Shell Formation and Bone Strength in Laying Hens

Effects of Age, Daidzein and Exogenous Estrogen

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Abstract

In Sweden almost 3.8% of all eggs are ruined due to cracked eggshells as shell quality declines with age during the laying period. This is a concern for food safety as well as an economical problem. Parallel with reduced shell quality the bone strength declines to an extent that the animal's welfare is threatened.

Laying hens of Lohmann Selected Leghorn (LSL) and Lohmann Brown (LB) were monitored during a complete production period. The first sign of declining shell quality was found in mid-production in both hybrids, and even before, in peak production, decreasing bone strength was detected. An imbalance between estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) in the shell gland and a reduced density of tubular glands are suggested as factors involved in the age-related decline of shell quality. Since the majority of eggshell calcium is absorbed in the duodenum, the dramatic drop in active carbonic anhydrase (CA) seen in duodenum at mid-production, may also contribute. In the shell gland CA is considered a key enzyme in the supply of the carbonate ions needed for shell formation. However, CA in the shell gland did not primarily seem to be involved in declining shell quality, in fact number of capillaries positive for CA activity increased with age. The calcium transporter PMCA was located in shell gland and duodenum but was not altered by age.

Boosting laying hens with a low continuous dose of estradiol at the end of the laying period improved shell quality in both hybrids, while a daidzein supplementation (50mg/kg feed) in the feed did not. Bone strength was unaffected by both treatments. CA activity in the shell gland may be regulated by estrogen and daidzein, possibly by acting via ERβ. However, a difference in sensitivity to daidzein and estradiol was found between the hybrids which complicates the interpretation of results and must be taken into a count when comparing studies from other investigations.

Keywords: Laying hens, shell gland, shell formation, shell quality, bone strength, duodenum, carbonic anhydrase, estrogen, phytoestrogen, estrogen receptor α, estrogen receptor β, plasma membrane calcium ATP-ase.

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Dedication

To my family

This report, by its very length, defends itself against the risk of being read.
Winston Churchill (1874-1965)

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Wistedt, A. Ridderstråle, Y. Wall, H. Holm, L. (2012). Effects of phytoestrogen supplementation in the feed on the shell gland of laying hens at the end of the laying period. *Animal Reproduction Science* 133(2012), 205-213.

II Wistedt, A. Ridderstråle, Y. Wall, H. Holm, L. Age related changes in the shell gland and duodenum in relation to shell quality and bone strength of laying hens. Submitted to *Animal Reproduction Science*.

III Wistedt, A. Ridderstråle, Y. Wall, H. Holm, L. Exogenous estradiol improves shell strength in laying hens at the end of the laying period, possibly through regulation of estrogen receptor ratio and carbonic anhydrase. Submitted to *Acta Veterinaria Scandinavica*.

Abbreviations

CA	Carbonic anhydrase
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
LB	Lohmann Brown
LH	Luteinizing hormone
LSL	Lohmann Selected Leghorn
PBS	Phosphate-buffered saline
PMCA	Plasma membrane Ca^{2+} ATP-ase
TRPV6	Transient receptor potential cation channel, subfamily V, type 6

1 Introduction and background

1.1 Introduction

A modern laying hen produces more than 20 kilogrammes of eggs during a production period and the amount of calcium for eggshell formation corresponds to almost a kilo during that time. It is hard to imagine how this rather small animal can cope with this effort, which requires a high ability to process feed for eggs and eggshell.

In commercial production, the hen arrives at the laying facility at puberty, at the age of around 16 weeks, and in Sweden the production period lasts until she is about 75 weeks of age. However, there is a trend to keep the hens even longer until 90 weeks of age. The production over a 12-month period is approximately 320 eggs (Lohmann Tierzucht Gmb, **C**uxhaven, Germany), almost one egg per day. The eggshell consists of 96% calcium carbonate and 4% organic matrix, and for each egg, about 2-2.5 g of calcium is required. The availability of calcium depends on intestinal absorption from feed, but the skeleton also acts as a source of calcium during the dark hours of the day, when intestinal absorption has ceased. At the end of the laying period the hen produces larger eggs with reduced shell quality (Al-Batshan *et al.*, 1994; Garlich *et al.*, 1984). The eggshell functions as a food package, and when a small crack in the eggshell passes through the control system undetected it can be considered a risk to food safety. In Sweden only, almost 3% or 3.8 million kilo eggs are ruined yearly due to cracked eggshells leading to large economical losses.

Another problem occurring in commercial laying hens is the loss of bone strength. As much as 30 % of laying hens experience fractures either during the production period or during the unloading of birds from the cages at the end of the production (Gregory & Wilkins, 1989). The progressive osteoporosis is initiated when the hen reaches sexual maturity and continues throughout the laying period (Wilson *et al.*, 1992). This makes progressive osteoporosis a

serious animal welfare problem (Webster, 2004; Whitehead & Fleming, 2000). Modern laying hens face a major physiological challenge when they have to produce large quantities of eggs and maintain the strength of their skeleton.

1.2 Background

In everyday terms, it is easy to forget that the yolk is a large egg cell and thereby reproduction is highly connected with egg production. The ovulatory cycle is defined as the interval between ovulations and for hens is about 25 to 28 hours. In Sweden the life of a laying hen in commercial egg production ends at 70-75 weeks of age. This is of course not an "old" hen, but at the end of the first production period egg weight increases while the eggshell weight does not change, which results in a decrease in shell quality (Nys, 1986).

In the adult bird oviduct function is maintained by estrogen together with progesterone and androgen (Etches, 1996). Estrogen is a steroid hormone strongly associated with female reproduction and female reproductive behavior. The laying hen is no exception. Phytoestrogens are plant-derived compounds which mimic endogenous estrogens and can be found in e.g. soya bean meal, a common protein source in feed to laying hens. Phytoestrogens may have detrimental or beneficial estrogenic effects on reproduction in laying hens.

The eggshell is formed by the shell gland, an expanded pouch-like part of the oviduct, and during shell formation the blood flow to the shell gland increases 4-5 fold (Wolfenson *et al.*, 1982). The major calcium absorption takes place in the first part of the intestine, duodenum. The passage of calcium in and out of the duodenum and shell gland cells involves several transcellular pathways and will be described in more detail below. The carbonate ions for shell formation are derived from metabolic carbon dioxide. As early as 1933 it was suggested that the enzyme carbonic anhydrase (CA) is essential in the process by catalyzing the conversion of carbon dioxide to bicarbonate ions (Meldrum & Roughton, 1933).

1.2.1 Reproductive tract of the laying hen and overview of egg formation

Ovary

In the normal female bird only the left ovary and oviduct develop during sexual maturation. The ovary is situated on the left side of the abdominal cavity close to the median line, attached to the dorsal wall by a fold of peritoneum, the mesovarium (Hodges, 1974). The ovary consists of follicles, of which a small number constantly grow in diameter as yolk transported from the liver is

incorporated. The five largest oocytes are arranged in a hierarchy and at ovulation the largest ovum is ovulated (Johnson, 2000).

Ovulatory cycle

Estrogen production from the small follicles in the ovary starts at puberty and induces the development of the oviduct, female-specific plumage and other secondary sexual characteristics (Etches, 1996). Estrogen also induces synthesis of egg white proteins in the magnum (Palmiter *et al.*, 1973; Oka & Schimke, 1969) and synthesis of progesterone (Joensuu, 1990; Mester & Baulieu, 1977). Before the onset of laying the estrogen concentration is very high in the young hen, this may be essential for the synthesis of yolk protein (Elbrecht *et al.*, 1984; Senior, 1974) and medullary bone for later eggshell formation (Senior, 1974).

The production of estrogen is maintained by the small follicles (F_n) in the ovary. There is a shift in production to androgen, in the second largest follicle (F_2) and prior to ovulation the largest follicle (F_1) starts to produce progesterone. Thus the LH surge prior to ovulation is not induced by estrogen as in mammals but progesterone (Etches, 1996). The circulating level of estrogen in plasma correlates to the ovulatory cycle and reaches a peak about six hour before ovulation (Johnson & van Tienhoven, 1980). The exact secretion pattern of estrogen during a production period is not clear and since the estrogen level varies during the ovulatory cycle and therefore varies during the day, the hour for sample-taking is crucial. A study where estrogen concentration was measured from 35 until 100 weeks of age showed no differences in estrogen levels between young and old hens, but at the first sign of molt a decrease in estrogen was detected (Braw-Tal *et al.*, 2004). Joyner and coworkers (1987) claim that as long as the hen produces eggs the estrogen level in plasma is the same, but it decreases in old non-laying hens. However, Tanabe and coworkers (1981) found that plasma estrogen shows a rise after one year and is negatively correlated to egg production.

Oviduct

The oviduct is highly convoluted, formed like a tube and suspended in the abdominal cavity attached dorsally by a ligament of peritoneal membrane. On the ventral side of the oviduct a similar ligament, not connected to the body wall with a muscular band along the oviduct (Etches, 1996). A schematic drawing of the oviduct is found in figure 1.

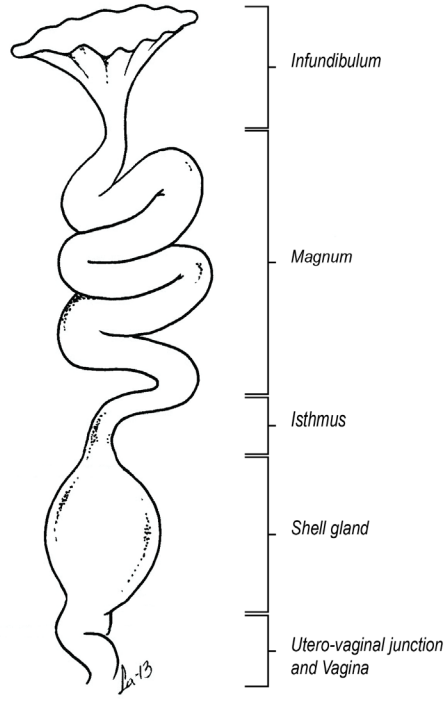

Figure 1. Schematic drawing of a bird oviduct showing the morphologically and functionally distinct regions.

The infundibulum, the first part of the oviduct, captures the ovum within 15 minutes after ovulation (Etches, 1996). Sperm storage sites, so called glandular groves, are located in the infundibulum, which is the site of fertilization. The short period during which fertilization can take place, before the first layer of albumen is deposited, is called "window of fertilization" and is only about 15 minutes long (Wishart & Horrocks, 2000). From here and onwards the ovum cannot be fertilized and is now called an egg. In the magnum, the second and longest part of the oviduct, the majority of the albumen is secreted during approximately 3 hours (Wyburn *et al.*, 1970). The two shell membranes are formed in the following part, the isthmus. The inner shell membrane surrounds the albumen and the outer shell membrane functions as a support for the eggshell. In the last part of isthmus the mammillary cores are formed which will function as crystallization sites and anchor the shell to the outer membrane (Solomon, 2010; Wyburn *et al.*, 1973; Baker & Balch, 1962). The egg stays in this part of the oviduct for approximately 1.5 hour (Draper *et al.*, 1972;

Simkiss & Taylor, 1971). It then enters the shell gland where the albumen is diluted in a process called plumping and the hard shell is deposited during the next 18-22 hours (Etches, 1996). This process will be described in more detail below. Finally, the cuticle, a waxy layer covering the pores in the shell, is formed (Simkiss & Taylor, 1971; Baker & Balch, 1962). The egg leaves the shell gland, passes the utero-vaginal sperm storage tubules, where the first storage of sperm occurs, and enters the vagina where it remains for a few minutes before oviposition through the cloaca.

1.2.2 Calcium sources for shell formation

Calcium from the feed

Shell formation is time consuming and in progress during the greater part of the day and during the dark hours the skeleton contributes with calcium for shell formation. A sufficient amount of calcium in the feed is a necessity for both shell formation and skeletal health and resorption of calcium takes place in duodenum. The duodenal mucosa has long leaf-shaped villi and tubular crypts of Lieberkühn. The epithelium lining the villi and crypts consists of a single layer of tall columnar cells and an underlying layer of lamina propria. Muscularis mucosa and submucosa are quite thin and surrounded by muscularis externa consisting of one circular and one longitudinal layer of smooth muscle (Hodges, 1974). A schematic drawing of the duodenum is found in figure 2.

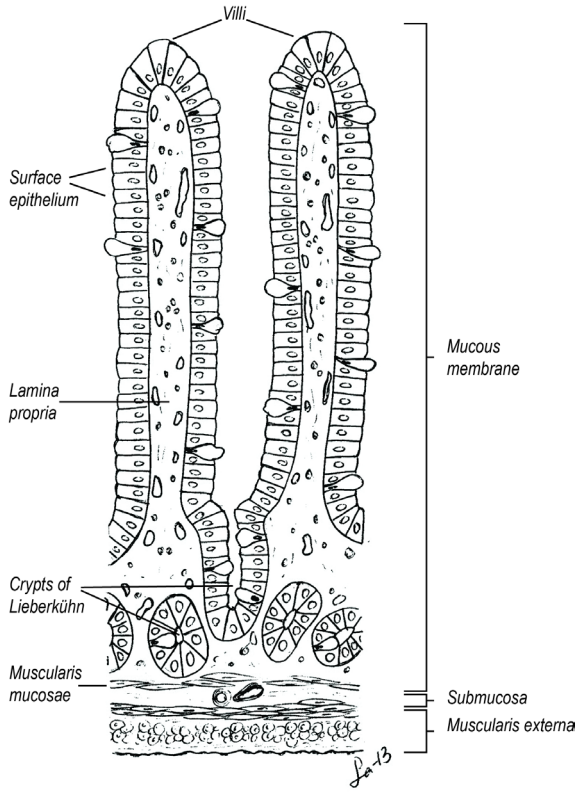

Figure 2. Schematic drawing showing morphological overview of the laying hen duodenum.

