treatment periods showed that soy supplementation did affect quality (Table 4 and Table 5). Eggs collected from the control groups were consistently lighter ($P<0.0001$ Period 1 and $P=0.37$ Period 2) and smaller ($P=0.0002$ Period 1 and $P=0.0049$ Period 2) than those from SS and SE treated layers. With the increased size there were no adverse effects noted in shell integrity. Moreover, an increase in eggshell thickness was detected at the end of Period 2 in both SE and SS, while SF had the thinnest eggshells ($P=0.0435$). Yolk quality was higher in eggs collected from the control groups as evidenced by a stronger vitelline membrane ($P=0.034$)(Table 5) and yellower yolk in SF eggs ($P<0.0001$)(Table 6).

DISCUSSION

Aging effects

Statistical analysis of the data collected during washout weeks show aging effects on egg quality. Egg weight increased as laying hens aged and corresponded with a decline in internal and external qualities. Data from the present study is in agreement with previous findings where increased size and weight were attributed to aging effects as internal components were altered to facilitate embryonic development [49][50][51][52][53][54]. Egg weight is an important trait because it is a non-destructible measure of egg quality [55][56]. According to Yasmeen et al. [57] and Suk and Park [58], heavier eggs are produced by older birds as the yolk: albumen ratio increases, negatively impacting internal quality characteristics.

Dependent on genetic strain and age, variations in the yolk: albumen ratio can lead to declining internal quality as hens age [59]. Data collected by Silversides,

illustrated that egg weight increased in aging hens as albumen height decreased [59][60]. Moreover, Tumova and Ledvinka [54] observed an inverse correlation between egg weight and albumen quality as hens aged. In some genetic lines selected for larger egg production, yolk index will increase with aging; however, aging can also favor smaller yolks as was evident in the current study [59].

Table 1 shows that there was a negative correlation between shell thickness and hen age as shells from eggshell from older hens were thinner than those from younger hens. Shell quality decreases as hens age due to their finite capacity of Ca deposition, therefore as egg size increases, shell percentage can decline [61] According to Safaa et al. [61], most egg losses are related to poor shell quality produced at the end of the production cycle [61]. Similarly, De Ketaelere et al. [58] and Suk and Park [62] also confirmed that shell thickness decreases as hens continue to age.

Housing effects

The current study found that production systems had a significant impact on shell quality and integrity. Eggs from BC system were larger while the eggshells were thinner and fundamentally weaker than eggs collected from the FR system. Research conducted by Hughes et al. [63], Van Den Brand et al. [53]), and Sekeroglu et al. [56] also indicated that eggs from FR systems were smaller than those from BC laying hens. According to Hughes et al. [63] the differences in egg size were due to the temperature variations between FR and caged production systems. Furthermore, Van Den Brand et al. [53] concluded that as hens from FR systems aged they produced eggshells with consistent thickness while eggshells from BC systems declined with hen age. These results are consistent with those reported by Kuckukylmaz et al. [64] and Mungai et al. [65] They

concluded that alternative rearing systems, allowing movement and access to direct sunlight, positively impacted eggshell quality by modulating mineral metabolism and enhancing Vitamin D synthesis [64][65].

Housing system also affected the color of the egg yolks with more yellow and redder yolks collected from FR layers with access to the outdoors than those from BC hens. While L* values representing darkness-lightness (0-100) were not significantly impacted, a* values representing green (-a) and red (+a), and b* values representing blue (-b) and yellow (+b) were affected. Color differences can be attributed to the amount of carotenoids, or fat soluble pigments, ingested by FR laying hens allowed to graze on grasses and fed vegetable products [65]. Similar results for egg yolk color were noted by Van Den Brand et al. [53] and Pištěková et al. [66]. Hasin et al. [67] also concluded that richer yolk colors were associated with nutritive factors and were preferred by consumers.

VMS was negatively affected during washout periods by free range housing systems. A market study conducted by Jones et al. [68] reported similar findings in vitelline membranes from cage-free eggs. After analyzing quality characteristics of 8 types of eggs (traditional, cage-free, organic, etc), the researchers concluded that eggs from caged production were of greater internal quality as demonstrated by a stronger and more elastic vitelline membrane ($P < 0.01$).

Soy Supplementation Effects

Tables 4 and 5 show that soy supplementation, both SS and SE, had positive effects on egg size and, unlike the effects of aging, had no adverse effects on shell

integrity. Additionally, at the end of period 2, thicker eggshells were produced by laying hens from SE treatment.

Effects of isoflavone supplementation have been examined in previous avian studies and have noted positive effects on egg quality in end-of-lay avian species [26][27[69]. Described in detail by Whitehead et al. [70], estrogen plays a vital role in the homeostatic mechanisms that control bone metabolism and subsequent eggshell formation thus, as hens age and circulating estrogen levels decline, a direct correlation is evident in bone and eggshell quality. Ni et al [26], Saito et al [27] and Zhao [60], found that isoflavone supplementation could ameliorate the effects of estrogen deficiency by directly binding to estrogen receptors amplifying its total systemic effect.

