sustainability

MDPI

Article

Geographical Variations in Egg Nutrient Density: A Comparative Study of Sustainable Layer Hen Systems in Ohio and Indiana in Late Fall and Early Winter Months

Julianna Adams [1], Rachel Van Duinen [1], Selin Sergin [1], Chad A. Bitler [2] and Jenifer I. Fenton [1,*]

[1] Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824, USA; adamsj86@msu.edu (J.A.); vandui20@msu.edu (R.V.D.); selin.sergin@emory.edu (S.S.)
[2] Greenacres Foundation, Inc., Cincinnati, OH 45242, USA; cbitler@green-acres.org
* Correspondence: imigjeni@msu.edu; Tel.: +1-5173533342

Abstract: Regenerative, pasture-raised layer hen systems create synergistic relationships between the hens, forage, soil, and weather, fostering a sustainable system. However, the influence of these factors on egg nutrient profile and quality may vary by region. This study took place over two years; samples were collected from a farm in Ohio during the first year and a farm in Indiana during the second year. Egg (n = 12) and forage (n = 3) samples were collected monthly from September to December. Fatty acids were quantified using gas chromatography-mass spectrometry, micronutrients were assessed in a commercial laboratory, and carotenoids and polyphenols were analyzed colorimetrically. Ohio eggs had significantly darker yolks (average yolk fan score: 8.5 ± 1.8 vs. 3.9 ± 2.0; $p < 0.0001$), a higher beta carotene and carotenoid content, and a lower n-6:n-3 fatty acid ratio across the season. Indiana eggs had a lower average total phenolic content (0.07 ± 0.00 mg GAE/g vs. 0.14 ± 0.10 mg GAE/g) and lower average total cholesterol (5.35 ± 2.9 mg/g vs. 10.99 ± 1.54 mg/g). Forage composition and soil parameters varied between farms. Regional variations in forage composition, soil nutrients, and regional weather can significantly influence the nutrient density of eggs produced in sustainable, pasture-raising systems. This research highlights how local environmental factors can shape the nutritional profiles of eggs in different regions. Future studies should explore this relationship in more regions.

Keywords: regenerative; pasture-raised; sustainable systems; poultry management; yolk; antioxidants; fatty acids

Academic Editor: Jaspreet Singh

Received: 4 March 2025
Revised: 28 March 2025
Accepted: 1 April 2025
Published: 4 April 2025

Citation: Adams, J.; Van Duinen, R.; Sergin, S.; Bitler, C.A.; Fenton, J.I. Geographical Variations in Egg Nutrient Density: A Comparative Study of Sustainable Layer Hen Systems in Ohio and Indiana in Late Fall and Early Winter Months. *Sustainability* **2025**, *17*, 3208. https://doi.org/10.3390/su17073208

1. Introduction

In recent years, consumer demand for sustainably produced food has increased interest in alternative agricultural practices, including regenerative and pasture-based farming systems [1,2]. These systems, which prioritize biodiversity, animal welfare, and soil health, have been championed for their potential environmental and economic benefits. Proponents of regenerative agriculture argue that it can enhance local ecosystems, reduce agricultural runoff, and provide more nutrient-dense food options, thereby benefiting both human health and the broader environment [3]. However, the degree to which these systems deliver on their promises—particularly in terms of nutritional value—remains a subject of ongoing debate.

Among the most prominent products marketed as part of regenerative agriculture are pasture-raised and free-range eggs, believed to be more nutrient-dense than their

conventionally produced counterparts. These eggs are perceived by consumers as healthier due to their higher content of beneficial fatty acids, vitamins, and minerals, attributed to hens' access to diverse forage and a natural diet [4,5]. Previous studies support these beliefs, demonstrating that systems that allow hens access to pasture produce eggs with higher levels of antioxidants, n-3 fatty acids, monounsaturated fatty acids, and total fat compared to eggs from commercial caged and cage-free systems [6,7]. However, little is known about the variations in nutrient density across different pasture-raising systems, particularly those located in distinct geographical regions. The notion that all pasture-raised systems are equal in terms of nutritional benefits has not been systematically examined.

Variations in egg nutrient composition are influenced by multiple factors, including pasture availability, hen diet, and seasonal changes. Studies have demonstrated that the intake of grasses and herbs significantly affects the nutritional profile of eggs [8]. In pasture-raising systems, seasonal fluctuations in grass availability directly impact egg quality, with higher grass intake leading to improved nutrient profiles during peak pasture seasons [9]. For example, pasture-based feeding systems increase egg yolk n-3 fatty acid content due to hens' increased grass consumption [9]. Nutrient content in the hen's diet also affects egg size, shell strength, yolk color, and carotenoid content [10]. Further, a study investigating seasonality found that egg yolk nutrients, including vitamin A, vitamin E, folate, choline, and calcium, varied significantly across the season [11]. These factors suggest that there may be important regional variations in the nutrient composition of pasture-raised eggs.

States across the United States are enacting legislation mandating that large-scale layer hen operations transition to cage-free management systems [12]. Currently, there are two raising claim labels regulated by the USDA: "cage-free" or "from free-roaming hens" and "free range" or "pasture-fed". "Cage-free", as defined by the USDA, allows hens to roam vertically and horizontally in indoor hen houses; they are not required to have access to outdoors. "Free range" eggs must be produced by hens that are able to roam freely indoors with continuous access to outdoor areas during their laying cycle; there is no minimum requirement of outdoor access [13]. The shift away from conventional caged systems may introduce greater variability and reduce consistency in egg production. Pasture-raising systems further amplify this variability, as factors such as forage availability, feed composition, weather conditions, and soil characteristics contribute to fluctuations in egg composition and quality. As a result, increased uncertainty is an inherent aspect of these alternative production methods.

The goal of this research is to investigate the geographical variations in egg nutrient density between two pasture-raising systems with similar management practices located in the Midwest: one in Southern Ohio and the other in Indiana. Specifically, this study compares the nutrient profiles of eggs, feed, forage, and soil from both systems over a period of four months (September through December). By analyzing these elements, we seek to understand how regional differences—such as local soil composition, climate, and plant biodiversity—may influence the nutrient density of eggs produced in pasture-raised systems. Given that these systems are marketed as healthier food options, it is critical to determine whether all pasture-raised eggs deliver the same nutritional benefits or if geographic location plays a significant role.

This research provides new insights into the variability of nutrient density in pasture-raised eggs, a topic that has implications for consumer purchasing decisions, as well as for the broader discussion surrounding sustainable agriculture.

2. Materials and Methods

2.1. Sample Characteristics

This study took place over two years. In 2022, samples were sourced from an Ohio farm (latitude: 39°13′ N, longitude: 84°20′ W; elevation: 290 m), and in 2023, samples were sourced from an Indiana farm (latitude: 39°34′ N, longitude: −85°20′ W; elevation: 282 m). Samples were collected from pasture-raising layer hen systems in which chickens had fenced access to routinely rotated pasture. Both systems were managed by the Greenacres Foundation and practiced rotational pasture raising management methods where they were rotated to a fresh ¼ acre (1011 m^2) plot every four weeks. At the start of the study, the flock consisted of roughly 300 layer hens. The flock size decreased in later months due to high predation. To account for the high predation rate after the first month, the original hens, which were all Comets, were repopulated with Black sex-linked hens. The same flock of Black sex-linked hens was used for the remainder of the study in both locations. Ohio hens were rotated to pastures following cattle grazing, whereas Indiana hens were rotated independently to fields with no prior livestock grazing.

Samples were collected monthly from September through December for a total of four months. Eggs (n = 36) and forage (n = 3) were sent to the laboratory each month. The same layer hen feed was fed at both farms and remained constant across the season. To ensure consistency, layer hen feed samples were collected from a well-mixed feed bin in the final month of each year for analysis. Triplicate feed samples (n = 3) were shipped to the laboratory. Upon arrival, layer hen feed was passed through a 1 mm screen in a Wiley mill (Arthur H. Thomas, Philadelphia, PA, USA) and subsequently purged with nitrogen and stored at −80 °C. The composition of the layer hen feed is shown in Table 1.

Table 1. Composition of the Layer Hen Feed.

