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K.K. Hansen
University of Nebraska-Lincoln

R.J. Kittok
University of Nebraska-Lincoln, rkittok1@aol.com

G. Sarath
USDA-ARS, Gautam.sarath@ars.usda.gov

C.F. Toombs
University of Nebraska-Lincoln, ctoombs1@unl.edu

N. Caceres
USDA-ARS

See next page for additional authors

PHYSIOLOGY AND REPRODUCTION

Estrogen Receptor-α Populations Change with Age in Commercial Laying Hens[1]

K. K. Hansen,*,[2] R. J. Kittok,* G. Sarath,† C. F. Toombs,* N. Caceres,† and M. M. Beck*,[3]

*Department of Animal Science and †USDA-ARS, University of Nebraska-Lincoln, Lincoln, Nebraska 68583

ABSTRACT Older hens in production lay larger but fewer eggs than younger birds, and the incidence of soft and broken shells is greater in older hens than younger. These changes are attributable at least in part to changing hormone profiles and diminished ability of the hen to transport calcium at the duodenum. In further exploration of this relationship, a study was conducted with three ages of Hy-Line W-36 birds: prelay pullets (PL; 19 wk, 0% production), peak-production hens (PP; 29 wk, ~93% production), and late-stage hens (LS; 71 wk, ~80% production). Hens from the PP and LS groups were palpated for presence of an egg in the shell gland; hens were then euthanized and tissues (kidney, shell gland, hypothalamus) were removed for quantification of estrogen receptor-α (ERα) populations via immunocytochemical and Western blot analyses. Localization of ERα by immunostaining in the shell gland showed differences among age groups; however, no differences were noted in localization of ERα between age groups in the kidney and hypothalamus. In both the kidney and the shell gland there was a decrease in the amount of ERα, as detected by immunoblotting, in the LS hens compared to PL and PP birds ($P < 0.05$). The results suggest that failure of calcium regulating mechanisms with age may be mediated at least in part through the reduced populations of estrogen receptors in certain critical tissues.

(*Key words*: estrogen receptor, hormone profile, hypothalamus, kidney, oviduct)

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INTRODUCTION

Egg production and shell and bone quality decrease as hens age. Older hens in production lay larger but fewer eggs than younger birds; the incidence of soft and broken shells is greater, and bones become brittle (Izat et al., 1985; Joyner et al., 1987; Al-Batshan et al., 1994). Williams and Sharp (1978) documented the widespread observation that the rate of lay declines in flocks of hens as they age and that this decline is in part the result of shortening sequences. The changes in egg production and shell and bone quality are attributable also to changing hormone profiles, decreased sensitivity of tissues to hormone action, and diminished ability of the hen to transport calcium at the duodenum (Al-Batshan et al., 1994; Hansen, 1998, 2002).

Mechanisms that regulate calcium underlie shell formation and hormone regulation, and the complex reciprocal relationship of calcium with estrogen is well documented. In addition to increases in egg size with age, decreases in shell quality are, of course, closely associated with calcium availability at the site of either its source (primarily diet, secondarily bone) or its delivery (shell gland). It has been shown that intestinal calcium absorption decreases with age in rats (Horst et al., 1978), humans (Schachater et al., 1960; Avioli et al., 1965, Bullamore et al., 1970) and chickens (Al-Batshan et al., 1994; Hansen, 2002). Age-related decreases in plasma estrogen concentrations in humans have been associated with a greater risk of osteoporosis for several years (Silverberg and Lindsay, 1987).

Commercial egg producers have long used molt, the naturally occurring annual or biannual process of feather replacement in many avian species, to improve egg production and shell quality in older laying hens. Scientists have shown that, in addition to these benefits, molting does in fact serve to rejuvenate the hen's reproductive system, to increase the synthesis of calcium-binding protein, Calbindin D28K (CaBP D_{28K}) (Berry and Brake, 1991), and to enhance calcium transport mechanisms (Al-Batshan et al., 1994) in gut tissue.