An extensive review of literature indicated that little data has been collected in peak production avian species, where isoflavone action would stimulate a different cascade of effects. Zhao et al. [71]found that daidzein supplementation in 100-day old Shaoxing ducks led to decreased egg production and decreased egg mass. The researchers concluded that isoflavones could act as an estrogen antagonist, competitively binding to ER's and ultimately interfering with the homeostatic biomechanisms regulating eggshell formation. Unlike the data collected by Zhao et al. [71], the current study found that isoflavone supplementation had positive effects on egg size and eggshell quality in young laying hens.

Although, the effects of isoflavone supplementation on VMS have not been previously examined, Table 3 shows it was affected by dietary manipulations. VMS was weaker in hens treated with both SE and SS, and strongest in the control group. The vitelline membrane is comprised of various intricate proteins, therefore, diet

manipulations can alter their synthesis and interactions [72]. Chukuwuka et al. [73] reported that certain antioxidants and tannins from grains such as sorghum could increase the permeability the vitelline membrane. Furthermore, gossypol a polyphenolic yellow compound from cotton seed meal, in large quantities was also shown to decrease VMS [74].

Yolk color listed in Table 5 was affected by soy treatments. Overall, yolks from layers treated with SF diets had redder yolks than those treated with both SS and SE diets. Though the direct effects of soy protein have not been examined in table eggs, extensive research indicates that isoflavones can be classified as benzopyran derivatives [75]. Benzopyran derivatives include flavonoids and anthocyanins, water soluble compounds phenolic compounds present in vascular plants that can act as coloring agents [76]. Therefore, though the exact mechanism still needs to be elucidated, isoflavones can affect coloring of egg yolks due to their ability to act as coloring agents either alone or with carotenoids [75].

CONCLUSIONS AND APPLICATIONS

1) Production system affects both internal and external quality characteristics. Laying hens from FR production systems produced smaller eggs with thicker and stronger shells than those from conventional BC systems. Internal quality was negatively affected by FR production as measured by VMS. Egg yolks from FR laying hens were redder and yellower than those raised in BC.

2) Supplementation with soy feed affected both internal and external quality characteristics. Eggs collected from laying hens treated with both SS and SE were larger and heavier than eggs collected from BC. Shell strength and thickness were also positively impacted by soy supplementation. Soy supplementation did not alter albumen quality but had significant undesirable effects on yolk quality as evidenced by color and VMS.

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REFERENCES AND NOTES

1) Stadelman, W.J. 1999. The Incredibly Functional Egg. Poult. Sci. 78: 807-811.

2) Position of the American Dietetic Association: Functional foods. 1999. JADA. 99: 1278-1285.

3) Surai, P.F., and N.H.C Sparks. 2001. Designer eggs: from improvement of egg composition to functional food. Trends Food Sci. Tech. 12: 7-16.

4) Fisinin, V.I., T.T. Papazyan, and P.F. Surai. 2008. Producing specialist poultry products to meet human nutrition requirements. Selenium enriched eggs. Worlds Poult. Sci. J. 64: 85-98.

5) Charoensiriwatana, W., P Srijantr, P. Teeyapant, and J. Wongvilairattana. 2010. Consuming idodine enriched eggs to solve the iodine deficiency endemic for remote areas in Thailand. Nutr. J. 9: 68-73.

6) Applegate, E. 2000. Introduction: Nutritional and functional roles of eggs in the diet. J. Am Coll. Nutr. 19; 495-398.

7) Patterson, P.H., K.W. Koelkbeck, D.D. Bell, J.B. Carey, K.E. Anderson, and M.J. Darre. 2001. Egg marketing in national supermarkets- Specialty eggs- Part 2. Poult. Sci. 80: 390-395.

8) Blank, C. 1997. Demand for organic eggs soars. Egg Industry. 100: 3-7.

9) USDA. 2012. Egg Market News Report. Agricultural Marketing Service, USDA, Washington, D.C.

10) Meister, K. 2002. The role of eggs in the diet: Update. Pages 5-27 in American Council on Science and Health.

11) Saitoh S., T. Sato, H, Haranda, and T. Matsuda. 2004. Biotransformation of soy isoflavone-glycosides in laying hens: intestinal absorption and preferential accumulation into egg yolk of equol, a more estrogenic metabolite of daidzein. Biochimica. et Biophysica Acta. 1674: 122-130.

12) Parisi, M. 2012. The microbiological and nutritional composition of an equol-enhanced egg produced in free-range and conventional production systems. PhD Diss. Clemson Univ., Clemson.

13) Bennetts, H.W., E.J. Underwood, and F.L. Shier. 1946. A specific breeding problem of sheep on subterranean clover pastures in Western Australia. Aust. J. Agric. Res. 22:131–8.

14) Liu, J., S.C. Ho, X.Y. Su, W.Q. Chen, C.X. Zhang, and Y.M. Chen. 2009. Effect of long term intervention of soy isoflavones on bone mineral density in women: a meta-analysis of randomized control trials. Bone. 44: 948-953.

15) Ishimi, Y. 2010. Dietary equol and bone metabolism in postmenopausal Japanese women and osteoporotic mice. J. Nutr. 140: 1373-1376.