Guaranteed Analysis	
Crude Protein (Min)	16.00%
Lysine (Min)	0.85%
Methionine (Min)	0.35%
Crude Fat (Min)	3.50%
Crude Fiber (Max)	9.00%
Calcium (Min)	3.25%
Calcium (Max)	3.75%
Phosphorus (Min)	0.70%
Salt (Min)	0.25%
Salt (Max)	0.75%
Selenium (Min)	0.3 ppm
Vitamin A (Min)	4000 IU/lb
Vitamin D3 (Min)	1500 IU/lb

Ingredients: Wheat Midds, Oats, Barley, Organic Non-GMO Soybean Meal, Calcium Carbonate, Fish Meal, Kelp Meal, Salt, Monocalcium Phosphate, Brewers Grain Yeast, Lactobacillus acidophilus, Enterococcus Faecium, Aspergillus Oryzae, Bacillus Subtilis, Bacillus Licheniformis, Yucca Schidigera, DL-Methionine, Vitamin A Supplement, Vitamin D3 Supplement, Vitamin E Supplement, Menadione Sodium Bisulfite Complex, Niacin, Riboflavin, D-Calcium Pantothenate, Pyridoxine Hydrochloride, Folic Acid, Zinc Amino Acid Chelate, Potassium Amino Acid Complex, Magnesium Amino Acid Chelate, Manganese Amino Acid Chelate, Copper Amino Acid Chelate, Vitamin B12 Supplement, Ferrous Sulfate, Manganese Oxide, Copper Sulfate, Sodium Selenite, Zinc Oxide, Choline Chloride, Ethylenediamine Dihydroiodide, Selenium Yeast.

Forage height and composition were evaluated prior to moving hens to fresh pasture each month. To do so, ten 0.5 m^2 hoops were randomly thrown into the pasture; species composition was determined by looking at the percent cover of each species within the hoop. These results were averaged to estimate the total pasture composition each month. Pre-graze forage height was determined by measuring the tallest forage in the center of each hoop. This procedure was repeated after the hens had been rotated off the plot to

estimate post-graze height. The pre-graze and post-graze heights were collected to estimate the foraging behaviors of the hens.

To analyze the nutrient composition of the pastures, forage samples were collected monthly by randomly placing nine 0.25 m^2 quadrants throughout the field and clipping to 1 cm stubble. The clippings were thoroughly mixed to create a homogeneous sample. Soil samples were collected before hens were given access to the pasture. To measure the soil composition of the pastures, an 8-inch soil probe was used to collect soil samples in a zig-zag pattern. One homogeneous soil sample was formed using 15–20 soil probes throughout the pasture. Forage and soil collection was repeated three times for a total of n = 3 replicates of forage and soil each month. Samples were stored in a –20 °C freezer until arrival at the laboratory. Upon arrival, forage was freeze-dried and passed through a 1-mm screen in a Wiley mill (Arthur H. Thomas, Philadelphia, PA, USA) and subsequently purged with nitrogen to be stored at −80 °C. Soil samples were sent directly for analysis upon arrival at the laboratory.

2.2. Physical Characteristics of Eggs

Each month, 24 of the 36 eggs were randomly selected, and physical characteristics were assessed using methods previously outlined [14,15]. Briefly, the weights of the eggs, albumen, shells, and yolks were recorded. A micrometer was used to measure albumen height on a flat surface. Albumen height was used to calculate the Haugh unit, a measure of egg quality, using a formula (Haugh unit = 100 × log (albumen height + 7.57 − 1.7 × egg weight$^{0.37}$)) [16]. Two methods were used to assess yolk color: a DSM yolk fan and a colorimeter. Yolk color was assigned on a scale from one to sixteen (1 = pale yellow to 16 = dark orange) using the DSM yolk fan (DSM Nutritional Products, Basel, Switzerland). The colorimeter provides a numerical value to the egg yolk color that allows it to be classified on a color scale [17]. Colorimeter values are expressed as Lab* values. L* represents the lightness of a color on a scale of 0–100 (0 = black, 100 = white); a* represents the position of the color on a red-green color scale (positive = red, negative = green); b* represents the position of the color on a yellow-blue color scale (yellow = positive, blue = negative). The yolk was then separated from the albumen into a silicone boat to be freeze-dried and subsequently ground into a fine powder. To create each sample, two yolks were combined, resulting in a total of n = 12 samples per month. These yolk samples were purged with nitrogen and stored at −80 °C until further analyses.

2.3. Proximate Analysis of Forage and Layer Hen Feed

Proximate analysis and mineral evaluation of both forage and layer hen feed were carried out at the DairyOne Forage Laboratory (Ithaca, NY, USA). Feed and forage moisture content was assessed using a forced air oven from adapted methods of AOAC 991.01 and 930.15 [18]. Crude protein, acid detergent fiber (ADF), lignin, crude fat, and ash contents were determined according to AOAC methods 990.03, 973.18, and 954.02 [18]. The neutral detergent fiber (NDF) content of forage and feed was measured using methods adapted from Van Soest et al. [19]. To analyze starch in forage and feed, enzymatic digestion of glucose was performed using glucoamylase. The resulting glucose was quantified indirectly through hydrogen peroxide equivalents using a YSI 2700 Select Biochemistry Analyzer. Metabolizable energy and total digestible nutrients (TDN) were calculated using formulas from the Nutritional Requirements of Dairy Cattle [20].

2.4. Fatty Acid Extraction and Methylation Procedure

Fatty acid extraction was conducted using an adapted method based on one previously described by Bronkema et al. [21]. Briefly, 400 mg of homogenized yolk, forage, or layer hen feed sample was added to an 8-mL solution containing 4:1 (v/v) ethyl acetate/methanol

with 0.1% butylated hydroxytoluene (BHT). Extraction took place in a CEM Mars 6 microwave (CEM Corp., Matthews, NC, USA) set to 55 °C for 15 min. The sample was filtered through a filter paper into a tube containing 3.5 mL of HPLC water. The sample was centrifuged, and the top layer was transferred to a new tube for methylation.

Fatty acid methylation was conducted using methods described by Sergin et al. [14] and adapted from Jenkins [22]. Briefly, two milligrams of the extracted oil were mixed with 500 µL of toluene and an internal standard of 20 µg of methyl-12-tridecanoate (U-35M, Nu-Chek Prep, Elysian, MN, USA) to create fatty acid methyl esters (FAMEs). Two milliliters of anhydrous potassium methoxide (0.5 N) were added to the sample and heated at 50 °C for 10 min for base-catalyzed methylation. Samples were cooled before adding 3 mL of 5% methanolic HCl. Samples were then reheated at 80 °C for 10 min for the acid-catalyzed methylation. HPLC water was added (2 mL), followed by two extractions of FAMEs using 2 mL of hexane each. The extracted FAMEs were then resuspended in 1 mL of isooctane and stored at −20 °C until analysis.

2.5. Fatty Acid Quantification

FAMEs were isolated using the Perkin Ekmer (Waltham, MA, USA) 680/600S gas chromatography-mass spectrometer (GC-MS) instrument in the electron impact mode using a HP-88 column (100 m, 0.25 mm inner diameter, 0.2 µm film thickness; Agilent Technologies, Santa Clara, CA, USA). Helium was used as the carrier gas (flow rate of 1 mL/min). Column parameters modified from Kramer et al. [23] were as follows: 80 °C for 4 min, 175 °C for 27 min (ramp at a rate of 13.0 °C/min), then to 215 °C for 35 min (ramp at a rate of 4.0 °C/min). Two injections (30:1 split and spitless) were conducted to quantify high and low concentration analytes, and 1 µL of the samples was injected at 250 °C for both injections. The MS recorded a mass range of m/z 20–400 with an electron energy of 70 eV in full scan mode. The MS transfer line and ion source were held at 180 °C. Fatty acids were reported as a percent of total fatty acids and g per 100 g of fresh egg yolk, layer hen feed, or forage.

A reference standard curve was created for the GC-MS using the Supelco 37 Component FAME Mix (Sigma-Aldrich, St. Louis, MO, USA) combined with mead acid, docosatetraenoic acid (DTA), n-3 docosapentaenoic acid (DPA), n-6 DPA, and palmitelaidic acid (Cayman Chemical, Ann Arbor, MI, USA). Conjugated linoleic acid (CLA) isomers were quantified using the CLA reference standard curve UC-59M (Nu-Chek Prep, Elysian, MN, USA). Branch chain fatty acids (BCFAs) were quantified using Mixture BR 3 (Larodan AB, Solan, Sweden).

