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Hen Age Dependency of Eggshell Refractive Index in Terahertz Frequency

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Research Article

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Abstract

Eggshell is a natural protective barrier and plays multiple functions for an egg. This study investigated the noble and fundamental biological phenomena of hen age dependency of eggshell optical properties and microstructure using Terahertz spectroscopy and scanning electron microscope (SEM) respectively. SEM was used for taking the cross-sectional ultrastructure image of eggshell. Images were taken with resolution of 256 · 256 pixels, dimension of 1280 · 960 pixels and magnification at 1000x. Images were processed to visualize the differences in eggshell structure. In case of younger birds` eggshell, course microstructure, less deep mammillary layer, but wider space between two mammillary cones are found and opposite characteristics were found for older aged birds. The RI (a measure of material density) of the eggshell was significantly (*p*-value < 0.05) higher for older hens (2.6) than eggshell from younger hens (2.4). This study might contribute to egg and poultry industry and for better understanding the variations in eggshell in avian protocols and corresponding mother birds' health and nutritional status for precision egg and poultry production management.

Introduction

Eggshell is a natural protective barrier of an egg (in avian and reptile kingdom) and the symbol of great tailoring capability of mother birds which plays multiple functions for eggs such as protecting internal contents from contaminants and UV radiation (Maurer *et al.* 2015; Khaliduzzaman *et al.* 2020), take part in respiratory gas exchanges and major mineral sources for developing embryos (Tuan & Ono 1986; Richards 1997). As laying hens age, their eggshell changes. These changes include a significant reduction in mammillary knob density, reduced palisade layer thickness, and an increased ratio of the mammillary layer to the overall shell, which leads to weaker, less organized, and more porous shells.

Quality of the eggshell as a protective barrier depends on internal microstructure of the shell, which influences the density or compactness of eggshell. The internal microstructure of the eggshell can be studied using scanning electron microscopic (SEM) image, as scanning electron microscope is one of the common methods for imaging the microstructure and morphology of the materials. In SEM, an electron beam with low energy is radiated to the material and scans the surface of the sample. Several different interactions occur as the beam reaches and enters the material, which lead to the emission of photons and electrons from or near the sample surface (Omidi *et al.* 2017). Several morphological features such as particle size, grain size distribution, pore size, line edge roughness, overall surface texture, structural arrangement and connectivity of features are common to analyz for SEM image to observe the micro-structural differences.

Although the mother chickens throughout their life gathered a lot of experiences of depositing calcite layers over the internal content of the egg with their increasing laying age, the mother flocks perhaps also suffer many limitations due to their increasing biological age. The limitations of the older mother birds might be reflected into the eggshell internal structure and thus can be traced back their nature and health status based on the terahertz optical properties (e.g., refractive index), architecture and microstructure of the eggshell. Terahertz (THz) spectroscopy has high sensitivity and specificity to intermolecular vibrations, enabling it to investigate the crystallinity of a material based on specified absorption bands (Guo *et al.* 2023). The terahertz pulse-based sensing technology in agricultural research is a new, under explored, part of the electromagnetic wave range that promises to be an extremely useful tool in egg and poultry research (Khaliduzzaman *et al.* 2021). Thus, eggshell perhaps contains many unique and characteristics information of mother chicken. Thus, it is imperative to apply a new technology like terahertz electromagnetic pulse to study the eggshell for extracting fundamental biological information.

Therefore, this manuscript discovered the novel and fundamental biological phenomena on hen

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s age dependency of eggshell density evidenced from RI in THz region and SEM images. This research reported the density (i.e., refractive index in terahertz electromagnetic wave) and internal microstructure are greatly differed with hen ages. As the density increases with ages, nature of the mother birds might be reflected into the eggshell internal quality and can be traced back their status in the future precision production management. This study might also contribute for better understanding the eggshell and its internal microstructure towards industrial grading of eggs and in precision poultry production system as well as for ecological and eggshell evolutionary studies in other avian species.

Materials and Methods

Materials

Light brown eggs (ROSS 308) from younger (32 weeks old: 32W) and older (53 weeks old:53W) hens were used for this experiment as shown in Fig. 1. The experiments were conducted at Graduate School of Agriculture, Kyoto University, Japan.