Calcium homeostasis in the hen is highly complex, as are the relationships between estrogen and calcium, and the exact mechanisms that link them have yet to be fully elucidated. Because any hormone action necessarily begins with the interaction of hormone and its receptor, it seemed

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[2]Present address: Eppley Institute for Cancer Research, 98685 University of Nebraska Medical Center, Omaha, NE 68198-6805.

[3]To whom correspondence should be addressed: mbeck1@unl.edu.

Abbreviation Key: ERα = estrogen receptor-α; LS = late-stage hens; PL = prelay pullets; PP = peak-production hens.

logical to begin an investigation of the action of estrogen on calcium homeostasis in the aging hen with that step in the regulatory cascade. Therefore the following study was conducted to localize and quantify estrogen receptor-α (ERα) in kidney, hypothalamus, and shell gland over the productive lifetime of the hen.

MATERIALS AND METHODS

Birds

We obtained Hy-Line W36 hens from a commercial layer operation, brought them to our facility, and allowed them to acclimatize for at least 1 mo prior to sampling at the following ages [prelay (PL), <19 wk of age, 0% production; peak production (PP), 27 to 29 wk of age, approximately 93% production; and late-stage production (LS) 71 wk of age, approximately 65% production]. The hens were housed individually in an environmentally controlled room at a constant temperature of 22°C. They were provided water and a layer diet (2,947 ME Kcal/kg feed, 3.8% Ca, 0.5% P, 17.0% protein) ad libitum. The photoperiod consisted of 16L:8D. Ten hens per age group were randomly selected for the study. All animal and experimental procedures were conducted with approval of the University of Nebraska-Lincoln IACUC.

Hens were euthanized by cervical dislocation shortly before oviposition (as determined by abdominal palpation) or, for PL pullets, at approximately the same time each day. For immunocytochemical localization of ERα, tissues (shell gland, kidney, hypothalamus) were excised, embedded in OCT medium[4] in plastic paraffin molds, and immediately frozen on dry ice and stored at –80°C until assayed. For quantification of ERα by Western blot, tissues (shell gland and kidney) were excised, immediately frozen in liquid nitrogen, and stored at –80°C until assayed. In all cases, samples were from similar segments of the respective tissue. Estrogen receptor-β was not included in this analysis, even though its message has been found in various tissues of the Japanese quail (Foidart et al., 1999). In spite of extensive effort, we were unable to obtain antibody to estrogen receptor-β and so were unable to include it in the study.

Experimental Protocol

Immunocytochemistry. The protocol for immunocytochemistry was previously described by Kwon et al. (1997). Frozen tissues were sectioned at 7 μm in a cryostat microtome[5] at –20°C. Tissue sections were thaw-mounted on precleaned glass slides; hypothalamic tissue sections were thaw-mounted on Ultra-stick glass slides,[6] and all sections were freeze-substituted for 2 d in anhydrous acetone at –80°C. The frozen sections were fixed in 3.7% formaldehyde in 0.1 *M* PBS (pH 7.4) for 10 min at 4°C. Sections were rinsed with PBS plus Triton X-100 and incubated in 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase. Sections were then treated with normal rabbit serum[7] to reduce background staining and an avidin/biotin block[7] to inhibit endogenous protein-bound biotin. The sections were then incubated with a rat monoclonal anti-human ERα antibody (H222) kindly provided by Geoffrey L. Greene, University of Chicago. Control sections were treated with rat immunoglobulin (IgG)[8] in place of the primary antibody at the same concentration (10 μg/mL) as the primary antibody. Sections were then processed with a Vectastain Elite ABC kit,[7] an avidin-biotin-peroxidase method. Peroxidase was visualized with a Vector SG substrate kit.[7] All sections were counterstained with Nuclear Fast Red. Images were obtained utilizing confocal microscopy, and grayscale images were obtained by neutralizing the counterstain.