The plasma concentration of calcium is sensed by the parathyroid glandular cells, responding with parathyroid hormone (PTH) secretion. A fall in plasma Ca^{2+} levels stimulates PTH secretion and a rise suppresses secretion (Brown, 1991). The primary targets for PTH in birds are bone and kidney (Ritchie & Pilny, 2008; Dacke, 2000). PTH increases the release of calcium from bone by stimulating osteoclast activity. In the kidney PTH increases reabsorption of calcium and decreases the levels of phosphate, which leads to an increased calcium-phosphate ratio and more calcium is available in the plasma. In the kidney PTH also up-regulates enzymes responsible for converting vitamin D_3 (cholecalciferol) to its biologically active form calcitriol ($1,25(OH)_2$-D_3) (Johnston & Ivey, 2002; Brown, 1991) and thereby increasing the absorption of calcium (Johnston & Ivey, 2002).

Across the epithelium there are two possible pathways for calcium. First, the paracellular process, a passive diffusion process that refers to the passage through tight junctions and the extracellular space between the cells. Then,

secondly, the transcellular process which is an active transport where calcium passes through both the apical and basolateral cell membranes. This path consists of three different processes (Wasserman, 2004). The epithelial calcium channel, TRPV6, which transfers calcium into the cell (Yang *et al.*, 2011; Hoenderop *et al.*, 2002), the intracellular protein calbindin D_{28k} which binds calcium with high affinity inside the cell, and finally the ATP-dependent membrane calcium pump (PMCA), which transports calcium out of the cell (Wasserman *et al.*, 1992; Carafoli, 1991). Each step in the transcellular pathway of Ca^{2+} is dependent upon vitamin D (Wasserman, 2004; Wasserman & Fullmer, 1995) and might also influence the paracellular pathway (Wasserman, 2004). There are exceptions, however, as estrogen can affect calcium absorption by up-regulation of calcium channels in the duodenum, presumably in a non-genomic manner and independent of vitamin D (Van Cromphaut *et al.*, 2003; Picotto *et al.*, 1996). Vitamin D_3 receptors are located in both the duodenum and the shell gland of laying hens (Yoshimura *et al.*, 1997).

Skeletal calcium

Laying hens have three types of bone tissue namely cortical, spongy (trabecullar bone or cancellous bone) and medullary bone. The cortical bone is the compact structural bone and spongy bone not as compact and located at the end-part of the long bones. The medullary bone develops at sexual maturation (Webster, 2004; Senior, 1974; Taylor & Moore, 1958) and is a kind of woven bone in the marrow cavities and the main storage of calcium for shell formation in the skeleton (Kim *et al.*, 2012). Medullary bone is found in large amounts in modern laying hens throughout the laying period (Fleming *et al.*, 1998) and the bone with the highest content of medullary bone is found in the long leg bones (Whitehead & Fleming, 2000).

Both cortical and spongy bone contribute to the strength of the skeleton. Indirectly the cortical and spongy bone contribute to eggshell formation since these types of bone are resorbed to maintain the medullary bone, which delivers one third of the total calcium needed for the eggshell (Buss & Guyer, 1984; Taylor & Moore, 1958). The amount of medullary bone is constantly remodeled and increasing in volume throughout the egg-laying period at the cost of cortical and spongy bone. The cortical and spongy bone losses start as early as at the onset of reproductive activity in female birds (Wilson & Thorp, 1998).

The word osteoporosis means “porous bone” and occurs when there is a mineral loss from the bone, particularly calcium, as well as architectural losses of normal bone structure. The World Health Organization (WHO) defines

human osteoporosis as a bone mineral density of 2.5 standard deviations or more below the mean peak in bone mass found in young healthy adults. Osteoporosis in laying hens is identified by a severe loss of structural bone causing bone brittleness and birds more susceptible to fractures (Whitehead & Fleming, 2000; Urist & Deutsch, 1960). It was first noticed in the mid-20th century, and associated with cage layer fatigue when hens began to be housed in cages (Webster, 2004). The mechanism behind the loss of structural bone in favor of medullary bone is driven by estrogen, since the loss of structural bone is progressive throughout the laying period but is reversed when the hens goes out of lay and estrogen levels decline (Whitehead & Fleming, 2000).

In high producing layers, osteoporosis is more common in conventional caged laying hens compared to layers in systems that provide better possibility for physical activity (Webster, 2004). In severe osteoporosis spontaneous bone fractures can occur, most commonly in the ribs, keel and thoracic vertebrae. Most of the caged laying hens suffer from some kind of fractures during their first laying period, which may take several weeks to heal, resulting in skeletal deformities. The most critical phase is at the end of a laying period when catching and handling of the birds during depopulation can lead to high incidences of new fractures [reviewed in (Webster, 2004)]. The reasons for osteoporosis are several and not so well defined, but the problem is partly genetic and a result of breeding for high productivity (Whitehead & Wilson, 1992).

The role of estrogen in calcium transport

Estrogen also plays an important role in calcium metabolism, especially during shell formation and an injection of exogenous estradiol increases plasma Ca^{2+} levels in laying hens (Bar *et al.*, 1996). Estrogen is hypothesized to affect Ca^{2+} transport in the duodenum by up-regulation of calcium channels (Van Cromphaut *et al.*, 2003; Picotto *et al.*, 1996) as mentioned above, but also by stimulating the conversion of vitamin D_3 to its biologically active form calcitriol (Castillo *et al.*, 1977), by up-regulation of $1,25D_3$ receptors and by synthesis of calbindin D_{28k} (Bar *et al.*, 1996; Wu *et al.*, 1994; Bar & Hurwitz, 1987; Tanaka *et al.*, 1978). However, estrogen does not affect calbindin synthesis in the shell gland (Bar *et al.*, 1996). Estrogen and androgens also regulate Ca^{2+} transport through cell membranes via PMCA and ion channels in the kidney, which has been shown in mice (Dick *et al.*, 2003). In the skeleton estrogen stimulates the deposition of calcium and has a stimulatory effect on osteoblasts making them produce medullary bone instead of structural bone (Whitehead, 2004).

1.2.3 Shell gland and the process of shell formation

The shell gland mucosa consists of a surface epithelium covering leaf-shaped folds and an underlying layer of lamina propria with tubular glands within the folds. The surface epithelium consists of a single layer of columnar cells, ciliated cells with nuclei located apically and non-ciliated cells with nuclei located basally. The layer of smooth muscle consists of a circular and longitudinal layer, the outer longitudinal layer is particularly well developed compared to anterior segments of the oviduct (Hodges, 1974). A schematic drawing of the shell gland is found in figure 3.

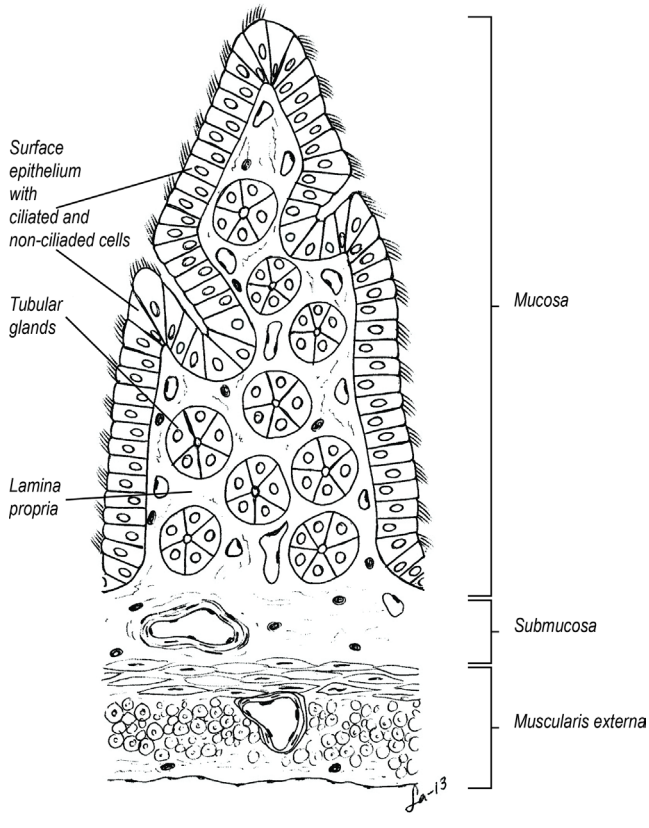

Figure 3. Schematic drawing showing morphological overview of laying hen shell gland

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The structure of the shell

The shell has an average thickness of approximately 0.3 mm and can be divided into three different layers that can be distinguished, the mammillary layer, the palisade layer and a vertical crystal layer closely connected to the cuticle (Hodges, 1974). The crystallized palisade layer is composed mainly of crystalline calcium carbonate arranged in columns perpendicular to the surface (Johnson, 2000). Each palisade column grows from one mammillary knob and during calcification these columns fuse forming the bulk of the "true shell" (Solomon, 2010). The shell gland cells secrete the compounds of a milieu which is saturated in calcium carbonate relative to calcite (Nys *et al.*, 1991) and the precursors of eggshell matrix (Dominguez-Vera *et al.*, 2000; Nys *et al.*, 1999; Gautron *et al.*, 1997). Organic matrix proteins help control the mechanical properties of the eggshell (Hincke *et al.*, 2010; Dominguez-Vera *et al.*, 2000) and the shape, size and orientation of the calcite crystals are due to the interaction of calcium carbonate and the organic matrix (Dominguez-Vera *et al.*, 2000; Nys *et al.*, 1999). Simplified, one can describe the organic matrix as reinforcement of the calcite crystals in the eggshell. Finally a cuticle covers the eggshell which minimizes penetration of e.g. microorganism (Board & Halls, 1973).

Shell gland secretion

When the egg enters the shell gland plumping occurs and the albumen is diluted by adding ions (Na^+, K^+ and Cl^-), water and glucose (Wyburn *et al.*, 1973; Simkiss & Taylor, 1971). The exact time when and where shell formation starts is somewhat controversial. As the plumping is completed, calcification increases as large amounts of calcium carbonate deposition occur and a hard shell is formed (Etches, 1996).

With more focus on the minerals of the eggshell and the formation of calcium carbonate, there is an increase in extracellular water transfer, including sodium and chloride, but the uterine fluid also contains calcium, potassium and bicarbonate ions (Nys *et al.*, 1999; Arad *et al.*, 1989). The content of Na^+, Cl^- and Ca^{2+} is high at the beginning of shell calcification and decreases progressively, while K^+ shows an inverse change (Nys *et al.*, 1999). Chloride ions diffuse passively back to the blood and Na^+ is exchanged for Ca^{2+} by the mucosal cells (Arad *et al.*, 1989). The transport of Na^+, Cl^-, K+, Ca^{2+} and HCO3- occurs against their electrochemical gradients and involves ion exchangers and ion channels (Nys *et al.*, 1999). The regulation of calcium secretion is strictly linked to the arrival of an egg and synchronized to ovulation (Nys *et al.*, 1999).

Evidence suggests that the surface epithelium provides the bulk of Ca^{2+} needed for shell formation (Etches, 1996; Solomon *et al.*, 1975; Gay & Schraer, 1971) and the ciliated cells contain the highest concentration of calcium (Holm *et al.*, 2003). Ultrastructural studies show that these cells produce secretory granules and the production of granules reaches its greatest intensity during the early stages of shell formation (Breen & De Bruyn, 1969). Recent findings implicate the calcium ion channel TRPV6 in shell formation (Jonchere *et al.*, 2012) and immunohistochemistry revealed that the protein is located in the surface epithelial cells, but the location to a specific type of surface epithelial cell was not evident (Yang *et al.*, 2013). However, the non-ciliated cells have been suggested to supply the eggshell with organic matrix and osteopontin, a well-known matrix protein, is present in these cells (Fernandez *et al.*, 2003).

The tubular gland cells are likely to produce a watery fluid, which may well include calcium since these cells contain calbindin-D_{28k} and show intense staining for PMCA at the apical membrane (Wasserman *et al.*, 1991). In addition, these cells contain numerous mitochondria which can provide the ATP necessary for active transport across the apical membrane (Breen & De Bruyn, 1969). However, it has been suggested that the tubular gland cells may have a dual function and also provide the HCO_3^- needed for shell formation. The production of HCO_3^- originates mainly from the hydration of CO_2 by CA, which is present in the tubular gland cells (Nys *et al.*, 1999; Eastin & Spaziani, 1978). The latter authors also noted that calcium may be transported across the mucosa with two different mechanisms, of which one is active transport functionally linked to luminal HCO_3^- secretion (Eastin & Spaziani, 1978).