2.6. Phenolics

Phenolics extraction was conducted using methods previously described by Sergin et al. [15] based on modified methods from Nimalaratne et al. and Chen et al. [24,25]. Briefly, 2 g of lyophilized egg yolk, forage, or layer hen feed was extracted using two sequential solvent extractions. The first extraction utilized 20 mL of a methanol/distilled water/acetic acid mixture [80:18:2 ($v/v/v$)] followed by 20 mL of an acetone/distilled water/acetic acid solution [80:18:12 ($v/v/v$)] in the second extraction. Samples were subsequently shaken and then centrifuged at 4 °C and 840× g. The supernatants were pooled. Furthermore, 100 µL of Folin-Ciocalteu agent and 800 µL of 5% sodium bicarbonate were then added to a gallic acid standard curve (1 mg/mL to 0.002 mg/mL) and subsequently added to 100 µL of supernatant. This was followed by a sample heating step for 30 min at 40 °C. Samples were cooled to room temperature before being plated in triplicates on a 96-well plate. Absorbance was measured at 765 using a microplate reader (Bio-Tek, Winooski, VT, USA) and the results were compared to a standard curve. The phenolic content was

reported as milligrams of gallic acid equivalents (GAE) per gram of fresh egg yolk, forage, or feed.

2.7. Egg Yolk Carotenoids

To analyze egg yolk carotenoids, samples were vortexed for two min to create a homogenous mixture of 0.5 g of freeze-dried egg yolk and 5 mL of acetone containing 0.05% BHT. This was followed by ultrasonication in a water bath for 5 min. Samples were then centrifuged at 1200× *g* for 15 min at 4 °C. A UV-Vis Double Beam Spectrophotometer (VWR, Radnor, PA, USA) at 450 nm against blank acetone was used to measure absorbance. Total carotenoid content was calculated with an ε of 140,663 L/mol based on methods previously described by Biehler et al. [26]. Results were expressed in micrograms of beta-carotene and carotenoids per gram of fresh egg yolk.

2.8. Forage and Layer Hen Feed Carotenoids

Two grams of ground layer hen feed or ground forage were mixed with 20 mL of 70% aqueous acetone in a conical tube. After shaking the mixture for 30 min, tubes were centrifuged at 840× *g* for 20 min at 4 °C. Then, the supernatant layer was moved to a fresh tube. An additional 20 mL of 70% aqueous acetone was added to the tube, and this process was repeated. The supernatants from both extractions were combined in glass cuvettes and used to assess chlorophyll and carotenoids on the spectrophotometer at three wavelengths (663, 646, 470 nm). Total carotenoids, chlorophyll A, and chlorophyll B were calculated using methods from [14] and formulas from [27].

2.9. Vitamin Analysis

Vitamin content was analyzed by the Veterinary Diagnostic Laboratory at Michigan State University (East Lansing, MI, USA) following AOAC method 2001.13. The vitamin content was analyzed by saponifying the lipid portion of the sample with a potassium hydroxide solution in ethanol, converting them to vitamin esters. Using hexane, the vitamins were excreted through phase separation. The hexane layer was subsequently evaporated, and the residual was re-dissolved in a solvent mixture of acetonitrile, methylene chloride, and methanol (70:20:10, *v*/*v*/*v*) for chromatographic analysis. The analysis was conducted on an Acquity BEH C18 column (1.7 µm, 2.1 × 50 mm). Vitamin quantification was assessed using the ApexTract method, and calibration curves were constructed with standards for retinol, beta-carotene, and alpha-tocopherol (Sigma Aldrich, St. Louis, MO, USA).

2.10. Egg Yolk Mineral Assessment

To digest the powdered egg yolk, 0.10 g of sample was combined with 3 mL of a concentrated nitric acid and perchloric acid mixture (60:40 *v*/*v*) in borosilicate glass tubes and left at room temperature for 16 h for predigestion. The mixture was then heated in a digestion block, starting at 120 °C, where it was maintained for 4 h. Following this, an additional 2 mL of nitric acid was added, and the samples were further heated at 120 °C for 2 h. The temperature was subsequently raised to 145 °C for an additional 2 h before finally being raised to 190 °C to evaporate the remaining liquid. After digestion, the samples were diluted with 10 mL of ultrapure water. The final digests were analyzed using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES, Thermo iCAP 6500 Series, Thermo Scientific, Waltham, MA, USA). For quality assurance, standard solutions were measured every 10 samples. Yttrium was introduced at a final concentration of 0.50 µg/mL as an internal standard to correct for matrix effects and ensure the reliability of the results. Results were expressed as ug per g of fresh yolk.

2.11. Egg Yolk Cholesterol Analysis

Cholesterol was extracted from 0.5 g of freeze-dried powdered egg yolk by dissolving the sample in 9 mL of 2% (*w*/*v*) NaCl solution. Each sample was vortexed for 2 min, followed by incubation at 37 °C for 2 h with shaking. After the extraction, 0.5 mL of the solution was further diluted with 9.5 mL of the same NaCl solution and vortexed for 1 min. The resulting mixture was then passed through a 0.45 µm syringe filter to separate the cholesterol. A 50-µL aliquot of the filtered and diluted solution, which is expected to contain 3–6 µg of cholesterol, was used for quantification. Cholesterol levels were measured using a colorimetric assay, following the protocol provided with the Cholesterol Quantification Assay Kit (Catalog #CS0005-1KT, Sigma-Aldrich, Burlington, MA, USA).

2.12. Soil Collection and Analysis

Soil samples (n = 3) were analyzed using the organic matter and general soil profile packages provided by a commercial laboratory associated with Michigan State University. Soil pH was measured with a standard pH meter. Organic matter and ash content were determined using the loss on ignition (LOI) method in a muffle furnace. Following LOI, the ash residue was analyzed for mineral content using inductively coupled plasma optical emission spectroscopy (ICP-OE, Thermo Scientific, Waltham, MA, USA).

2.13. Weather Collection

Temperature and precipitation were collected daily over the study period using the National Centers for Environmental Information: National Oceanic and Atmospheric Administration website. Daily values were averaged to report the monthly data. This database was also used to collect 30-year averages (1991–2020) [28].

2.14. Statistical Analysis and Manuscript Preparation

Means and standard deviations were calculated to summarize all numeric data by month and location. A two-way ANOVA was conducted, followed by Sidak's correction to account for multiple comparisons. This assessed how egg and soil nutrients varied by location at each level of month and any potential interactions between these factors. This analysis was completed using GraphPad Prism Version 7.0. Additionally, a one-way ANOVA was separately conducted to specifically evaluate seasonal variation (month-to-month differences) within each location. To determine whether egg physical characteristics, yolk nutrients, and forage nutrients varied significantly by month, a one-way ANOVA followed by Tukey's HSD post hoc test with corrections for multiple comparisons was conducted using RStudio v2024.09.1 + 394 (R Core Team, Vienna, Austria) for each location. Results were considered significant at $p < 0.05$. Values under the lower limit of detection were treated as zeroes.

Next, a Spearman correlation analysis was conducted using R Studio to investigate the relationships between forage nutrients, egg nutrients, and weather data using packages: ggplot2, reshape2, Hmisc, RColorBrewer, corrplot, showtext, and readxl. This analysis aimed to identify significant relationships and dependencies between these variables. Parameters for the correlation analysis are previously described by Krusinski et al. [29].

OpenAI was used for proofreading and organization during the preparation of this manuscript.

3. Results

3.1. Weather

The temperature and precipitation patterns across the season in Ohio and Indiana are shown in Figure 1. Weather patterns in Indiana and Ohio during the study period were

generally consistent with the respective 30-year historical averages. For both locations, monthly precipitation levels were slightly above the 30-year averages, while average temperatures were marginally below these long-term trends.

