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Effects of Housing and Calcium-Deficient Diet on Health and Egg Production in Bovans Brown Hens.

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EFFECTS OF HOUSING AND CALCIUM-DEFICIENT DIET ON HEALTH AND EGG PRODUCTION IN BOVANS BROWN HENS

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LIST OF ABBREVIATIONS

$1,25\text{-}(OH)_2D_3$	1,25-Dhydroxycholecalciferol
ACTH	Adrenocorticotropic Hormone
aP	Available Phosphorus
BMC	Bone mineral content
BMD	Bone mineral density
BW	Body weight
Ca	Calcium
CORT	Corticosterone
CRH	Corticotrophin-Releasing Hormone
d	Day
DA	Dopamine
DEXA	Dual energy X-ray absorptiometry
EP	Epinephrine
FPW	Fat Pad Weight
H: L	Heterophil to Lymphocyte ratio
HPA	Hypothalamic-pituitary-adrenal axis
HPLC	High Performance Liquid Chromatography
L: D	Lighting period : Dark period
LW	Liver Weight
NE	Norepinephrine
P	Phosphorus
Wk	Week

ABSTRACT

Hu, Jiaying. M.S., Purdue University, December 2013. Effects of housing and calcium-deficient diet on health and egg production in Bovans Brown hens. Major Professor: Heng-wei Cheng.

Public concern about the welfare of hens kept in conventional cages has become an important issue worldwide. The conventional cage system has been banned by the European Union since 2012. Several housing systems, including free range, have been developed as welfare friendly alternatives to the conventional cage system. Experiment 1, the study examined the effects of housing environment, conventional cages vs. floor pens, on hens' health and egg production. A total of 84 19-wk-old Bovans Brown hens were randomly assigned into 2-bird cage with 12 replications (n=12), providing 968 cm^2 floor space per hen or 10-bird floor pen with 6 replications (n=6), providing 3711 cm^2 floor space per hen for 8 wks. The floor pens were furnished with perches, nest boxes, and wood shavings litter. Egg production was recorded up to 26 wk of age. Egg weight, egg quality, and shell quality were measured in two consecutive days at wk 22, 24, and 26, respectively. Body weight, liver weight, abdominal fat pad weight, plumage condition and foot health were measured at wk 26. Mineralization of the tibia, femur and humerus, liver fat, and heterophil to lymphocyte (H: L) ratio were also analyzed. Results showed there were no environmental effects on daily egg production, plumage condition, and feet

hyperkeratosis of hens ($P > 0.05$, respectively). Compared to hens housed in floor pens, caged hens had longer claws ($P < 0.0001$). In addition, caged hens had greater egg weight at 22 wk of age ($P = 0.001$); while floor pen housed hens had cumulative higher %shell and shell thickness ($P = 0.002$ and $P = 0.02$, respectively). Floor pen housed hens also had greater BMD (bone mineral density) and BMC (bone mineral content) in the measured bones than those of the caged hens ($P < 0.05$). The H: L ratio, an immunological response parameter and stress indicator, was higher ($P = 0.002$) in caged hens than hens housed in floor pens. Overall, the results suggest that furnished floor pens may be a favorable alternative housing system to conventional cages for improving hen welfare; however the cages still have certain advantages for egg production.

Calcium (Ca) and Phosphorus (P) are the most wide-distributed minerals in chicken body, which is mostly provided through diet. It is estimated by National Research Council (NRC, 1994) that the Ca requirement of brown laying hens is about 36g/kg for 110 g/hen/d feed intake to maintain physical and physiological homeostasis. Deficiency in Ca results in reducing eggshell quality and leading to skeletal abnormalities such as osteoporosis in layers and lameness in broilers. Experiment 2, the study was to determine the effects of Ca-deficient diet on hens' health and egg production. A total of 120 20-wk-old Bovans Brown hens were randomly assigned into twelve 10-bird floor pens. After 2 wks acclimation to the environment, hens of half pens were treated with a Ca-deficient diet and other half hens were fed with a commercial layer diet for 4 wks. Each floor pen provides 3711 cm^2 floor space per hen, and was furnished with perches, nest boxes, and

wood shaving litter. During the 4 wk trail, experimented hens were fed with a diet contain 0.9% of Ca, while control hens were fed with a diet contain 3.3% of Ca. Eggs were collected and recorded for daily production; and egg and shell traits were analyzed at wk 22, 24, and 26. Eating, drinking, and perching behaviors were observed using scan sampling weekly from wk 22 to 26. Body weight, liver weight, and abdominal fat pad weight were measured at wk 26. Bone parameters including BMD and BMC of the tibia, femur, and humerus, bone ash, and bone Ca and P concentrations; and stressor indicators including liver fat, plasma epinephrine and norepinephrine, and H: L ratio, were also analyzed. Results showed that accumulative daily egg production and both shell thickness and shell percent were lower in Ca-deficiency fed hens compared to controls ($P = 0.02$ and $P < 0.001$, respectively), but there were no treatment effects on egg weight and size ($P > 0.05$). No difference was observed in hen body weight between treatments, but the liver weight and liver fat was higher in hens fed regular diet ($P = 0.05$ and $P = 0.003$, respectively). Drinking behavior was not affected by treatment ($P = 0.77$) but eating and perching activities were higher in control hens ($P < 0.0001$ and $P = 0.01$, respectively). Both BMD and BMC in the retrieved bones were lower in Ca-deficiency diet fed hens than controls ($P < 0.05$, respectively). The immunological parameters including H: L ratio and the plasma EP and NE were no affected by treatment ($P > 0.05$, respectively). Short-term deprivation of dietary Ca in Bovans Brown hens at the onset of egg laying had negative impact on egg production, shell quality, and skeletal mineralization, which will cause further economic loss and hen welfare issues. The data further evidences that

dietary supplement of Ca is one of the critical factors to maintain chicken health and production.

CHAPTER 1. LITERATURE REVIEW

1.1 Definition of Animal Welfare

Farm animals have been playing an important role in human society for several thousand years. During the domestication process, farm animals experienced multiple changes in their living environments resulting in morphological and physiological alterations. Compared to their wild counterparts, under artificial selection, these changes greatly shifted their priorities and interests which can range from daily nutrition to reproduction (Boessneck, 1985; Cheng, 2010; Broom, 2011). In general, animals' inability to adapt to their environments causes a great impact on their welfare (Weiss, 1971; Broom, 2011). To farmers, an animal experiencing good welfare is synonymous to excellent animal health and productivity. Initially, an animal's productivity was the major indicator for evaluating its welfare, i.e., high productive performance equals good animal welfare (Broom, 1991; Hewson, 2003). However, in the modern intensive animal industry, high productivity in animals do not necessarily indicate the animals' welfare is at a good status, because farm animals have been selected for high production over thousands of generations and producers have shifted their interests in animal production from the individual animal to a group of animals (Matheny and Leahy, 2007).

Animal welfare includes both physical and mental health and focuses on how an animal copes with its living conditions (Hewson, 2003; Broom, 2011). Although animal welfare

has become a critical issue of the animal industry worldwide, there are no accurate measurements used for evaluating animal welfare (Barnard, 2007; Hubbard and Scott, 2011). Commonly, animal welfare is linked to the relationship between human and animals, including the animal's feelings, needs, and their abilities to perform natural behaviors within specialized environments provided by humans (Hubbard and Scott, 2011). Emotional feelings, regarding a physical or mental sensation, are part of an animal's coping mechanisms. For example, once animals are unable to adapt to a stressor, such as high environmental temperature, restriction in freedom of movement, or social competition, they will experience negative emotional feelings such as fear and pain. Although feelings are a critical component when assessing the welfare of animals, they, unlike human beings, are unable to report verbally their sensory feelings and emotional experience. So multiple factors should be included in evaluating animal welfare. For example, the physiological alterations occurred in caged hens due to behavioral and physical restrictions have been considered as a critical measure of hens' health and welfare (Dawkins, 2004; Bracke and Hopster, 2006). Previous studies have shown that the decreased mobility of laying hens housed in conventional cages have negative effects on the layers' skeletal system, with a high probability of suffering from osteoporosis (Duncan et al., 1992; Webster, 2004; Jendral et al., 2008).

1.2 Broiler Welfare

Broilers and layers are two major kinds of commercial chickens representing the meat and egg sectors of the poultry industry, respectively. Similar to other animals, poultry welfare has been assessed based on mortality, behavior, production, physical, and

physiological health. Commercial broiler chickens have been subjected to intense genetic selections. In the past 50 years, broiler growth rate has increased by over 300%; broilers reach 2-3 kg of BW in about 42 days. However, due to rapid growth in such a short period of time, many broilers suffer from impaired locomotion with lameness, which could be so severe that some are unable to walk (Julian, 1998; Dawkins et al., 2004). In addition, broiler chickens maintained at a high stocking density on littered floor systems may have negative implications for their welfare (Hall, 2001). Numerous studies have been conducted to determine the best stocking density as well as to identify methods to control growth rate and improve skeletal health in broilers (Cravener et al., 1992; Hall, 2001; Dawkins et al., 2004; Leone and Estevez, 2008). Recently, legislation and guidelines have been implemented for maintaining broiler chickens in a more welfare-friendly environment, examples for stocking density recommendations include the European Union standard of a maximum of 33 kg of BW/m^2 of floor space (European Commission, 1999/74/EC) and 38 kg of BW/m^2 of floor space in the United States (National Chicken Council, 2010).

1.3 Laying Hen Welfare

1.3.1 Layer Housing System Design

Modern egg laying strains of chickens have experienced many quantitative and qualitative alterations in their physical and physiological characteristics over thousands of generations during domestication. However, after generations of selection for traits of economic importance to the egg industry, laying hens still have a high motivation to express some of their natural behaviors similar to their ancestors and wild counterparts

such as roosting, perching, and nesting (Price, 1999; Newberry et al., 2001). It is important to meet hen's behavioral, physical, and physiological requirements when providing them with a new environment (Achterbosch, 2008; Lay et al., 2011). Unlike broilers for whom the littered floor system is most commonly used, the housing environments for layers are more varied. Currently, there are four major housing systems used by the egg industry: conventional cages, enriched cages, cage free systems (floor pen, barn, and aviary), and outdoor free range system.

1.3.1.1 Conventional and Enriched Cages

The conventional or battery cage has been widely adopted since the 1950s. It has been considered as the most effective system for cost-effective egg production (Tactacan et al., 2009; Pohle and Cheng, 2009b; Lay et al., 2011). Approximately 95% of the commercial eggs in the United States and almost 90% of the world's eggs are produced in conventional cages (UEP, 2010). Conventional cages are also considered to be the best system for prevention of infectious disease, especially for those diseases that are transmitted through the feces as the hen's fecal material falls through the wired floor of the cage to reduce cross contamination among the hens (Hulzebosch, 2006). In general, the risk of injury due to feather pecking is low in caged hens because the living group size is smaller compared with other housing systems (Tauson, 2005; Blokhuis et al., 2007; Shimmura et al., 2011). However, conventional cages have been criticized for its poor welfare assessment (Dawkins, 2004). Chickens kept in conventional cages cannot perform many of their natural behaviors, and they lack freedom of movement which can lead to non-infectious diseases such as cage layer fatigue and osteoporosis. As the name

implies, the hens become so weak that they are no longer able to stand and collapse in the cage (Couch, 1955). Hens with cage layer fatigue experience paralysis, bone brittleness, and perhaps death. Cage layer fatigue does not usually occur in other housing systems. More typically, hens experience osteoporosis rather than cage layer fatigue which is characterized by decreased mineralized structural bone as hens aged (Whitehead and Fleming, 2000; Silversides et al., 2012). Recent recommendations on stocking density have given hens more space, with minimums established at 550 cm^2 (85 in^2) per hen in the European Union and 432 cm^2 (67 in^2) per hen in the United States (Achterbosch et al., 2008). The greater space allocations in cages hopefully provide with more freedom of movement to improve skeletal health. To better meet hen's behavioral needs, enriched or furnished cages have been developed, providing with perches, nest box, nail trimmers, and a scratch pad area. Hens housed in enriched cages have better bone mineralization than hens in conventional cages (Webster, 2004; Achterbosch, 2008; Valkonen et al., 2010; Lay et al., 2011) because of the increase in perching activity (Hughes et al., 1993; Newberry et al., 2001; Hester et al., 2013). Shervin et al. (2010) reported that chickens kept in conventional cages were heavier and had more fat deposits than chickens housed in other systems. The lipid accumulation has been recognized as a result of chronic stress (Puvadolpirod and Thaxton, 2000a; Patterson and Abizaid, 2013). Several stress parameters were improved when hens were offered housing systems with increasing complexity such as the furnishings offered in enriched cages. Egg production is similar between conventional and enriched cages (Appleby et al., 2002; Phole and Cheng, 2009b). Furnished cages may be a more welfare friendly alternative to the conventional

cage and offer a compromise between cage and non-cage systems (Pohle and Cheng, 2009 a,b).