Tissue Homogenization and Preparation for Western Blot Analysis. All procedures were performed at 4°C. Tissues were individually homogenized in a volume (6 mL/g tissue) of extraction buffer containing 50 m*M* Tris-HCl (pH 8.0), 500 m*M* KCl, 2 m*M* DTT, 1 m*M* EDTA, and 0.05% protease inhibitor cocktail[8] in a 15-mL polypropylene tube using a Polytron homogenizer.[9] The homogenate was filtered through cheesecloth into a microcentrifuge tube and centrifuged at 14,000 rpm for 15 min at 4°C. The aqueous phase between the upper fat layer and the pellet was collected into a fresh microcentrifuge tube and recentrifuged at 14,000 rpm for 15 min at 4°C. The resulting supernatant was collected into a fresh microcentrifuge tube and stored at –20°C prior to being used in Western blot analysis. Protein concentrations were calculated using the BCA Protein Assay kit[10] with bovine serum albumin as the standard. Concentrations of protein averaged 3.732 μg/μL for shell gland and 6.85 μg/μL for kidney.

Avian tissue contains a large amount of lipid that can interfere with protein separation; this lipid was precipitated out by adding ~900 μL of ice-cold acetone in 50-μL increments to an aliquot of extracted tissue (Hansen, 2002). The final solution was incubated on ice for 20 min with gentle mixing by inversion at 5-min intervals. The solution was centrifuged at 14,000 rpm for 15 min at 4°C, and the acetone was poured off and allowed to evaporate completely, leaving the pellet. Pellets were resuspended in Laemmli sample buffer[11] plus 350 m*M* DTT and subsequently heated at 95°C for 5 min before electrophoresis.

Western Blot Analysis. Prestained molecular weight markers[11] were included, and rat uterine tissue (100 μg) was used as the positive control. The amounts of protein loaded per tissue were as follows: shell gland, 150 μg total protein; kidney, 200 μg total protein. Proteins were separated by SDS-PAGE by using 10% gels with 5% stacking gels in a vertical gel electrophoresis system.[12] Gels were

[4]Tissue-Tek, Elkhart, IN.
[5]Damon/IEC Division, Needham Heights, MA.
[6]Gold Seal Products, Portsmouth, NH.
[7]Vector, Burlingame, CA.
[8]Sigma Chemical Co., St. Louis, MO.
[9]Brinkmann Instruments, Westbury, NY.
[10]Pierce, Rockford, IL.
[11]Bio-Rad Laboratories, Hercules, CA.
[12]GibCo BRL, Life Technologies, Gaithersburg, MD.

run at 150 V until the dye band reached the end of the stacking gel and then at 200 V until the dye band reached the end of the gel. Proteins were transferred from the polyacrylamide gels to 45-μm nitrocellulose membranes[11] by electroblotting in a Semi-Dry blotting apparatus[13] at a constant setting of 14 V for 1.5 h. Following transfer, gels were incubated with Coomassie blue stain to stain remaining proteins as a method of ensuring equal loading of proteins within tissue type across wells. In addition, duplicate gels were also run and subsequently stained with Coomassie blue as another check for equal loading across wells.

We used Coomassie blue, a general protein stain, instead of running a housekeeping protein as reference for this particular study. Our objective was to ensure, to the extent possible, that any differences in ERα staining caused by uneven protein loading could be accounted for. In addition, housekeeping protein concentrations can vary with age and other physiological conditions, making their use as an absolute reference problematic (G. Sarath, unpublished data; B. White, 2002, University of Nebraska, personal communication).