1.2.4 The role of carbonic anhydrase

Besides shell formation CA also take part in several other fundamental physiological processes such as respiration, acid-base balance, bone resorption, calcification and a variety of processes involving ion, gas and fluid transport (Chegwidden & Carter, 2000). CA is an enzyme family consisting of at least 16 isoforms (reviewed in (Esbaugh & Tufts, 2006), of which thirteen are considered active in mammals (Hilvo *et al.*, 2008). CA isozymes show a great variation in sub-cellular and tissue localization (Chegwidden & Carter, 2000), catalytic activity and vary in sensitivity to inhibition (Hilvo *et al.*, 2008).

Carbonic anhydrase in shell gland

The bicarbonate ions required for shell formation are mainly produced by the glandular cells from metabolic CO_2 in a reaction catalyzed by CA $CO_2 + H_2O \leftrightarrow HCO^{3-} + H^+$ (Nys *et al.*, 1999). It was found that inhibition of CA results in soft-shelled or even shell-less eggs (Lundholm, 1990; Benesch *et al.*, 1944) and that CA activity in the duodenum is lower in hens laying soft-shelled eggs (Nys & de Laage, 1984). In the shell gland CA activity increases at sexual maturity (Nys *et al.*, 1986) and at the end of the first laying period CA activity decreases (Snapir & Perek, 1970) and presumably a decrease in available carbonate ions occurs. In 1976 as many as nine isozymes were reported in laying hens (Grunder & Hollands, 1976). Today several of them have been linked to shell formation. In the shell gland of laying hens Dunn and coworker found gene expression of isozyme CAII and CAVIII (2009). According to Jonchere et al. isozyme CAII, IV and VII are expressed in the shell gland, and bicarbonate ions are likely produced from CO_2 by CAII, since CAII is over-expressed during eggshell mineralization compared to CAIV and VII (2012). The major localization of CA activity in the shell gland is in the lateral membranes of the tubular gland cells and in the endothelia of some but not all capillaries (Holm *et al.*, 2006; Berg *et al.*, 2004).

The localization of CA activity in the shell gland of other avian species differs somewhat. In ostrich and quail shell glands, staining for CA activity is present only in the cell membrane of tubular glands and no staining of the capillary endothelium was detected (Holm *et al.*, 2001; Holm & Ridderstråle, 1998). In guinea fowl, known to produce very strong eggshells, the enzyme is found in the membranes of surface epithelial cells and tubular gland cells, which also have some cytoplasmic enzyme and in endothelial cells of many capillaries (Knutsson & Ridderstrale, 1982).

Carbonic anhydrase in duodenum

The levels of CAII in erythrocytes are much higher in high producing hens compared to low producing hens and roosters (Nishita *et al.*, 2011a), and CA III in erythrocytes is correlated to high egg production (Nishita *et al.*, 2011b).

The role of CA in the duodenum is to serve as a defense zone between ventriculus and jejunum, a mechanism to protect enterocytes from the acid chyme (Sjöblom, 2011; Kaunitz & Akiba, 2006; Mizumori *et al.*, 2006). High expression of cytosolic and membrane-bound CA (CAIX, XII and XIV) is found in rat duodenal epithelial cells and membrane-bound CA (CAIV) in capillary endothelium (Kaunitz & Akiba, 2006). In the avian duodenum the same isozymes (CAII, IV and VII) are expressed as in the shell gland of laying

hens, CAII is predominantly expressed and has a major function in the hydration of CO_2 to produce HCO^-_3 in duodenum (Jonchere *et al.*, 2012).

Regulation of carbonic anhydrase

In reproductive organs, it appears that CA activity is under the control of hormones. Under the influence of progesterone, CA increases in rabbit uterine homogenate (Hodgen & Falk, 1971; Lutwak-Mann, 1955) and CA increases under the influence of estrogen in guinea-pigs (Hodgen & Falk, 1971). Several other investigations have reported that CA is up-regulated by estrogen treatment in the rat uterus (Caldarelli *et al.*, 2005) and mouse uterus (Barnett *et al.*, 2008) and have a novel role in uterine bicarbonate transport, important for successful reproduction in mice (He *et al.*, 2010). Embryonic exposure of eggs from domestic hens and Japanese quail with synthetic estrogen or an estrogenic environmental contaminant, resulted in adult birds with disrupted distribution of CA activity in shell gland (Holm *et al.*, 2006; Halldin *et al.*, 2003).

1.2.5 Estrogen receptors

The biological actions of estrogen (17β-estradiol) are mediated via estrogen receptors (ER). A classical estrogen action pathway is when estrogen diffuses into the cell and binds to the receptor located in the nucleus to regulate gene transcription, resulting in a physiological response. This response occurs over the course of hours. Estrogen can act more quickly, within seconds or minutes, via a non-genomic mechanism through ER located in or adjacent to the plasma membrane, with cellular responses such as increased levels of Ca^{2+} or NO (Deroo & Korach, 2006). The ER exists in two main forms, referred to as alpha and beta (ERα, ERβ), encoded by separate genes, *ESR1* and *ESR2*, and found on two different chromosomal locations (Morani *et al.*, 2008; Deroo & Korach, 2006). The first ER found (Jensen *et al.*, 1972) was renamed ERα when a second receptor, ERβ, was detected in rat ovary and prostate in 1996 (Kuiper *et al.*). Both receptors have been localized in laying hens (Ball *et al.*, 1999), and in reproductive organs nuclear ERα has been localized in the shell gland (Hansen *et al.*, 2003; Isola, 1990; Joensuu & Tuohimaa, 1989). Expression of ERα seems to be predominant compared to ERβ in reproductive organs during sex differentiation in quail and in ovaries of adult domestic hens (Hrabia *et al.*, 2008; Mattsson *et al.*, 2008).

In tissues expressing both receptors there are indications that ERα and ERβ have opposite functions, thus in mouse mammary gland epithelium ERα elicits and ERβ suppresses proliferation (Morani *et al.*, 2008). In human duodenal epithelial cells the ratio of ERα and ERβ within a cell determines the biological response or the sensitivity towards estrogen (Böttner *et al.*, 2013).

1.2.6 Phytoestrogens

Phytoestrogens are estrogenic compounds in plants able to evoke biological responses by activating ERα and ERβ (Dusza *et al.*, 2006; Kuiper *et al.*, 1998). Phytoestrogens are high affinity ligands especially for ERβ, but the doses that are biologically active differ between species (Dusza *et al.*, 2006; Kuiper *et al.*, 1998). Isoflavones, the estrogenic compounds present in soya and legumes, seem to act as antagonists in a high estrogen environment and as agonists in a low estrogen environment (Hwang *et al.*, 2006).

Reproductive disturbances such as impaired ovarian function have been found in ewes grazing forages with clover (Adams, 1995; Curnow *et al.*, 1948). However, health benefits are reported in humans after consumption of phytoestrogen. It has been suggested that humans eating a typical Japanese diet, with a high intake of dietary phytoestrogens, may show a reduced risk of breast and prostate cancer, compared to humans on a typical Western diet (Adlercreutz *et al.*, 1991). Protective effects of phytoestrogens in humans have also been reported on menopausal symptoms (Baird *et al.*, 1995) and osteoporosis (Potter *et al.*, 1998).

Soya bean or soya bean meal are commonly used as a protein source for layers. The most common isoflavones in soya are genistein, daidzein, glycetin and coumestrol (Adams, 1995). In laying hens, daidzein is transformed into its metabolite equol which is a more active form (Saitoh *et al.*, 2004). The biological potency compared to estradiol is >coumestrol, >genistein, >equol >daidzein (Murkies *et al.*, 1998). The concentration of daidzein in soya beans varies greatly and range between 240 to over 1500µg/g fresh weight [reviewed in (Reinli & Block, 1996)]. The estrogenicity of daidzein has been investigated in chicken by Ni and coworkers (2010) who found that high doses of daidzein up-regulate ERβ mRNA in chicken hepatocytes in the absence of estradiol. The effect of daidzein decreases with increasing amounts of estradiol, and with a high dose of estradiol daidzein functions as an inhibitor. Varying concentrations of daidzein together with different concentrations of endogenous estrogen in live animals may lead to completely different effects. However, a supplement of daidzein improves the laying performance during post peak laying of Shaoxing duck (Zhao *et al.*, 2005) and in ISA layers the amount of cracked eggs decreases and eggshell thickness and egg production increases (Ni *et al.*, 2007).

1.2.7 Age related changes in laying hens

At the end of the laying period the production gradually declines and the hens would eventually enter molt. In Swedish egg production the hens will be replaced before molt.

Several physiological changes coincide at the end of the first laying period. The hens produce larger and heavier eggs and at the same time the eggshell weight does not change (Nys, 1986). Several shell quality parameters have been confirmed to decline with age such as percentage of shell, shell thickness (Al-Batshan *et al.*, 1994) and shell index (g/dm^2), and in parallel the number of cracked eggshells increase (Nys, 1986). The declining shell quality is also connected to a reduction of the attachment force and breaking strength of the shell membranes (Kemps *et al.*, 2006). Also the organic matrix of the shell undergoes changes due to aging affecting the microstructure of the shell and older hens show a greater variability in structural properties compared to young hens (Rodriguez-Navarro *et al.*, 2002).

Even if shell quality declines with age, the concentrations of Ca^{2+} in plasma and calbindin in the shell gland and duodenum are the same in young and old hens (Bar & Hurwitz, 1987) and the duration of shell formation does not change with age (Nys, 1986). Abe and coworker (1982) suggest that decreasing shell quality in older hens is associated with disorders of vitamin D metabolism, but vitamin D receptors in the shell gland gradually increase at sexual maturation and then remain at that level throughout production and even during molt (Yoshimura *et al.*, 1997). However, the production of vitamin D_3 and its concentration in plasma is higher in young hens compared to old if they are fed a diet with a low content of calcium, suggesting that the ability to adapt to changes in calcium content in the feed decreases with age (Bar & Hurwitz, 1987). It is also known that intestinal calcium absorption decreases with increasing age in laying hens (Al-Batshan *et al.*, 1994) and that there are lower levels of ERα in both the kidney and the shell gland (Hansen *et al.*, 2003).

The progressive loss of bone strength in hens starts early but continues throughout the production period with no time to recuperate (Wilson *et al.*, 1992) causing osteoporosis to be most severe at the end of the laying period (Whitehead & Fleming, 2000).

Some differences between hybrids in age-related changes of eggshell and bone have been reported. When comparing one brown and one white egg-laying hybrid at the end of the laying period, egg production of the white hens were greater, compared to the brown hens. This is probably due to brown hybrids often have a longer interval between oviposition, and at the same time they were more resistant to broken bones (Riczu *et al.*, 2004). In another study white eggs had a greater decrease in eggshell quality compared to brown eggs at the end of production (Silversides & Scott, 2001).

1.3 Aims of the thesis

The general aim of this thesis was to investigate key-factors associated to shell formation and age related reduction in eggshell quality in commercial laying hens.

A more detailed aim of the present thesis was therefore to:

- investigate how capillary density, plasma calcium, localization of CA activity, ERα, ERβ and PMCA is altered by age in the shell gland and duodenum
- determine how these factors are related to shell quality and bone strength and if they are regulated by estrogen
- test the hypothesis that bone strength and shell quality can be improved by an estrogen implant and if this effect can be achieved by supplementing the feed with natural phytoestrogen

2 Materials and methods

2.1 Research plan

Three experiments were conducted at Lövsta research facility of SLU in Uppsala, Sweden, between 2008 and 2010. Due to a mistake made by the commercial feed manufacturer regarding mineral content the first experiment planned to be run during a whole production period had to be stopped at half-time. The experiment then had to be repeated from the beginning in a new batch of layers, which caused us to shift order between the experiments from the original plan. Below the experiments are described in chronological order Experiment I-III. For more detailed information on *e.g.* experimental procedures analysis and materials used, please see specific papers (Paper I-III).

The animals used in the three experiments were laying hens of a white hybrid Lohmann Selected Leghorn (LSL) and a brown hybrid Lohmann Brown (LB) (Gimranäs AB, Sweden). These hybrids are commonly used in commercial egg production in Sweden, where the white hybrids are more common. The birds were held in the university poultry research facility under conditions similar to commercial egg production. They were housed in furnished cages as described by Wall and Tauson (2002). The cages fulfilled the Swedish Animal Welfare Directives and the Uppsala Local Ethics Committee approved all three experiments.

2.1.1 Experiment I

This study was set up using a controlled amount of phytoestrogen in the feed to a total of 64 laying hens of the two hybrids during 12 weeks at the end of a production period. The aim was to monitor how or if the isoflavone daidzein influences the shell gland during shell formation. The calculated amount of daidzein was 2 mg/kg body weight, approximately the same as a conventional diet containing soya. The main variables of interest were shell quality, localization of PMCA, ERα, ERβ and CA activity in tissue from shell gland together with general morphology.