Terahertz spectra acquisition

Terahertz time domain spectroscopy was used to measure the eggshell RI of the eggshells in 0.5-2.0 THz (potential frequency for egg research) from younger (32 weeks old) and older (53 weeks old) parent flocks' eggs. As shown in Fig. 2, transmission measurement of sampled eggshells was performed using femtosecond laser-based terahertz time-domain spectroscopy (THz-TDS, model: TPS spectra 3000, TeraView Ltd., UK). Actual RI of the eggshells (n = 30) was obtained from the device by inputting actual shell thickness during transmission measurement. The eggshell refractive index measured by the THz-TDS based on Snell's law (Bernier *et al.* 2013).

In the terahertz region the refractive index (n) depends on how compact the material (e.g. crystallinity, density) is and the moisture content of the material (Khaliduzzaman *et al.* 2020). The higher the refractive index is, the higher the reflection intensity and lower the speed of the light will be when passing

through the medium.

The Eq. 2 is used to calculate the actual refractive index of eggshell which has been derived from Snell`s law of light refraction shown in Eq. 1. Considering non-perpendicular incidence of electromagnetic wave, the relation between optical path and material (i.e., eggshell) thickness (*T*) can be explained by Eq. 2 (Khaliduzzaman *et al.* 2021).

$$n_1 sin\text{Œ}\prod_1 = n_2 sin\text{Œ}\prod_2$$

1

$$P = \frac{2nT}{cos\text{Œ}\prod_2} = \frac{2n_2 T}{\sqrt{1-\left(\frac{n_1}{n_2}\, sin\text{Œ}\prod_1\right)^2}}$$

2

where,

P= optical path in eggshell

c= speed of light in vacuum (mm/s)

n= refractive index of eggshell

T=actual thickness of eggshell (mm)

θ_1 = incident angle of THz pulse at air-eggshell interface

SEM Imaging of eggshell

The scanning electron microscope, SEM 10 kV (TM4000 Plus, HITACHI, JAPAN) was used for taking the cross-sectional ultrastructure image (8 bit) of eggshell of the palisade and mammillary layers. After breaking the eggshell, the sampling shell was taken from the center of the egg. SEM images were taken with resolution of 256 · 256 pixels (px), image dimension of 1280 · 960 pixels (99.219 µm/px) and magnification at 1000x (Fig. 3).

SEM image processing

Image processing techniques such as edge detection (e.g., canny), image gray thresholding and binarization were applied to the SEM images to investigate the differences in shell internal microstructure (Fathi & Taghizadeh Rahmat Abadi 2024). Several common but useful morphological features such as particle size, grain size distribution, pore size, line edge roughness, overall surface texture, structural arrangement and connectivity of features were analyzed for SEM image of both classes to observe the micro-structural differences. The ROI were selected from the SEM images interactively with rectangular shape to illuminate the background. Two sample *t*-test was used to see the statistically significant differences (p-value < 0.01) between two age groups on morphological properties of eggshell. In Scanning Electron Microscopy (SEM), morphology describes the physical features of a sample, while homogeneity and energy are statistical descriptors used in image texture analysis often via the Gray-Level Co-occurrence Matrix (GLCM) to quantify the sample's surface properties.

Results and Discussion

Clear and significant differences in optical properties such as eggshell refractive index and eggshell internal microstructure were found between eggshells of two age group of birds, as shown in Fig. 4–5. Eggs from younger (32 weeks old: 32W) and older (53 weeks old:53W) mother birds were used for their experiment. In case of younger birds` eggshell, the mammillary layer was found less deep to the palisade layer, but wider space or gap between two mammillary cones and opposite characteristics were found for older aged birds (Fig. 5).

The material density of the palisade layer and mamillary nob was significantly higher (p-value < 0.05) for older birds which were confirmed by the refractive index (RI) of the eggshell in Terahertz frequency (Fig. 4). The refractive index of eggshell in THz region is an indicator of the density (e.g., material compactness) of crystalline material. Therefore, the density as well as microstructure of the eggshell depends on the hen's age. Generally, as laying hens further age, their eggshell microstructure deteriorates, resulting in lower stiffness, decreased breaking strength, and increased fracture susceptibility.

It was found that the density (i.e., refractive index in terahertz electromagnetic wave) and internal microstructure were greatly differed with hen ages, 53W vs 32W. The higher RI indicates the less abundant of organic matrix-matter in the older flock eggshell. It has been confirmed the density of eggshell from difference ages of mother birds from the following SEM images (after binarization), shown in Fig. 5. These differences might be due to the available resource limits (e.g., lower organic matrix) as older hens suffer various constraints with their increasing biological age. In the contrary, older mother birds perhaps achieved the higher capability for finely depositing eggshell calcite during eggshell formation.