Membranes were blocked with 5% dry milk in Tris-buffered saline with 0.05% Tween-20 (TBST, pH 7.5) for 1 h. After washing with TBST, membranes were agitated for 16 to 20 h in a 5 μg/mL solution of rat monoclonal anti-human ERα (H222; Geoffrey L. Greene, University of Chicago, Illinois) in 5% milk TBST. Membranes were then washed and treated with anti-rat IgG-peroxidase conjugate, diluted 1:17,000 in antibody buffer, and agitated for 1 h. The membrane was washed with TBST followed by an additional washing with TBS (Tris-buffered saline). Immunoreactive proteins were visualized using SuperSignal West Pico[10] chemiluminescence. Because ERα analysis by Western immunoblotting has never been reported previously in the hen, each tissue type had to be optimized with regard to amount of protein loaded and assay conditions, including concentrations of primary and secondary antibody, blocking agent, and chemiluminescence signal enhancer. For each tissue, ~15 gels were required to achieve optimal conditions and ensure repeatability of band separation relative to the known rat uterine standards.

Western blots were electronically scanned with a Visioneer PaperPort flat bed scanner (300 dpi resolution) and saved as 500 × 330 pixel TIFF images. Coomassie-stained gels were scanned with Odyssey Infrared Imaging System.[14] These digital images were then analyzed using Scion Image[15] on a Gateway PC according to a procedure similar to that reported by Vierck et al. (2001). Before measuring the intensity of specific bands, the background density of all blots was subtracted. Each specific band was then measured with Scion Image with a constant measurement area of 20 × 25 pixels (275 pixels) around the protein bands. The number obtained for each band of the immunoblot was divided by the number obtained for the respective band in the Coomassie-stained gel. This gave a relative protein density for each sample.

Statistical Analysis

The experimental design was completely randomized, with age as the fixed effect and hen as the experimental unit. Western immunoblot data were analyzed by ANOVA implemented in Proc GLM of SAS software (SAS Institute, 2000). The differences among means were determined using the least significance difference (LSD) test.

RESULTS

Representative immunocytochemistry images of the three tissues are shown in Figures 1, 3 and 5. For each image, the left side of each panel is the color image captured with confocal microscopy. The right side of each panel is the corresponding grayscale image obtained with neutralization of counterstain. Each panel is labeled according to the age group it represents, with the nonspecific binding panel serving as the control. In the nonspecific binding incubations, the first antibody was replaced with rat IgG.

In the shell gland (Figure 1), the majority of ERα immunostaining was concentrated in the epithelium. However, there were differences among age groups and production status regarding the type of epithelial tissue that was positively stained. In the PP and LS birds, the ERα immunostaining was localized to the luminal epithelium, whereas in PL birds there was positive staining in both the luminal and the glandular epithelial layers. No ERα immunostaining occurred in the NSB sections.

Immunoblotting of ERα in shell gland tissue of the three ages of birds is shown in Figure 2a, and densitometric measurements are shown graphically in Figure 2b. The major band at 66 kDa in rat uterine tissue has been noted previously (Hiroi et al., 1999). The 67-kDa band found in shell gland tissue in this study corresponds to this rat receptor band and is the same size as the avian estrogen receptor cloned by Krust et al. (1986) and Maxwell et al. (1987). The amount of ERα protein immunostaining in the shell gland was decreased in LS hens compared to PL and PP hens ($P < 0.05$); the greatest amount of ERα immunostaining was in the PL birds (Figure 2b; $P = 0.0492$).

The kidney sections were all cross sections taken from cortical tissue. ERα immunostaining was localized to the tubules (arrows, Figure 3). There were no apparent differences in location of ERα between the different ages of laying hens. There was some very slight background binding in the kidney even with use of the avidin/biotin blocking kit, perhaps because of the high concentration of endogenous protein-bound biotin in avian kidney tissue (Cooper et al., 1997). No ERα immunostaining occurred in the NSB sections.

The ERα immunoblot from kidney tissue is shown in Figure 4a and graphically in Figure 4b. Again, both rat uterine tissue and avian kidney tissue had a major band at ~66 or 67 kDa, respectively. Densitometric analysis (Figure 4b) of the kidney immunoblots demonstrated a de-

[13]Owl Separation Systems, Portsmouth, NH.
[14]Li-Cor, Lincoln, NE.
[15]Scion Image, Frederick, MD.