16) Weaver CM., and L.C. Legette. 2010. Equol, via dietary sources or intestinal production may ameliorate estrogen deficiency-induced bone loss. J. Nutr. 140: 1377-1379.

17) Setchell, K.D.R., N.B. Brown, and E. Lydeking-Olsen. 2002. The clinical importance of the metabolite equol- a clue to the effectiveness of soy and its isoflavones. J. Nutr. 132: 3577-3584.

18) Atkinson C., H.E. Skor, E.D. Fitzgibbons, D. Scholes, C. Chen. K. Wähälä, S.M. Schwartz, and J.W. Lampe. 2003. Urinary equol excretion in relation to 2 hydroxysterone and 16α-hydroxyestrone concentrations: an observational study of young to middle-aged women. J. Steroid Biochem. 86: 71-77.

19) Aso, T. S, Uchiyama, Y. Matsumara, M. Taguchi, M. Nozaki, T. Kiyoshi, B. Ishizuka, T. Kubota, J. Minzuma, and J. Ohta. 2012. A natural S-equol supplement alleviates hot flushes and other menopausal symptoms in equol nonproducing postmenopausal Japanese women. J. Womens Health. 21: 92-100.

20) Lund, T.D., C. Blake, A.N. Hamaker, and E.D. Lephart. 2011. Equol an isoflavonoid: potential for improved prostate health, in vitro and in vivo evidence. Reprod. Biol. Endocrin. 9: 4-13.

21) Lampe, J.W., S.C. Karr, A.M. Hutchins, and J.L. Slavin. 1998. Urinary equol excretion with a soy challenge: Influence of habitual diet. Proc. Soc. Exp. Biol. Med. 217: 335-339.

22) Setchell, K.D.R., and S.J. Cole. 2006. Method of defining equol-producer status and its frequency among vegetarians. J. Nutr. 136: 2188-2193.

23) Marrian, G.F., and G.A.D. Haslewood. 1932. Equol, a new inactive phenol isolated from the ketohydroxyoestrin fraction of mare's urine. J. Biochem. 26:1227–32.

24) Adlercreutz, H., P.I. Musey, T. Fostis, C. Bannwart, K. Wahala, T. Makela, G. Brunow, and T. Hase. 1986. Identification of lignans and phytoestrogens in urine of chimpanzees. Clin. Chim. Acta. 158: 147-154.

25) Uehara A., K Ohta, K. Sakai, K, Suzuki, S .Watanabe, and H. Adlercretuz. 2001. Dietary fructopolysaccharides modify intestinal bioavailability of a single does of genistein and dadizein and affect their urinary excretion and kinetics in blood of rats. J. Nutr. 131: 787-795.

26) Ni, Y., Q. Zhu, Z. Zhou, R. Grossman, J. Chen, and R. Zhao. 2007. Effect of dietary daidzein on egg production, shell quality, and gene expression of ER-α, GH-R and IGF-IR in shell glands of laying hens. J. Agric. Food Chem. 55: 6997-7001.

27) Sahin N., M. Onderci, T.A. Balci, G. Cikim, K. Sahin, and O. Kucuk. 2007. The effect of soy isoflavones on egg quality and bone mineralization during the lay laying period of quail. 48: 363-369.

28) Ahmadi, F., and F. Rahimi. 2011. Factors affecting quality and quantity of egg production in laying hens: A review. World Appl. Sci. J. 12: 372-384.

29) Starter feed- 16% Crude Protein (CP) and 1050 Metabolizable energy (ME) kcal/lb

30) Battery Cage- Soy Free 18 CP with 1,300 ME/lb mixed at University of Georgia Poultry Department, 110 Cedar Street Athens, GA 30602

31) Free-Range- Standard Soy Feed-18% CP with 1,250 ME/lb Layena Purina Mills, LLC. PO Box 66812, St. Louis, MO 63166

32) Battery Cage Standard Soy Feed- 19% CP 1,250 ME/lb Newberry Feed and Farm Inc, Carolina Choice Feed. 2431 Vincent St, Newberry, SC 29108

33) Free Range-Soy Free- 19% CP with 1,220 ME/lb Organic Soy Free Layer Feed. 801 Second St., Waynesborough, VA 22980

34) Soy Germ- Frutarom Soylife. Frutarom USA, Inc., 9500 Railroad Ave. North Bergen, NJ, 07407

35) Yannakopoulos, A.L. and A.S. Tserveni-Gousi. 1986. Quality characteristics of quail eggs. Br. Poult. Sci. 27: 171-176.

36) Jones, D.R. and M.T. Musgrove. 2005. Effects of extended storage on egg quality factors. Poult. Sci. 84: 1774-1777.

37) TA.XT *Plus* Texture Analyzer 123 Stone Mill Lane, Marietta, GA 30064

38) Kul, S., and I. Seker. 2004. Phenotypic correlations between some external and internal egg quality traits in Japanese Quail (Coturnix coturnix japonica). Int. J. Poult. Sci. 3: 400-405.