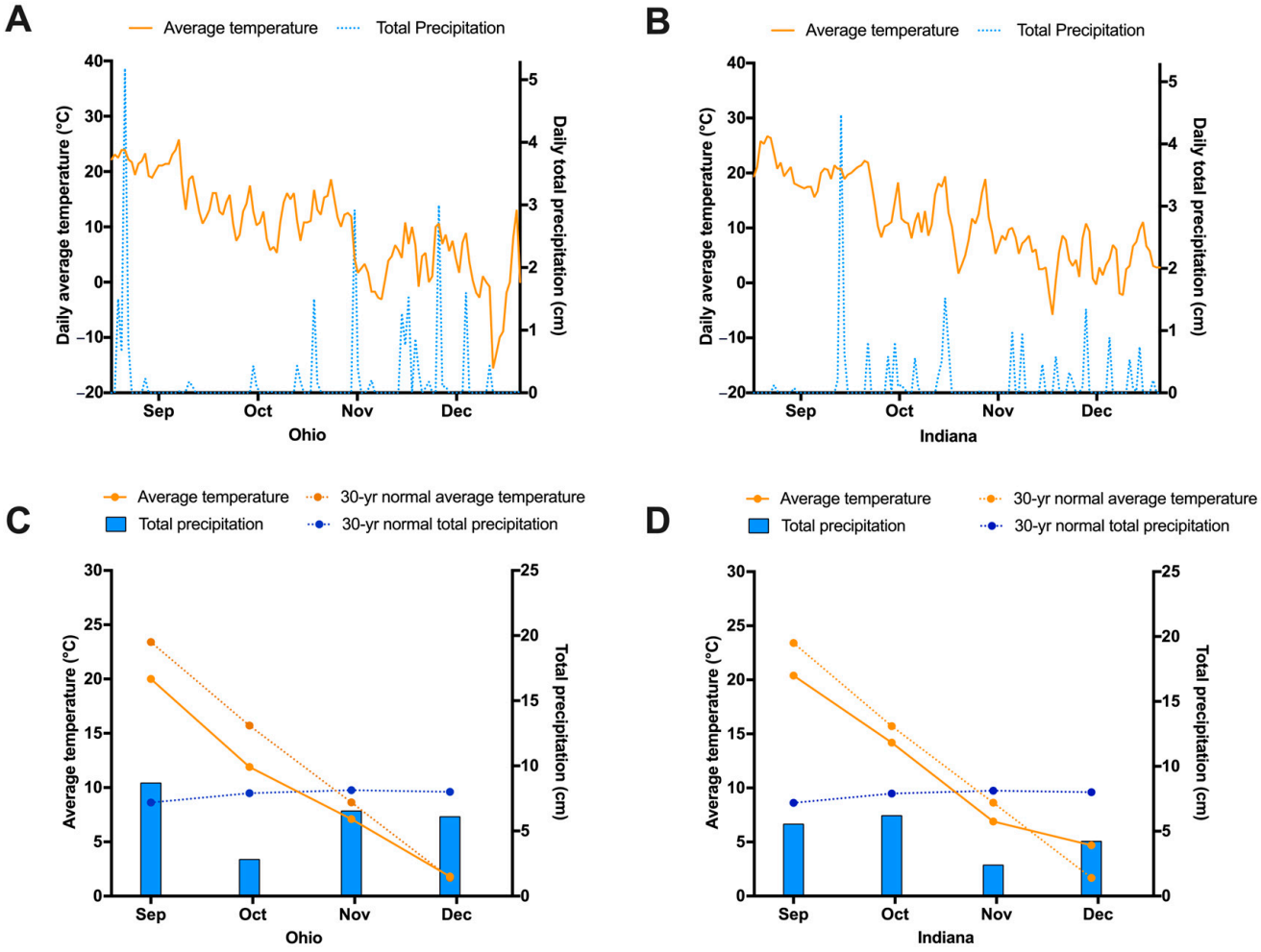

Figure 1. Temperature and precipitation patterns across the grazing season. (**A**) Daily average temperatures and total daily precipitation for Ohio. (**B**) Daily temperature and total daily precipitation for Indiana. (**C**) Monthly average temperatures and total monthly precipitation compared to their respective 30-year average for Ohio. (**D**) Monthly average temperatures and total monthly precipitation compared to their respective 30-year average for Indiana.

Daily weather observations indicated similar seasonal trends in both locations, with a gradual decline in temperatures from late fall through early winter. Ohio experienced higher rainfall totals in the later months (November and December).

3.2. Soil Composition

The soil composition and nutrients across the season in Ohio and Indiana are shown in Figure 2. Soil pH levels exhibited significant geographical variation between the Ohio and Indiana sites in September ($p < 0.001$). From November through December, both systems experienced a steady decline in soil pH, reflecting seasonal changes. The concentration of organic matter was significantly different between the two locations in all months studied except December ($p < 0.001$). Organic matter content followed a contrasting trend between the two states. Ohio's values were higher in September and October, declining in later months, while Indiana's organic matter increased steadily, reaching its highest level in December ($4.0 \pm 0.3\%$).

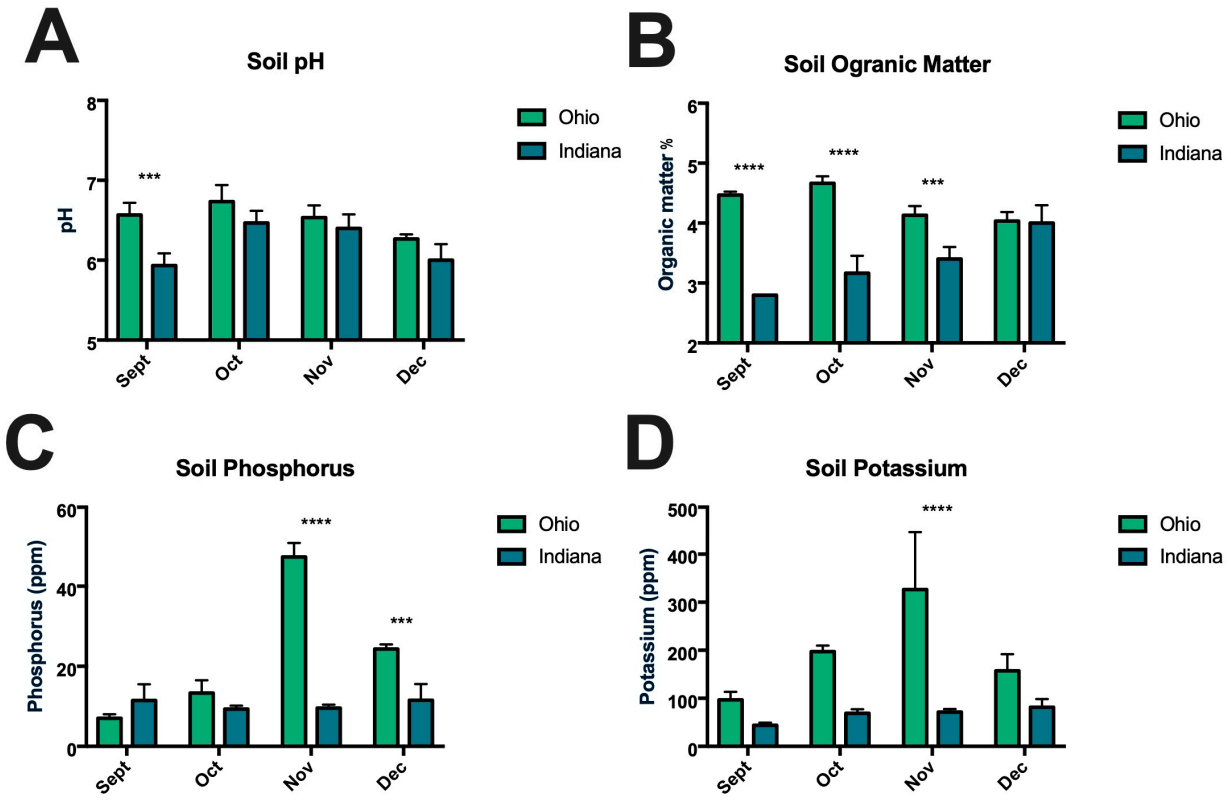

Figure 2. Soil nutrients across the season in Ohio and Indiana. (**A**) Soil pH across the season. (**B**) Organic matter content across the season. (**C**) Soil phosphorus across the season, measured in parts per million (ppm). (**D**) Soil potassium across the season, measured in ppm. Results of a two-way ANOVA with Sidak's multiple comparisons to compare Ohio versus Indiana at each level of month, values are shown as mean and standard deviation ('****' $p < 0.0001$, '***' $p < 0.001$).

The complete soil mineral analysis can be found in Table S1. Soil phosphorus levels demonstrated a remarkable peak in Ohio during November (47.33 ± 3.51 ppm), which remained significantly elevated through December. Phosphorus in Indiana showed no significant differences across the season ($p < 0.67$), peaking at (11.50 ± 4.07 ppm). Soil potassium levels were significantly different between the Ohio and Indiana locations in November ($p < 0.0001$). Potassium concentrations were higher in Ohio, with a pronounced peak in November (326.33 ± 121.71 ppm), whereas Indiana showed a gradual increase, peaking in December (81.30 ± 17.2 ppm).