To fulfill hens' behavior demands and improve their welfare, the conventional cage system had been abandoned in the Europe Union since 2012. Egg producers must provide a minimum of at least 750 cm^2 (116 in^2) of floor space per hen with a perch, nest, and scratch area inside the cage (European Commission, 1999/74/EC). In the United States, although the conventional cage is still acceptable, the United Egg Producers (UEP, 2008) started a voluntary certification program to improve hen welfare by reducing stocking density with a minimum of 432 cm^2 (67 in^2) of floor space per hen for White Leghorn and 490 cm^2 (76 in^2) for Brown layers. In addition, the UEP and the Humane Society of the United States (HSUS) had signed an agreement requiring United States egg producers to convert hen housing systems from conventional cages to enriched cages providing 800 cm^2 (124 in^2) per hen in a 15-year period (UEP-HSUS, undated).

1.3.1.2 Cage Free Systems

Several cage free housing systems are used currently by commercial egg producers such as single level floor pens and aviaries. Compared to furnished cages, the floor pen is an advanced enriched housing environment that provides litter for foraging and dust bathing and ample space for freedom of movement (Leone and Estévez, 2008). The deep litter system is considered to be safe for the chickens and eggs and acts as a source of food as well (Pistekova et al. 2006). Hetland and Svihus (2007) found that hens were able to consume litter when housed in a paper material pen, and both the nutrient and non-nutrient substrates affected the balance of hens' diet and their ability to digest the feed.

The additional space, with perch availability, allows hens to exhibit their natural behaviors such as foraging, stretching, perching, wing flapping, and walking without restriction, which benefits the health of hen's skeletal system (Sherwin et al., 2010; Vlakonen et al., 2010; Lay et al., 2011). The multi-tiered aviary, that utilizes the entire space of the hen house, provides hens with additional freedoms of movements such as short flight and access to higher perches, but at the same time, the hens could be infested with parasites and experience crash landings leading to bone fracture (Berg, 2001; Flaten and Lien, 2006). Compared to the conventional cage system, littered floor and aviaries produce larger amounts of ammonia emissions, a potential unhealthy living condition for chickens, resulting in negative effects on respiratory health and egg production (Roberts, 2004; Achterbosch, 2008; Ribikauskas et al, 2010).

1.3.1.3 Free-Range (Outdoor)

Non-cage systems have provided chickens with space for exhibiting a wide range of behaviors, but the free-range system allows chickens to display the most diverse behaviors during their lifespan (Lay et al., 2011). As an example, free-range hens have the capability to find and utilize edible materials during outside foraging. Based on the foraging materials consumed, chickens adapt their digestive system quickly to the new sources of food (Horsted and Hermansen, 2007). In contrast, the increase in freedom of movement and natural light could lead to abnormal behaviors such as cannibalism (El-Lethey et al., 2000; Berg, 2001; Fossum et al., 2009). Furthermore, toxins from pesticides and herbicides and parasite infestation can increase with outdoor access (Lay et al., 2011).

1.3.1.4 Organic Poultry Industry

Organically grown animal products such as eggs and milk represent a rapidly growing market domestically and internationally. It has been reported that the global sales of organic food and drink reached 58 billion US dollars in 2010, which has expanded over three-fold in ten years ($17.9 billion in 2000) (Willer and Kilcher, 2012). According to the organic industry survey conducted by the Organic Trade Association in 2000, the United States organic market grew at about 8% in 2010. Especially in the egg industry, organic egg sales grew by an average annual rate of 19% from 2000 to 2005 (NBJ, 2006). The surveys of consumer perception have shown that the majority of consumers link organic foods with improvements in animal welfare and animal health as no additional chemicals or biological additives are used in organic farming (Onyango et al., 2007; Van Loo et al., 2010).

In the organic poultry production system, outdoor access, that provides an area mainly covered by grass (at least 4 m^2 per chicken), is required for the entire rearing period of chickens, or at least, during the warm season based on the location of farms (Berg, 2001). Allowing chickens to display natural behaviors is a key concept in organic farming (Berg, 2001; Lund and Rocklinsberg, 2001). The loose housing provides freedom for hens to move, leading to improvements in bone strength (Appleby, 1993). According to the management guideline or requirements of the organic industry, antibiotics or chemotherapeutics are not allowed for use on organic farms, which can increase the frequency of diseases and result in spikes in mortality caused by infectious agents and

parasites (Berg, 2001). However, the Salmonella load was lower in certified-organic broilers compared to conventionally raised broilers (Alali et al., 2010).

1.3.2 Production Response to Various Housing Environments

Over the last 50 years, conventional cages have been the most economical way to produce table eggs and the most efficient system for preventing diseases (Van Horne and Achterbosch, 2008). For the commercial egg industry, production efficiency is one of the most important assessments used for evaluating the effects of housing environment on animal well-being. Some studies have reported that egg production is relatively higher in the conventional cages than other housing systems (Abrahamsson et al., 1996; Tauson et al., 1999; Hetland et al., 2003; Valkonen et al., 2010). But, other studies reported that egg production was not different between conventional cages and alternative housing systems (Scott and Silversides, 2000; Singh et al., 2009). In addition, egg production is also affected by environment-associated stress (Thaxton, 2004). Egg production, for instance, was reduced in the hens exposed to a hot (Mashaly et al., 2004) or cold (Hester et al., 1996) environment.

Some studies indicated there was no difference in egg weight in hens between conventional and furnished cages (Pohle and Cheng, 2009b; Neijat et al., 2011). In contrast, Singh et al. (2009) reported that heavier eggs were produced in hens housed in floor pens as compared to those in conventional cages, and the difference may have been due to the differences in their BW; hens kept in floor pens had heavier BW than the chickens housed in cages. In contrast, Yakabu et al. (2007) reported that hens laid heavier eggs in conventional cages as compared to floor pens.

Housing-associated differences in the frequency of occurrence of diseases, infection, and parasite can affect hen health differently, which eventually impacts egg production and mortality (Lay et al., 2011).

1.3.3 Bone Development Affected by Housing Systems

Compared to conventional cages, enrichments installed in alternative housing systems have positive effects on the chicken's skeletal system, such as improving bone strength so as to reduce suffering from bone fracture (Appleby et al., 1988; Jendral et al., 2008; Tactacan et al., 2009; Lay et al., 2011). Skeletal health is improved by increasing movement associated with increased available space and providing perches in the alternative housing systems (Vits et al., 2005; Struelens et al., 2009). Hen welfare has been improved by increasing space allowance, promoting exercise, and allowing hens to display their natural behavioral repertoires (Jendral et al., 2008; Tactacan et al., 2009; Silversides et al., 2012). Currently, alternative housing systems have become more preferable than the conventional cage system and as mentioned previously, the conventional cage system is now forbidden in European countries. However, in some of the alternative systems where enrichments are provided, birds may injure themselves, such as broken keel bones when interacting with the perch (Ferrante, 2009).

1.3.4 Body Condition and Behavioral Response to Housing Environment

Maintaining good body condition is a critical factor in meeting satisfactory standards in welfare. In poultry, the conditions of hyperkeratosis and claw length are two major concerns when assessing hen foot health. Hyperkeratosis is the hypertrophy of the corneus layer of the skin on both toes and footpad. The frequency of hyperkeratosis is

increased when chickens spend more time grabbing the cage wire floor or compressing of the toes and footpad on the wire floor or perches (Weitzenbürger et al., 2006, Enneking et al., 2012). For floor systems and aviaries, high litter moisture and ammonia level can affect foot condition leading to foot-pad dermatitis or bumble foot (Wang et al., 1998; Dawkins et al., 2004).

Caged hens are unable to trim their claws as effectively as in non-cage systems (Glatz et al., 2002). Excessive claw growth occurs if hens do not have access to abrasive materials for trimming their nails such as a scratch pad (or sand bath) in furnished cages (Appleby et al., 1993; Abrahamsson and Tauson, 1997, Glatz et al., 2002). Untrimmed claws can break off more easily, leading to open wounds and greater susceptibility to infection (Glatz et al., 2002; Hester et al., 2012).

A chicken's exterior appearance can be measured by plumage condition. Feather condition, as an indicator of welfare condition, is affected by the pecking behaviors of chickens (Sherwin et al., 2010). Plumage can also be affected by rubbing against enrichments, the wired cage partition, or other hens. As an example, poorer feather scores of caged hens at the end of lay occurred in the neck, breast, wings, and tail due to abrasion against perches and/or between the bodies of perching hens as compared to hens in cages without perches (Tauson et al., 1984; Hester et al., 2012)

A chicken's physical condition can also affected by stocking density. Limited resources such as feeder space in caged hens result in stocking at a high density may increase aggressive behavior (El-Lethey et al., 2000; Sedlackova et al., 2004). The risk of injury due to feather pecking increases with increment increases in group size (Hughs et al.,

1997; Shimmura et al., 2011). Hens housed in a perch-installed environment exhibited less pecking behavior than those housed in conventional cages without perches. The main reason for the difference in pecking was that the chickens spent more time perching than pecking with the end result being improved feather condition (Barnett et al, 1997).

1.4 Bone Plasticity and its Associated Skeletal Health in Egg Laying Hens

Cage layer fatigue has been described as bone brittleness and paralysis in laying hens (Grumble, 1959; Whitehead and Fleming, 2000). It has been noticed shortly after the laying hens were assigned to conventional cages (Webster, 2004). The affected hens are suffering from welfare problem due to bone fractures, and, in some cases, it eventually causes hen death (Urist and Deutsch, 1960; Webster, 2004). The cage layer fatigue is highly related to the development of osteoporosis (McCoy and Reilly, 1996; Whitehead and Fleming, 2000; Webster, 2004). Osteoporosis in laying hens is defined as a decrease in mineralization of structural bone, leading to susceptibility to fragility and fracture (Whitehead and Fleming, 2000). Osteoporosis is a common skeletal problem in hens during the period of high egg production (Urist and Deutsch, 1960; Webster, 2004). A survey conducted in UK reported that approximately 29% of caged hens had broken bones at least once during their lifetime (Gregory and Wilkins, 1989). Fractured bones due to osteoporosis are a major concern in the poultry industry (Riczu et al., 2004; Kim et al., 2007; 2012).

There are three distinctive types of bones in layers, which are cortical, medullary, and cancellous bones (Van de Velde et al., 1985; Kim et al., 2007; 2012). Cortical bone is synonymous with compact bone, which facilitates bone's main functions to support the

body, protect organs, and store chemical elements which is mainly Ca (Kim et al., 2012). Cancellous bone is also referred to as trabecular bone. Among the 3 types of bones, it has a higher surface area but is less dense and stiffer. It is typically at the ends of long bones, proximal to the joints, and within the interior of vertebrae (Gomez, 2002). Medullary bone is unique for matured female laying hens. It develops at the onset of sexual maturity and acts as a labile source of Ca during egg shell formation (Wilson and Duff, 1991; Dacke et al, 1993; Whitehead, 2004; Fleming, 2008). The hen skeleton experiences rapid remodeling during egg shell formation (Whitehead, 2004; Kim et al., 2012) which is mediated by osteoblasts and osteoclasts which are the bone-forming and bone-resorbing cells, respectively (Matsuo and Iris, 2008; Vries et al., 2010; Kim et al., 2012; Jiang et al., 2013).

1.5 Effects of Nutrition on Calcium Metabolism

Calcium is the most widely distributed mineral in the body and is in great demand from the daily diet in chickens (Vries, 2010). Hens actively laying eggs require Ca for maintaining the integrity of the skeleton as well as for egg shell formation. A laying hen needs about 10 to 15 times more Ca than mammals of equivalent body size (Graveland and Berends, 1997). Calcium stored in the skeleton is in the form of hydroxyapatite (Whitehead and Felming, 2000) and in the egg shell it is deposited as calcium carbonate (Bolukbasi et al., 2005; Bar, 2009). Calcium also serves as a regulator (or a messenger) playing critical roles in muscular contraction and many other biochemical reactions in an organism (Matos, 2008). In chickens, following Ca intake from the diet, part of the absorbed Ca is transferred directly to the shell gland for shell formation and part is stored

in the skeletal system, from where it can be released and transported to the shell gland during egg production (Gilbert, 1983). Dacke et al. (1993) and Bar (2009) reported up to 40% of the egg shell Ca is derived from bone reserves. Chickens are able to maintain a satisfactory level of production if they are provided a diet with an adequate level of dietary Ca. The NRC (National Research Council, 1994) estimates that the Ca requirement for brown laying hens is 36 g/kg of feed based on 110 g of feed consumed/hen/d. For white hens consuming a similar quantity of feed, the Ca requirement is approximately 35 to 38 g/kg of feed (NRC, 1994; Roland et al., 1996, Ahmad et al., 2003, Narvaez-Solarte et al., 2006).