2.1.2 Experiment II

This study extended during a laying period and the aim was to monitor age related changes during a production period. The study included a total of 40 hens of the two hybrids, hens in early production at 21 weeks of age, hens in peak-production at 29 weeks of age, hens in mid-production at 49 weeks of age and hens at the end of production at 70 weeks of age. The main variables of interest were shell quality, bone strength and calcium concentration in plasma, localization of PMCA, ERα, ERβ and CA activity in tissue from shell gland and duodenum together with general morphology.

2.1.3 Experiment III

This study was set up using an implant of 17-β-estradiol in laying hens at the end of a production period. The study included a total of 40 hens and was conducted between 70 and 72 week of age. The aim in this study was to find out if an exogenous compound similar to endogenous estrogen affects shell quality and shell formation in laying hens. The main variables of interest were shell quality, calcium concentration in plasma, bone strength and localization of PMCA, ERα, ERβ and CA activity in tissue from shell gland and duodenum together with general morphology.

2.2 Egg production and eggshell measurements

Eggs were collected each morning and the number of eggs and egg weight were recorded. Laying % was calculated as number of eggs/day and hen x 100. In experiment I and III laying % was calculated for the hens included in the study. In experiment II, a laying % was calculated for the whole production group and used as a reference for normal production.

Shell quality was measured on eggs collected shortly before and during the experiments. The quality parameters were shell deformation, shell breaking strength, shell weight, shell thickness including shell membranes and shell thickness excluding shell membranes. For more details, see paper I-III.

2.3 Total calcium in plasma and bone strength

Blood samples were collected from the wing vein and held on ice until further processing. The samples were centrifuged at 3000 rpm at 4^{o}C for 10 minutes to separate the plasma. The plasma was stored at -70^{o}C until analysis of total calcium.

At sacrifice right leg and wing were frozen. Before testing the bones were thawed and free dissected from skin, ligaments and muscles. The bones were tested to breaking point measured in Newton on a three-point electromechanical testing machine. For more details, see paper II-III.

2.4 Tissue preparation

All hens were killed by an intravenous injection of pentobarbital sodium (100mg/ml, Apoteket AB, Umeå, Sweden) in the wing vein and the body weight was recorded for each bird.

The oviduct and duodenum were rapidly removed. The oviduct was free dissected from the mesoviductus and the length was measured from the vaginal orifice to the fimbriated infundibulum, and the location of the egg was recorded. The shell gland was cut open lengthwise and pieces were taken from the middle part. Duodenal samples were taken immediately distal to the duodenal loop. Tissue pieces from both shell gland and duodenum were fixed in glutaraldehyde for CA histochemistry and in paraformaldehyde for immunohistochemistry. For more details, see paper I-III.

2.5 Histochemistry

To be able to localize CA in tissues we used a histochemical method described by Ridderstråle (1991; 1976). This method shows the localization of all active CA isozymes present in the tissue. Immunohistochemistry, as an alternative for localization, requires specific antibodies for each different isozyme and is therefore more time consuming and expensive.

For this purpose the tissues were embedded in a water-soluble resin (Historesin® by Leica Historesin, Heidelberg, Germany). Sections (2µm) were incubated floating on the surface of an incubation medium made up by mixing 17 ml of solution I and 40 ml of solution II immediately before start. Solution I contained 10 ml 0.2 M $CoSO_4$, 60 ml 0.5 M H_2SO_4 and 100 ml 1/15 M KH_2PO_4 and solution II 0.75 g $NaHCO_3$ dissolved in 40 ml distilled water. The CO_2 produced due to the catalytic activity of CA leaves the floating sections and causes local increases in pH at sites with CA. A cobalt-phosphate-carbonate complex is formed which is transformed to an insoluble black precipitate of cobalt-sulphide by treating the sections with a solution containing $(NH_4)_2S$. Controls for CA specificity were run in the presence of the CA inhibitor acetazolamide (10^{-5} M) in the incubation medium.

2.6 Immunohistochemistry

For localization of ERα a monoclonal antibody H222 (provided by Dr. Joe Pitts, University of Chicago, Ben May Institute for Cancer Research, Chicago, USA) was used in paper I as described for chicken tissue by (Isola, 1990; Greene *et al.*, 1984). However, this antibody is no longer available and was replaced in paper II and III with a monoclonal rabbit anti-ERα (clone 60C, Millipore, USA) previously used by Oliveira and coworkers (2011) in rooster epididymis, tissue also used as a positive control in all our incubations.

For localization of ERβ a mouse monoclonal antibody (MCA 1974ST, Serotec, Düsseldorf, Germany) was used on shell gland (paper I-III) and duodenum (paper II-III) with procedures according to Norrby et al. (2013).

For localization of the calcium transporter PMCA, a mouse monoclonal antibody (5F10 ab2825, Abcam 330, Cambridge Science Park, Cambridge, CB4 0FL, UK) used on shell gland (paper I-III) and duodenum (paper II-III) according to Wasserman et al. (1991).

2.7 Image analysis

To exclude bias, all slides were coded before examination and were evaluated by one person (A. Wistedt).

Digital images of sections were taken with a Nikon Microphot-FXA microscope. For evaluation of CA activity and morphology in shell gland, the top of mucosal folds were chosen. The total number of capillaries/mm^2 and number of CA positive capillaries/mm^2 were recorded. For evaluation of mucosal height of duodenum, the height was measured from the submucosa to the top of the villus. All measurements were analyzed using an image analysis software (Elcipse Net, version 1.20, Developed by Laboratory Imaging, Prague, Czech Republic).

The immunohistochemical staining of ERα, ERβ and PMCA, location were described and staining intensity was scored on a scale from 0-3. Histochemical staining of CA activity in duodenum, localization were described and intensity scored on a scale from 1-3. In the shell gland, the tubular gland density was scored as dense or not dense.

2.8 Statistical analysis

In paper II, the density of tubular glands was analyzed by chi-square contingency test using GraphPad Prism (ver. 5.02, GraphPad Software, Inc., CA, USA). Remaining parameters in papers I-III were subjected to analyses of variance either by using The general linear model (Proc GLM) or the Mixed Procedure (proc Mixed) of SAS® software version 9.2 (SAS Institute Inc., Cary, NC, USA). The statistical models included the fixed effects of hybrid, treatment (diet or implant), age or period and interaction between fixed effects. Results are given as mean ± s.e unless otherwise stated. A difference was considered significant at $p < 0.05$.

In the analyses of production and egg quality traits each group, i.e. hens housed in the same cage, was treated as one experimental unit. In the analysis of body weight, organ measurements, morphological data, total calcium and bone strength each hen was treated as an experimental unit. Body weight was included as a covariate in the analyses of bone strength and total calcium. Egg weight was included as a covariate in shell weight and shell weight was included as a covariate in the analyses of shell thickness.

3 Results

3.1 Egg production and eggshell quality

In experiment II, where egg production was monitored during a complete laying period, the laying percentage decreased with age after peak production and was higher for the white hens compared to brown hens during the laying period (fig 4a). Egg weight increased with age and brown eggs were heavier compared to white eggs during the laying period (fig 4b). Similarly, eggs from hens laying brown eggs were heavier compared to white eggs also in experiment I and III at the end of the laying period. A supplement of daidzein in the feed or treatment with estradiol did not affect laying percentage or egg weight.

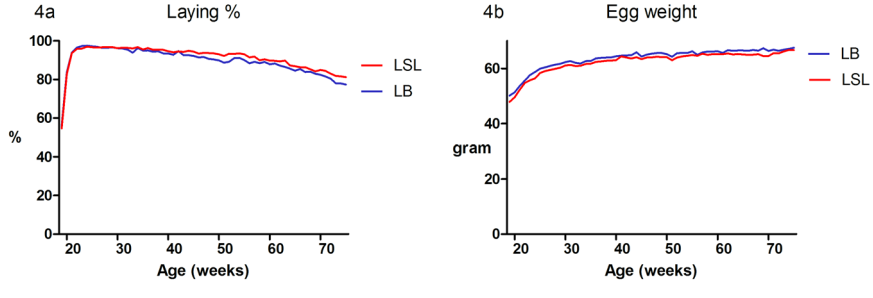

Figure 4. Production data during a production period (57 weeks) of LSL hens (n=61) and LB hens (n=61). (a) Laying percentage, number of eggs/day and hen x 100. The laying percentage of LSL hens were higher compared to LB hens ($p<0.001$). (b) Egg weight in gram, the eggs from LB hens were heavier compared to eggs from LSL hens ($p<0.001$). Results are expressed as mean.

Shell quality decreased with increasing age, when shell deformation increased and breaking strength decreased (fig 5a-b). Shell weight decreased from peak production to mid-production (29 vs.49 weeks of age), more details are available in paper II.

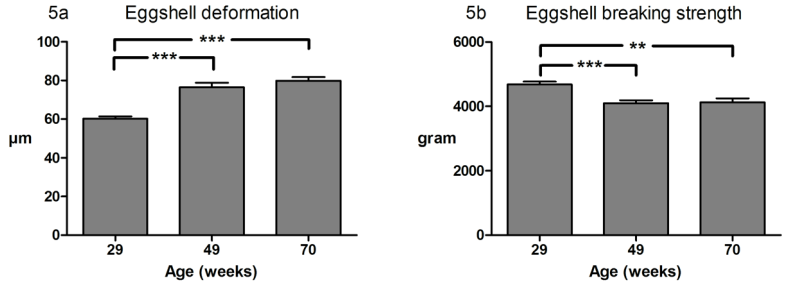

Figure 5. Eggshell quality measurement of eggshell from LSL and LB hens (n=2) at three different ages during the laying period. (a) Shell deformation, after a load of 1,000 g was applied on the egg. (b) Breaking strength, the load that needs to break the eggshell. Results are expressed as mean ± SE. ** (p<0.01), *** (p<0.001).

Treatment with a low dose of exogenous estradiol improved shell quality, especially in eggs from the brown laying hybrids, where shell deformation decreased and shell weight and shell thickness, with and without membranes increased. In white eggs shell thickness without membranes improved and tended to improve with membranes (table 1). Shell quality was not affected by a supplement of daidzein in the feed in any of the hybrids.

Table 1. *Shell quality measurements of eggs from LSL and LB hens treated with estradiol or placebo at 70-72 weeks of age. Values are expressed as mean ± SE.*

Eggshell quality	**Lohmann Selected Leghorn (LSL)**			**Lohmann Brown (LB)**		
	Placebo (n=2)	**Estradiol (n=2)**	**P value**	**Placebo (n=2)**	**Estradiol (n=2)**	**P value**
Shell Deformation (µm)	74 ± 0.9	73 ± 0.9	n.s	74 ± 1.2	69 ± 0.7	0.0002
Shell breaking strength (g)	4077 ± 71	4026 ± 58	n.s	4263 ± 79	4388 ± 71	n.s
Shell weight (g) *	5.94 ± 0.04	5.96 ± 0.04	n.s	6.09 ± 0.04	6.38 ± 0.04	<.0001
Shell thickness incl membranes (mm) *	0.386 ± 0.002	0.390 ± 0.002	0.0749	0.400 ± 0.002	0.409 ± 0.002	0.0014
Shell thickness excl membranes (mm) *	0.357 ± 0.002	0.362 ± 0.002	0.0373	0.372 ±0.002	0.378 ± 0.002	0.0415

* Egg weight was included as a covariate in the analysis of shell weight and shell weight was included as a covariate in the analysis of shell thickness, and these values are expressed as LSmean ± SE.

3.2 Bone strength and plasma calcium

Breaking strength of bone

The bone strength of the femur decreased already at peak production (29 week of age) and was lowest at mid-production followed by a small recovery seen at the end of production (fig 6a). The bone strength of the humerus decreased somewhat later during mid-production (fig 6b).

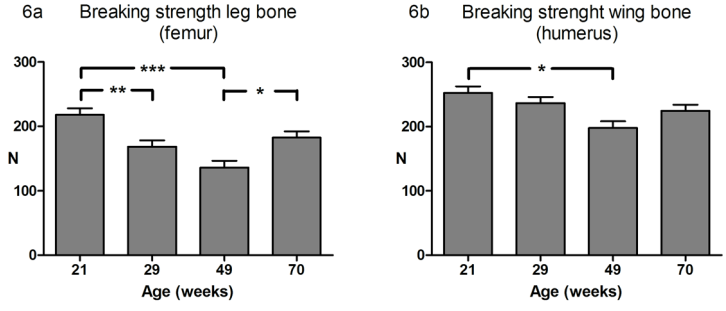

Figure 6. Breaking strength in Newton tested by three-point-bending. Leg and wing bone of LSL and LB hens (n=10) at four different ages during the laying period. (a) Leg bone (femur) (b) Wing bone (humerus). Results are expressed as LSmean ± SE. * (p<0.05), ** (p<0.01), *** (p<0.001).

The bone strength of the femur was not affected by estradiol treatment and the bone strength in the tibia showed no effect of daidzein supplementation (not shown).

Total calcium in plasma

Total calcium concentration in plasma increased with age (fig 7a). Daidzein supplementation of the feed decreased calcium concentration in plasma (fig 7b). The calcium concentration in the plasma was not affected by treatment with estradiol (fig 7b).