39) Kırıkçı, K., A. Günlü, O. Cetin, and M. Garip. 2007. Effect of hen weight on egg production and some egg quality characteristics in the partridge (Alectoris graece). Poult. Sci. 86: 1380-1383.

40) Kirunda, D.F.K. and S.R. McKee. 2000. Relating quality characteristics of aged eggs and fresh eggs to vitelline membrane strength as determined by texture analyzer. Poult. Sci. 79: 1189-1193.

41) Raji, A.O. J. Aliyu, U. Igwebuike and S. Chiroma. 2009. Effects of storage methods and time on egg quality traits of laying hens in a hot dry climate. J.Agr. Biol. Sci. 4: 1-7.

42) Jin Y.H., K.T Lee, W.I. Lee and Y.K. Han. 2011. Effects of storage temperature and time on the quality of eggs form laying hens at peak production. Asian-Aust. J. Anim. Sci. 24: 279-284.

43) Tilki, M., and M. Saatci. 2004. Effects of storage time on external and internal characteristics in partridge (Alectoris graeca) eggs. Revue Med. Vet. 155:561-564.

44) Lyon, C.E., G.W. Newell, and R.D. Morrison. 1972. Vitelline membrane strength and egg yolk mottling. Poult. Sci. 51: 480-487.

45) Minolta Chroma Meter CR-300. PO BOX 1364, Maarssenbroek 3600, The Netherlands.

46) Clemson University Agricultural Service Laboratory 171 Old Cherry Road, Clemson, SC 29634.

47) U.S. Environmental Protection Agency. 1994. Determination of trace elements in waters and wastes by inductively coupled plasma-mass spectrometry, Method 200.8 U.S. Environmental Protection Agency, Environmental Monitoring Systems Lab, Cincinnati, Ohio.

48) SAS. 1999. SAS/STAT User's Guide. Release 8.0 Edition. SAS Institute Inc., Cary, NC.

49) Finkler, M.S., J.B. Van Orman, and P.R. Sotherland. 1998. Experimental manipulation of egg quality in chickens: influence of albumen and yolk on the size and body composition of near-term embryos in a precocial bird. J. Comp. Physiol. B. 168: 17-24.

50) Silversides, F.G. and T.A. Scott. 2001. Effect of storage and layer age on quality of eggs from two lines of hens. Poult. Sci. 80: 1240-1245.

51) Silversides, F.G., T.A. Scott, D.R. Korver, M. Afsharmanesh, and M. Hruby. 2006. A study on the interaction of xylanase and phytase enzmes in wheat based diets fed to commercial white and brown egg laying hens. Poult. Sci. 85: 297-305.

52) Silversides, F.G., D.R. Korver, and K.L. Budgell. 2006. Effect of strain of layer and age at photostimulation on egg production, egg quality and bone strength. Poult. Sci. 85: 1136-1144.

53) Van Den Brand, H.K., Parmentier, and B .Kemp. 2004. Effects of housing system (outdoor vs. cages) and age of laying hens on egg characteristics. Brit. Poult. Sci. 45: 745-752.

54) Tumova, E., and Z. Ledvinka. 2009. The effect of time of oviposition and age on egg weight, egg components and eggshell quality. Arch. Geflügelk. 73: 110-115.

55) Farooq, K.A.M., F.R. Durrani, K. Sarbiland, and N. Chaud. Predicting egg weight, shell weight, shell thickness, and hatching chick weight of Japanese quails using various egg traits as regressors. Int. J. Poult. Sci. 2: 164-167.

56) Sekeroglu, A., M. Sarica, E. Demir, Z. Ulutas, M. Tilki, M. Saatci, and H. Omed. 2010. Effects of different housing systems on some performance traits and egg qualities of laying hens. J. Anim. Vet. Adv. 9: 1739-1744.

57) Yasmeen F., S. Mahmood, M.Hassan, N. Akhtar, and M.Yaseen. 2008. Comparative productive performance and egg characteristics of pullets and spent layers. Pakistan. Vet. J. 28: 5-8.

58) Suk, Y and C. Park. 2001. Effects of broiler breeder age and length of egg storage on albumen characteristics and hatchability. Poult. Sci. 80: 855-858.

59) Silversides F.G., and K. Budgell. 2004. The relationship among measurements of egg albumen, pH, and whipping volume. Poult .Sci. 83: 1619-1623.

60) Scott, T.A., and F.G. Silversides. 2000. The effect of storage and strain of hen on egg quality. Poult. Sci. 79:1725-1729.

61) Safaa, H.M., M.P. Serrano, D.G. Valencia, M. Frihka, E. Jimenez-Moreno, and G.G. Mateos. 2008. Productive Performance and Egg Quality of Brown Egg-Laying Hens in the Late Phase of Production as Influenced by Level and Source of Calcium in the Diet. Poult. Sci. 87: 2043-2051.

62) De Ketelaere, B., T Govaerts, P. Coucke, E. Dewil, J. Visscher, E. Decuypere, and J. De Baerdemaeker. 2002. Measuring the eggshell strength of 6 different genetic strains of laying hens: Techniques and comparisons. Brit. Poult. Sci. 43: 238-244.