3.3. Forage Compositon

The species composition across the season in Ohio and Indiana is shown in Figure 3. Species composition displayed distinct patterns between Ohio and Indiana over the study period. Pre-graze and post-graze height were recorded as an estimate of hen forage intake. In Ohio, fescue (*Festuca*) was a prominent species across all months, accompanied by clover (*Trifolium*), litter, aster (*Aster*), dock (*Rumex*), and foxtail (*Setaria*). Species diversity in Ohio was highest in September with 20 different species, a peak that remained elevated into October. Species diversity declined in November and December.

In Indiana, narrow-leaf plantain (*Plantago lanceolata*) dominated species cover in September. As the months progressed, species diversity increased, with narrow-leaf plantain (*Plantago lanceolata*) remaining a prominent species in October alongside other forage species. By November and December, the species composition shifted to include fescue (*Festuca*), litter, aster (*Aster*), and bare ground as the most notable components.

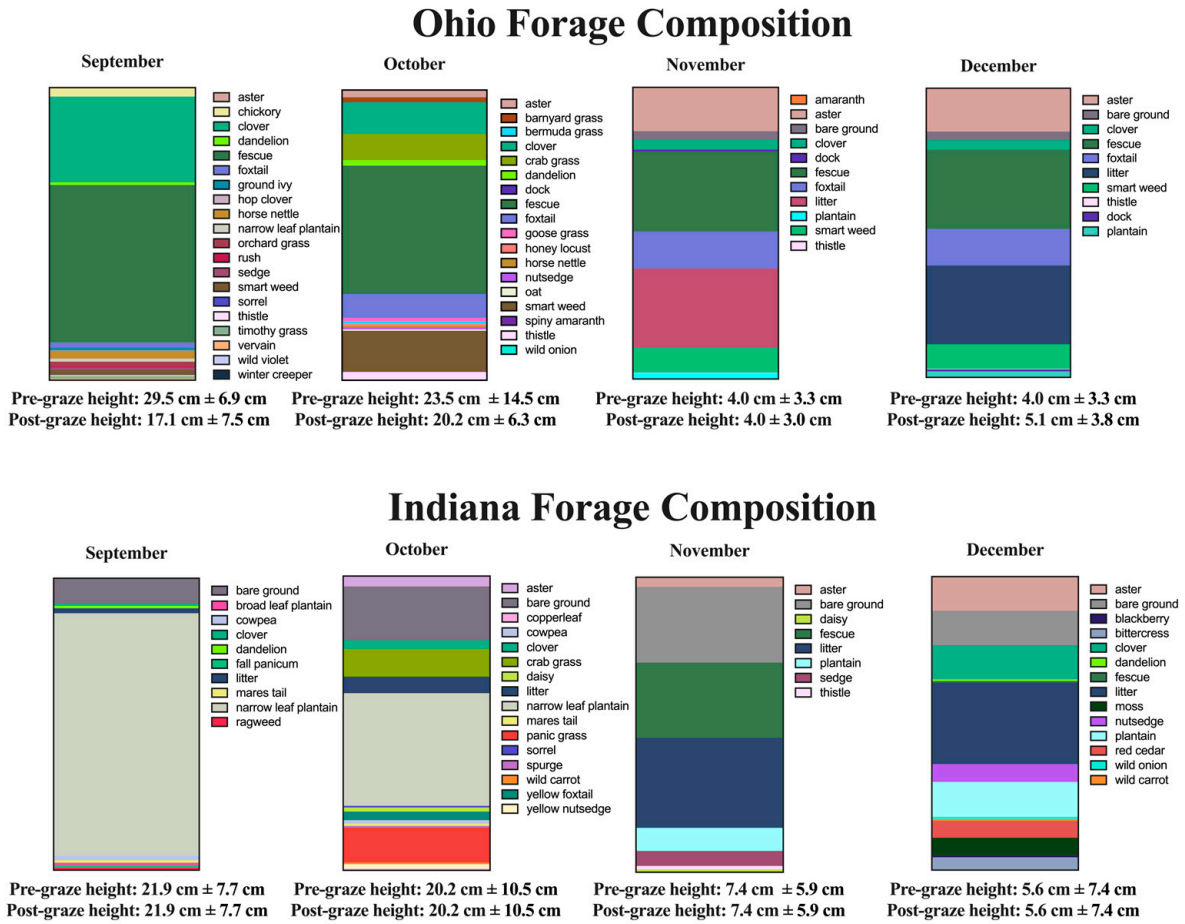

Figure 3. Species composition for each month in Ohio and Indiana. Each species is represented by a distinct color, with the size of each section indicating its percent coverage of pasture. Forage height is reported as mean ± standard deviation. The pre-graze and post-graze heights are reported as an estimation of forage intake.

Both locations saw the biggest decrease in pre- and post-graze heights in September and October. In the later months, these heights either did not vary or slightly increased, indicating seasonal trends in grazing impact. Increased consumption of forage by layer hens likely contributed to the larger variation in pre-graze height and post-graze height observed in September and October. Fatty acid profile, antioxidants, and proximate analysis of forage are reported in Tables S2–S4.

3.4. Physcial Characteristics of Eggs

The physical characteristics of eggs across the months for the Ohio and Indiana systems are shown in Tables 2 and 3. Egg weights in both locations varied significantly across the season. In Indiana, eggs reached their peak weight in December (65.68 ± 6.26 g). In Ohio, eggs reached their peak weight in November (60.38 ± 4.94 g). Both systems demonstrated an increase in egg yolk weight over time, with December eggs having the heaviest yolks; Indiana eggs had a yolk weight of 17.14 ± 1.89 g, while Ohio eggs had a yolk weight of 14.73 ± 1.09 g. Throughout the year, eggs from Indiana consistently exhibited higher yolk weights compared to those from Ohio. In Ohio, yolk color reached its peak in December with a mean value of 9.54 ± 1.38, while Indiana's yolk color peaked earlier in November at 4.13 ± 2.47. The average Haugh unit in the Ohio farm was 81.93 ± 7.73, with the highest value being recorded in October and the lowest value being recorded in December. The Indiana farm had a lower average Haugh unit across the season of 77.93 ± 8.74,

with the highest value being recorded in November and the lowest value being recorded in December.

Table 2. Physical Characteristics of the Ohio System Eggs by Month.

Parameter	Sept	Oct	Nov	Dec	*p*-Value [2]
Egg weight (g)	53.39 ± 6.69 b [1]	57.73 ± 6.70 a	60.38 ± 4.94 a	57.31 ± 4.24 ab	<0.001
Shell weight (g)	5.28 ± 0.79 c	5.45 ± 0.82 bc	5.93 ± 0.41 ab	6.12 ± 0.49 a	<0.001
Yolk weight (g)	12.02 ± 1.88 c	13.15 ± 1.65 bc	13.88 ± 1.22 ab	14.73 ± 1.09 a	<0.001
Dried yolk weight (g)	6.10 ± 0.99 b	6.71 ± 0.93 ab	7.11 ± 0.69 a	7.31 ± 0.61 a	<0.001
Albumin weight (g)	36.10 ± 4.71 b	39.13 ± 4.92 ab	40.57 ± 3.99 a	36.46 ± 3.26 b	<0.001
Albumin height (μm)	7.04 ± 1.44 ab	7.73 ± 1.13 a	6.63 ± 1.09 b	5.55 ± 0.99 c	<0.001
Haugh unit	85.07 ± 9.43 ab	88.29 ± 6.51 a	80.56 ± 7.65 b	73.81 ± 7.34 c	<0.001
Yolk color fan [3]	7.33 ± 1.88 b	8.38 ± 2.80 ab	8.79 ± 0.88 a	9.54 ± 1.38 a	0.001
Colorimeter [4] (L)	68.86 ± 2.98 a	66.07 ± 3.94 b	67.68 ± 1.21 ab	65.78 ± 2.35 b	<0.001
Colorimeter (a)	14.58 ± 5.02 b	17.74 ± 6.33 ab	18.20 ± 1.56 a	17.34 ± 3.33 ab	0.026
Colorimeter (b)	60.57 ± 3.78 b	69.83 ± 4.75 a	61.46 ± 3.69 b	59.52 ± 4.10 b	<0.001

[1] Means ± standard deviation (n = 24 eggs pooled to form n = 12 replicates). [2] Results of one-way ANOVA; a–c, means within a row with different letters significantly differ ($p < 0.05$). [3] Yolk color fan was measured on a scale of 1–16 (1 = pale yellow to 16 = dark orange). [4] Colorimeter values used to determine egg yolk color as indicated by L*, a*, and b*. L* represents the lightness of yolks on a scale of 0 to 100; a* represents yolk color on a red-green color scale, a positive value indicates red and negative value indicates green. b* represents yolk color on a yellow-blue color scale, a positive value indicates yellow, and a negative value indicates blue.