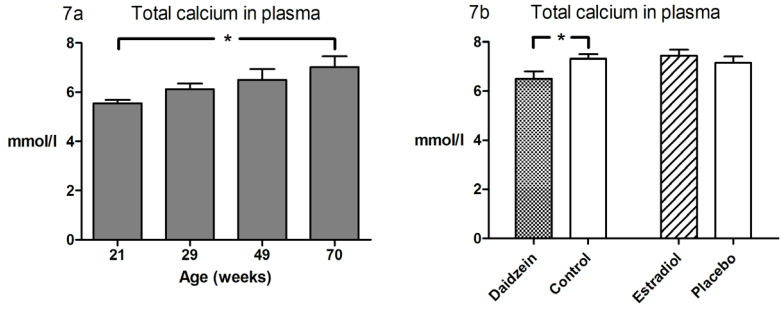

Figure 7. Total plasma calcium concentration in mmol/l. (a) LSL and LB hens (n=10) at four different ages during the laying period. (b) LSL and LB hens (n=32) fed a daidzein supplemented diet or control diet at the end of the laying period, and from LSL and LB hens (n=20) treated with estradiol or placebo at 70-72 weeks of age. Results are expressed as mean ± SE. * ($p<0.05$)

3.3 Body weight and oviductal length

In all three experiments the mean body weight of the brown laying hens was significantly higher compared to the white laying hens. This difference is in agreement with the values given by the breeding company (Lohmann Tierzucht Gmb, **C**uxhaven, Germany). During the laying period brown hens continued to increase in body weight up to 49 weeks of age, while the white hens did not change in body weight during the laying period.

The oviductal length increased in both hybrids and had reached full length at 49 weeks of age. Estradiol treatment or a daidzein supplementation in the feed did not affect body weight or oviductal length.

3.4 Morphological evaluation

3.4.1 Capillary and tubular gland density in shell gland

The total numbers of shell gland capillaries/mm^2 at the top of the mucosal fold increased from 21 to 29 weeks of age and was thereafter constant in both hybrids (fig 8a). The total numbers of capillaries was not affected by estradiol treatment or daidzein supplementation in the feed (fig 8b).

The density of tubular glands in the shell gland was lowest at 49 week of age, and was not altered by estradiol treatment or daidzein supplementation in the feed.

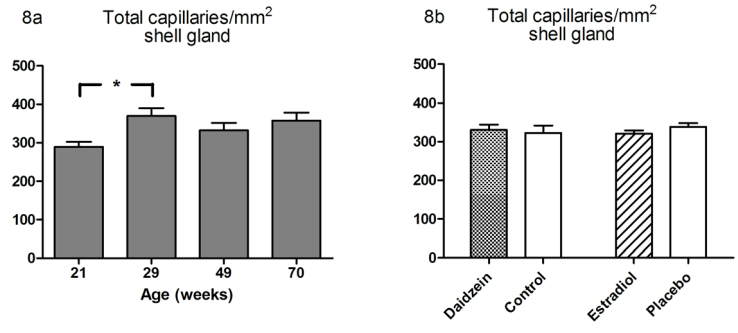

Figure 8. Total number of capillaries/mm^2 in shell gland mucosal fold of laying hens. (a) LSL and LB hens (n=10) at four different ages during a laying period. (b) LSL and LB hens (n=36) fed a daidzein supplemented diet or control diet at the end of the laying period, and LSL and LB hens (n=20) treated with estradiol or placebo at 70-72 weeks of age. Results are expressed as mean ± SE. * ($p<0.05$)

3.4.2 Duodenal mucosal height

During the laying period the height of the mucosa of duodenum in brown hens was higher at the age of 49 weeks, whilst the height of the mucosa in the white hens did not differ during the laying period.

3.4.3 Carbonic anhydrase distribution

The histological method used to analyze CA activity results in a black precipitate at sites of enzyme activity. Sections incubated with the CA inhibitor contained no significant staining.

Shell gland

The shell gland surface epithelium was unstained in all hens regardless of age, hybrid, feed or treatment. Inconsistent weak membrane-bound staining for CA activity was seen in tubular gland cells, except at 29 weeks of age and in brown hens also at 70 weeks of age when no staining was detected. Estradiol treatment increased membrane-bound CA activity in the white hens at the end of the laying period, but daidzein supplementation had no effect on CA activity in the tubular gland cells.

The capillary endothelium showed intense membrane-bound staining for CA in the shell gland. The number of capillaries/mm^2 with CA at the top area of mucosal folds increased with increasing age in both hybrids (fig 9). The number of CA positive capillaries increased in the brown hens fed a daidzein supplement (fig 10a) and increased in the white hens treated with estradiol (fig 10b), thus white hens were unaffected by daidzein and brown hens unaffected by estradiol treatment.

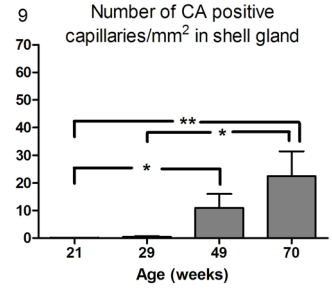

Figure 9. Number of carbonic anhydrase positive capillaries/mm^2 at four different ages during the laying period in shell gland mucosal fold of LSL and LB hens (n=10). Results are expressed as mean ± SE, * (p<0.05), ** (p<0.01).

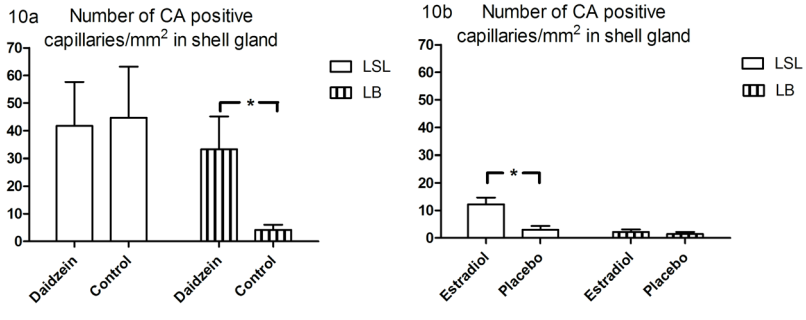

Figure 10. Number of carbonic anhydrase positive capillaries/mm^2 in shell gland mucosal fold. (a) LSL hens (n=16) and LB hens (n=16) fed a daidzein supplemented diet or control diet at the end of the laying period. (b) LSL hens (n=10) and LB hens (n=10) treated with estradiol or placebo at 70-72 weeks of age. Results are expressed as mean ± SE. * (p<0.05).

Duodenum

Strong staining for CA activity was found in the brush border and lateral cell membranes of surface epithelial cells. These cells also contained moderate cytosolic staining. The staining of surface epithelial cells gradually decreased towards the top of the villi. Capillary endothelium with intense membrane-bound staining was found in villi, crypt region and muscularis. In the crypts of Lieberkühn the apical cell membrane was unstained, lateral cell membrane staining varied from unstained to intense and cytosolic staining from unstained to moderate. At 49 weeks of age a marked decrease in staining intensity occurred and only weak or no staining could be seen (fig 11a, c, d). A similar pattern was observed in the brown layer hens treated with estradiol (fig 11b).

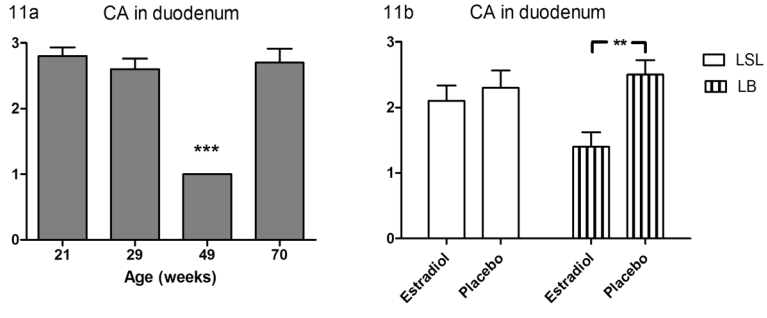

Figure 11. Intensity scoring of carbonic anhydrase activity in duodenum. (a) LSL and LB hens (n=10) at four different ages during a laying period. (b) LSL hens (n=10) and LB hens (n=10) treated with estradiol or placebo at 70-72 weeks of age. Staining intensity scored as 1-3. Score 1: weak or no staining. Score 2: moderate staining. Score 3: strong staining. Results are expressed as mean ± SE. ** (p<0.01), *** (p<0.001).

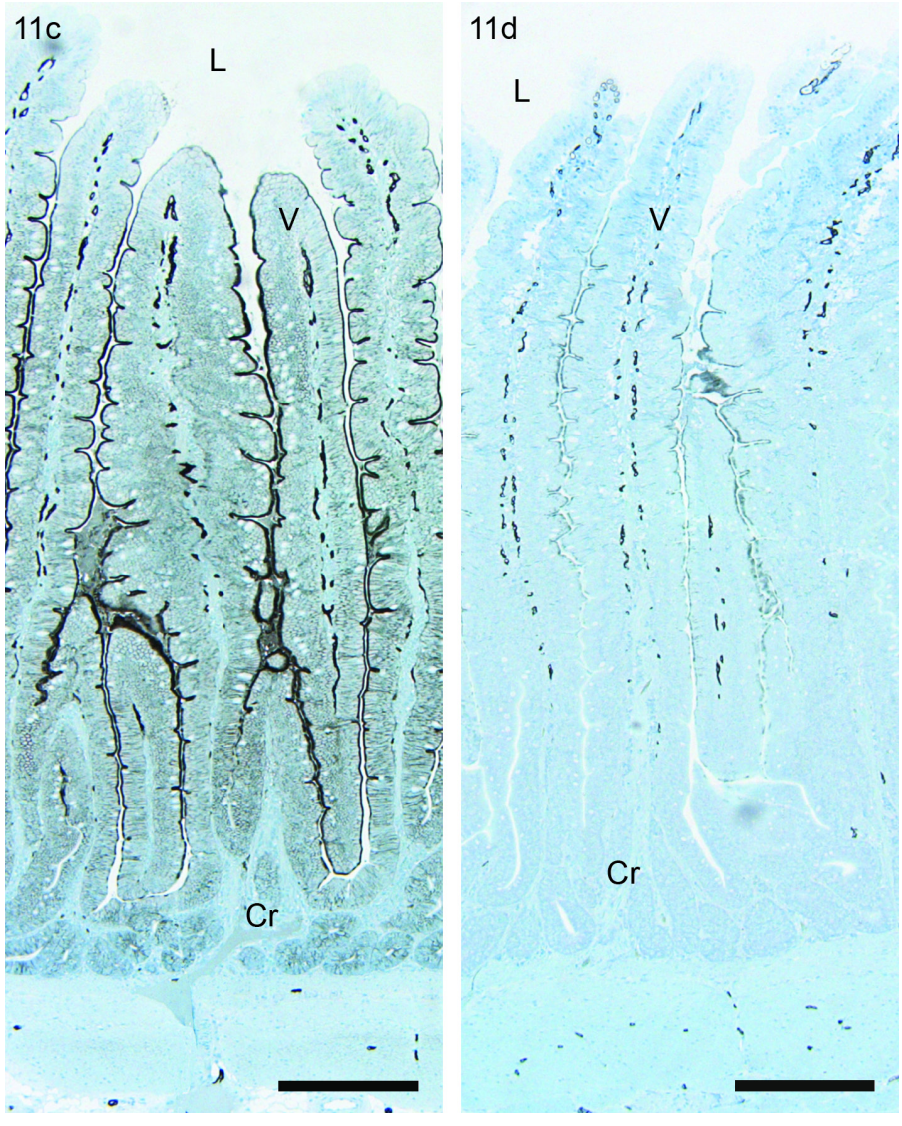

Figure 11. Black staining showing localization of carbonic anhydrase (CA) activity in duodenum of layer hybrids. (c) LB hen at age 29 weeks. Surface epithelium of villi (V) with intense staining for CA activity in lateral cell membranes and brush border, and moderate cytosolic staining. Note capillaries in villi lamina propria with intense membrane-bound staining of endothelial cells. In the crypts of Lieberkühn (Cr), lateral cell membranes are intensely stained and the cytosol weak to moderate. (d) LSL hen at age 49 weeks. Surface epithelium (V) shows none or very weak staining for CA activity in lateral cell membranes and moderate staining of brush border, cytosolic staining is very weak or absent. Note capillaries in villi lamina propria with intense membrane-bound staining of endothelial cells. No staining of cell membranes or cytosol in crypts of Lieberkühn (Cr). Weak azure blue counterstain. L = lumen, V = villus, Cr = crypts of Lieberkühn. Bar = 200µm

3.4.4 Estrogen receptor alpha

Shell gland

The staining for ERα showed some differences between the two antibodies H222 and clone 60C, mainly in the surface epithelium of the shell gland. H222 was found in the apical and occasionally basal cell membranes in both ciliated and non-ciliated cells and with weak to moderate staining in nuclei and cytosol. When using clone 60C the nuclei and cell membranes were unstained and a weak to moderate cytosolic granular staining was found in the apical part of both non-ciliated and ciliated cells.