This manuscript discovered the novel and fundamental biological phenomena on hen`s age dependency of eggshell density for the first time. This research reported the density (i.e., refractive index in terahertz electromagnetic wave) and internal microstructure are greatly differed with hen ages.

Eggshell from younger hens only showed significantly (p-value < 0.05) higher texture homogeneity as well as texture energy compared to older hens as shown in Fig. 6. Texture homogeneity is a statistical measure of how similar or smooth the pixels in an SEM image are to one another. High homogeneity indicates a uniform surface with minimal variation in gray-level intensities (e.g., a smooth, flat surface) whereas low homogeneity indicates a rough or noisy surface with frequent and significant changes in pixel intensity (e.g., porous structures or multi-phase mixtures) (Głowska *et al.* 2024).

Texture energy measures the uniformity or orderliness of the image's gray-level distribution.

High energy occurs when the image contains a few dominant, repetitive gray-level patterns (i.e., high order) whereas low energy value occurs when gray levels are distributed more randomly or across many different intensities (i.e., disorder). Therefore, a reduction in mammillary knob density, reduced palisade layer thickness, which might lead to weaker, less organized, and more porous shells.

As the density increases with ages, nature of the mother birds might be reflected into the eggshell internal quality and can be traced back their health status in near future. These fundamental findings might have wide application in industrial grading of eggs, precision poultry production system and avian biological studies.

Conclusions

This manuscript discovered the novel and fundamental biological phenomena on hen`s age dependency of eggshell density and developed protocol for the first time. This letter reported that the density (i.e., refractive index in terahertz electromagnetic wave) and internal microstructure are greatly differed with hen ages. Eggshell internal architecture is influenced by hen age, eggshell from older mother birds have higher refractive index (i.e., density), Coarse grain in eggshell ultrastructure is observed in case of younger birds, laying experience is a matter in calcite deposition during eggshell formation, and older birds suffer resource limitation. These fundamental biological findings with engineering tools have the potential to be applied not only to poultry sector but also to many other scientific fields (e.g., avian and reptile biology, ornithology, developmental biology, evolutionary biology) that encompasses about 18,000 species of birds and 10,000 species of reptile worldwide. Moreover, this finding would enable us to study and explore many undiscovered functions of eggshell in the near future. Because the variation in mother bird age dependency of eggshell microstructure may influence offspring development, since it is a major source of minerals; take part in respiratory gas exchanges and protects against UV radiation (sun/light exposure). Therefore, this research might contribute for better understanding the temporal and spatial variations of eggshell in avian biological context.

Declarations

Funding Declaration

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Ethics Declaration

This experiment was conducted according to the ethical standard of Kyoto University (Approval number: 28–59).

Competing interests

Alin Khaliduzzaman is on the editorial board member of this journal.

Author contributions

A.K. made the experimental and research plan. A.K and K.S. conducted the experiments. Y.O., K. S. and N. K. provided the technical guidelines time to time. A.K prepared the draft manuscript, and all authors revised the manuscript. N.K was the host professor of the laboratory and project administrator.

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Figures



(a) Experimental eggs (32 weeks) (b) Experimental eggs (53 weeks)

Figure 1

Experimental egg sample from two age groups of mother birds (ROSS308)