1.5.1 Calcium and Medullary Bone

In female birds, the formation of medullary bone is stimulated by the action of estrogens shortly after sexual maturity, and this process remains during the laying cycle (Dacke et al, 1993; Whitehead, 2004). The increased level of estrogen triggers the activity of osteoblasts to produce medullary bone (Whitehead, 2004). During the egg-laying cycle, osteoclasts accelerate the resorption of medullary bone for Ca supply for egg shell formation (Dacke et al, 1993; Kim et al., 2012). Compared to structural bone, medullary bone is well vascularized and is easily accessible with a higher metabolic rate (Hurwitz, 1965; Dacke et al, 1993; Whitehead, 2004). Under long-term Ca deficiency, medullary bones are restored by the continued resorption of structural bone through increasing the functions of osteoblasts. The gradual structure bone loss will lead to osteoporosis (Dacke et al., 1993; Whitehead and Fleming, 2000; Vries, 2010).

1.5.2 Calcium and Egg Parameters

Egg production and egg quality traits of laying hens are highly related to Ca metabolism (Keshavarz and Nakajima, 1993; Van Den Brand et al., 2004). The daily demand for Ca for egg shell formation is approximately 10% of the total body reserve of Ca (Gilbert, 1983). Gilbert and Blair (1974) reported that hens fed a diet containing 0.05% Ca virtually ceased laying eggs (production < 4%), and those fed a diet containing 0.5% Ca maintained egg production between 20 and 30%. Schreiweis et al. (2003) also reported hens were unable to sustainably regular egg production with a low Ca diet (2.1%). In addition, Roland et al. (1985) observed a gradual decrease in egg production in hens fed Ca levels ranging from 5.0 to 2.5% during the first laying cycle.

The level of Ca in a diet affects shell quality traits (Rennie et al, 1997; Schreiweis et al., 2003; Whitehead, 2004; Safaa et al., 2008). Hurwitz and Bar (1965) found a decrease in shell thickness and shell weight of eggs obtained from hens consuming a diet containing 1.7% Ca as compared to hens consuming 3.7% Ca. Schreiweris et al. (2003) and Cufadar et al. (2011) also determined that hens consuming a low Ca diet laid eggs with poor shell quality. Under conditions of a Ca deficiency, in order to maintain Ca homeostasis, hens reduce their Ca usage by lowering egg production and shell Ca excretion (Hamilton and Cipera, 1981; Schreiweris et al, 2003; Williams, 2005).

Hens fed a diet with a high level of Ca (4.64 g of Ca/hen per day) improved egg production, egg mass, and shell quality, but not the interior egg quality traits (albumen height and yolk color) as compared to hens fed control levels of Ca (4.08 g of Ca/hen per day, Safaa, 2008). In contrast, Schreiweris et al. (2003) reported that feeding a high Ca

diet (4.80 g of Ca/hen daily) did not improve shell quality beyond the control-fed hens that consumed 3.19 g of Ca/ hen per day. Keshavarz and Nakajima (1993) also reported that increasing dietary Ca consumption above 3.75 g/hen per day did not improve shell quality. In addition, an excess level of Ca may interfere with other nutrients, such as zinc, thereby causing a deficiency of other mineral elements (Maynard et al., 1979).

1.5.3 Genetic Selection for Calcium Metabolism

A commercial line of White Leghorn hens selected for high egg production was further selected for bone strength. Bishop et al. (2000) reported that there was no relationship between production performance and bone quality traits in hens from the 5th generation of the selected line. By comparing 37 different lines of chickens (12 commercial broiler, 12 commercial layer, and 13 traditional chickens of which one was the J-line Brown Leghorn), Hocking et al. (2009) reported that, although the commercial layer lines had been selected for a high egg production, there was no differences in bone size, shape, or quality compared to the traditional lines at 6, 8, and 10 wk of age. However, at 55 wk of age, the tibia from the layer line was lighter, less stiff, less dense, and weaker than the tibia from the traditional lines (Hocking et al., 2003). The heavier, stronger, and more radiodense tibias of broilers as compared to egg laying lines at 6, 8, or 10 wk of age suggested that bones of broilers mature earlier than layers and traditional lines (Hocking et al., 2009).

1.5.4 Other Nutritional Factors

Phosphorus is needed for laying hens mostly for skeletal integrity, but it is also a crucial factor in metabolism (Vries et al., 2010). A deficiency in P results in bone loss, and is

likely associated with osteoporosis (Wilson and Duff, 1991; Whitehead, 2000). In a chicken egg, only a small amount of P is required for egg shell formation with most of the P found in yolk (Vries et al., 2000). The metabolism of P and Ca are interrelated (Ahmad and Balandar, 2004). Any bone mobilization to fulfill Ca requirements results in elevated concentrations of plasma P and excretion of bone P, especially during times of shell formation (Hurwitz and Bar, 1965; Kebreab, 2009). Phosphorus absorption is oppositely linked to the dietary Ca supply. High levels of dietary Ca result in a high plasma Ca with a low P absorption, whereas low plasma Ca results in increased P absorption (Hurwitz and Bar, 1965; Vries et al., 2010). The available P requirement for White Leghorn hens between 18 and 50 wk of age was 0.13% or 0.14 g of available P/hen per day when a diet contained 4.0 - 4.2 g of Ca/hen per day at 110 g of feed/hen daily (Van der Klis et al., 1997).

The calcification of bone and egg shells is regulated by the active form of vitamin D, 1,25-dihydroxycholecalciferol [1,25 $(OH)_2D_3$] (Vaiano et al., 1994; Edwards, 2000; Bolukbasi et al., 2005). A vitamin D_3 dependent Ca-binding protein has been found in the intestinal and uterine of laying hens, which is involved in actively transporting Ca between the organs and blood (Edwards, 2000; Bolubasi et al., 2005). Laying hens given a dietary supplement of vitamin D3 (3,000 IU/kg), as compared to ones without supplement (0 IU/kg), had higher levels of Ca in both egg shell and plasma (Bolukbasi et al., 2005). In addition, a dietary supplement of 1,25-$(OH)_2D_3$ decreased tibial dyschondroplasia in broilers (Rennie et al., 1993; Thorpe et al., 1993; Edward, 2000) and

also decreased the incidence of rickets in turkeys fed Ca-deficient diets (Sanders and Edwards, 1991; Edward, 2000).

1.6 Bone Quality Assessments

Various methods have been used to assess bone quality in poultry. Bone ash (g/g dry weight), bone mineral density (BMD), bone mineral content (BMC), and bone breaking strength are the most popular indicators used for evaluating bone quality in chickens. Ashing of bone is a fundamental method to assess bone mineralization. After using high temperatures to remove the organic material, the remaining dry weight of the fat-free bone is approximately the weight of its mineral content (Onyango et al., 2003; Schreiweis et al., 2005; Kim et al., 2012). Chickens fed a Ca-deficient diet have smaller bone sizes and lower % ash compared to control chickens fed normal diets (Hurwitz and Bar, 1966; Zhang and Coon, 1997). Bone ash is positively related to BMD (Yan and Keen, 2005). The correlation coefficients of bone ash concentration with bone breaking force or bone density were 0.69 and 0.58, respectively (Zhang and Coon, 1997). However, both the mineral and organic matrix of bones are mobilized during bone loss and resorption, so % ash may remain unchanged under conditions of a Ca deficiency (Ham and Leeson, 1961; Cheng and Coon, 1990; McCoy and Reilly, 1996).

Dual energy x-ray absorptiometry (DEXA) has been widely used to diagnose osteoporosis in humans (Adams, 2013). Use of DEXA as an accurate tool for measuring bone mineralization in chickens has been validated both in vivo and in vitro (Onyango et al., 2003; Schreiweis et al., 2003; 2004; Hester et al., 2004). As examples, both BMD and

BMC (from DEXA scans) were highly correlated with bone ash weight, with correlation coefficients of 0.77 and 0.94, respectively ($P < 0.0001$, Hester et al., 2004).

1.7 Stress Response

Animals can adapt to their environments with physical and behavioral changes in order to maintain physiological and psychological homeostasis. However, stimulation, beyond an animal's adaptive capability, leads to stress causing bio-functional changes and pathological symptoms (Thaxton, 2004). Animals can be stressed from uncomfortable experiences such as fear, hunger, isolation, pain, transportation, and or abrupt changes in their living environment (Zulkifli and Han, 2010). There are genetic variations in the capability of chickens to adapt their environments. For example, broilers adapt more fast to a novel environment than laying hens and red jungle fowl (Zulkifli and Han, 2000; Saito et al., 2004; Tomonaga and Noda, 2007).

Stress stimulates the activity of the hypothalamic-pituitary-adrenal (HPA) axis in chickens, which regulates releasing of corticotrophin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) (Gomez et al., 1996; Bakshi and Kalin, 2000; Ehlert et al., 2001). In general, when homeostasis in chickens is disturbed by a stressor, CRH is released from the hypothalamus. The released CRH stimulates the release of ACTH from the pituitary gland. Ultimately, ACTH stimulates corticosterone (CORT) released from the adrenal glands (Puvadolpirod and Thaxton, 2000a; Ehlert et al., 2001). In addition, catecholamines [dopamine (DA), epinephrine (EP) and norepinephrine (NE)], as neurotransmitters and hormones, are also involve in the stress response. Increasing

plasma catecholamines, such as DA and EP, are closely related to emotional stress such as fearfulness and pain in chickens (Torrellas et. al., 1981; Sanchez et al., 2003). Numerous studies have been conducted to test the effects of various housing environments on the stress response with many showing no effect on plasma CORT (Barnett et al., 2009; Pohle and Cheng, 2009b; Tactacan et. al., 2009). In addition, caged hens as compared to hens kept on littered floor had shown higher (Sherwin et al., 2010), lower (Gibson et. al., 1986), or no change in plasma CORT concentrations (Craig and Craig, 1985). The conflicting data from different investigations could be related to different, name a few, species, genetic strains, and stressors used as well as duration and frequency of stressor presentation.

Chronically elevated glucocorticoids cause a redistribution of reserved fat, an increase in central abdominal fat accumulation (MacFarlane et. al., 2008). Stress also causes lipid accumulation in the liver (Puvadolpirod and Thaxton, 2000a). The liver, rather than adipose tissue, is the major site of fatty acid synthesis in chickens (Leveille, 1969). Increasing fat deposit in the liver leads to fatty liver syndrome, which is commonly seen in various species, especially in caged layers (Scheele, 1997; Jiang et al., 2013). Fatty liver is a chronic disease, which affects hen health and results in a reduction in egg production (Butler, 1976). A change of fat metabolism may affect skeletal health in laying hens as well (Liu et al., 2004; Jiang et al., 2013).

Heterophils, known as neutrophils in mammals, are the most abundant granulocytes in response to acute inflammation in avian species (Harmon, 1998). Like neutrophils in mammals, the main role of the heterophils is to engulf and destroy foreign materials

through phagocytosis at the inflammatory lesion site (Montali, 1988). Lymphocytes are the largest population among all types of circulating leukocytes in birds. There are 2 types of lymphocytes: T- and B-cells. In immature avian species, lymphocytes are concentrated in the central lymphoid organs and tissues, such as the thymus and the bursa of Fabricus (Sharma, 1991).

Heterophil (H) to lymphocyte (L) ratio (H: L), as a common immunological stress indicator in birds, has a correlation with elevated ACTH and other stress-associated immune parameters such as the changes in adrenal gland and spleen weight (Beuving et. al., 1989; Mumma and Thaxton, 2006; Puvadolpirod and Thaxton, 2000a,b). Gross and Siegel (1983) reported an increase in circulating numbers of H with a concomitant decrease in L; and therefore, an increase in the H: L ratio in the stressed fowl. Numerous studies have used H: L ratios to evaluate an animal's stress response (Al-Murrani et al., 1997; Puvadolpirod and Thaxton, 2000a). An increase in the H: L ratio occurred in laying hens housed in conventional cages compared to hens kept in enriched cages (Tactacan et al., 2009). Campo et al. (2008) reported that chickens housed in deep litter with access to an outdoor area had a lower H: L ratio as compared to those without outdoor access. In addition, H: L ratio, as well as other immune responses, in chickens is affected by multiple environmental factors such as housing complexity (Singh et al., 2009), rearing condition (Moe et al., 2010), lighting schedule (Ertas and Sahin, 2002), and room temperature (Mashaly et al., 2004; Campo et al., 2008).

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CHAPTER 2. EFFECT OF FLOOR PENS VS. CONVENTIONAL CAGES ON HEN WELFARE

2.1 Introduction

Approximately 95% of commercial eggs in the United States and 90% of the eggs in the world are produced by hens housed in conventional cages (UEP, 2010). Conventional cages which are barren without furnishment, such as a nest, perches, and scratch pad, have been widely used since the 1950s. They have several benefits for hen welfare such as a low level of aggression and cannibalism because of the small group size, low mortality, high egg production, and clean eggs (Appleby, 1998; Rodenburg et al., 2005; Tactacan et. al., 2009). However, conventional cages have also been criticized for its negative effects on hen welfare such as lack of freedom in movement, other behavioral restrictions such the inability to perch or nest, and skeletal health issues such as osteoporosis (Wilkins et al., 2004; Fleming et al., 2004, 2006; Tactacan et. al., 2009; Silversides et. al., 2012). In addition, there is a considerable body of physical, morphological, and behavioral evidence that the conventional cages results in greater stress in housed hens as compared with those kept in alternative housing systems such as enriched floor pens because hens cannot exhibit many of their natural behaviors (Tactacan et. al., 2009). Numerous studies have shown that alternative housing systems that provide for more hen space allowance and greater opportunity for hens to exercise

have improved bone strength (Valkonen et al., 2010; Silversides et. al., 2012), but unfortunately these non-caged hens have more bone breaks due to crash landings and bumping into objects (Blokhuis et al., 2007).