Strong staining for ERα with both H222 and Clone 60C was found in the nuclei of tubular gland cells, with weak to moderate cytosolic staining. Smooth muscle cells had only weak nuclear staining. Generally the endothelium was devoid of staining in both capillaries and larger vessels, except for a few stained nuclei in larger vessels when clone 60C was used. The erythrocytes were found to be negative with both antibodies.

The staining intensity for ERα decreased in nuclei of tubular gland cells and cytosolic granules in surface epithelial cells with age (fig 12a-b). Estradiol treatment decreased the staining intensity of ERα in the white laying hens (fig 13). Daidzein supplementation had no detectable effect on the localization or staining intensity for ERα. A summary of staining intensity is found in table 2.

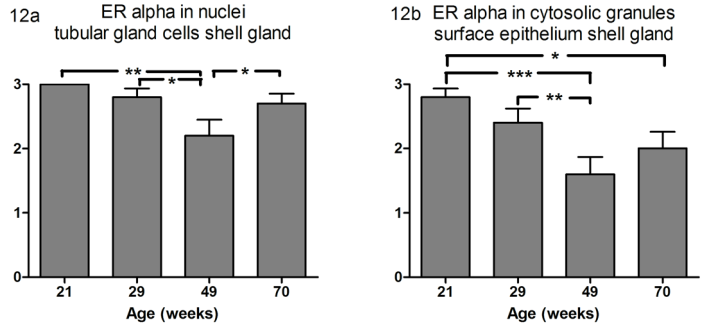

Figure 12. Intensity scoring of immunohistochemical staining for estrogen receptor α (ERα) with Clone 60C in shell gland mucosal fold of LSL and LB hens (n=10) at four different ages during a laying period. (a) ERα in nuclei of tubular gland cells. (b) ERα in granules in surface epithelial cells. Intensity scoring from 0-3. Score 0: no staining. Score 1: weak staining. Score 2: moderate staining. Score 3: strong staining. Results are expressed as mean ± SE. * (p<0.05), ** (p<0.01), *** (p<0.001).

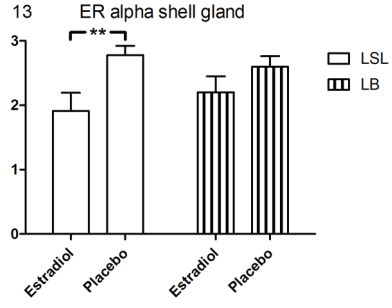

Figure 13. Intensity scoring of immunohistochemical staining for estrogen receptor α (ERα) in shell gland of LSL hens (n=10) and LB hens (*n*=10) treated with estradiol or placebo at 70-72 weeks of age. Intensity scoring from 0-3. Score 0: no staining. Score 1: weak staining. Score 2: moderate staining. Score 3: strong staining. Results are expressed as mean ± SE. ** ($p<0.01$).

Duodenum

Immunohistochemical staining for ERα in the duodenum was performed with clone 60C. Weak staining for ERα was found in nuclei of smooth muscle cells and some nuclei of cells in the lamina propria. There was no detectable difference in location or intensity between age groups or treatment with estradiol. A summary of staining intensity is found in table 2.

Table 2. *Scoring intensity of immunohistochemical staining for estrogen receptor alpha (ERα) in shell gland mucosal fold and duodenum from laying hens. 0 = No staining, + = weak staining, ++ = moderate staining, +++ = strong staining, — = not evaluated*

ER α	Cell structure	Hybrid	Experiment I (H222)		Experiment II (Clone 60C)				Experiment III (Clone 60C)	
			Daidzein	Control	21 wk	29 wk	49 wk	70 wk	Estradiol	Placebo
Shell gland										
Surface epithelium	Nuclei	LSL & LB	+/++	+/++	0	0	0	0	0	0
	Cytoplasm	LSL & LB	+/++	+/++	+++	+++	+/++	++/+++	+/++	+/++
	Membrane	LSL & LB	+++	+++	0	0	0	0	0	0
Tubular gland cells	Nuclei	LSL & LB	+	+	+++	+++	++	+++	+++	+++
	Cytoplasm	LSL & LB	+	+	+/++	+/++	+/++	+/++	+	+
	Membrane	LSL & LB	++	++	0	0	0	0	0	0
Lamina propria	Nuclei	LSL & LB	+/++	+/++	—	—	—	—	—	—
Capillary and vascular endothelium	Nuclei	LSL & LB	0	0	0/+	0/+	0/+	0/+	0/+	0/+
Smooth muscle cells*	Nuclei	LSL & LB	+/++	+/++	+/++	+/++	+/++	+/++	+/++	+/++
Duodenum										
Smooth muscle cells*	Nuclei	LSL & LB	—	—	+	+	+	+	+	+

* In muscularis and vessels.

3.4.5 Estrogen receptor beta

Shell gland

The strongest staining of the shell gland for ERβ was found in nuclei of non-ciliated cells in the surface epithelium. Ciliated cells varied more showing strong, moderate, weak and even unstained nuclei. The staining in the ciliated cells was more pronounced in young hens (21 weeks of age) compared to hens in production (29, 49, 70 weeks of age). Cytosolic staining for ERβ was found in the surface epithelium and tended to decrease with age. Daidzein supplementation in the feed and estradiol treatment increased the cytosolic staining of epithelial cells of brown hens (fig 14a).

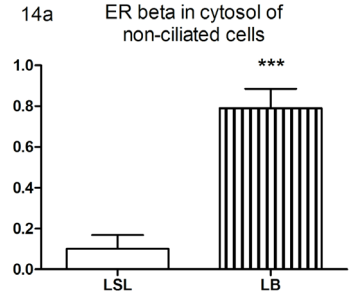

Figure 14a. Amount of non-ciliated cells with immunohistochemical staining for estrogen receptor β (ERβ) in shell gland of LSL hens (n=20) and LB hens (n=20) treated with estradiol or placebo at 70-72 weeks of age. Results are expressed as mean ± SE. ***(p<0.001).

Moderate to strong staining for ERβ was found in the nuclei of tubular gland cells and weak to moderate cytosolic staining. Cytosolic staining in brown hens was strongest at peak-production (29 weeks of age) compared to 21, 49 and 70 weeks of age. Cytosolic staining in white hens did not change with age and was not affected by estradiol treatment or daidzein supplementation. Strong nuclear staining was also found in some connective tissue cells, smooth muscle cells of muscularis and larger blood vessels. ERβ was present in endothelial nuclei of capillaries and larger blood vessels. The staining increased with age in both hybrids and was strongest at mid-production (49 weeks), no effects were seen after estradiol treatment or daidzein supplementation. Erythrocytes were negative. A summary of staining intensity is found in table 3 and fig 14b.

Duodenum

In the duodenum weak to moderate staining for ERβ was found in the nuclei of the surface epithelium, crypts of Lieberkühn and smooth muscle cells. A weak cytosolic staining was found in crypts of Lieberkühn and the surface epithelium. The staining intensity of both nuclei and cytosol gradually decreased towards the top of villus (fig 14c). There were no detectable differences in the intensity or localization of ERβ between different hybrids, age groups or treatment with estradiol.

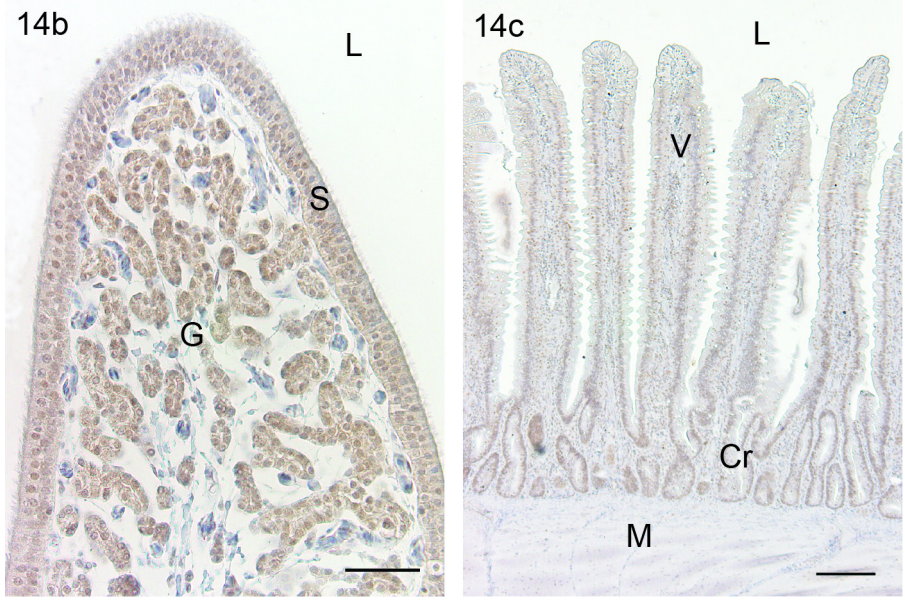

Figure 14. Immunohistochemical localization of estrogen receptor beta (ERβ) in laying hens. (b) Shell gland of LB hen at 49 weeks of age. Strong nuclear staining in non-ciliated cells and weak or occasionally unstained nuclei of ciliated cells in surface epithelium (S). Tubular gland cells (G) show strong nuclear staining for ERβ. Weak to moderate cytosolic staining in surface epithelium (S) and tubular gland cells (G). Bar=50µm. (c) Duodenum of LSL hen at 21 weeks of age. Moderate nuclear staining in surface epithelium of villi (V), crypts of Lieberkühn (Cr) and weak staining in smooth muscle cells (M). Staining gradually decreasing towards the top of the villus. Weak hematoxylin counterstain in both images. L = lumen. Bar= 133µm.

Table 3. *Scoring intensity of immunohistochemical staining for estrogen receptor beta (ERβ) in shell gland mucosal fold from laying hens. 0 = No staining, + = weak staining, ++ = moderate staining, +++ = strong staining, — = not evaluated*

ER β Shell gland	**Cell structure**	**Hybrid**	**Experiment I**		**Experiment II**				**Experiment III**	
			Daidzein	**Control**	**21 wk**	**29 wk**	**49 wk**	**70 wk**	**Estradiol**	**Placebo**
Surface epithelium Non-ciliated cells	Nuclei	LSL & LB	+++	+++	+++	+++	+++	+++	+++	+++
	Cytoplasm	LSL	++	++	+/++	+/++	+/++	+/++	++	++
		LB	+++	+++					+++	+++
	Membrane	LSL & LB	0	0	0	0	0	0	0	0
Ciliated cells	Nuclei	LSL & LB	+/++	+/++	++	0/+/++	0/+/++	0/+/++	0/+/++/+++	0/+/++/+++
	Cytoplasm	LSL	++	++	+/++	+/++	+/++	+/++	+/++	+/++
		LB	+++	+++						
	Membrane	LSL & LB	0	0	0	0	0	0	0	0
Tubular gland cells	Nuclei	LSL & LB	++	++	+++	+++	+++	+++	++/+++	++/+++
	Cytoplasm	LSL	+	+	+	+	+	+	++	++
		LB				++				
	Membrane	LSL & LB	0	0	0	0	0	0	0	0
Lamina propria	Nuclei	LSL & LB	++	++	++/+++	++/+++	++/+++	++/+++	+++	+++
Capillary and vascular endothelium	Nuclei	LSL & LB	++	++	++	++	+++	+++	+++	+++
Smooth muscle cells in muscularis	Nuclei	LSL & LB	++	++	++/+++	++/+++	++/+++	++/+++	+++	+++

3.4.6 PMCA

Shell gland

Positive immunohistochemical staining for PMCA was found in the apical membranes of tubular gland cells and somewhat weaker in surface epithelial cells. The intensity and localization of the staining was not affected by estradiol treatment, daidzein supplementation or by age (fig 15c).

Duodenum

Strong staining for PMCA was found in the baso-lateral membranes of the surface epithelial cells and a weak baso-lateral membrane staining in the cells of Lieberkühn crypts. The staining was strongest at the top of the villi and gradually decreased towards the base (fig 15d). In the brown laying hens staining intensity at the base of the villi increased with age, was strongest at mid-production (49 weeks of age) and decreased again towards the end of production (fig 15a), while the staining intensity in the white hens was high already at 21 weeks of age and tended to decrease (fig 15b). During the whole laying period the staining intensity of the crypts of Lieberkühn was stronger in the white hens. Estradiol treatment did not affect the staining intensity or localization of PMCA in duodenum.