63) Küçükyilmaz, K.,B. Mehmet, E.N. Herken, M. Çinar, A. U. Çath, E. Bintaş, and F. Çöven. 2012. Effects of rearing systems on performance, egg characteristics and immune response in two layer hen genotype. Asian-Aust. J. Anim. Sci. 25: 559-568.

64) Hughes, B.O., P. Dun, and C.C. McCorqudale. 1985. Shell strength of eggs from medium-bodied hybrid hens houses in cages or on range in outside pens. Br. Poult. Sci. 26: 129-139.

65) Mugnai, C. A. Dal Bosco, and C. Castellini. 2009. Effects of rearing system and season on the performance adn egg characteristics of Ancona laying hens. Ital. J. Anim. Sci. 8: 175-188.

66) Pištěková V., M. Hovorka, V. Večerek, E. Straková, and P. Suchý. 2006. The quality comparison of eggs laid by laying hens kept in battery cages and in a deep litter system. Czech. J. Anim. Sci. 51: 318-325.

67) Hasin, B.M., A.J.M. Ferdaus, M.A. Islam, M.J. Uddin and M.S. Islam. 2006. Marigold and orange skin as egg yolk color promoting agents. Int. J. Poult Sci. 5: 979-987. 2006.

68) Jones, D.R., M.T. Musgrove, K.E. Anderson, and H.S. Thesmart. 2010. Physical quality and composition of retail shell eggs. Poult. Sci. 89: 582-587.

69) Zhao R., Y. Wang, Y. Zhou, Y. Ni, L. Lu, R. Grossman, and J. Chen. 2004. Dietary daidzein influences laying performance of ducks (Anas platyrhynchos) and early post-hatch growth of their hatchlings by modulating gene expression. Comp. Biochem. Physiol. 138: 459-466.

70) Whitehead, C.C. 2004. Overview of bone biology in the egg-laying hen. Poult. Sci. 83: 193-199.

71) Zhao, R.Q., Y.C. Zhou, Y.D. Ni, L.Z. Lu, Z.R. Tao, W.H. Chen and J.Chen. 2005. Effect of daidzein on egg-laying performance in Shaoxing duck bredders during different stages of the egg production cycle. Brit. Poult. Sci. 175-181.

72) Shimizu1 T., D. G.Vassylyev, S. Kido, Y. Doi and K. Morikawa. 1994. Crystal structure of vitelline membrane outer layer protein I (VMO-I): a folding motif with homologous Greek key structures related by an internal three-fold symmetry. EMBO J. 13: 1003-1010.

73) Chukwuka, O.K., I.C. Okoli, N.J. Okeudu, A.B.I Udedibie, I.P. Ogbuewu, N.O. Aladi, O.O.M Ihesiulor, and A.A. Omede. 2011. Egg quality defects in poultry management and food safety. Asian Journal of Agricultural Research 5: 1-16.

74) Berry J.O., G.W. Newell, D.P. Holder, G.V. Odell, and D.E. Bee. 1986. The effect of cottonseed products and selected feed additives on egg yolk discoloration. Poult. Sci. 47: 783-794.

75) Delgado-Vargas, F.,A. R. Jiménez, and O. Paredes-López. 2000. Natural pigments: carotenoids, anthocyanins, and bealains-characteristics, biosynthesis, processing and stability. Crit. Rev. Food Sci. and Nutr. 40: 173-289.

76) Koes, R. E. K., F. Quattrocchio, and J.N.M. Mol. 1994. The flavonoid biosynthetic pathway in plants: function and evolution, BioEssays. 16: 123–132.

Table 1: Feed Analysis on Dry Matter Basis[1]

	BATTERY CAGE			FREE-RANGE		
Based on 100% dry matter	SS[2]	SE	SF	SS	SE	SF
crude protein %	18.8	19.7	18.4	18.6	19	18.1
fat %	2.5	2	7.7	3.3	3.2	3
P %	0.72	0.77	1.49	0.79	0.83	1.09
K %	0.9	0.9	0.54	1.14	1.13	0.71
Ca %	3.3	3.2	6.44	4.25	3.33	4.9
Mg %	0.19	0.2	0.22	0.35	0.34	0.24
S %	0.24	0.23	0.33	0.28	0.27	0.26
Zn ppm	180	138	136	172	134	194
Cu ppm	19	22	16	20	14	12
Mn ppm	110	124	135	161	152	140
Fe ppm	271	267	162	174	159	615
Moisture %	10.7	10.2	10.1	10.3	9.7	11.2
Ca/P	4.61	4.16	4.32	5.37	4.01	4.48
Dry matter %	89.3	89.8	89.9	89.7	90.3	88.8

[1] Feeding study as designed by Michelle Parisi

[2]SE represents standard soy feed content with added soy germ in the amount of 5 g soy-germ/100 grams feed; SS indicates standard soy diets with 25% soy content; SF represents soy-free diet

Table 2. Eight week feeding study [12]