Table 3. Physical Characteristics of the Indiana System Eggs by Month.

Parameter	Sept	Oct	Nov	Dec	*p*-Value [2]
Egg weight (g)	57.71 ± 3.43 b [1]	57.45 ± 12.11 b	63.9 ± 7.01 a	65.68 ± 6.26 a	0.001
Shell weight (g)	5.16 ± 0.60 b	5.4 ± 0.68 ab	5.70 ± 0.70 a	5.65 ± 80 ab	0.018
Yolk weight (g)	13.83 ± 1.44 c	15.45 ± 2.21 b	16.1 ± 1.77 ab	17.14 ± 1.89 a	<0.001
Dried yolk weight (g)	7.00 ± 0.79 c	7.70 ± 1.15 bc	8.24 ± 0.87 ab	8.60 ± 1.02 a	<0.001
Albumin weight (g)	38.72 ± 2.92 ab	36.63 ± 11.78 b	42.1 ± 5.67 a	41.88 ± 4.38 ab	0.023
Albumin height (μm)	6.24 ± 0.91 b	5.9 ± 1.31 b	7.31 ± 1.51 a	5.79 ± 0.89 b	<0.001
Haugh unit	78.91 ± 6.47 ab	76.32 ± 10.91 b	83.47 ± 10.15 a	73.02 ± 7.41 b	0.001
Yolk color fan [3]	3.92 ± 1.64 a	3.54 ± 2.13 a	4.13 ± 2.47 a	4.00 ± 1.62 a	0.768
Colorimeter [4] (L)	71.94 ± 2.98 a	71.57 ± 2.21 a	67.21± 4.51 b	71.52 ± 2.63 a	<0.001
Colorimeter (a)	9.63 ± 3.73 a	6.94 ± 3.57 b	9.98 ± 4.17 a	6.88 ± 2.59 b	0.002
Colorimeter (b)	54.07 ± 5.92 a	49.52 ± 8.36 a	50.49 ± 7.23 a	52.34 ± 5.85 a	0.113

[1] Means ± standard deviation (n = 24 eggs per month). [2] Results of one-way ANOVA; a–c, means within a row with different letters significantly differ ($p < 0.05$). [3] Yolk color fan was measured on a scale of 1–16 (1 = pale yellow to 16 = dark orange). [4] Colorimeter values used to determine egg yolk color as indicated by L*, a*, and b*. L* represents the lightness of yolks on a scale of 0 to 100; a* represents yolk color on a red-green color scale, a positive value indicates red and a negative value indicates green. b* represents yolk color on a yellow-blue color scale, a positive value indicates yellow, and a negative value indicates blue.

3.5. Egg Yolk Color and Antioxidants

Yolk color, beta carotene content, and carotenoid content in both locations are shown in Figure 4. Egg yolk color was significantly darker in Ohio across all months ($p < 0.0001$). Analysis of yolk nutrient composition revealed that beta-carotene and total carotenoids were consistently higher in Ohio compared to Indiana during all months of the study. These differences were statistically significant in October, November, and December ($p < 0.01$). Notably, beta-carotene and carotenoids in Ohio increased over the season, reaching their highest concentrations in December, while Indiana showed a decrease in these antioxidants over the same period. Beta-carotene and carotenoid content across the season is reported in Table S5.

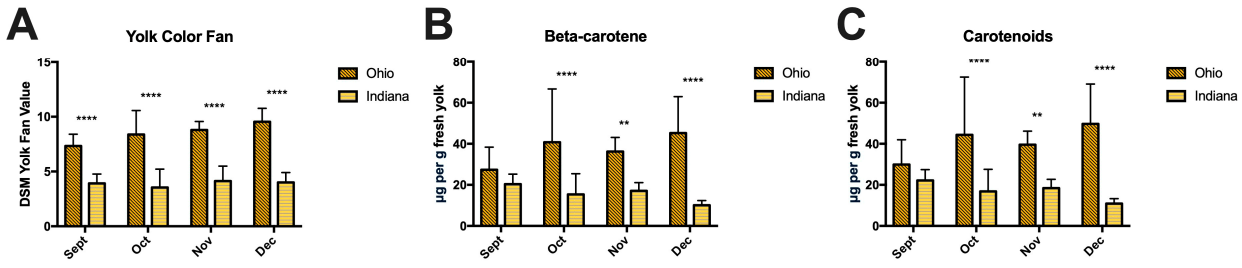

Figure 4. Changes in yolk color, beta-carotene, and carotenoids across the season between the Ohio and Indiana farm (n = 24 eggs pooled to form n = 12 replicates). (**A**) Changes in yolk color are determined by DSM yolk fan. (**B**) Changes in yolk beta-carotene content. (**C**) Changes in egg yolk carotenoid content. Results of a two-way ANOVA with Sidak's multiple comparisons to compare Ohio vs. Indiana at each level of month, values are shown as mean and standard deviation. Asterisks demonstrate significant differences between locations ('****' $p < 0.0001$, '**' $p < 0.01$).

3.6. Egg Yolk Vitamins and Phenolic Content

The total phenolic content, total vitamin E content, and total vitamin A content in both locations across the season are shown in Figure 5. Vitamin A was significantly higher in September and October in Ohio ($p < 0.0001$). Vitamin A concentrations in Ohio eggs exhibited a significant decline from September to December ($p < 0.05$). Levels were highest in September (10.80 ± 3.88 µg/g FW) and lowest in December (2.85 ± 0.39 µg/g FW). Indiana eggs showed a variable and significant fluctuation in vitamin A concentrations, with levels peaking in November (7.62 ± 2.16 µg/g FW) and reaching a low in October (3.20 ± 1.16 µg/g FW) ($p < 0.05$).

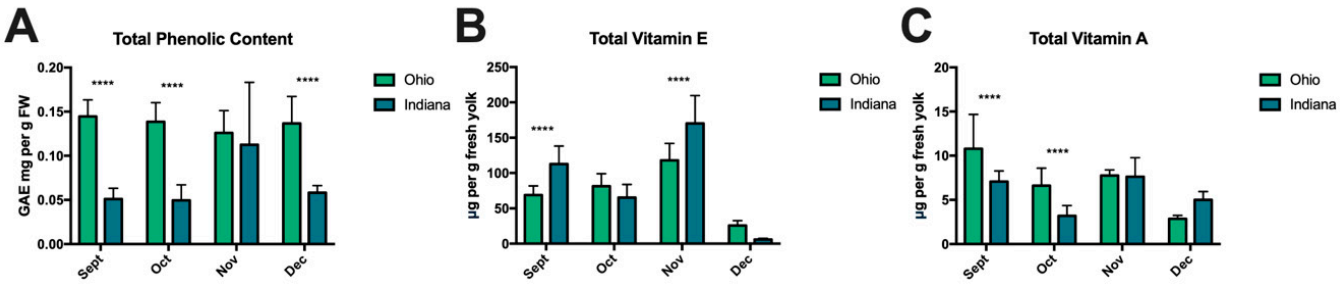

Figure 5. Changes in total phenolic content, total vitamin E, and total vitamin A across the season between the Ohio and Indiana farm (n = 24 eggs pooled to form n = 12 replicates). (**A**) Changes in phenolic content, expressed as gallic acid equivalents (GAE). (**B**) Changes in vitamin E content. (**C**) Changes in vitamin A content. Results of a two-way ANOVA with Sidak's multiple comparisons to compare Ohio vs. Indiana at each level of month, values are shown as mean and standard deviation. Asterisks demonstrate significant differences between locations ('****' $p < 0.0001$).

Vitamin E was significantly higher in Indiana in September and November ($p < 0.0001$). In Ohio, Vitamin E showed a distinct peak in November (118.06 ± 23.89 µg/g FW), followed by a sharp reduction in December (25.72 ± 6.90 µg/g FW). In Indiana, Vitamin E content was highest in November (170.3 ± 39.35 µg/g FW) but decreased dramatically in December (5.97 ± 1.73 µg/g FW).