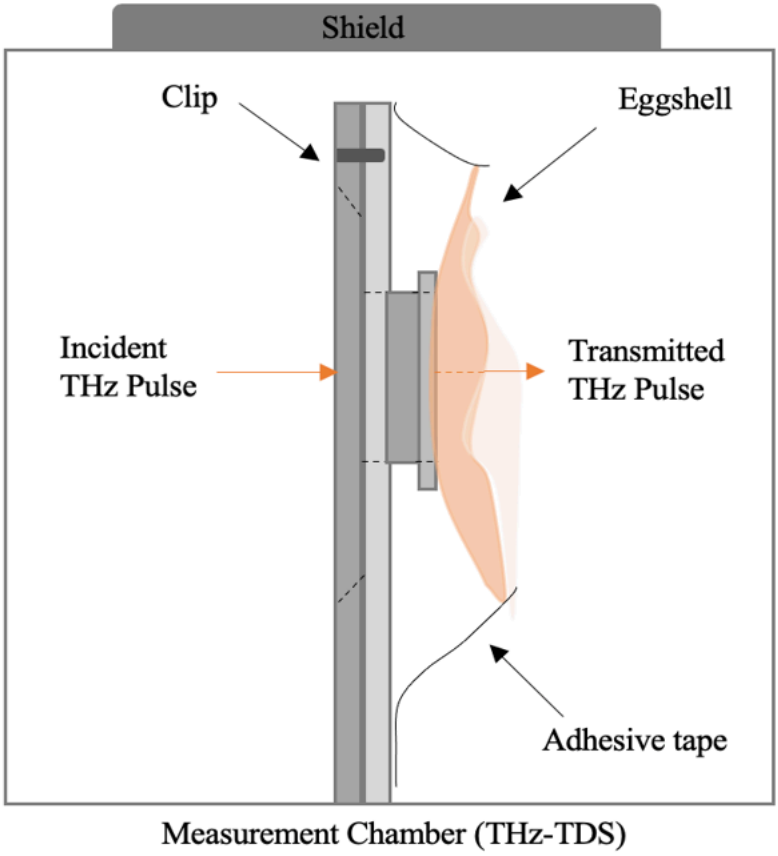
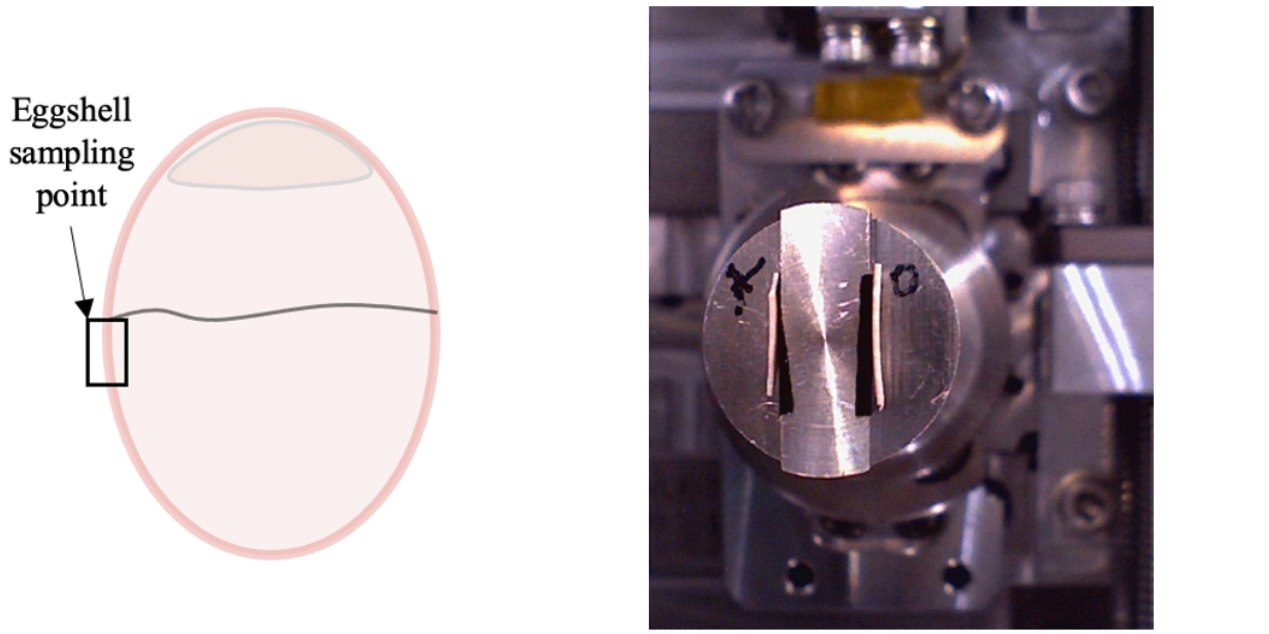


Figure 2

Placement of eggshell in THz-TDS measurement chamber during transmission measurement.



(a) Eggshell sampling position (b) Placement of eggshell in SEM

Figure 3

Eggshell sampling and setting in the chamber of SEM 10 kV before electronic microscopic image acquisition