The standards and guidelines for improving commercial hen welfare have been established in the European countries (Directive, 1999/74/EC). As of 2012, all traditional conventional cages were replaced by enriched cages (with a perch, nest, nail trimmers, and scratch pad) or alternative housing systems such as floor pens or aviaries. In the United States, the conventional cage system is still acceptable, but in 2008, the United Egg Producers (UEP) started a voluntary certification program to implement the floor space at a minimum of 430 cm^2 per hen for White Leghorn hens and 490 cm^2 for hens that lay brown eggs. There are a number of recent studies comparing hen welfare in different housing systems (Barnett et al., 2009; Pohle and Cheng, 2009; Tactacan et al., 2009; Sherwin et al., 2010; Lay et al., 2011). Compared with conventional cages, furnished cages (Pohle and Cheng, 2009; Tactacan et al., 2009) and floor pens (Sherwin et al., 2010) provide more space for hens with an increased opportunity to perform their natural behaviors and reduce social stress. In non-cage systems, the birds have more space as individual and as a group and have ample opportunities to avoid negative social interactions. Furthermore, functional areas such as perches, nests and litter are more separated in aviary systems than in cages, so that birds that perform different types of behavior do not disturb each other (Rodenburg and Koene, 2007).

My hypothesis is that littered floor pens enriched with nests and a perch as compared to conventional cages will increase egg production, bone mineralization, and reduce social

stress by providing hens more space, freedom of movement, and increase in exercise. The objective of this study was to examine the effect of floor pens vs. conventional cages on hen welfare traits in Bovans Brown laying hens from 19 to 26 wk of age.

2.2 Materials and methods

2.2.1 Birds and managements

Bovans Brown hens were reared at a pullet farm (Organic Valley, LaFarge, WI) following the organic management protocol of Centurion Poultry, Inc. (Lexington, GA). The beaks of chicks were trimmed at 7 d of age using hot blade beak trimming (UEP, 2010). At 18 wk of age, 100 hens were transferred to the Purdue University Poultry Research Farm and kept in conventional cages or furnished floor pens for 1 wk to adapt to their new environment. The experiment began when pullets were 19 wk of age and was terminated 8 wk later when hens were 26 wk of age. A non-organic laying hen diet (2,654 kcal/kg of ME, 16.6% CP, 3.8% crude fat, 3.85% Ca and 0.26% available P) and water were provided for ad libitum. Light cycle was begin at 14: 10 (L: D) at 18 wk of age and increasing by 1/2 hour each week to 16:8 (L: D), and the room temperature was maintain at an average of 22^{o}C throughout the experiment period. The experimental protocol was approved by the Purdue Animal Use and Care Committee (PACUC Number: 02-010-11).

2.2.2 Housing:

At 19 wk of age, 84 of the 100 hens were used based on their BW and physical appearance. The hens were randomly assigned to 12 cages (51 cm × 38 cm, L × W) each containing 2 hens (n=12) or 6 floor pens (2.44 m× 1.52 m, L× W) with 10 hens each (n=6). Each conventional cage provided 968 cm^2 of floor space and 18.9 cm^2 of feeder

space per hen with two nipple drinkers. The floor pens were furnished with one wood perch (1.52 m × 6.3 cm × 3.8 cm, L × H × W), one nest boxes (58.42 cm × 29.21 cm × 40.64 cm, L × H × W), and wood shavings (5 cm deep), providing 3,711 cm^2 of floor space, 11.2 cm of drinker space, 12.7 cm of feeder space, 237.4 cm^2 of nest allowance and 15.2 cm of perch space per hen.

2.2.3 Production Parameters and Egg Quality:

Two randomly selected hens per pen and one per cage were weighted and recorded at the beginning and the end of the experiment. Eggs were collected and recorded daily. The hen-day egg production per pen was calculated as (number of eggs produced per pen during the 7 days / number of live hens per pen at the same period) x 100. The d the first egg was laid for each cage or floor pen was recorded. The d when 50% egg production (total eggs divided by hen number x 100) was achieved for each cage or floor pen was also recorded. Four eggs were collected randomly from each floor per and all eggs laid by hens in cages were collected for 2 consecutive days at 22, 24, and 26 wk of age. Egg weight, shell weight, and shell thickness were measured as described by Klingensmith and Hester (1985). The length (maximum height) and width (maximum diameter) of each egg was measured in triplicate with a vernier micrometer (Coolant Proof Micrometer Series 293, Mitutoyo, U.S.A.) and averaged for each egg (Narushin et al., 2003); the shape index of each egg was calculated (width/length x 100). Egg shells collected at 26 of age were ashed at 600 ^oC for 6 h, and shell Ca was determined via atomic absorption spectrometry (Association of Official Analytical Chemists, 1990; Perkin-Elmer, 1992).

2.2.4 Physical and Physiological Sampling:

At 26 wk of age, 12 hens from floor pens (2 hens/pen) and 12 hens from conventional cages (1 hen/cage) were randomly sampled. The hens were sedated using an intravenous injection of sodium pentobarbital (30mg/kg of BW), and then a 10 mL blood sample was collected from each hen by cardiac puncture. Individual BW was determined followed cervical dislocation. The liver and abdominal fat pad were collected and weighed. The liver was dried at 105 ^{o}C for 24 h (Bax et al., 2012). Relative weights of the liver and abdominal fat pad mass were calculated using the following formula: weight of the liver (g) or abdominal fat pad (g)/BW (kg). Liver fat was determined using the Soxtec Extraction Unit (Bax et al., 2012; Jiang et al., 2013).

Feather conditions of the neck, breast, back, wings, vent and tail were measured using a 1 to 4 point scoring system, with 1 being the worst condition and 4 representing the best condition with few worn or otherwise deformed feathers (Tauson et al., 2005; Fahey and Cheng 2008; Hester et al., 2012). The foot pad and all toes were scored for hyperkeratosis condition using a 1 to 4 scoring system of Tauson et al. (1984). A score of 1 represented severe hyperkeratosis with deep and large epithelial lesions of the foot-pad and toes, while a score of 4 represented healthy feet and toes with no lesions (Tauson et al., 1984). The scores of both feet and feathers were averaged for each hen. The 8 nail lengths of each hen were measured by a flexible measuring tape, and a mean of the measures were calculated per hen.

2.2.5 Bone Quality Traits:

The right wing, thigh, and drum were collected from sampled hens at 26 wk of age. The right humerus, femur, and tibia were scanned using dual energy x-ray absorptiometry (DEXA) to quantitate bone mineral density (BMD) and bone mineral content (BMC, Onyango et al., 2003; Schreiweis et al., 2003, 2004; Hester et al., 2004). Following the DEXA scans, all soft tissues together with the cartilage cap were removed from the bones. The diaphysis was separated from the 2 epiphyses of each bone. All three parts (2 epiphyses and 1 diaphysis) were dried, defatted, and ashed at 600 ^{o}C for 6 h (Association of Official Analytical Chemists, 1990, method 972.15; Angel et al., 2005; 2006). Based on previous studies, bone ash is positively correlated to BMD and bone breaking strength (Zhang and Coon, 1997; Hester et al., 2004), the same parameter was analyzed in the current study. Bone ash P and Ca concentrations were measured by spectroscopy (Rounds and Nielsen, 1993; Newkirk and Classen, 1998) and atomic absorption spectroscopy (Perkin-Elmer, 1992), respectively.

2.2.6 Heterophil to Lymphocyte Ratio

The heterophil to lymphocyte (H: L) ratio was determined from 2 blood smears per hen at 26 wk of age (Houwen, 2000). Blood smear slides were air dried and stained with Wright's stain (Walberg, 2001; Fahey and Cheng, 2008; Felver-Grant et al, 2012). One-hundred leucocytes, including granular (heterophil, eosinophils, basophils) and nongranular (lymphocytes, monocytes), were counted from each slide (i.e., 200 white cells per bird) using oil immersion microscopy with 4,000 X magnification. The

heterophil to lymphocyte ratio was calculated by dividing the total number of heterophils by lymphocytes per bird.

2.2.7 Statistical Analysis

Data from the completely randomized design was subjected to an ANOVA (Steel et al., 1997) using the MIXED model procedure of SAS Institute (2008). The treatment of housing system was considered a fixed effect. An ANOVA with repeated measures over the age of the hen was used for body weight, egg production, egg weight, egg size parameters, shell thickness, % shell, the ash P and Ca concentration. A one-way ANOVA was performed on relative fat weight and abdominal fatpad weight. The BW was used as a covariate for BMD and BMC. The Tukey-Kramer was used to differentiate means for age by treatment interactions. The variability of least square means was reported as the SEM. The probability level for statistical difference is at $P < 0.05$.

2.3 Results

2.3.1 Production Parameters and Egg Quality

Daily egg production was not different between hens housed in conventional cages and floor pens ($P = 0.70$, Table 1). The first egg laid was earlier for hens of the floor pens than the caged hens ($P = 0.02$), while type of housing did not affect the age when 50% egg production was achieved ($P = 0.09$, Table 1). The cumulative egg size traits of length, width, and the shape index measured over 3 ages were not different between hens housed in floor pens as compared to cages, while % shell and shell thickness of eggs were poorer in cages than the floor pens ($P = 0.002$ and 0.02, respectively, Table 1). The egg weight was higher in caged hens than that of hens housed in the floor pens at 22 wk of age with

no differences at 24 and 26 wk of age ($P = 0.04$, treatment by age interaction, Figure 1). No difference was observed in the % egg shell ash and shell Ca concentrations between eggs laid by hens in the 2 housing systems (data not presented, $P = 0.48$ and 0.21, respectively).

2.3.2 Physical and Physiological Parameters

One bird housed in the floor pen was sick and was removed from the study at 20 wk of age. The BW at 19 wk ($P = 0.09$) and 26 wk ($P = 0.44$) of age and the liver weight ($P = 0.92$) were not affected by treatment; however, abdominal fat pad weight ($P = 0.04$) and liver fat content ($P = 0.01$) were higher in caged hens than those kept in floor pens (Table 2). Hens' plumage and hyperkeratosis scores were similar between treatments (Table 3). Nail lengths were longer ($P < 0.0001$) in caged hens than hens housed in floor pens (Table 3). Hens housed in cages had a greater H: L ratio than that of hens housed in floor pens ($P = 0.002$, Table 3).

2.3.3 Bone Quality Traits:

Hens kept in floor pens had greater BMD and BMC than hens housed in cages at 27 wk of age (Table 4, humerus, $P < 0.0001$; femur, $P = 0.01$; and tibia, $P = 0.002$). Dried femur and tibia weights were similar between hens of the 2 housing systems; however, the dried humerus weight retrieved from hens kept in floor pens was heavier than those from caged hens ($P = 0.02$, Table 4). In addition, the % ash of the humerus and tibia was higher in the floor pen hens ($P = 0.03$ and $P = 0.01$, respectively; Table 6). Differences in bone P concentrations was observed in the diaphysis of the tibia only with a higher percentage in

the hens from floor pens ($P = 0.007$, Table 6). There was no difference in % Ca in the bone ash of hens from the 2 treatments (Table 6).

2.4 Discussion

There was no difference in egg production between the hens from cages and floor pens even though the floor pen hens had 3.8 times more floor space than caged hens. In the current study, floor pens were ineffective in improving egg production, which is opposite to our hypothesis that, compared to caged hens, egg production will be improved in the hens kept in floor pens as which provide hens with more space and opportunities to display natural behaviors. One of the reasons could be the study was too short, an 8 wk trait, to exhibit the effect of housing environment on egg production. Previous research with conventional cages has shown that hens respond favorably to increase space by increasing the number of eggs laid (Adams and Craig, 1985; Davami et al., 1987). Perhaps, another reason for a lack of response in egg production between hens housed in cages vs. floor pens in the current study was the generous space offered to the hens. The caged hens in the current study received almost double size of the space per hen recommended by the poultry industry (United Egg Producers, 2010). With White Leghorns in conventional cages, there is a point where providing space allocation above 554 cm^2 (86 in^2) does not result in further improvement in egg production (Bell et al., 2004). However, less information is available on the effect of caged space allocation on egg production in brown hybrids. Nevertheless, the 968 cm^2 space allocation per caged Bovans Brown hen used in the current study was most likely exceeded any predicted the

point whereby egg production is no longer increased by providing more space. Similarly, studies on space allocation needs of floor pen hens are lacking.