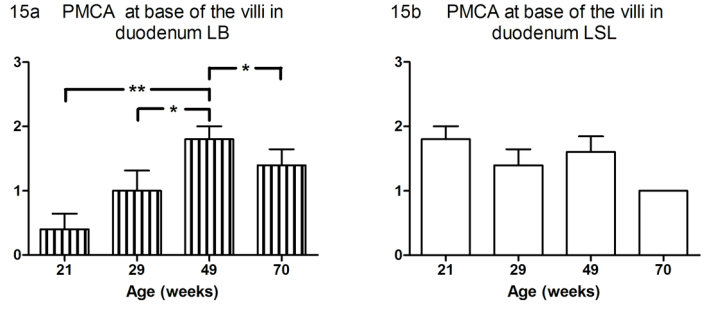

Figure 15. *Intensity scoring of immunohistochemical staining for PMCA in duodenum in LSL and LB hens at four different ages during a laying period. (a) PMCA in LB hens (n=5) at the base of the villi and crypts of Lieberkühn in duodenum. (b) PMCA in LSL hens (n=5) at the base of the villi and crypts of Lieberkühn in duodenum. Score 0: no staining. Score 1: weak staining. Score 2: moderate staining. Score 3: strong staining. Results are expressed as mean ± SE. * (*p<0.05), ** (p<0.01).*

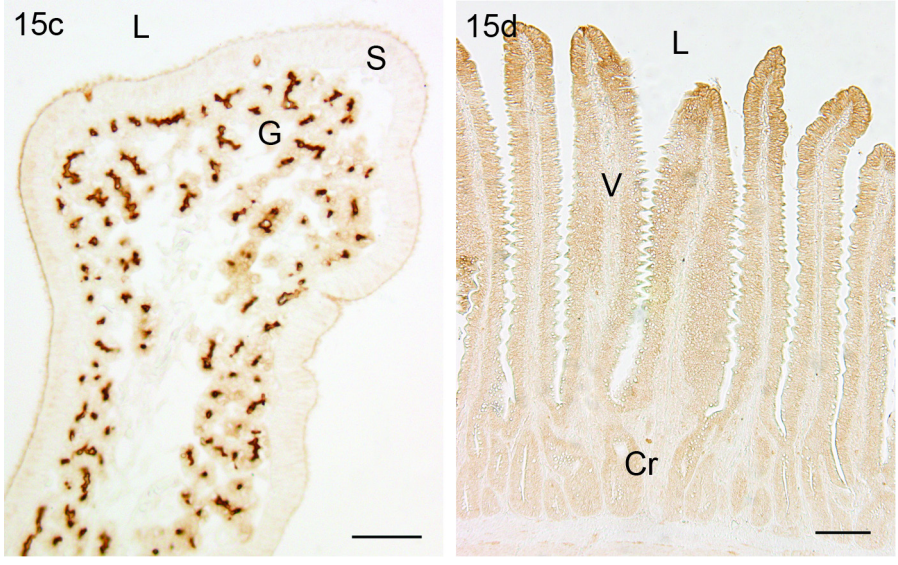

Figure 15. Immunohistochemical localization of plasma membrane calcium ATPase (PMCA) in laying hens. (c) Shell gland of LSL hen at 21 weeks of age, show strong membrane-bound staining for PMCA in the apical part of tubular gland cells (G) and weak apical membrane staining of surface epithelial cells (S). Bar=50µm (d) Duodenum of LSL hen at 21 weeks of age, strong staining for PMCA in basolateral membranes of villi (V) and weaker staining in crypts of Lieberkühn (Cr). Bar=133µm. L = lumen.

4 Discussion

During all three experiments, the main focus has been shell formation. How key-factors associated to shell formation alter during the production period, under the influence of phytoestrogen in the feed or treatment with exogenous estradiol at the end of the laying period. The skeleton contributes one third of the calcium for eggshell formation and since osteoporosis is a welfare concern for the egg producing industry, bone strength has been evaluated in parallel, but with no intention of covering every aspect of this complex problem.

Both hybrids used in this thesis produced eggs with reduced shell weight and shell quality in mid-production, at the age of 49 weeks. Shell quality was registered as both shell deformation and breaking strength and neither of those parameters deteriorated any further during the later stages of the production period. Shell weight even tended to recover somewhat at 70 weeks of age. Parallel to the reduced shell quality, bone strength was reduced over time with the first signs of osteoporosis detected as early as 29 weeks in the leg (femur). The lowest bone strength was recorded at 49 weeks in both leg and wing (humerus), but the leg appeared to have gained some strength at 70 weeks. The ability to store calcium in the skeleton decreases with age (Fleming *et al.*, 1998) while egg production is still high and egg weight increases, as seen in our project as well as others. The results presented in this thesis indicate that modern laying hens, represented by one white laying (LSL) and one brown laying (LB) hybrid, reach a physiological imbalance in mid-production, when the demands for both shell formation and skeletal strength cannot be upheld, but that they manage to stay at this level until the end of the laying period, at least in the production system used in this thesis.

The eggshell consists mainly of $CaCO_3$ and the ion precursors are transported to the shell gland by the blood. During shell formation blood flow through the shell gland increases five-fold (Wolfenson *et al.*, 1982). The number of capillaries in the shell gland increased in the young hens as they reached peak production and thereafter stayed constant during the production

period. Earlier findings from our laboratory show that hens with experimentally induced eggshell thinning have reduced number of capillaries in the shell gland (Berg *et al.*, 2004), suggesting that capillary density in the shell gland is of importance for eggshell formation. However, since capillary density remained constant as the birds age it is not likely to be a factor involved in age related reduction in eggshell quality. In our experiments we found a difference between the hybrids where white laying hens had higher capillary density in the shell gland compared to brown laying hens. A higher number of capillaries indicates an increase in the capacity to provide the shell gland with ion precursors Ca^{2+} and HCO_3^- for shell formation. The overall production measured as laying percentage of the white laying hybrids was also higher when compared to the brown birds. Although it is well known that angiogenesis in mammalian uterine tissues is regulated by estrogen [reviewed in (Herve *et al.*, 2006)] no effect on capillary density was detected after estrogen treatment or after feeding the hens with a supplement of daidzein.

A link between CA and eggshell formation was presented as early as 1944 when Benesch and coworkers (1944) found that feeding a CA inhibitor to laying hens results in thin-shelled or even shell-less eggs. Even if later experiments have shown that inhibition of CA in the shell gland mucosa (Eastin & Spaziani, 1978) actually affects shell formation, this early experiment was the result of oral administration of the inhibitor. As presented in this thesis, abundant staining for CA was found in the duodenum of both layer hybrids. It is therefore likely that oral administration of a CA inhibitor initially may have a local effect on the digestive tract, resulting in reduced uptake of calcium from the feed and subsequently thinner eggshells.

It has been known since the mid-eighties that CA is present in the laying hen duodenum (Nys & de Laage, 1984) but to our knowledge its localization to specific cell types has not been presented earlier. We found strong membrane-bound staining for CA in especially the surface epithelium of villi and in capillary endothelial cells. Cytosolic staining of enterocytes was moderate. The activity changed with age and showed a marked drop in mid-production, which coincided with the declining shell quality seen at 49 weeks. Nys and de Laage (1984) found that CA activity in the duodenum is lower in hens laying soft-shelled eggs compared to hens laying normal eggs, providing additional evidence that duodenal CA may be one factor involved in an age related reduction in shell quality. The fact that CA activity in the brown hens in our study was reduced in the duodenum following estrogen treatment, while shell quality improved in the brown eggs, may suggest otherwise. However, the reduction of CA was in the crypts only and the absorption of calcium is mainly in the villi and increases towards the top (Walters & Weiser, 1987; Bikle &

Munson, 1986). Interestingly the brown hybrids had stronger staining for CA in the brush border, lining the villi surface epithelium, compared to the white laying hens. The actual process of calcium absorption and the precise role of CA were beyond the scope of this thesis to cover. The role of CA in the mucosal defense, protecting the enterocytes from the acidic chyme in the duodenum, has been extensively studied in mammals (Sjöblom, 2011; Mizumori *et al.*, 2006). An interesting find is that a Ca^{2+} rich fluid in the duodenal lumen will elicit the same response from the enterocytes as luminal acidification. This will result in an increased production of HCO_3^- as Ca^{2+} is absorbed (Akiba *et al.*, 2009) and provides a possible link between CA and duodenal calcium absorption.

In the shell gland CA was detected in both capillaries and membrane-bound in tubular glands, as has been presented earlier from our group (Holm *et al.*, 2006; Berg *et al.*, 2004). The most favored hypothesis about the function of tubular gland cells is that they are responsible for providing the HCO_3^- needed for shell formation and that CA is involved in this process (Jonchere *et al.*, 2012; Mongin & Carter, 1977; Simkiss & Taylor, 1971). In our study the overall density of the tubular glands decreased at 49 weeks, which coincided with reduced shell quality, lending some additional support for this proposed function. The distribution of tubular gland CA in all three experiments was, however, limited to weak membrane-bound staining that was patchy and somewhat inconsistent. Treatment with estradiol, but not daidzein, increased the distribution of tubular gland CA in the white hybrids and improved shell quality, but shell quality of the brown eggs also improved with no effect on tubular gland CA in the bird.

We found that the number of capillaries positive for CA activity in the shell gland increased at 49 weeks of age, i.e. in the middle of the production period. These findings are somewhat surprising since results from our previous experiments indicate that the number of capillaries with CA activity is lower in older hens (Holm *et al.*, 2006; Berg *et al.*, 2004). Feeding laying hens with a controlled amount of daidzein increased the number of CA positive capillaries in the brown laying hens but not the white laying hens. The white hens on the other hand reacted with an increase in number of CA positive capillaries and also the distribution of tubular gland CA, when they were treated with estradiol, this time the brown laying hens were unaffected. Several investigations show that CA may be up-regulated by estrogen (He *et al.*, 2010; Barnett *et al.*, 2008; Caldarelli *et al.*, 2005; Pincus & Bialy, 1963), and since ERs were detected in both tubular gland cells and capillary endothelia there is at least a possible route for this effect. The exact role of capillary CA in the shell gland remains to be determined, but since shell gland HCO_3^- is derived

from metabolic CO_2, and at least some of this CO_2 must be transported by the blood, it is reasonable to assume that capillary CA may be involved in facilitating the diffusion of CO_2 across the endothelium. Capillary CA may also influence the micro circulation by mediating changes in extra- and intracellular pH, as seen in the rat retina (Reber *et al.*, 2003).

In the present thesis the localization of ERβ was described for the first time in the laying hen shell gland and both ERs can now be presented, including how they are altered during a production period and affected by treatment with estradiol or a daidzein supplement. The antibodies used for the detection of ERα resulted in some inconsistency regarding the results presented in paper I compared to papers II and III. At the time of writing this thesis an extended evaluation of this discrepancy is being performed and as a precaution only the results which clearly overlap between the two antibodies will be included in the discussion.

In tubular gland cells both ERα and ERβ were found, with the most distinct staining in the nuclei and weaker cytosolic staining. Age related thinning of the eggshell has been associated with decreasing levels of ERα (Hansen *et al.*, 2003) and in experiment II we detected a significant decrease in tubular gland ERα in mid-production (49 weeks). Although this is earlier than described by Hansen and co-workers (2003) it did coincide with the reduction in shell quality detected in our experiment. Nuclear ERβ was strong and did not change with age. In tissues expressing both receptors, ERα and ERβ may have opposite functions. In mammary gland epithelial cells from mice, ERα elicits proliferation, while ERβ is believed to suppress proliferation (Morani *et al.*, 2008). According to Böttner et al. (2013), ERβ can be considered a dominant negative regulator of ERα and the ratio between ER's within a cell may determine its sensitivity to estrogens and therefore the biological response. Since the function of tubular gland cells may involve both calcium and bicarbonate transport, the change in ratio between the two ERs seen at 49 weeks may be one cause behind the reduced shell quality. At 72 weeks, treatment with a low continuous dose of estradiol, but not daidzein supplementation, improved shell quality in both hybrids. The staining for ERα was decreased in the white laying hens, which may seem contradictory, but the improvement of shell quality in the white birds was not as convincing as in the brown hybrids.

In the surface epithelium both nuclear and cytosolic ERβ was detected. The nuclear staining differed somewhat between ciliated and non-ciliated cells but did not show any changes over time that could indicate an effect on shell quality. The staining intensity for cytosolic, or possibly granular, ERα decreased in mid–production. Since the surface epithelium is likely to be

involved in the provision of both matrix proteins and the bulk of calcium needed for the shell, these results may have a connection to the decreased shell quality seen at 49 weeks. Interestingly, in the oldest hens (paper I and III) the cytosolic ERβ is stronger in the brown hybrids. In paper I they produced bigger eggs with thicker shells and in paper III the estrogen treatment improved several shell quality parameters in the brown eggs.

In the duodenum ERβ was located in nuclei and cytosol of the enterocytes and the only co-localization with ERα was in nuclei of smooth muscle cells. Böttner and co-workers (2013) consider ERβ the primary receptor mediating effects of estrogen in the digestive tract of mammals. Results in this thesis suggest that this may well be the case also in laying hens. Estrogen is clearly involved in calcium absorption acting both through its nuclear receptors in the traditional manner [reviewed in (Beck & Hansen, 2004)] and in a non-genomic manner acting via extra-nuclear ER (Van Cromphaut *et al.*, 2003; Picotto *et al.*, 1996). According to several investigations duodenal calcium absorption decreases in laying hens as they grow older (Al-Batshan *et al.*, 1994; Garlich *et al.*, 1984) and boosting with exogenous estrogen may counteract this effect (Hansen *et al.*, 2004). In this study no effect of endogenous estradiol treatment could be detected on calcium availability in plasma, but the dose was much lower compared to the study made by Hansen and co-workers (2004). In addition, to be certain that there is an effect on the absorption in the duodenum blood samples need to be collected from the portal vein, which was not an option in our experimental setup. From the results presented in this thesis it is not likely that ERβ or PMCA is involved in age-related reduction in eggshell quality since neither was altered by age, with one exception. In the brown hybrids PMCA increased at the base of the duodenal crypts at 49 weeks. In general the staining for PMCA in duodenal mucosa did show a gradual difference from the base to the top of villi, with the strongest staining at the top. This follows the same pattern as absorption i e more calcium is absorbed at the top of the villi than at the base (Walters & Weiser, 1987; Bikle & Munson, 1986) so it is doubtful that this change had any real impact on calcium absorption in the brown laying birds.