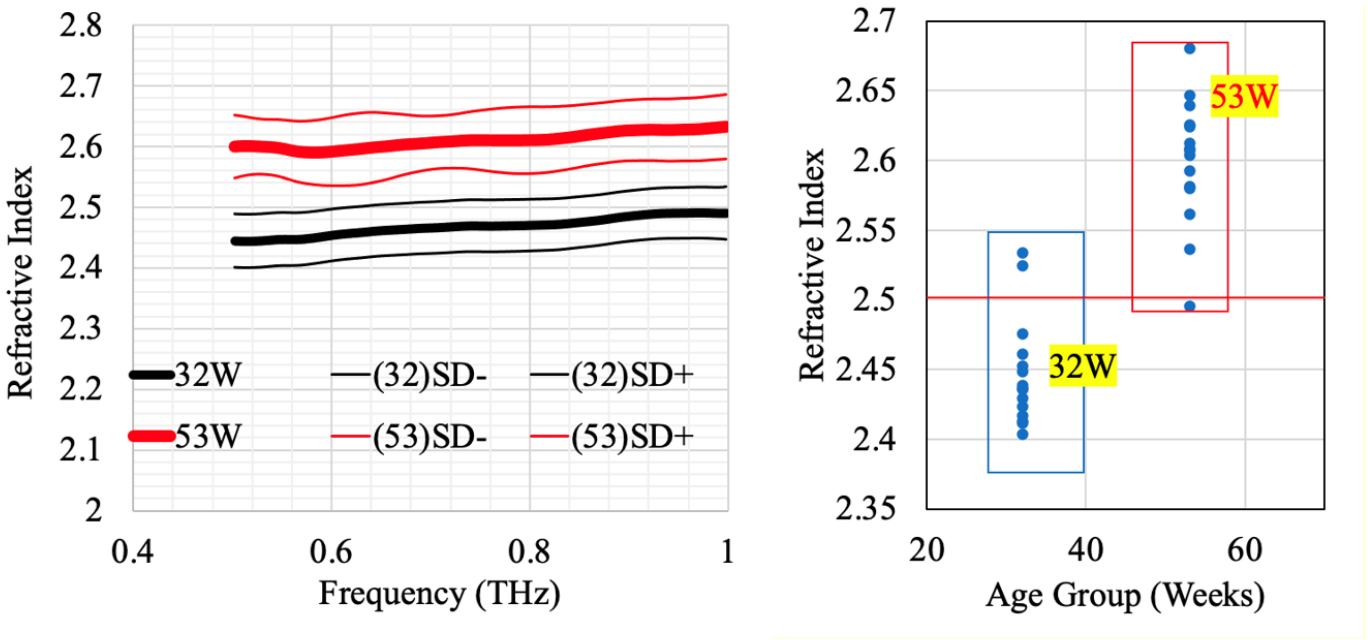


Figure 4

RI of eggshells from younger (32W) and older (53W) hens (0.5~1.0 THz). SD = standard deviation