Housing/Diet Group	TRIAL 1		TRIAL 2	
	20 weeks of age	24 weeks of age	25 weeks of age	28 weeks of age
BATTERY CAGE (BC) BC Group-1	BCSF[1] Diet	BCSS[2] Diet	BCSF Diet	BCSE Diet[3]
BC Group-2	BCSF Diet	BCSE Diet	BCSF Diet	BCSS Diet
BC Group-3	BCSF Diet	BCSF Diet	BCSF Diet	BCSF Diet
FREE-RANGE (FR) FR Group-1	FRSF[4] Diet	FRSS[5]	FRSF Diet	FRSE[6]
FR Group-2	FRSF Diet	FRSE Diet	FRSF Diet	FRSS Diet
FR Group-3	FRSF Diet	FRSF Diet	FRSF Diet	FRSF Diet

1) BCSF refers to battery caged soy free layer feed

2) BCSS refers to battery caged standard soy feed

3) BCSE refers to battery caged soy enhanced feed

4) FRSF refers to free-range soy free feed;

5) FRSS refers to free-range standard soy feed

6) FRSE refers to free-range soy enhanced feed

Table 3. Egg quality parameters at washout weeks of feeding study

	20 WEEKS[1]		**25 WEEKS**[2]		**SOURCES OF VARIATION**	
	Battery Cage	**Free Range**	**Battery Cage**	**Free Range**		
	SF[3]	SF	SF	SF	Age Effects	Housing Effects
Weight (g)	48.1 ± 6.53	48.4 ± 4.13	52.0 ± 3.61	52.8 ± 4.11	P<0.0001[4]	NS[5]
Shell Index	75.8 ± 3.17	73.7 ± 3.79	75.9 ± 2.78	74.7 ± 3.18	NS	P=0.0002
Shell Thickness (mm)	0.351 ± 0.006	0.355 ± 0.005	0.299 ± 0.006	0.303 ± 0.005	P<0.0001	NS
Shell Strength (g)	4026.0 ± 1111.4	4334.0 ± 912.2	3949.8 ± 728.8	4008.9 ± 857.9	NS	NS
AH (mm)	7.04 ± 1.44	7.29 ± 1.79	5.73 ± 1.16	5.76 ± 1.42	NS	NS
HU	86.4 ± 9.38	87.9 ± 9.79	77.0 ± 8.69	76.4 ± 10.9	P<0.0001	NS
Yolk Index	45.3 ± 3.55	44.8 ± 3.75	42.9 ± 2.73	42.5 ± 2.59	P=0.0002	NS
VMS (g)	4.25 ± 0.72	3.85 ± 0.56	4.22 ± 0.66	3.92 ± 0.59	NS	P=0.0002

[1]SE represents standard soy feed content with added soy germ in the amount of 5 g soy-germ/100 grams feed; SS indicate standard soy diets with 25% soy content; SF represents soy-free diet

[2]Probability value for General Linear Model of SAS

[3] NS=Not significant at P>0.05

Table 4. Egg quality parameters at end of treatment period 1

	24 Weeks						SOURCES OF VARIATION	
	Battery Cage			Free Range				
	SF[1]	SS	SE	SF	SS	SE	**Treatment**	**Housing**
Weight (g)	50.0 ± 2.54	54.8 ± 2.89	53.6 ± 3.08	52.4 ± 4.73	54.4 ± 5.2	52.9 ± 2.52	P<0.0001[2]	NS[3]
Shell Index	75.0 ± 1.88	77.0 ± 2.85	77.4 ± 2.39	75.2 ± 2.99	77.1 ± 1.64	76.1 ± 2.21	P<0.0002	NS
Shell Strength	3743.9 ± 1013.6	3609.7 ± 808.9	3970.0 ± 1148.8	4516.3 ± 1020.5	4379.4 ± 894.8	4055.2 ± 869.4	NS	P=0.024
Shell Thickness	0.294 ± 0.008	0.305 ± 0.007	.306 ± .0008	0.306 ± 0.008	0.316 ± 0.005	0.310 ± 0.009	NS	NS
AH (mm)	5.49 ± 1.47	5.82 ± 1.36	4.49 ± 0.95	5.13 ± 1.84	5.00 ± 1.43	5.47 ± 0.92	NS	NS
HU	75.1 ± 10.78	76.1 ± 9.04	65.9 ± 8.48	71.0 ± 16.0	68.2 ± 16.6	74.4 ± 7.66	NS	NS
Yolk Index	41.8 ± 3.58	39.8 ± 4.32	43.1 ± 2.42	40.1 ± 2.89	42.3 ± 3.73	42.4 ± 2.77	NS	NS
VMS (g)	4.85 ± 2.30	3.75 ± 0.74	3.91 ± 0.47	3.71 ± 0.56	4.15 ± 0.52	3.45 ± 0.62	NS	NS

[1]SE represents standard soy feed content with added soy germ in the amount of 5 g soy-germ/100 grams feed; SS indicates standard soy diets with 25% soy content; SF represents soy-free diet