Some studies have reported that egg production is relatively higher in the conventional cages than littered floor pen (Abrahamsson et al., 1996) and aviary systems (litter-Lövsta with two tiers and Marielund with three tiers) (Tauson et al., 1999). However, the similar egg production has also been found between floor pen system and conventional cages (Anderson and Adams, 1994; Singh et al., 2009).

Similar to the egg number, egg size i.e., egg weight and length and width, was unaffected by the housing system. In contrast, an increase in egg weight noted at 22 wk of age in caged hens as compared to hens kept in floor pens, which was most likely due to larger caged hens at the beginning of the study (caged hens vs. floor pen hens at 19 wk of age, 1,821 g vs. 1,699 g, $P = 0.09$). By 26 wk of age, the differences in BW between caged and floor pen hens had dissipated ($P = 0.44$, Table 2) with no differences in egg weight. It is well known that hen BW is positively correlated with egg weight (Siegel, 1962; Festing and Nordskog, 1967; Di Masso et al., 1998).

In the current study, BW was not affected by housing environment. In contrast, some of previous studies reported that hens' BW is affected by housing designs. For example, Sherwin et al. (2010) reported that hens from the conventional cage were the heaviest compared to those kept in enriched cages, barn, and free range systems. Taylor and Hurnik (1994) found aviary hens were significantly lighter than those in cages. However, Singh et al. (2009) reported that birds kept on the floor were heavier than caged birds.

In the current study, the floor pen hens have earlier onset of egg laying (i.e., d of 1st egg) than the caged hens, which was considered as an early sexual maturity. The reason for hens kept in floor pens reached sexual maturity earlier than caged hens is unclear, but it could be related to less stress. It is well known that stress causes circulating lymphocytes to decrease relative to heterophils leading to a higher H:L ratio in stressed chickens (Gross and Siegel, 1983; McFarlane and Curtis, 1989). In the current study, hens in floor pens as compared to caged hens had a lower H: L ratio at 26 wk of age. However, no H: L ratio measurements were made at the beginning of the study when hens were coming into egg production to verify if floor pen hens were actually experiencing less stress at this earlier age. The effect on sexual maturity was short–term and did not persist to age at 50% lay as there were no differences in egg production in hens between housing systems a few days later. A further study is needed to evidence if floor pens cause less stress in Brown hens than conventional cages.

As shown in current study, shell traits were of lower quality for caged hens as compared with hens in floor pens, similar to the results of Singh et al. (2009) who reported increased egg shell weight and shell thickness for hens of floor pens as compared to conventional cages. The increased activity and exercise of hens in floor pens as compared to cages perhaps led to more effective intestinal absorption, transport, and uterine utilization of Ca during shell formation. It concur the statement of Abrahamsson et al. (1998), who observed higher classified aviary eggs as having greater shell thickness and greater percent of shell compared with eggs from conventional cages. Hughes et al. (1985)

also found free-range eggs had greater shell thickness and stronger shells compared with conventional cages.

Hens kept in conventional cages had a higher abdominal fat pad weight and liver fat than those housed in floor pens at 26 wk of age, creating the potential for caged hens to develop fatty liver syndrome later in life. Hens that accumulate excessive fat deposits in the liver are unable to export liver lipoprotein to adipose and other organs (Butler, 1976). It is not uncommon for the fat content of livers to be as high as 70% but is more typically at 40% of dry matter in hens with fatty liver syndrome (Whitehead, 1979). Fatty liver decreases egg production and can cause sudden death in hens when the liver capsule ruptures. Necropsy results show a hemorrhagic liver that is yellow, friable, soft, and enlarged (Butler, 1976). Similar to the current findings, Garlich et al. (1975) reported that laying hens housed in conventional cages were at higher risk for developing fatty liver disorder than those kept on littered floor. Hens on littered floor of the current study had 3.8 times more space than conventionally caged hens providing these cage-free hens with opportunities for running, walking, scratching, foraging, and flapping of wings to minimize fat deposition, and perhaps reduced stress levels as indicated by lowered H:L ratios in floor as compared to caged hens. Chronic stress in chickens can cause lipid accumulation in organs such as the liver (Puvadolpirod and Thaxton, 2000). In addition, stress causes elevation of glucocorticoid hormones which can trigger redistribution of reserved fat, increasing central abdominal fat accumulation (MacFarlane et al., 2008). The perfect feather scores of hens in the current study for hens in cages and floor pens were expected as the hens were still early in their egg production cycle and were not old

enough for feathers to become worn or torn. In addition, there were no feather pecking problems with either housing system of the current study to worsen feather scores as pulled feathers from severe pecking which can adversely affect plumage quality (Sherwin et al., 2010). In addition, as hens age, plumage quality deteriorates due to the hen's body rubbing on furnishments like a perch or the wired side partitions of cages (Tauson et al., 1984; Hester et al., 2012).

Similar to the plumage condition, type of housing did not affect hyperkeratosis scores at 26 wk of age perhaps due to the young age of the hens when they were scored and the excellent litter quality that was maintained in the floor pens during the 8 wk treatment. The near perfect scores for hens in cages and floor pens of the current study were indicative of excellent foot health. Hyperkeratosis is characterized by hypertrophy of the corneus layer of the skin on both toes and the footpad. The incidence of hyperkeratosis increases with time spent grasping a perch or standing on caged wired floors because of increased compression on the toes and footpad (Weitzenbürger et al., 2006, Enneking et al., 2012). In addition, too much moisture and ammonia in the litter causes inflammation of the foot-pad and toes leading to hyperkeratosis and foot-pad dermatitis (Wang et al., 1998; Dawkins et al., 2004). Similar to our result, Tauson et al. (1999) reported excellent foot scores for hens at 35 wk of age with no differences due to type of housing, i.e., floor pen, conventional cage, and aviary; however, by 55 wk of age, hens in conventional cages had poorer foot scores.

Nails of 26-wk-old hens were longer in caged hens than those housed in floor pens, which is in agreement with previously published results (see the review of Glatz, 2002).

Conventionally caged hens are unable to trim their nails as effectively as hens in non-cage housing due to lack of litter or scratch areas. Excessive nail growth occurs if hens do not have access to abrasive materials for trimming their nails such as a scratch pad or nail trimmers in furnished cages (Appleby et al., 1993; Abrahamsson and Tauson, 1997, Glatz et al., 2002). Nails that are excessively long can break more easily, leading to open wounds and greater susceptibility to infection.

One of the major concerns of hens kept in conventional cages is the impact of level of inactivity on skeletal health. Lack of exercise impairs skeletal integrity in hens leading to severe loss of bone mineralization and strength causing hens to be highly susceptible to bone fracture during egg laying (Whitehead and Fleming, 2000; Vits et al., 2005). Bone mineralization from DEXA scans is positively correlated to bone breaking strength (Schreiweis et al., 2005) and negatively correlated to fracture incidence (Mazucco and Hester, 2005). In this study, the increased weight of the humerus, the % ash of the epiphysis of the tibia and humerus, the bone mineralization of the humerus, femur, and tibia in 26 wk-old-hens housed in floor pens as compared with those kept in conventional cages are all indicators of improved skeletal health. The stimulus of walking, running, wing flapping, scratching, and jumping on and off the perch contributed to the improved skeletal integrity of the hens in littered floor pens. Similar results by Silversides et al. (2012) showed that hens kept in floor pens had greater bone density than that of caged hens.

Stress associated with housing environment is an important factor for assessing animal welfare. The H: L ratio has been used as an immunological response parameter and stress

indicator in animal research including poultry (Gross and Siegel, 1983; Shini et, al., 2010). For example, Felver-Gant et al. (2012) reported that H: L ratio increased in hens followed heat stress. In addition, an increase in H:L ratios occurred in hens following social stress (Craig and Muir, 1996; Hester et al., 1996). Hens housed in conventional cages had a higher H: L ratio compared with free-ranged hens (Shini, 2003; Moe et al., 2010) and hens in modified cages (Shini, 2003). The higher H: L ratio of 26-wk-old caged hens of the current study may indicate that conventional cages caused more stress than floor pens. If caged hens were more stressed, it did not affect their reproductive abilities as far as egg production was concerned as the number of eggs laid which was similar between housing systems. However, shell deposition, which is an integral component of avian reproduction, was negatively affected by conventional cages as indicated by thinner shells and less % shell of eggs laid by caged hens as compared to the eggs of hens in floor pens. Glucocorticoids released by the adrenal glands in response to stress can impair calcium utilization (Siegel, 1995).

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Table 2.1 The effect of floor pens vs. conventional cages on egg production, egg size parameters, and shell quality traits of Bovans Brown laying hens

Item	Hen-day egg production (%)	1st egg (d)	50% egg production (d)	Egg weight (g)	Egg length (mm)	Egg width (mm)	Egg shape index[1]	Shell (%)	Shell thickness (mm)
Floor pen	65.19	136[b]	144.8	59.9	56.5	43.7	77.47	9.86[a]	0.38[a]
Conventional cage	64.13	142[a]	143.5	60.1	56.3	43.7	77.62	9.73[b]	0.37[b]
SEM	0.03	2	0.7	0.5	0.5	0.5	0.3	0.04	0.002
n[3]	56	9	9	130	130	130	130	130	130
P-value	0.70	0.02	0.09	0.19	0.29	0.57	0.75	0.002	0.02

[a-b] Least square means within floor pens and conventional cages lacking common superscript differ ($P < 0.05$).
[1]Shape index = egg width/ egg length x 100.
[2]Values represent the least square means averaged over 56 days of egg production.

Table 2.2 The effect of floor pens vs. conventional cages on BW, fat pad weight, liver weight, and liver fat of Bovans laying hens at 26 wk of age.

Item	BW (g)		Relative abdominal fat pad mass (g/kg)	Relative dry liver mass (g/kg)	Liver fat content (%)
	19 wk of age	26 wk of age			
Floor pen	1,699	1,851	33.7[b]	26.2	23.5[b]
Conventional cage	1,821	1,910	42.1[a]	25.7	34.0[a]
SEM	60	59	2.7	0.9	2.8
n[2]	12	12	12	12	12
P-value	0.09	0.44	0.05	0.77	0.01

[a-b]Least square means within the floor pen and conventional cage housing systems lacking common superscript differ ($P < 0.05$).
[2]Average number of observations per least square mean during the 8 wk of the experiment.

Table 2.3 The effect of floor pen vs. conventional cages on feather score, hyperkeratosis score, nail length, heterophil to lymphocyte ratio of Bovans Brown laying hens at 26 wk of age.

Item	Mean Feather Score[2]	Mean Hyperkeratosis Score[3]	Mean nail length (cm)	Heterophil	Lymphocyte	Heterophil:Lymphocyte Ratio
Floor Pen	4	3.88	0.87[b]	19.7[b]	73.8[a]	0.27[b]
Conventional cage	4	4.00	1.11[a]	22.0[a]	71.9[b]	0.31[a]
SEM	-	0.06	0.02	0.7	0.6	0.01
n[1]	-	12	12	12	12	12
P-value	-	0.16	< 0.0001	0.002	0.04	0.002

[a-b] Least square means within the conventional cage system and the floor pen environment lacking common superscript differ ($P < 0.05$).

[1]Average number of observations per least square mean during the 8 weeks

[2]Scores for feather condition ranged from 1 to 4, with 1 being the worst condition and 4 representing the best condition.

[3]Score for foot hyperkeratosis ranged from 1 to 4, with 1 represented severe hyperkeratosis and 4 represented healthy feet.

Table 2.4 The effect of floor pen vs. conventional cages on Bone Mineral Density and Bone Mineral Content of Bovans Brown laying hens at 26 wk of age.

	Density			Content		
Item	Humerus	Femur	Tibia	Humerus	Femur	Tibia
	g/cm^2			g		
Floor Pen	0.19^a	0.24^a	0.23^a	1.74^a	2.72^a	3.34^a
Conventional cage	0.15^b	0.20^b	0.19^b	1.38^b	2.20^b	2.68^b
SEM	0.01	0.01	0.02	0.01	0.01	0.01
n[1]	12	12	12	12	12	12
P-value	< 0.0001	0.01	0.002	< 0.0001	0.01	0.001

[a-b] Least square means (adjusted for BW) within the conventional cage system and the floor pen environment lacking common superscript differ ($P < 0.05$).

[1]Average number of observations per least square mean during the 8 wk of the experiment.

Table 2.5 The effect of floor pens vs conventional cages on bone weight, ash, P and Ca of Bovans Brown laying hens at 26 wk of age.