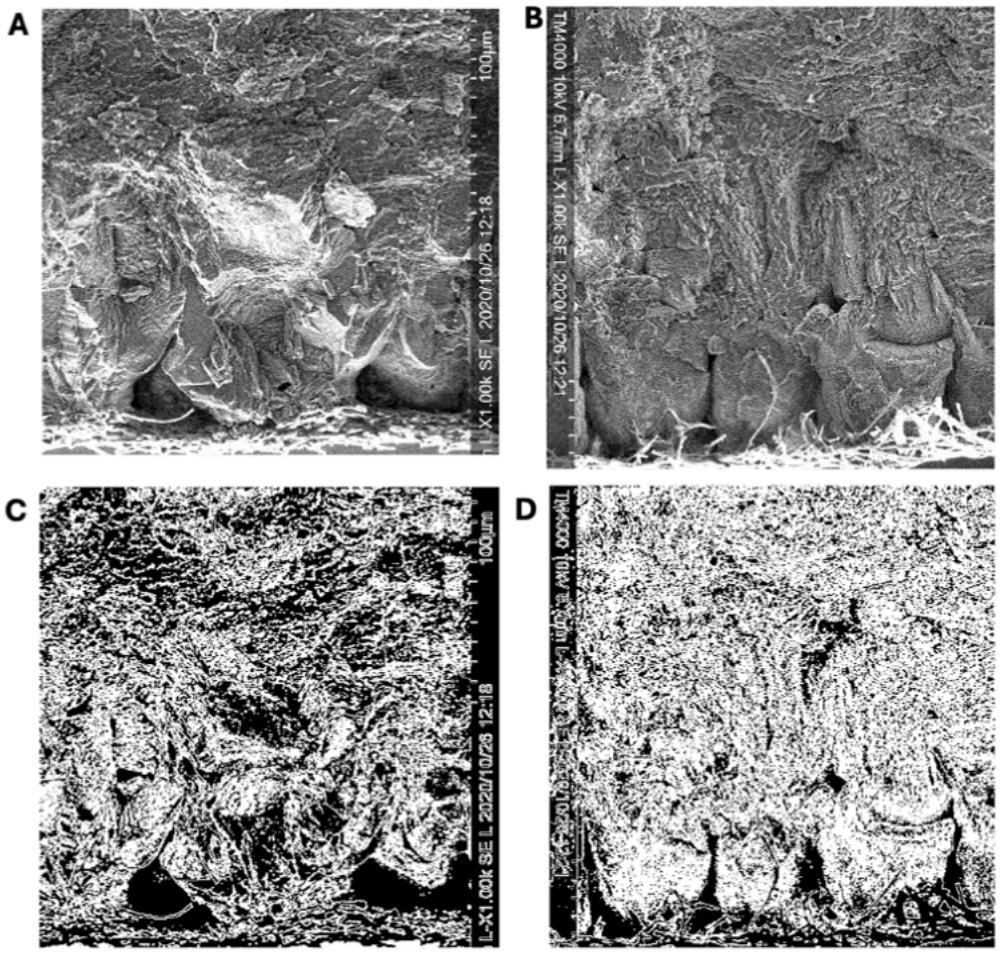
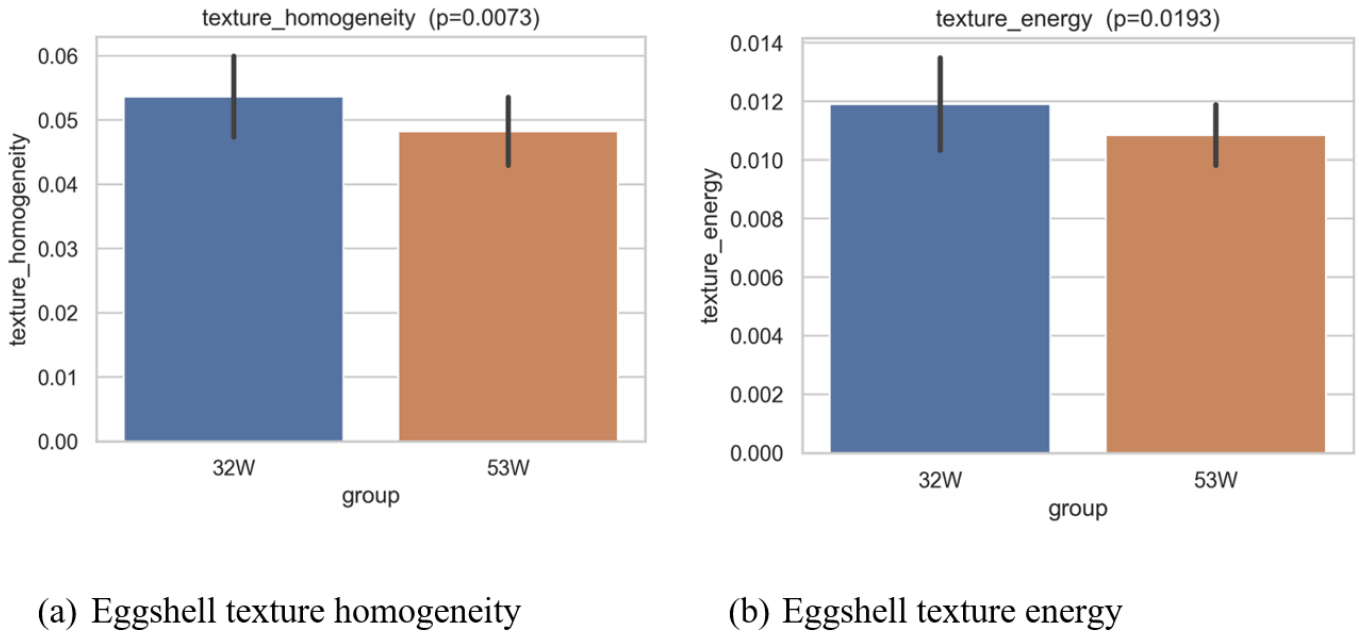


Figure 5

SEM images and edge detected images of eggshell from older (53w) and younger (32w) hens. A) original SEM image of eggshell from younger bird, B) eggshell image from older bird, C) Canny edge detected image (32W) and D) Canny edge detected image (53W). Finer grain crystalline microstructure at palisade and mamillary nobs are observed in case of older flocks (53 weeks old).



(a) Eggshell texture homogeneity (b) Eggshell texture energy

Figure 6

Significant difference in eggshell microstructure morphology (a) texture homogeneity (p-value=0.007), (b) texture energy with standard deviation (p-value= 0.019) (32 W vs 53 W).