Osteoporosis in laying hens is a complex problem that has accentuated with the use of cages and breeding for increased productivity (Webster, 2004; Whitehead & Wilson, 1992). It can be defined as a progressive loss of structural bone for the benefit of medullary bone, which provides one third of the calcium needed to produce eggshell (Wilson & Thorp, 1998). There are several techniques to measure osteoporosis, but to gain as much information as possible from single bones, three-point breaking strength was used in this thesis since it does not only evaluate the strength of the structural and spongy

bone, it is also positively correlated to the amount of medullary bone present (Fleming *et al.*, 1998). In this thesis the first sign of weaker bone was detected already at peak production (29 weeks of age) in femur (leg) which was even lower at mid-production (49 weeks), but appeared to recover somewhat towards the end of the laying period. The strength of the humerus (wing) decreased slightly later in mid production (49 weeks of age). Physical activity affects bone strength in a positive manner making the housing system an important factor to consider. Birds housed in conventional cages generally improve bone strength of the legs but not the strength of the wings since the space for flying or flapping their wings is limited (Silversides *et al.*, 2012). However, only the small movement onto a perch in the cage improves the bone strength of the humerus (Abrahamsson & Tauson, 1993). The fact that our birds were housed in commercially available furnished cages with five birds instead of the usual eight, gave our birds a little more space and could therefore have a positive effect on bone strength.

Phytoestrogens have been shown to have beneficial effects on bone strength in mice and humans (Morito *et al.*, 2001; Ishimi *et al.*, 2000; Potter *et al.*, 1998). However, our preliminary results reveal that daidzein in the feed did not have any effect on bone strength at the end of the laying period and neither had a low dose of estradiol. Even with a much higher dose (100mg/kg) of daidzein during a period of 12 weeks no effect on bone mineralization was detected (Gu *et al.*, 2013) suggesting that phytoestrogens may not be as beneficial to laying hens as to mammals, at least not beneficial regarding osteoporosis. Daidzein, at this dose did, however, improve eggshell quality (Gu *et al.*, 2013) and in a study by Ni and co-workers (2007) 10mg/kg feed of daidzein had a positive effect on both egg production and shell quality. In our study a dose of 50 mg/kg feed did not improve the shell quality or production in either of the hybrids, but we found that the two hybrids reacted differently to daidzein, as capillary CA increased in the LB hens but not in LSL. These results and the fact that the other investigations used other hybrids, suggest that there may be differences in sensitivity between breeds. Equol is a metabolite of daidzein which is produced by intestinal bacteria in some but not all human adults. Only 25-30% of the adult population of Western countries can produce equol, compared to 50-60% of adults from Japan, Korea or China (Setchell & Clerici, 2010). Daidzein is transformed into equol in white leghorn layer hybrids (Saitoh *et al.*, 2004). If this phenomenon shows the same variability between different layer hybrids as among humans it does provide one possible explanation to the differences in effects between these experiments. The discrepancy seen between our study and several others regarding improvement of shell quality and egg production might be the basal diet in these studies,

which most often contained various amount of soya bean meal or legumes. It is difficult to determine if the response is due to daidzein or not since the amount of daidzein in soya bean may vary greatly (Reinli & Block, 1996) and most likely affect the outcome.

5 Conclusions

- In this thesis age related reduction in shell quality was detected already in mid-production for both layer hybrids. Thereafter the birds appeared to cope, since shell quality was maintained throughout the laying period. Bone strength started to decrease even earlier and the first sign was found in peak production, with the lowest quality measured in mid-production. The bone strength did recover somewhat at the end of the laying period. A feed supplement of daidzein or a low continuous dose of estradiol did not improve bone strength.
- The presence of ERβ in the shell gland and duodenum are novel findings in domestic hens. The localization or intensity of ERβ labeling was not altered during the laying period and was not affected by a feed supplement of daidzein or a low dose of exogenous estradiol, at the end of the laying period. When evaluated in parallel with ERα, an altered ratio between the two receptors in the shell gland coincided with declining shell quality and is suggested as one possible factor behind age-related reduction in eggshell quality.
- Daidzein, a potent activator of ERβ mediated processes, did not affect shell quality or egg production when added as a feed supplement in a soya free diet. However, a difference in sensitivity to daidzein was noted between the two hybrids, as capillary CA activity was increased in the LB hens but not LSL.
- Boosting laying hens with a low continuous dose of estradiol improved shell quality in both hybrids without affecting body weight, egg production or plasma calcium. Furthermore, the results show that shell gland CA in both tubular glands and capillaries may be regulated by estrogen, possibly by acting via ERβ, but that differences occur between hybrids since the effect was noted only in the white birds.

- Tubular gland density of the shell gland decreased in mid-production and coincided with decreasing shell quality. Capillary density is of importance for providing the precursors for shell formation but after an initial increase at peak production the density was maintained and therefore not primarily involved in the subsequent reduction of shell quality.
- CA catalyzes the first step in carbonate ion formation and the number of CA positive capillaries increased with age. CA activity in tubular gland cells in the shell gland was inconsistent and the role of CA could not be connected to decreasing shell quality found with age.
- The localization of CA activity in duodenum has now been verified in domestic hens and a dramatic drop in CA activity coincided with decreasing shell quality found in mid-production. The calcium transporter PMCA was localized in both duodenum and shell gland, but did not seem to be involved in the age-related decrease in shell quality.
- Even though one can expect similar breeding goals for layer hybrids this thesis shows physiological differences between hybrids that complicate the interpretation of results and must be taken into account when comparing data from other investigations.

6 Svensk sammanfattning

Våra moderna värphöns hybrider har en anmärkningsvärd förmåga att omvandla foder till ägg. I kommersiell äggproduktion anländer hönorna till stallet då de har blivit könsmogna vid ca 16 veckors ålder och stannar fram till 70-75 veckors (~1,5år) ålder, vilket motsvarar en värpperiod. Under en produktionstid på 12 månader lägger hönan ungefär 320 ägg vilket motsvarar nästan ett ägg om dagen eller runt 20 kilo ägg per höna. Skalet till äggen består till 96% av kalciumkarbonat och ca 4% organiskt material. Till varje ägg går det åt ca 2-2,5g kalcium som hönan absorberar från fodret i första delen av tunntarmen (duodenum) och lagrar i skelettet. När hönan närmar sig slutet av värpperioden försämras successivt äggets skalkvalitet. Parallellt med skalförsämringen blir hönans skelett skörare vilket ökar risken för skelettdeformationer eller benbrott.

Det är lätt att glömma att äggproduktion har med reproduktion att göra och att varje ägg på frukostbordet har börjat med en ägglossning hos hönan. Ägglossningen styrs av hormoner, framför allt progesteron och östrogen. Östrogen har även en koppling till kalciummetabolism vilket gör det ytterligare intressant för skalbildningen. I vetenskapliga studier med östrogena miljögifter har man kunnat visa att dessa har med skalförtunning att göra där bland annat enzymet karbanhydras (CA) påverkas. Enzymet katalyserar reaktionen $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$ och deltar i bildandet av karbonatjoner till skalet. Det finns även forskning som visat att östrogenreceptorer, cellens kontaktstrukturer för hormonet, minskar med ålder hos höns.

I min avhandling har jag studerat faktorer som kan ha betydelse för den försämring av skalkvalitet som sker hos värphöns mot slutet av värpperioden. Hur dessa faktorer förändras under värpperioden och när det i så fall sker. Jag har även undersökt om man kan utnyttja växtöstrogen som finns naturligt i vissa typer av fodermedel, eller om en låg kontinuerlig dos av ett östrogen som

är mer likt det kroppsegna, kan påverka skalkvalitet och benstyrka hos hönsen i slutet av värpperioden.

Tre experiment genomfördes med två vanligt förekommande värphönshybrider, en brun Lohmann Brown (LB) och en vit Lohmann Selected Leghorn (LSL). Hönorna stallades in i inredda burar och utfodrades under normala produktionsbetingelser i Sveriges Lantbruksuniversitets (SLU) försöksstall för fjäderfä.

Försök I: Hönorna utfodrades med ett tillskott av daidzein, som är ett växtöstrogen som finns i soja. Hönorna fick under en 12 veckors period i slutet av värpperioden antingen daidzein i fodret eller enbart ett kontroll foder.

Försök II: Hönorna följdes under en hel värpperiod, från unga höns till slutet av värpperioden för att få en bild av hur olika faktorer förknippande med skalkvalitet och benstyrka normalt förändras under värpperioden.

Försök III: Hönorna injicerades i slutet av värpperioden med en pellet under huden som utsöndrade en låg dos av östrogen under 14 dagar eller med en placebopellet.

Under alla försök mättes äggproduktion, äggskalens kvalitet, benstyrka, täthet av kapillärer i skalkörtel och koncentration av kalcium i plasma. Lokalisering av CA, östrogenreceptorerna alfa och beta (ERα, ERβ) samt en membranbunden kalciumtransportör (PMCA) utfördes på vävnad från skalkörtel och duodenum.

Under försök II kunde jag konstatera att skalkvaliteten hade försämrats redan i mitten av värpperioden vid 49 veckors ålder, då äggproduktionen fortfarande var hög. Denna kvalitetsnivå kunde dock hönorna bibehålla värpperioden ut. Redan innan, så tidigt som vid 29 veckors ålder, upptäcktes första tecknet på att benstyrkan av lårbenet hade försämrats. Den lägsta benstyrkan uppmättes på både lårben och överarmsben i mitten av värpperioden. Mot slutet av värpperioden kunde en förbättring av benstyrkan i lårbenet noteras.

Lokaliseringen av ERβ förändrades inte under värpperioden och kunde inte kopplas till försämringen av skalkvaliteten. ERβ påverkades inte av foder med daidzein eller behandling med östrogen. Däremot kunde vi konstatera att med en sammanslagen bild av både ERβ och ERα syns en förändring i proportionen mellan de två receptorerna i skalkörteln, då ERα minskar samtidigt som skalkvaliteten försämras.

I skalkörteln finns körtelceller som bidrar med framför allt karbonatjoner, men även kalcium joner till skalet. Vid mitten av värpperioden var densiteten av dessa körtlar som lägst vilket sammanföll med försämrad skalkvalitet. Via kapillärerna i skalkörteln sker transporten av de byggstenar som behövs för

skalbildningen. Tätheten av kapillärer i skalkörteln ökade från unga höns till högproducerande höns och var därefter konstant genom hela värpperioden.

Aktiviteten av CA i skalkörtel hos värphöns är lokaliserad till körtelcellerna och kapillärerna. Antalet kapillärer med CA aktivitet ökade med åldern, men var förhållandevis liten mot det totala antalet kapillärer. CA-aktiviteten i körtlarna förekom fläckvis och kunde inte kopplas till försämringen av skalkvaliteten.

Att fodret innehåller tillräckliga mängder kalcium är avgörande för skalbildning. Den huvudsakliga absorptionen av kalcium sker i duodenum. Lokaliseringen av CA aktivitet i duodenum hos värphöns är nu klargjord och aktiviteten minskade dramatiskt i mitten av värpperioden, vilket också sammanfaller med försämringen av skalkvaliteten. För att transportera kalcium vidare från cellerna i duodenum finns bland annat PMCA, en membranbunden kalcium transportör. I den här avhandlingen kunde vi inte finna något samband mellan PMCA och försämrad skalkvalitet varken i skalkörtel eller i duodenum.

De hönor, av båda hybriderna, som fått en låg kontinuerlig dos av östrogen i slutet av värpperioden fick en förbättrad skalkvalitet, men det påverkade inte benstyrkan. Den låga dosen av östrogen påverkade inte heller äggproduktion, kroppsvikt eller kalciumnivåerna i plasma. Däremot ökade östrogen aktiviteten av CA i körtlarna och kapillärerna samt minskade ERα i skalkörteln. Denna förändring skiljer sig mellan hybriderna då förändringen skedde enbart hos de vita hönsen. Ett tillskott av växtöstrogenet daidzein i fodret påverkade inte skalkvalitet, äggproduktion eller benstyrka i den här studien. Däremot kunde vi konstatera att daidzein ökade antalet kapillärer med CA aktivitet, men enbart hos de bruna hönsen.

Känsligheten för både östrogen och daidzein skiljer sig alltså mellan hybriderna. Trots att man kan tänka sig att det hos våra värphönshybrider finns liknande avelsmål har jag i den här avhandlingen funnit ett flertal intressanta fysiologiska skillnader mellan de två hybriderna, vilket var oväntat. Hybridskillnaderna förser oss med viktig kunskap om alternativa fysiologiska förlopp som vi måste ta hänsyn till, samtidigt som det komplicerar tolkningen av både egna och andras resultat.

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