Item	Bone weight (g)	Bone ash (%)	Bone P (%)		Bone Ca (%)	
			Diaphysis	Epiphysis	Diaphysis	Epiphysis
Tibia						
Floor pen	6.67	47.18[a]	11.1[a]	10.8	35.7	35.3
Conventional cage	6.53	42.01[b]	10.6[b]	10.4	36.6	34.2
SEM	0.23	0.01	0.1	0.3	0.7	1.7
n	12	12	12	12	12	12
P-value	0.69	0.03	0.01	0.25	0.21	0.54
Femur						
Floor pen	5.49	48.04	10.7	10.5	35.3	35.1
Conventional cage	5.28	42.81	10.5	10.8	37.1	35.6
SEM	0.22	0.02	0.2	0.3	1.1	1.1
n	12	12	12	12	12	12
P-value	0.52	0.09	0.36	0.28	0.12	0.62
Humerus						
Floor pen	3.11[a]	57.84[a]	10.7	10.4	35.6	36.0
Conventional cage	2.62[b]	55.06[b]	10.6	10.5	37.0	34.7
SEM	0.18	0.01	0.3	0.2	1.0	1.7
n	12	12	12	12	12	12
P-value	0.02	0.03	0.63	0.70	0.22	0.49

[a-b] Least square means (adjusted for BW) within the conventional cage system and the floor pen environment lacking common superscript differ ($P < 0.05$).

[1]Average number of observations per least square mean during the 8 wk of the experiment.

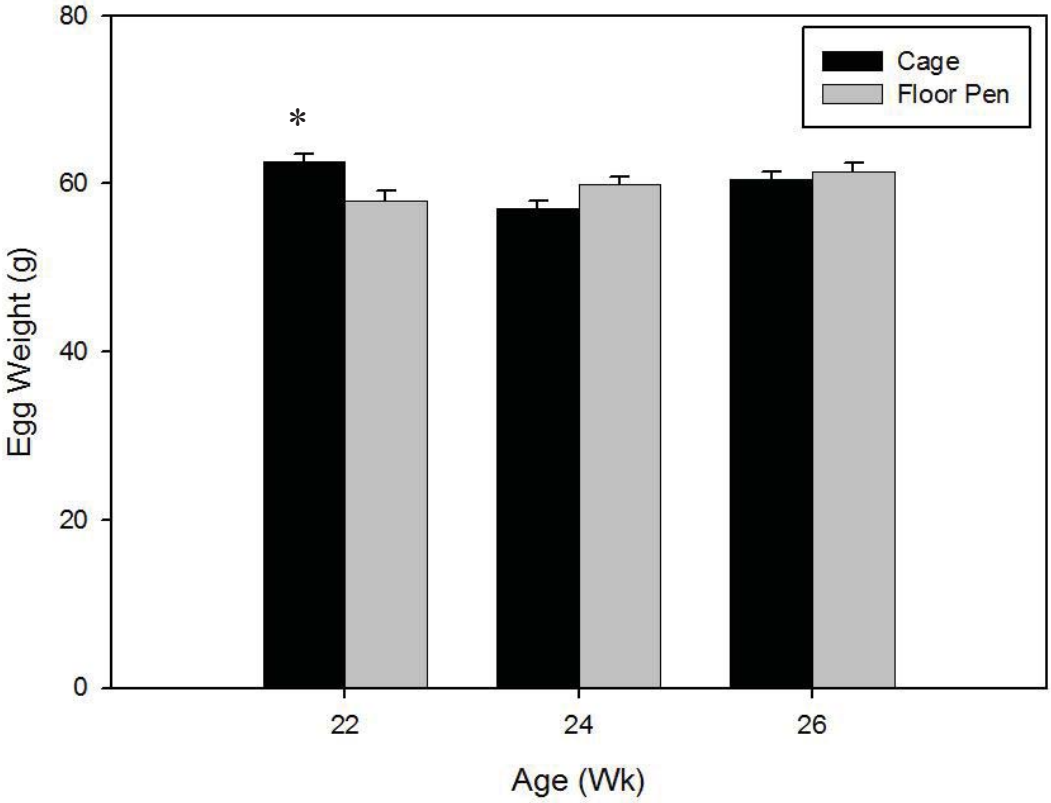

Figure 2.1 The effect of housing environments on egg weight of Bovans Brown laying hens at 22, 24, and 26 wk of age. Values are least square means ± SEM. The asterisk (*) indicates a difference in egg weight ($P = 0.04$) between the eggs laid in conventional cages as compared to floor pens at 22 wk of age.

CHAPTER 3. EFFECT OF CALCIUM-DEFICIENT DIET ON HEN WELFARE

3.1 Introduction

Calcium (Ca) and phosphorus (P) are the 2 most widely distributed minerals in chickens' body. Although bones serve as a mineral reservoir in chickens, retrieval of Ca and P from the diet plays an essential role in maintaining biological homeostasis. Calcium, for example, is in a great demand for egg shells of laying hens (Graveland and Berends, 1997; Vries et al., 2010). The NRC (National Research Council, 1994) estimates that the Ca requirement for brown laying hens is 36 g/kg of feed and 35 - 38 g/kg of feed for White Leghorn hens based on 110 g of feed consumed/hen/day.

Egg shell formation places a huge demand of Ca on hens. Each egg shell contains approximately 2 g of Ca, which is equivalent to about 10% of total body Ca (Taylor, 1970). Currently, a commercial hen lays about 300 eggs per year. At this level of production, a hen secretes a great quantity of Ca into egg shells, which is equivalent to more than 20 times of its body's total level of Ca (Graveland and Berends, 1997). The metabolism and turnover of Ca in egg laying strains of chickens are extraordinarily high compared to other vertebrae animals.

Dietary Ca is highly correlated to bone mineralization in animals, especially in laying hens (Schreiweis et al., 2003; Whitehead, 2004; Fleming, 2008; Vries et al., 2010). Hens have a unique bone remodeling pattern among the 3 types of bones, medullary, cortical,

and cancellous, which is associated with the sexual mature process and the egg laying cycle (van de Velde et al., 1985; Kim et al., 2007, 2012). As laying hens approach sexual maturity, the amount of medullary bone is quickly increased under estrogen stimulation (Fisher and Schraer, 1982) as a labile source of Ca during egg shell formation (Whitehead, 2004). In general, dietary Ca is the major source for egg shells. Hens are capable of maintaining physiological homeostasis and egg production for a short period of time under a suboptimal nutrient condition (Vries et al, 2010). However, hens' reproductive ability is disturbed if the Ca deficiency is prolonged, resulting in a decrease in egg production and egg quality, such as producing soft-shelled and cracked eggs, and osteomalacia subjecting hens to a great susceptibility to bone fracture (Bar, 2008; Vries et al., 2010; Kim et al., 2012). Under the situation of a deficiency of dietary Ca, hens resort to using Ca reserved in bones during egg shell formation, in particular, in medullary bone (Whitehead and Fleming, 2000; Fleming, 2007). Any malfunction in Ca metabolisms, such as impaired absorption from the gastrointestinal tract, bone remodeling, and Ca transport and utilization within the shell gland of the oviduct can lead to deterioration in the quality of egg shells. When bone resorption becomes excessive, the volume of structural bone is reduced, causing skeletal fragility, osteomalacia, in hens (Clunies et al., 1992; Whitehead and Fleming, 2000; Budgell and Silversides, 2004; Julian, 2005; Fleming et al., 2006; Fleming, 2008; Vries et al., 2010; Cufadar, 2012).

Numerous studies have examined the effects of dietary Ca on the quality of egg shells (Safaa et al., 2008; Neijat et al., 2011; Cufadar, 2012). Egg shell quality has been used as an important indicator for evaluating hens' health status and the economics of egg production. Bain (1997) reported that up to 8% of all eggs were broken or damaged

during the handling from producers to consumers, which could be, as least in part, due to thin egg shells. The quality of egg shells can be reduced in hens fed a Ca-deficient diet (Roberts et al., 2004).

Pre-lay diets (around 1% Ca) have been used in pullets for about 2 wks prior to lay first egg (Commercial Management Guide of Hy-Line, 2011) or before reaching 2% lay (ISA Brown Commercial Management Guide of Hendrix Genetics Company, 213) for stimulating the development of medullary bone. It is unknown the impact on egg production if pullets are fed a pre-lay diet longer than 2 wks during the sexual maturing process; especially, its effects on the stress response system as that hens can tolerant a short-term of suboptimal nutrient condition. Therefore, the objective of this study was to determine the effect of a short-term of Ca-deficient diets on egg production, bone mineralization, stress response, and behavior of Bovans Brown hens during sexual maturity.

3.2 Material and methods

3.2.1 Birds, Treatment, and Management

A total of 120, 20-wk-old beak-trimmed organically reared Bovans Brown hens (Organic Farm Supplies, Cashton, WI) were transported to the Purdue Poultry Farm. After 2 wks of acclimation to the new housing environment, a hypocalcemic trial was initiated for 4 wks (from wk 23 to 26). The hens were randomly assigned to 2 diets, treated hens fed a Ca-deficient diet (CP = 18%, Ca = 0.6-1.1% and av P = 0.30%, CP analysis = 15%, Ca analysis = 0.98, Total P analysis = 0.53, Lebanon, IN) and a commercial layer diet (CP = 18.3%, Ca = 4.2% and av P = 0.30%, CP analysis = 18%, Ca analysis = 3.31, Total P analysis = 0.50 West Lafayette, IN) for control hens. Each treatment contained 6 floor

pens (244× 152 cm, L× W) with 10 hens per pen. The floor pens were furnished with wood shavings (5 cm deep) as bedding, one square wood perch (152 × 3.8 × 6.3 cm, L × W × H) installed at mid-length of each pen 20 cm above the littered floor surface, and one nest box (58.42 × 40.64 × 29.21 cm, L × W × H), which provided 3,709 cm^2 of floor space, 11.2 cm of drinker space, 12.7 cm of feeder space, 237.4 cm^2 of nest allowance, and 15.2 cm of perch space per hen. Feed and water were provided ad libitum. Light cycle began at 15:00 (L: D) at 20 wk of age. The photoperiod was increased by 1/2 h each wk up to 16: 8 (L: D). Room temperature was maintained at an average of 22 ^oC throughout the experimental period. The experimental protocol was approved by the Purdue Animal Use and Care Committee (PACUC Number: 02-010-11).

3.2.2 Production Parameters and Egg Quality

Eggs were collected daily. Accumulative egg production was calculated for each pen as: (hen-day egg production for the period / number of egg produced during period /number of hens-days in the period) x 100. The day of the first egg was laid in each pen was recorded. Four eggs per pen were randomly collected on 2 consecutive days to determine egg weight, shell thickness, and % shell at 22 (before treatment), 24 (2 wks after treatment), and 26 wk (4 wks after treatment) of age using the method reported previously (Klingensmith and Hester, 1985). The length (maximum height) and width (maximum diameter) of each sampled egg were measured in triplicate using a Vernier micrometer (Coolant Proof Micrometer Series 293, Aurora, IL) and averaged for each egg; the shape index was calculated afterwards (width/length x 100) (Narushin et al., 2003). Egg shells collected at 26 wk of age were ashed at 600 ^oC for 6 h, and shell Ca

was determined via atomic absorption spectrometry (Association of Official Analytical Chemists, 1990; Perkin-Elmer, 1992).

3.2.3 Behavior Observation

Behavioral observations were conducted weekly on Wednesday morning at 0900 h and Thursday afternoon at 1500 h using the 10 min scan sampling method (Mack et al., 2013). Eating, drinking, and perching behavior of all hens within a pen were recorded. The percentage of each behavior was calculated using the following formula: the number of hens eating, drinking, or perching/the number of hens in the pen x 100. For each behavior, the data collected during the 2 days were averaged. All behaviors were observed and recorded by individuals trained in observing and analyzing poultry behavior.

3.2.4 Blood and Organ Sampling

At 26 wk of age, 2 hens per pen were randomly taken for sampling. The hens were sedated using an intravenous injection of sodium pentobarbital (30mg/kg of BW), and then a 10 mL blood sample was collected from each hen by cardiac puncture. Individual BW was recorded followed by cervical dislocation. The liver and abdominal fat pad were collected and weighed. The liver was dried at 105 ^{o}C for 24 h (Bax et al., 2012). Relative weights of the liver and abdominal fat pad mass were calculated using the following formula: weight of the liver (g) or abdominal fat pad (g) / BW (kg). Liver fat was determined using the Soxtec Extraction Unit (Bax et al., 2012; Jiang et al., 2013).

3.2.5 Bone Quality Traits

The right wings and legs were collected from the sampled hens at 26 wk of age, and the right humerus, femur, and tibia were scanned using dual energy X-ray absorptiometry

(DEXA) to quantitate bone mineral density (BMD) and bone mineral content (BMC) (Onyango et al., 2003; Schreiweis et al., 2003, 2004; Hester et al., 2004).

Following DEXA measurements, all soft tissues together with the cartilage cap were removed from each bone. Each bone was subsequently cut into epiphyses (top 25% of length) and diaphysis (mid-region at 50% of length) sections (van Wyhe et al., 2012). All three parts (i.e. 2 epiphyses and 1 diaphysis) were boiled in diethyl ether for 6 h to extract fat (Bax et al., 2012; Jiang et al., 2013). After bones were dried overnight at room temperature, the defatted bones were ashed at 600 ^{o}C for 6 h. The ash weight and % ash were determined (Association of Official Analytical Chemists, 1990; Angel et al., 2006). The levels of P and Ca in bone ash were measured by spectroscopy (Rounds and Nielsen, 1993; Newkirk and Classen, 1998) and atomic absorption spectroscopy (Perkin-Elmer, 1992), respectively.