[2]NS=Not significant at P>0.05

[3]Probability value for General Linear Model of SAS

TABLE 5. Egg quality characteristics at end of treatment period 2

28 Weeks							SOURCES OF VARIATION	
	BATTERY CAGE			FREE RANGE				
	SF[1]	SS	SE	SF	SS	SE	**Treatment**	**Housing**
Weight (g)	53.9 ± 4.33	56.9 ± 3.04	56.3 ± 3.31	55.5 ± 3.87	56.2 ± 3.45	56.5 ± 3.58	P=0.037[2]	NS[3]
Shell Index	74.1 ± 2.76	76.9 ± 1.87	76.4 ± 2.68	74.8 ± 2.69	75.1 ± 2.84	76.2 ± 2.37	P=0.0049	NS
Shell Strength	4078.1 ± 552.4	4203.2 ± 1176.4	4616.6 ± 771.0	4272.1 ± 720.8	4202.2 ± 957.6	4380.2 ± 324.7	NS	NS
Shell Thickness	0.340 ± 0.012	0.389 ± 0.007	0.371 ± 0.006	0.388 ± 0.007	0.389 ± 0.006	0.377 ± 0.008	P=0.0435	P=0.0079
AH (mm)	5.81 ± 1.11	4.84 ± 1.41	5.08 ± 1.33	5.12 ± 1.37	5.96 ± 1.10	5.35 ± 1.05	NS	NS
HU	76.5 ± 9.31	66.7 ± 12.8	69.5 ± 10.5	69.8 ± 11.6	77.2 ± 7.69	72.1 ± 8.40	NS	NS
Yolk Index	42.5 ± 3.67	40.5 ± 4.73	40.6 ± 3.14	41.5 ± 4.81	39.9 ± 3.54	40.9 ± 3.47	NS	NS
VMS	3.93 ± 0.45	3.53 ± 0.78	3.68 ± 1.33	4.20 ± 1.10	3.66 ± 2.84	3.81 ± 0.39	P=0.034	NS

[1]SE represents standard soy feed content with added soy germ in the amount of 5 g soy-germ/100 grams feed; SS indicates standard soy diets with 25% soy content; SF represents soy-free diet
[2]Probability value for General Linear Model of SAS
[3]NS=Not significant at P>0.05

Table 6. Yolk Color

		L*	**a***	**b***
Battery Cage	SF[1]	55.1 ± 0.56	1.79 ± 0.16	25.3 ± 0.44
	SS	58.4 ± 0.65	-1.75 ± 0.11	25.2 ± 0.57
	SE	58.5 ± 0.56	-1.31 ± 0.25	25.2 ± 0.36
Free Range	SF	58.7 ± 0.98	2.27 ± 0.51	26.3 ± 0.78
	SS	58.2 ± 0.75	0.78 ± 0.36	27.1 ± 0.76
	SE	54.8 ± 0.90	2.86 ± 0.37	26.2 ± 0.59
Sources of Variation		***Probability Values***		
Housing		NS[2]	P<0.0001[3]	P=0.0087
Treatment		NS	P<0.0001	NS

[1]SE represents standard soy feed content with added soy germ in the amount of 5 g soy-germ/100 grams feed; SS indicates standard soy diets with 25% soy content; SF represents soy-free diet
[2]NS=Not significant at P>0.05
[3]Probability value for General Linear Model of SAS

Table 7. Eggshell mineralization values

		P%	K%	Ca%	Mg%	S%	Zn ppm	Mn ppm	Fe ppm	Na ppm
Battery Cage	SF	0.120 ± 0.013	0.107 ± 0.013	30.73 ± 2.069	0.293 ±0.030	0.184 ± 0.048	2.100 ± 1.197	0.000 ± 0.000	4.400 ± 3.098	981.5 ± 130.6
	SE	0.095 ± 0.173	0.045 ± 0.010	29.30 ± 4.804	0.275 ± 0.044	0.143 ± 0.035	2.000 ± 0.816	0.000 ± 0.000	1.500 ± 0.577	837.8 ± 129.4
	SS	0.095 ± 0.129	0.045 ± 0.006	29.83 ± 2.939	0.276 ± 0.026	0.178 ± 0.057	2.500 ± 1.915	0.00 ± 0.00	3.250 ± 2.061	878.5 ± 81.43
Free-Range	SF	0.107 ± 0.011	0.045 ± 0.005	31.52 ± 0.863	3.170 ± 9.076	0.148 ± 0.018	1.000 ± 0.000	0.000 ±0.000	2.100 ± 0.568	933.4 ± 40.48
	SE	0.100 ± 0.014	0.050 ± 0.008	30.44 ± 2.690	0.315 ± 0.042	0.165 ±0.049	1.250 ± 0.500	0.500 ± 1.000	5.500 ± 4.509	896.0 ± 98.65
	SS	0.0975 ± 0.005	0.048 ± 0.005	30.77 ± 0.525	0.303 ± 0.015	0.170 ± 0.216	1.00 ± 0.000	0.250 ± 0.500	5.250 ± 4.031	899.8 ± 34.78
Sources of Variation		**Probability Values**								
Treatment		NS	NS	NS	NS	NS	NS	NS	NS	NS
Housing		NS	NS	NS	NS	NS	P=0.001	NS	NS	NS