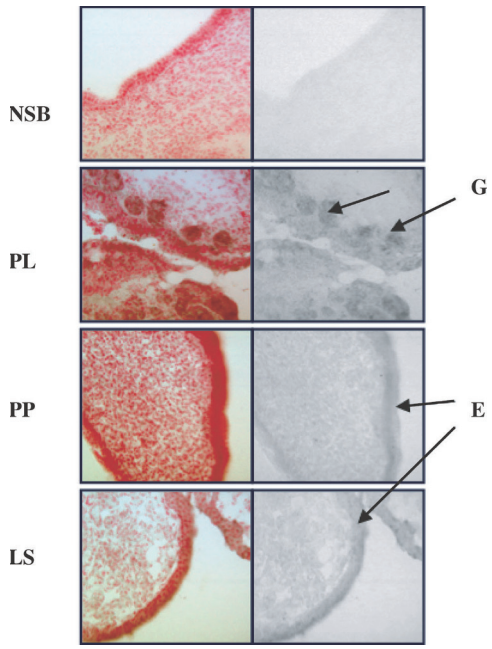

FIGURE 1. Immunocytochemistry results for localization of estrogen receptor-α in longitudinal sections of shell gland from three different ages of laying hens: prelay (PL) hens, peak-production (PP) hens, and late-stage production (LS) hens. Panels on the right represent the immunocytochemical results and panels on the left are the same sections with Nuclear Fast Red counterstain. Nonspecific binding of the tissue is presented in the upper panel (NSB). Glandular (G) and luminal epithelium (E) are denoted on the figure.

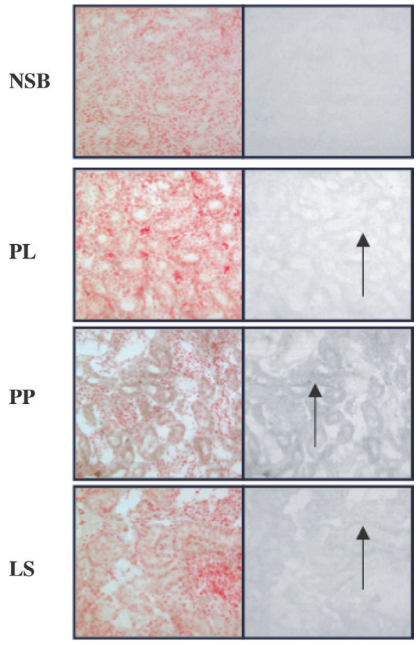

FIGURE 3. Immunocytochemistry results for localization of estradiol receptor-α in cross sections of kidney from three different ages of laying hens: prelay pullets (PL), peak-production (PP) hens, and late-stage production hens (LS). Panels on the right represent the immunocytochemical results, and panels on the left are the same sections with Nuclear Fast Red counterstain. Nonspecific binding of the tissue is presented in the upper panel (NSB). Staining was localized in the convoluted tubules (arrows).