3.2.6 Heterophil to Lymphocyte Ratio

The heterophil to lymphocyte (H: L) ratio was collected from 2 blood smears per hen at 26 wk of age (Houwen, 2000). Blood smears were air dried and stained with Wright's solution (Walberg, 2001; Fahey and Cheng, 2008; Felver-Grant et al., 2012). One hundred Leucocytes, including granular (heterophil, eosinophils, and basophils) and nongranular (lymphocytes and monocytes), were counted from each slide (i.e., 200 white cells per hen) using oil immersion microscopy with 4,000 X magnification. The H: L ratio was determined by dividing the number of heterophils by lymphocytes.

3.2.7 High Performance Liquid Chromatography Assay for Plasma Catecholamines

Plasma catecholamine [epinephrine (EP) and norepinephrine (NE)] were analyzed in duplicate using a plasma catecholamine analysis kit (ESA, Inc., Chelmsford, MA, Cheng

et al., 2001; Cheng and Fahey, 2009; Felver-Gant et al., 2012). Briefly, plasma samples were acidified and deproteinzed using 100 µL of 4 M percholoric acid. Afterwards, the samples were centrifuged at 13,000 x g for 10 min at 4^{o} C. The supernatant was collected and placed on an alumina column including an internal standard, deihydroxybenzylamine. The columns were vibrated for 10 min and rinsed and eluted with the solutions supplied by the company (ESA). The samples were injected into the mobile phase (25 µM EDTA, 75 mM Na_2HPO_4, 10% CH_3CN, 1.7mM octanesulfonic acid, and 100 µL/L triethanolamine at a pH of 3, adjusted using phosphoric acid) at a flow rate of 1.3 mL/min. The EP and NE concentrations were calculated from a standard reference curve using the standards provided. Concentrations were calculated as nanograms per milliliter (ng/mL).

3.2.8 Statistical Analysis

Data from the completely randomized design was subjected to an ANOVA (Steel et al., 1997) using the MIXED model procedure of SAS Institute (2008). The effect of diet was considered a fixed effect. An ANOVA with repeated measures over the age of the hen was used for egg production, egg weight, egg shape index, shell thickness, % shell, % ash, ash P and Ca concentration, and behavior. The shell thickness and % shell of wk 22 of age were used as a baseline, the egg and shell data from 24 and 26 wk of age (i.e. 2 and 4 wks of treatment) was divided by the data from 22 wk (before treatment) of age, and the percent increased or decreased were used to determine the diet effect on these traits. A one-way ANOVA was performed on body weight, relative fat weight and abdominal fat pad weight. The BW was used as a covariate for bone mineralization. The Tukey-Kramer was used to differentiate means for age by treatment interactions. The experiment unit

was the number of pens per treatment. The variability of least square means was reported as the SEM. Statements of significance is at a $P < 0.05$.

3.3 Results

3.3.1 Production Parameters and Egg Quality

Total 1,939 eggs form the treated hens and 2,378 eggs from the control group were laid during the 4 wks experimental period. Hen-day egg production, % shell, and shell thickness were lower in treated hens compared to controls (Table 1. $P = 0.02$, $P < 0.001$, and $P < 0.001$, respectively). In addition, compared to their egg shell parameters prior to treatment (22 wk of age), treated hens but not controls had a reduced % egg shell weight (Figure 1) at both 2 wks (24 wk of age, $P < 0.0001$) and 4 wks (26 wk of age, $P < 0.0001$) during the treatment; and reduced egg shell thickness at 2 wks only (Figure 2, $P < 0.01$). There were treatment by age interactions for % egg shell weight ($P = 0.01$) and egg shell thickness ($P = 0.04$) in low Ca fed hens. However, there was no treatment by age interaction for hen-day egg production ($P = 0.49$).

There was no dietary Ca effect on day the first egg was laid (Table 1, P = 0.85). In addition, egg weight, length, width, and shape index were similar between hens fed a Ca-deficient diet and commercial diet (Table 1).

3.3.2 Physical and Physiological Parameters

Hens' BW and the relative abdominal fat pad weight were not affected by dietary treatment (Table 2); however, liver weight ($P = 0.05$) and the liver fat ($P = 0.003$) were higher in control hens than hens fed the Ca-deficient diet. The H: L ratio (Table 3), plasma concentrations of EP and NE, and the EP: NE ratio were similar between treated and control hens (data did not shown, $P = 0.79$, 0.80, and 0.69, respectively).

3.3.3 Bone Traits

At 26 wk of age, compared to control hens, treated hens had a lower BMD of the humerus ($P = 0.03$) and femur ($P = 0.04$) but not the tibia ($P = 0.07$, Table 4). The BMC of all measured bones was also lower in treated hens (humerus, $P = 0.03$, femur, $P = 0.01$, and tibia, $P = 0.02$). Dried weights of the tibia ($P = 0.03$), femur ($P = 0.02$), but not the humerus ($P = 0.77$) were lighter in treated hens compared to controls (Table 5). However, there was no treatment effects on the % ash and bone ash Ca levels but treated hens had higher levels of % P in bone ash of the femur diaphysis ($P = 0.02$) and humerus epiphysis ($P = 0.01$) .

3.3.4 Behavior

Compared to controls, treated hens exhibited less perching ($P = 0.0001$) and eating ($P = 0.01$) behaviors with similar levels of drinking (Table 6). There were no treatment by age interactions onthe observed behaviors ($P = 0.99$, 0.74, and 0.76, respectively).

3.4 Discussion

Egg production and shell quality in Bovans Brown hens were reduced when fed a Ca-deficient diet (0.98% Ca) for 4 wks during their sexual maturation, from 23 to 26 wk of age. Similar to the present findings, Roland et al. (1995) reported that egg production in hens was gradually declined during the first laying cycle as the levels of dietary Ca were reduced, ranging from 5.0 to 2.5%,. Gilbert and Blair (1974) also reported that egg production was suddenly and remarkably reduced in 32 wk-old White Leghorn hens fed Ca-deficient diets for 6 wks. In that study, hens fed a diet containing 0.05% Ca virtually ceased egg laying (production rate < 4%); while those on a diet containing 0.5% Ca maintained egg production at a level of 20 to 30%. In the current study, however, egg

production of treated hens was reduced but did not experience such a precipitous drop. The different results between the current study and reported by Gilber and Blair may be related to the differences in the levels of Ca (1% vs. 0.05 and 0.5%) and the period of consuming low Ca diets (4 wks vs. 6 wks). Similarly, Schreiweis et al. (2003) reported that egg production was not affected in White Leghorn hens fed a low Ca diet (2.1%) from 32 to 58 wk of age. In addition, Cheng and Coon (1990) and Lim et al. (2003) did not observe any effects of Ca intake on egg production in hens fed Ca ranged from 2.0 to 4.5% and 3.0 to 4.0%, for 6 wks and 30 wks, respectively.

In the current study, the low Ca diet had no effect on egg weight and egg size parameters, which is similar to the results reported previously (Hamilton and Cipera, 1981; Roland et al., 1995; Schreiweis et al., 2003). However, egg weights were significantly reduced in hens with a severe dietary Ca deficiency (0.05% Ca for 6 wks) (Gilbert and Blair, 1974). Although egg weight and egg size are not affected in hens by consuming low levels of dietary Ca, shell quality is adversely affected as appeared in the current and previous studies (Hurwitz and Bar, 1965; Rennie et al., 1997; Schreiweis et al., 2003; Whitehead, 2004; Safaa et al., 2008; Cufadar et al., 2011; Kim et al., 2012). Hurwitz and Bar (1965) reported, for example, a reduction of shell thickness and shell weight in hens fed a diet containing of 1.7% Ca as compared to hens consumed a diet with 3.7% Ca. In laying hens, Ca deposited in egg shells is highly correlated with the Ca reserved in their body (Whitehead and Fleming, 2000). In order to maintain adequate egg shell quality, the amount of Ca reservoir within the hen's body is elevated before laying the first egg (Roberts, 2004) and replenished during the egg laying cycle (Kim et al., 2012). In the current study, hens were unable to maintain a positive Ca balance following fed the Ca-

deficient diet for 4 wks (from 23 to 26 wk of age). In treated hens, % shell weight was declined at both 2 and 4 wks following treatment, shell thickness was also decreased at 2 wks (Figures 1 and 2). Under hypocalcaemia, a Ca-deficient condition, hens use the Ca reserved in bones to fulfill the Ca demand during egg shell formation, and this could result in toward the recovery of shell thickness, but at the same time cause the changes in skeletal system as currently seen in hens at both 2 and 4 wks following treatment (described below).

Similarly to previous studies, under a hypocalcaemic condition hens attempt to maintain Ca homeostasis by first reducing egg production and the quantity of egg shell (Hamilton and Cipera, 1981; Schreiweris et al., 2003; Williams, 2005). Another mechanism used by hens to maintain Ca homeostasis is to increase the release of reserved Ca from bones by enhancing bone remodeling (Hurwitz and Bar, 1966; Schreiweis et al., 2003).Previous studies have evidenced that extensive bone resorption occurs under a hypocalcemic condition (Whitehead and Fleming, 2000; Whitehead, 2004; Kim et al., 2012). In the current study, bone mineralization was decreased in hens consumed a Ca-deficient diet for 4 wks. The BMD of treated hens was lower in all of the measured bones except the tibia which was numerically low without significant. Similarly to the current findings, previous studies had reported that Ca deficient diets reduced bone mineralization in chickens (Hurwitz and Bar, 1966; Schreiweis et al., 2003). During the onset of laying eggs, increased secretion of estrogen triggers osteoblasts to increase the formation of medullary bone, (Whitehead, 2004; Vries, 2010; Kim et al., 2012). Under long-term Ca deficiency, hens use structural bone to replace the Ca reserved in medullary bone. This remolding process of skeletal system results in a net loss of structural bone, leading to

osteomalacia and eventually osteoporosis with a condition of paralysis known as cage layer fatigue (Wilson and Duff, 1991; Whitehead and Fleming, 2000; Whitehead, 2004; Vries，2010).

Ash weight and its percentage have also been used as indicators of bone mineralization (Zhang and Coon, 1997; Schreweis et al., 2003). However, under hypocalcemia, both the ash and non-ash components of bones are mobilized during resorption so % ash may be unchanged (Ham and Leeson, 1961; Cheng and Coon, 1990; McCoy and Reilly, 1996; Zhang and Coon, 1997). Similar results were found in the current study. In addition, treated hens, compared to control hens, had a lower ash weight in the femur but not in the tibia and humerus (data not presented). Defatted dried bone weights of the tibia and femur but not the humerus were also lower in hens fed a low Ca diet for 4 wks (Table 5). Ca-deficiency diet induced different changes in dried weights among the measured bones were perhaps due to the differences of the bones' characteristics: the femur and tibia are considered medullary bones in female adult birds, while the humerus is generally categorized as a pneumatic bone, although its medullary component has been found in some strains of hens (Taylor and Moore, 1954; Whitehead, 2004). Specifically, the ends of the humerus along the endosteal surfaces where the bone is highly vascularized can have a medullary component, but the hollow diaphysis where the blood supply is minimal has little medullary bone fraction (Clunies et al., 1992). Therefore, those bones with a heavy medullary component such as the tibia and femur are more sensitive to the changes in dietary Ca than pneumatic bones such as the humerus (Schreiweis et al., 2003). In the current study, the Ca-deficient diet induced the changes in the bones was further determined by the DEXA scan. Bone mineral content was reduced in all of the measured

bones. The poor mineralization of the skeleton may cause osteomalacia in hens as result of feeding a Ca-deficient diet for 4 wks. The weaker bones of hens prevented the hens from perching as found in the study (see below). A severe skeletal damage, cage layer fatigue, can be induced in hens by a prolonged Ca deficiency, in which hens are so weak that they collapse inside the cages (Grumbles, 1959; Webster, 2004). In the present study, egg-eating was also observed in some Ca-deficient diet fed hens but not in controls. This behavior could be speculated due to the low Ca reservoir in the hens' body.

Liver weight and liver fat were reduced in Ca-deficient diet fed hens compared to control hens, which may indicate that treated hens' liver function was reduced in processing of low density lipoproteins as an integral component of egg yolk. Decreased egg output in hypocalcemic hens reduced the demand of lipoproteins for yolk, resulting in down regulation of liver metabolism (Schjeide et al., 1963; Roger et al., 1975). Avian liver is an important organ of lipid metabolism, and is the major site of fatty acid synthesis (Leveille, 1969). Excessive feed intake can lead to higher lipid concentration in the liver (Forenbaches et al., 1965; Lepkovsky and Furuta, 1971; Polin and Wolford, 1976). The lower liver weight and liver fat in the hypocalcemic hens may be explained by their reduced eating, which is in agreement with the findings from other studies (Lepkovsky and Furuta, 1971; Grobas, 1999; Julian, 2004) in which accumulations of liver fat were correlated with increased feed intake.