[1]SE represents standard soy feed content with added soy germ in the amount of 5 g soy-germ/100 grams feed; SS indicates standard soy diets with 25% soy content; SF represents soy-free diet

[2]NS=Not significant at P>0.05

[3]Probability value for General Linear Model of SAS

CHAPTER 4

CONCLUSION

4.1 Summary

Previous research indicates that isoflavone supplementation fed to laying hens can result in equol deposition into table eggs (Saitoh, 2007). By creating equol enhanced eggs, consumers could have a direct source of the metabolite completely independent of bacterial populations. However, diet manipulations can result in various physiological effects in laying hens. Therefore, the purpose of the research reported in this thesis was to examine how isoflavone supplementation via soybean meal fed to laying hens affected various quality factors including bone composition and bone strength as well as internal and external egg characteristics. Additionally, effects of production system were also examined since free-range and sustainable systems are increasingly emphasized both by animal welfare groups and consumers (Holt et al., 2011). The collected data shows that both housing system and soy supplementation significantly affected physical and chemical characteristics of femurs and eggs collected from laying hens.

4.2 Bone Quality

A positive impact on bone quality was observed in laying hens housed in FR production systems. Femoral mass was greater in FR hens as indicated by longer bones. Bone mineral content was also greater in FR indicating enhanced mineral deposition.

Within BC systems, isoflavone supplementation did not exhibit any protective effects on bone as there were no significant differences in bone characteristics. Conversely, within FR housing systems where mechanic loading stimulates bone turnover, various beneficial effects were associated with the SE feed. Layers fed SE diets

had longer, heavier and stronger bones than hens given SS and SF diets. Similarly, mineral deposition was also greater in layers fed SE diets. This data illustrates that exercise combined with isoflavone supplementation has positive effects in peak production laying hens. As exercise stimulates the rate of bone remodeling, isoflavones have protective effects further regulating bone resorption and deposition.

4.3 Egg Quality

The data collected in the current study indicates that exercise positively influenced egg quality, specifically shell quality. While eggs collected from BC laying hens were larger, their eggshells were thinner and weaker than those collected from their FR counterparts. Yolk colors from FR eggs were also more yellow and red than those from the BC system.

Similar to the femoral data, isoflavone supplementation also had notable beneficial effects on egg quality. Eggs collected from both soy treatments, SS and SE, were larger and heavier than those collected from SF treated hens. More importantly, the adverse effects noted in large eggs gathered from aged layers, were absent indicating that shell quality was not negatively impacted. Moreover, shell thickness was greater in eggs from SE and SS diet. Conversely, internal quality was negatively impacted as evidenced by yolk color and vitelline membrane strength.

4.4 Future Applications

The collected data indicates that soy supplementation has positive effects on bone integrity and external egg quality, two physiological pathways highly dependent on estrogen and calcium homeostasis. While past studies indicate that isoflavones can have agonistic effects in estrogen deficient states, the current study indicates they have a

different mechanistic role in bone remodeling and egg formation when estrogen levels are sufficient and metabolism is enhanced (Ni et al., 2007; Sahin et al., 2007). As suggested by Hwang et al., (2006), isoflavones specifically equol, may act competitively bind to ER's in osteoclast, slowing the rate of bone resorption and acting to maintain skeletal integrity. By slowing the rate of resorption, both structural and MB bone could be maintained longer ameliorating the effects of high estrogen in laying hens. Though Zhao et al., (2005) indicated that isoflavones competitively binding to ER's could cause adverse effects on eggshell quality, the collected data shows that shell integrity was maintained and further enhanced in the soy supplemented groups.

Though beneficial effects were noted in this particular study, further research needs to be conducted to determine the exact isoflavone mechanisms when estrogen levels are adequate. By conducting a long-term study on a larger laying hen population, the effects of isoflavones on endogenous estrogen could be further understood. While protective effects were detected in the current study, following a laying hen population as they age and as estrogen levels vary would help clarify agonist versus antagonistic mechanisms of dietary isoflavones and their metabolites on structural and MB bone and eggshell formation. Furthermore, soy supplementation had adverse effects on yolk quality as evidenced by decreased VMS in eggs from both SE and SS diets. This data indicates that isoflavones can alter the protein synthesis vital to vitelline membrane integrity and VMS. Therefore, additional studies also need to be conducted to determine the mechanism by which isoflavones interfere with protein synthesis. Finally, economic analyses of cost to benefit ratio would need to be conducted to determine the feasibility of incorporating high levels of soy into the diet of laying hens. Based on data from the

present study, increasing rations of soy in the diet of laying hens may be a viable option for free-range hens that have gone out-of-lay (egg quality is no longer paramount), but the hens are still destined to be processed for food in the future.

Overall, the data indicates that isoflavone supplementation had beneficial effects on various bone and egg quality characteristics and could eventually be utilized by the poultry industry to create equol-enhanced eggs. Layers given SE diets had greater bone integrity signifying that high soy diets could potentially be used by the poultry industry to curb the incidence of bone fractures. Stronger shells observed in SE diets could also help decrease the amount of cracked and downgraded eggs.