Total phenolic content was higher across the season in Ohio and significantly higher in September, October, and December ($p < 0.001$). In Ohio, total phenolic content remained relatively stable across the season. In Indiana, total phenolic content peaked in November at (0.11 ± 0.07 mg GAE/g FW) but remained relatively stable in the other sampling months ($p < 0.05$). Total vitamin A, vitamin E, and total phenolic content values are reported in Table S5.

3.7. Egg Yolk Fatty Acids and Total Cholesterol

Variation in yolk fatty acid and total cholesterol between the locations is shown in Figure 6. The fatty acid profiles of egg yolks from the Ohio and Indiana systems exhibited significant variation across the sampling months. Both total monounsaturated fatty acids (MUFA) and total saturated fatty acids (SFA) were significantly higher in Indiana across the season ($p < 0.01$). Indiana's MUFA and SFA levels followed a trend of stable decline over the late fall and early winter season, whereas Ohio's levels remained relatively stable throughout the same period. Both systems recorded their highest SFA concentrations in September. Total polyunsaturated fatty acids (PUFA) were significantly higher in Indiana during September and December ($p < 0.01$). However, Ohio exhibited relatively stable PUFA levels over the season compared to the declining trend observed in Indiana. Total n-3 fatty acids were consistently higher in Ohio for all months, with significantly greater levels observed in October and November ($p < 0.01$). Both locations showed a seasonal decline in total n-3 FA. Total n-6 fatty acids (n-6 FA) were higher in Indiana for all months, with significant differences in September, October, and December ($p < 0.001$). The n-6:n-3 ratio was higher in Indiana across all months, with significant differences observed in October, November, and December ($p < 0.0001$). Indiana exhibited an increasing n-6:n-3 ratio over the season, while Ohio maintained relatively stable ratios throughout the period. Complete fatty acid profiles for both locations are reported in Table S6.

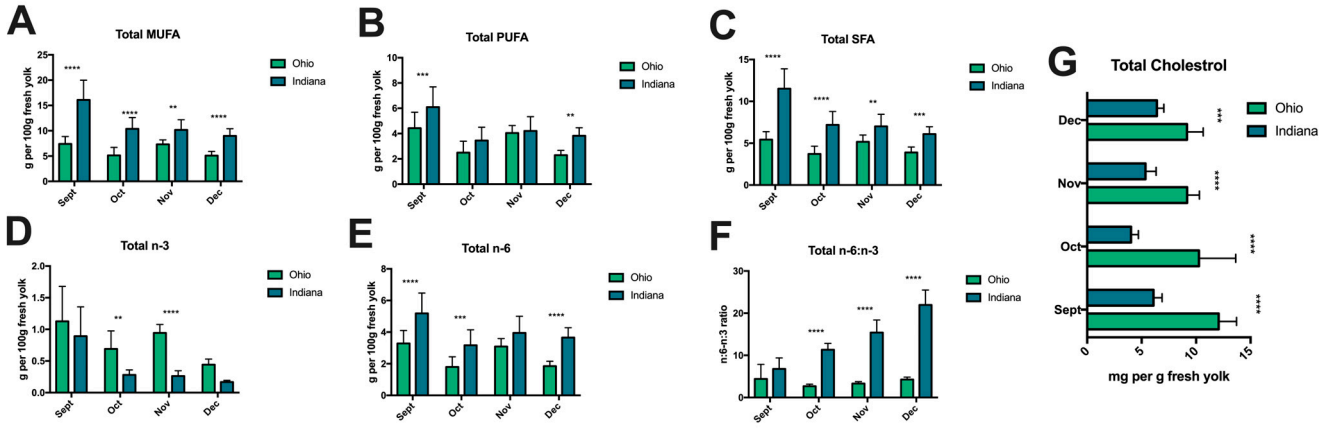

Figure 6. Changes in total yolk fatty acids across the season between the Ohio and Indiana farm ($n = 24$ eggs pooled to form $n = 12$ replicates). (**A**) Total monounsaturated fatty acids (MUFA) (**B**) Total polyunsaturated fatty acid (PUFA) (**C**) Total saturated fatty acids (SFA) (**D**) Total n-3 fatty acids (**E**) Total n-6 fatty acids (**F**) Total n-6:n-3 ratio. (**G**) Total cholesterol. Results of a two-way ANOVA with Sidak's multiple comparisons to compare Ohio vs. Indiana at each level of month, values are shown as mean and standard deviation. Asterisks demonstrate significant differences between locations ('****' $p < 0.0001$, '***' $p < 0.001$, '**' $p < 0.01$).

Total cholesterol was significantly higher in Ohio across the season ($p < 0.001$). In Ohio, total cholesterol content was significantly reduced in November and December (9.19 ± 1.12 and 9.17 ± 1.49 mg/g FW, respectively) compared to September and October (12.08 ± 1.64 and 11.53 ± 1.42 mg/g FW, respectively) ($p < 0.001$). Total cholesterol is reported in Table S5.

3.8. Correlation Analysis Between Yolk and Forage Nutrients

A correlation analysis demonstrating relationships between weather, forage nutrients, and egg nutrients is shown in Figure 7. Both locations showed a positive significant correlation between forage total carotenoids and chlorophyll A and chlorophyll B ($p < 0.05$). In Ohio, the n-3 PUFA content in forage was significantly positively correlated with the total phenolics in the egg yolk. In Indiana, the n-3 PUFA content in forage was significantly

positively correlated with beta-carotene and total carotenoids in the egg yolk. Yolk color was unrelated to total forage carotenoids, chlorophyll A, and chlorophyll B in Ohio, but negatively correlated in Indiana. In Ohio, temperature was negatively correlated with yolk color and forage vitamin E. Both locations observed a negative correlation between temperature and yolk weight.

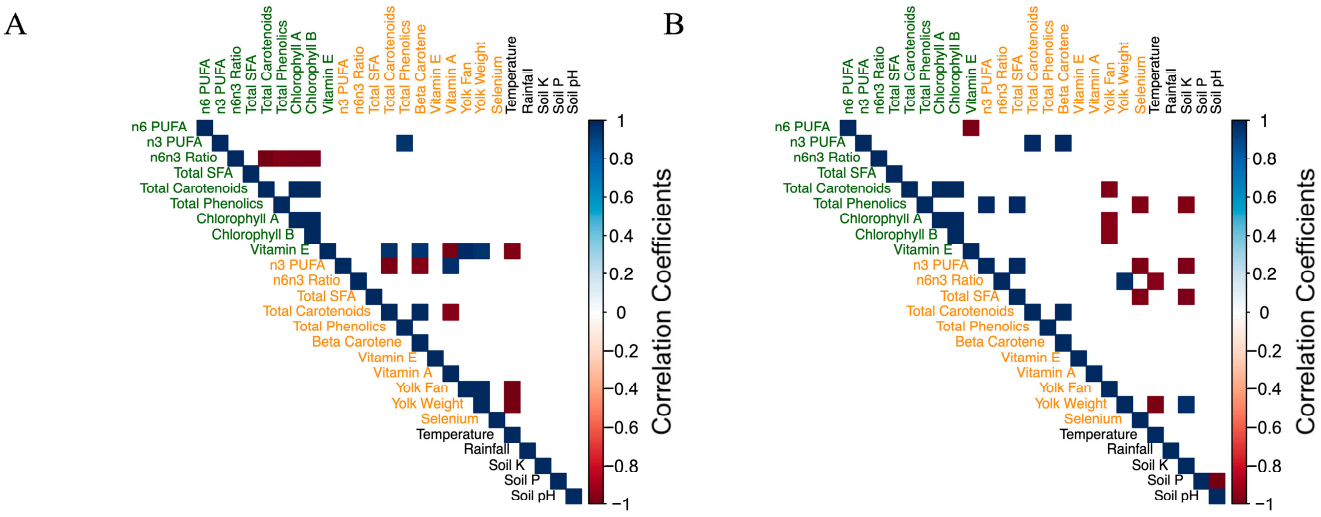

Figure 7. Spearman correlation matrix illustrating relationships across monthly averages of egg nutrients, forage nutrients, temperature, and precipitation. (**A**) Correlation matrix for the Ohio system. (**B**) Correlation matrix for the Indiana system. The color intensity represents the strength of the correlation depicted: Blue represents a positive correlation with R coefficient values between 0 to 1, while red represents a negative correlation value between 0 and –1. Boxes without color indicate no significant correlation ($p < 0.05$). Text colors distinguish between sample types: green is assigned to forage nutrients, orange to egg nutrients, and black is seasonal and soil changes. Total n-3; total omega-3 fatty acids, total n-6; total omega-6 fatty acids, total SFA; total saturated fatty acids, soil P; soil phosphorus, soil K; soil potassium.