There was no difference in BW and relative abdominal fat pad weight between treated and control hens. Low Ca diets caused different effects on BW, decrease, no change, and increase, have been reported previously. The differences may be related to the differences of strains of hens used in the studies, the length of feeding a low Ca diet and its Ca levels.

Schreiweis et al. (2003) reported that BW was not different between hens fed a Ca-deficient diet (Ca = 1.8%)and control hens fed a commercial layer diet for 4 wks, from 32 to 35 wk of age. However, Roland et al. (1985) reported an increase in fat pad weight, liver fat, and BW in some strains of hens fed a low level of dietary Ca (Ca = 1.7%). To meet Ca demand, hens over consumed feed, and therefore overload energy, leading to the accumulation of fat and a heavy BW (Roland et al., 1985).

A lLow-Ca diet has been recognized as a stressor in multiple species of animals including rodents (Zemel and Sun, 2008; Takaya et al., 2009) and zebra finch (Snoeijs et al., 2005). However, it is unclear if a low-Ca diet has a similar negative effect in laying hens. In the current study, there was no difference in H/ L ratio and the concentrations of EP and NE between treated and control hens. Both H/ L ratio and catecholamines (EP and NE) have been used as stress indicators when assessing animal welfare including laying hens (Beuving et. al., 1989; Puvadolpirod and Thaxton, 2000; Mumma and Thaxton, 2006). In addition, an increase in H: L ratio occurs in chickens when exposed to various stressors (Gross and Siegel, 1983; Maxwell et al., 1992; Zulkifi et al., 1995; Hocking et al., 1996; Al-Murrani et. al., 1997; Puvadolpirod and Thaxton, 2000; Webster., 2003.). Similarly, the concentrations of EP and NE were increased in animals in response to emotional stress such as fearfulness and pain (Sanchez et al., 2003). In the current study, hens were fed a low Ca for 4 wks when the H: Lratio and circulating EP and NE concentrations were measured. In a further study, the effects of the period of a low Ca treatment, shorter and longer than 4 wks, on hens' stress response should be tested.

3.5 Conclusions

Short-term (4 wks) deprivation of dietary Ca in Bovans Brown hens at the onset of egg laying had negative impact on egg production, shell quality, and skeletal mineralization. Hens in a hypocalcemic status affected their perching ability due to poor skeletal mineralization. These results emphasize the need for providing hens with an adequate level of dietary Ca throughout the egg laying cycle to avoid the development of osteomalacia and its associated deleterious effects on hen well-being.

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Table 3.1 The effect of a Ca-deficient diet on egg production, egg size parameters, and shell quality traits of Bovans Brown laying hens from 23 to 26 wk of age.

Item	Hen-day egg production[2] (%)	1st egg (d)	Egg weight (g)	Egg width (mm)	Egg length (mm)	Egg shape index[2]	Shell (%)	Shell thickness (mm)
Ca-deficient diet	41.5[b]	136.0	59.3	43.6	56.4	77.2	8.99[b]	0.34[b]
Regular diet	44.8[a]	135.8	59.1	43.5	56.6	77.5	9.94[a]	0.38[a]
SEM	1.01	0.8	0.9	0.1	0.3	0.7	0. 1	0.01
n[3]	28	12	12	130	130	130	130	130
P-value	0.02	0.85	0.81	0.33	0.51	0.75	< 0.001	< 0.001

[a-b] Least square means within a column lacking common superscript differ ($P < 0.05$).

[1]Values represent the least square means averaged over 28 days of egg production.

[2]Shape index = egg width/egg length x 100.

[3] Average number of observations per least square mean during the 4 wk of the experiment.

Table 3.2 The effect of dietary Ca on BW, fat pad weight, liver weight, and liver fat content of Bovans Brown laying hens at 26 wk of age

Item	26wk BW (g)	Relative abdominal fat pad mass[1] (g/kg)	Relative liver mass[1] (g/kg)	Liver fat content (%)
Ca-deficient diet	1,853	34	23.7[b]	17.8[b]
Regular diet	1,856	32	26.1[a]	23.5[a]
SEM	59	3	0.8	1.7
n[2]	12	12	12	12
P-value	0.96	0.37	0.05	0.003

[a-b] Least square means within a column lacking common superscript differ ($P < 0.05$).

[1]Relative abdominal fat pad weight or liver weight = absolute fat pad weight or liver weight (g) / BW (kg).

[2.] Average number of observations per least square mean during the 4 wk of the experiment.

Table 3.3 The effect of a Ca-deficient diet on heterophil to lymphocyte ratios of Bovans Brown laying hens at 26 wk of age.

Item	Heterophil	Lymphocyte	Heterophil:lymphocyte ratio
Ca-deficient diet	20.0	70	0.28
Regular diet	19.7	73	0.27
SEM	0.3	3	0.01
n[1]	12	12	12
P	0.75	0.25	0.22

[1] Average number of observations per least square mean during the 4 wk of the experiment.

Table 3.4 The effect of a Ca-deficient diet on bone mineralization of Bovans Brown laying hens at 26 wk of age.

Item	Bone mineral density			Bone mineral content		
	Humerus	Femur	Tibia	Humerus	Femur	Tibia
	g/cm^2			g		
Ca-deficient diet	0.17[b]	0.20[b]	0.20	1.56[b]	2.24[b]	2.86[b]
Regular diet	0.19[a]	0.24[a]	0.22	1.70[a]	2.68[a]	3.27[a]
SEM	0.01	0.01	0.01	0.06	0.16	0.16
n[1]	12	12	12	12	12	12
P-value	0.03	0.04	0.07	0.03	0.01	0.02

[a-b] Least square means (adjusted for BW) within a column lacking common superscript differ ($P < 0.05$).

[1] Average number of observations per least square mean during the 4 wk of the experiment.

Table 3.5 The effect of a Ca-deficient diet on bone traits of the tibia of Bovans Brown laying hens at 26 wk of age

Item	Bone weight (g)	Bone ash (%)	Bone P (%)		Bone Ca (%)	
			Diaphysis	Epiphysis	Diaphysis	Epiphysis
Tibia						
Ca-deficient diet	6.0[b]	46.8	11.0	10.8	36.1	35.5
Regular diet	6.7[a]	50.0	11.1	10.5	35.9	35.0
SEM	0.2	0.02	0.3	0.3	1.0	1.2
n[1]	12	12	12	12	12	12
P-value	0.03	0.23	0.58	0.32	0.83	0.73
Femur						
Ca-deficient diet	4.8[b]	47.2	11.0[a]	10.6	35.6	34.4
Regular diet	5.5[a]	50.4	10.6[b]	10.5	35.8	35.7
SEM	0.2	0.03	0.1	0.2	0.9	1.1
n[1]	12	12	12	12	12	12
P-value	0.02	0.23	0.02	0.95	0.82	0.26
Humerus						
Ca-deficient diet	2.9	58.9	10.9	10.8[b]	35.6	35.1
Regular diet	3.0	59.9	10.6	10.4[a]	35.4	35.2
SEM	0.1	0.02	0.3	0.2	1.0	1.0
n[1]	12	12	12	12	12	12
P-value	0.77	0.60	0.28	0.01	0.77	0.95

[a-b] Least square means within the femur diaphysis or the humerus epiphysis lacking common superscript differ ($P < 0.05$).

[1] Average number of observations per least square mean during the 4 wk of the experiment.

Table 3.6 The effect of Ca-deficient diet on perching, eating, and drinking of Bovans Brown laying hens from 22 to 26 wk of age.

Item	Perching (%)[1]	Eating (%)[1]	Drinking (%)[1]
Ca-deficient diet	18.9[b]	33.6[b]	10.1
Regular diet	52.4[b]	49.2[a]	10.7
SEM	4.3	3.2	1.3
n[2]	30	30	30
P	0.0001	0.01	0.77

[a-b] Least square means within a column lacking common superscript differ ($P < 0.05$).
[1] Behavior preference = the number of individual hens displaying the specific behavior / the number of hens per pen * 100.
[2] Average number of observations per least square mean during the 4 wk of the experiment.

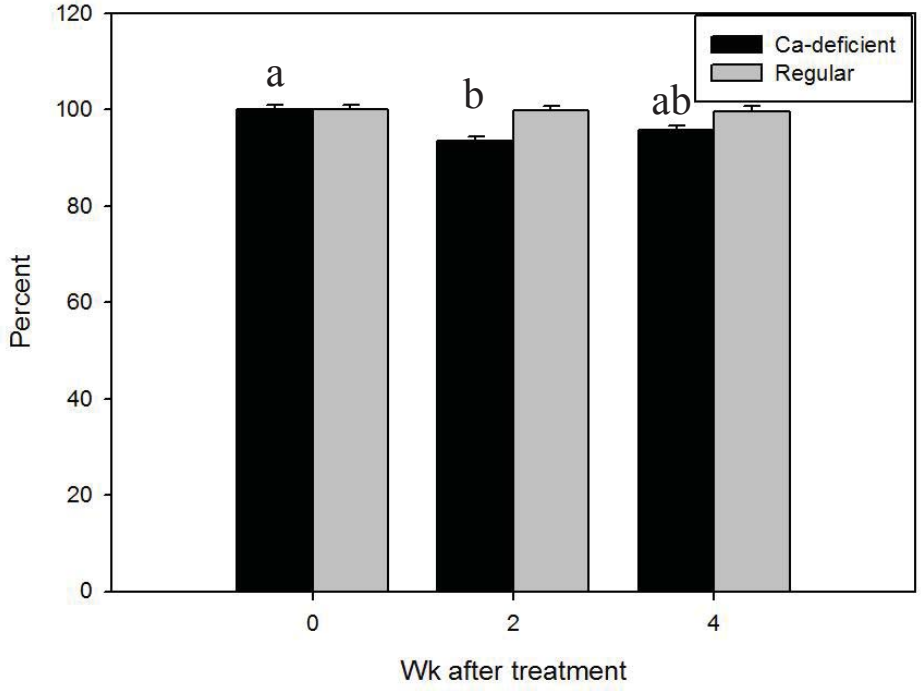

Figure 3.1 The effect of Ca-deficient diet on eggshell thickness of Bovans Brown laying hens before and 2 and 4 wk (wk 24 and 26 of age) during treatment. Within treatment, different letters denote a difference in shell thickness ($P < 0.05$) with age. Values are least square means ± SEM.

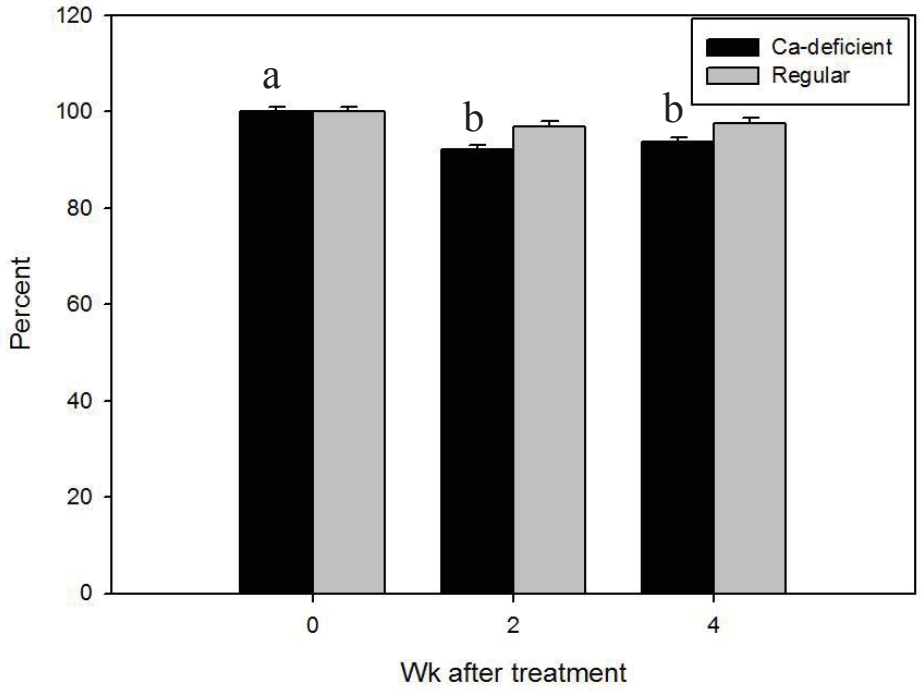

Figure 3.2 The effect of Ca-deficient diet on % eggshell weight of Bovans Brown laying hens before and 2 and 4 wk (wk 24 and 26 of age) during treatment. Within treatment, different letters denote a difference in % eggshell weight ($P < 0.05$) with age. Values are least square means ± SEM.