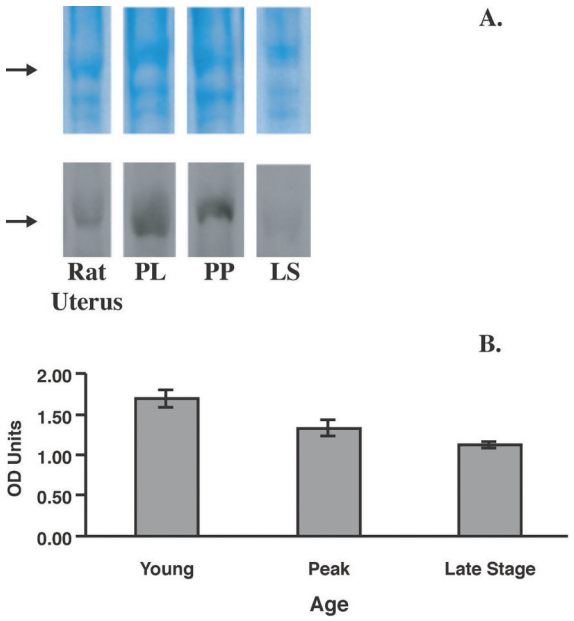

FIGURE 2. Western blot analysis of estrogen receptor-α (ERα) in avian shell gland tissue. A. Upper panel: a representative gel stained with Coomassie blue; the protein band corresponding to location of ERα is indicated by an arrow. Lower panel: representative immunoblot showing 67 kDa ERα band in rat uterine tissue and in shell gland tissue from prelay pullets (PL), peak production hens (PP), and late-stage hens (LS). B. ERα in the LS hens was less than in either of the other two groups ($P < 0.05$) and in PP compared to PL birds ($P < 0.05$), as normalized by optical density (OD) of the ERα band relative to the OD of the corresponding protein band in the Coomassie-stained gel.

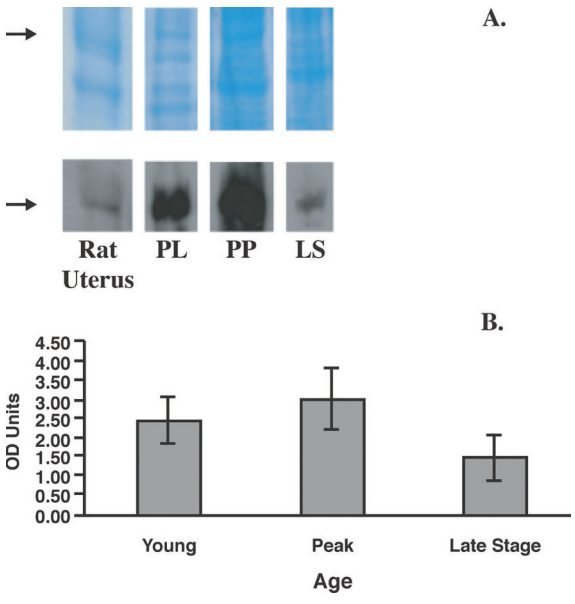

FIGURE 4. A. Western blot analysis of estrogen receptor-α (ERα) in avian kidney tissue. Upper panel: representative gel stained with Coomassie blue; the protein band corresponding to location of ERα is indicated by an arrow. Lower panel: representative immunoblot showig a 67-kDa ERα band in rat uterine tissue and in shell gland tissue from prelay pullets (PL), peak-production hens (PP), and late-stage hens (LS). B. ERα immmunostaining in the LS hens was less than in eiher of the other two groups ($P < 0.05$); in PP hens immunostaining was slightly higher ($P = 0.1152$) than in PL pullets, as normalized by optical density (OD) of the ERα band relative to OD of the corresponding protein band in the Coomassie-stained gel.

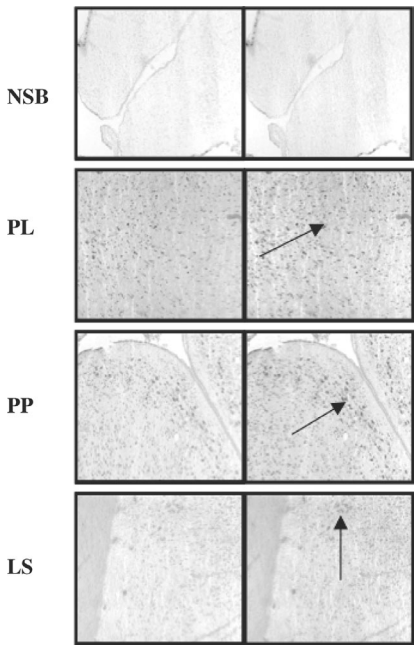

FIGURE 5. Immunocytochemistry results for localization of estradiol receptor-α in hypothalamus from three different ages of laying hens: prelay pullets (PL), peak-production hens (PP), and late-stage production hens (LS). Panels on the right represent the immunocytochemical results, and panels on the left are the same sections with Nuclear Fast Red counterstain. Nonspecific binding of the tissue is presented in the upper panel (NSB). Staining was localized in the cell nucleus (arrows). This figure is in color in the online version of this article.

crease in LS hens compared to PL and PP ($P < 0.05$). The greatest amount of ERα immunostaining in the kidney was in PP hens.

We chose to normalize the amount of ERα as measured by optical densitometry against the densitometry obtained from total protein in the respective Coomassie-stained band because of the inherent variability in amount of protein loading between wells and because it was important, within age group, to account for that variability. We acknowledge that there is a valid alternative methodology—namely, to run a housekeeping protein as an indicator to ensure that apparent age-related changes are real. However, for a variety of reasons, including the fact that housekeeping proteins can also change with age, we chose to normalize against total protein in the band within which the ERα protein is found.

All ERα immunostaining in the hypothalamic tissue appeared to be specifically localized in the cell nucleus (arrows, Figure 5) in all ages of hens. In this tissue, the intensity of staining did appear to decrease with increasing age; however, this was not quantified. Again, there was no ERα immunostaining in the NSB sections. The hypothalamic tissue was not subjected to analysis by Western immunoblotting because it is not a calcium regulating tissue in the way that the kidney and shell gland are.

DISCUSSION

To the best of our knowledge, there is no prior information regarding ERα immunostaining of calcium-regulating tissues during aging in the laying hen. This is also the first report of analysis of ERα by Western immunoblotting. The monoclonal ER antibody H222 has been shown to bind only the hormone-binding receptor region in human and chick tissues (Greene et al., 1984), and it has been used successfully in avian tissue (Joensuu and Tuohimaa, 1989; Kwon et al., 1997).

As hens age, there are apparent breakdowns in their ability to maintain optimum calcium levels (thin eggshells; fragile, easily broken bones); data from our laboratory (Hansen, 2002) and others (Al-Batshan et al., 1994) have shown a profound effect of age on the ability of duodenal tissue to uptake ^{45}calcium. Our results suggest that this breakdown may be mediated at least in part through reduced populations of estrogen receptor in kidney and shell gland.

The kidney is believed to be the primary site at which estrogen sets off the cascade of events leading to calcium uptake by the gut (Forte et al., 1983; Martz et al., 1985; Sommerville et al., 1989; Elaroussi et al., 1993). Because of the very high demand for calcium by the hen during the period of peak production, it seems likely that estrogenic action on the kidney would be maximal in this period. The very high ERα immunoblotting in PP birds in this study is consistent with this idea, and both PL pullets prior to lay and LS hens well after egg production declines would have less need for estrogen receptors in the kidney.

The localization of ERα in the glandular epithelium of the shell gland of the PL birds is consistent with earlier studies; Isola (1990) reported ER immunostaining in the glandular epithelium of the estrogen primed chick oviduct. In addition, Joensuu and Tohimaa (1989) noted a redistribution of estrogen receptor immunostaining from stromal cells and undifferentiated epithelial cells to glandular epithelial cells in chicks that had been treated with exogenous estrogen as well as in pullets at puberty; birds at 19 wk of age had ERα immunostaining only in differentiated tubular gland cells. In contrast, ERα immunostaining in PP and LS hens in this study was primarily localized to the surface epithelium of the shell gland, where estrogen would act to maintain the tissue and to enable the calcium flux for shell formation.

While the significant reductions in estrogen receptor populations in these two very significant calcium-regulating tissues with increasing hen age certainly provide some insight into the well-documented decreases in shell quality and skeletal integrity, it is highly unlikely that this would be the complete answer. The development of cage layer osteoporosis is attributable to a number of cooperating factors. Future studies will focus on further examination and characterization of the estrogen receptor populations and will take a biochemical approach into the mechanisms of estrogen action at these and other calcium regulating sites in the laying hen. It is our hope that these studies will provide useful information not only for the poultry industry and possible future management practices but also for the biological and physiological community as well.

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