4. Discussion

Weather patterns, including temperature and precipitation, influence farming systems by altering soil nutrient availability, forage nutrients and composition, and foraging behavior and nutrient deposition of the hens. The correlation analysis revealed several nutrients and egg characteristics negatively correlated with temperature. Weather patterns were similar between the two states, but Indiana remained slightly warmer at the end of the season. Layer hens in Ohio may have consumed more feed at the end of the season to compensate for the colder temperatures, leading to a higher influence of the layer hen feed on the nutrient profile of eggs. Optimal temperatures for layer hens range from 19 to 22 °C, as identified by Kim [30]. Cooler conditions likely increased feed intake to meet thermoregulatory energy demands, potentially leading to the layer hen diet playing a stronger role in the nutrient composition of the eggs in the later season [30–32]. Furthermore, the correlation analysis revealed that in Ohio, temperature was negatively correlated with yolk weight, yolk fan color, and forage vitamin E. In Indiana, a negative correlation was also observed between yolk weight and temperature. This is consistent with several studies that have found heat stress to reduce egg weight and lighten egg color. In Indiana, the n-6:n-3 ratio was negatively correlated with temperature. Previous studies have demonstrated that heat stress is able to induce lipid changes in egg yolk; for example, high temperatures can increase triglyceride levels in egg yolks [33–35]. Further, the correlation matrix revealed a negative correlation between temperature and forage vitamin E in Ohio. It is well described that heat stress tends to increase vitamin E content in plants as a defense mechanism to

combat the oxidative damage caused by stress [36,37]. However, the correlation analysis revealed the opposite relationship in the forage of the Ohio system. These findings suggest that feed consumption and several characteristics, including yolk weight, yolk color, forage vitamin E, and yolk n-6:n-3 ratio, were likely influenced by temperature.

Eggs from free-range systems may have higher egg quality compared to eggs from conventional systems [38]. However, important variations in egg physical characteristics were observed between the two pasture-raised farms in this study. Both farms observed an increase in egg weight in later months, which may reflect seasonal trends. Heat stress has been demonstrated to reduce egg weight [39]; lower egg weights at the beginning of the laying season may be due to warmer temperatures. Further, the Haugh unit, a measure of egg quality, declines with hen age. A younger hen age may be why slightly higher Haugh units were observed in Ohio, rather than geographical differences.

The soil composition between the two farms varied significantly. Organic matter was significantly higher from September through November in Ohio. Soil pH generally showed a decline across the season in both locations. Soil pH was significantly higher at the Ohio farm in September and remained slightly higher across the season. Further, Ohio soil phosphorus and potassium showed a significant and marked increase in November. The pH of soil is the most important factor for forage growth and availability of nutrients to plants. A soil pH of 6.0 to 7.0 is optimal for plant growth [40]. Indiana soil fell below this optimal range in September with a pH of 5.93. Phosphorus availability is maximized at a soil pH from 6 to 7. Potassium decreases with any increase in soil pH [41]. The correlation analysis reinforced this, revealing a negative correlation between soil pH and soil potassium concentration in Indiana. Further, the significantly higher organic matter observed in September and November in Ohio could be due to the cattle grazing on the pastures prior to layer hens. There was no prior cattle grazing on the Indiana farm. Cattle manure has the potential to increase soil organic matter; an essential characteristic in nutrient availability and water holding capacity of soil [42]. Overall, differences in soil composition, including pH, organic matter, potassium, and phosphorus content, likely influenced nutrient availability for forage that was available to layer hens in both systems, which could have an indirect influence on the nutrients available to the layer hens.

Yolk fan score, beta-carotene content, and total carotenoid content were higher in eggs from Ohio than those in Indiana. Our study found that yolk color was significantly darker in all examined months within the Ohio layer hen system. Yolk color, the most important sensory attribute for consumers [43,44], is primarily influenced by the carotenoid content of the feed [45,46]. Carotenoids, such as beta-carotene, are fat-soluble pigments with beneficial properties for human health. Due to the solubilization of yolk lipids, they are an important carrier of carotenoids in the human diet and more bioavailable than carotenoids from leafy green vegetables [46]. The geographical difference in yolk color and carotenoid content in this study may be due to differences in pasture access, composition, or consumption by the layer hens. It is well studied that pasture access has the potential to increase carotenoid content [47]. However, the correlation analysis demonstrated that yolk color was not related to carotenoid content in Ohio. In Indiana, yolk color was negatively correlated with carotenoid content, suggesting that pasture was not rich in carotenoids. While both the Ohio and Indiana systems offered pasture access, the forage species available varied between the two locations. Since both Ohio and Indiana hens had access to the same layer hen feed, the differences in forage composition likely contributed to the observed variation in yolk color and nutrient density. Moreover, the bioavailability of carotenoids in the forage may have differed, influenced by factors such as forage variety, maturity, and local environmental conditions.

Further, there were important differences in egg yolk vitamin A, vitamin E, and phenolic content between Indiana and Ohio across the study. Interestingly, even though both locations were fed the same feed supplemented with vitamin A, egg yolk vitamin A was significantly higher in the Ohio system in September and October, indicating alternative influences on vitamin A content. Vitamin E, however, was significantly higher in Indiana in September and November. Likely, specific forage species influenced the vitamin A and E content of the egg yolks. Due to the high lipid content of egg yolks, we would expect there to be a clear transfer of fat-soluble vitamins, like vitamins E and A, from the layer hen diet. However, several studies have documented an adverse effect of vitamin A on vitamin E absorption in egg yolks, suggesting an interaction between the bioavailability of these two vitamins in layer hens [48–50]. In the correlation analysis, forage vitamin E was negatively correlated with egg yolk vitamin A in Ohio, suggesting that higher forage vitamin E content of certain forages may have influenced vitamin A uptake into eggs in Indiana. Ohio egg yolks had a significantly higher total phenolic content in September, October, and December. Polyphenols are secondary plant metabolites that have antioxidant properties beneficial for human health. Like vitamin A and E content, specific forage species may have been the drivers of this variation. For example, Iqbal found higher polyphenolic content in tall fescue as compared to narrow-leaf plantain, two prominent species observed in Ohio and Indiana [51]. Overall, vitamin A, E, and total phenolic content fluctuated across the season and varied significantly between the egg yolks at the two different farms, potentially reflecting the influence of pasture.

Geographical variation in yolk fatty acid content was observed between the two systems. Egg yolk total MUFAs, PUFAs, SFAs, total n-6 fatty acids, and the n-6:n-3 ratio were higher in the Indiana system. Previous studies show little variation in cholesterol content between different production methods [6]. Our study, however, revealed significantly higher total cholesterol content in the Ohio system ($p < 0.001$). Importantly, our findings indicate that eggs from the Ohio system had a more favorable n-6-n-3 ratio of approximately 3.7:1, on average. This is consistent with the higher n-6 fatty acids and lower n-3 fatty acids in the Indiana system across the season. The optimal n:6-n-3 ratio should be approximately 1:1 to 4:1 to maintain optimal balance in the human diet [52]. A lower n-6:n-3 ratio is more favorable for human health [53]. The differences in PUFA, MUFA, and SFA content further demonstrate the regional variation in egg yolk fatty acids. SFAs varied only slightly within each system but varied significantly between systems. There was a higher total fatty acid content in Indiana across the season. Indiana's total fatty acid content ranged from 19.0 to 33.8 g per 100 g of fresh weight of egg yolk across the sample collection. Conversely, total fatty acid in Ohio ranged from 11.4 to 17.4 g per 100 g fresh weight of egg yolk. These differences likely led to higher MUFAs, PUFAs, SFAs, total n-6 fatty acids, and n-6:n-3 ratio in Indiana eggs. Previous studies have demonstrated that forage availability can influence fatty acid profiles, specifically high concentrations of clover and chicory in pasture can lower SFAs and increase PUFAs [54]. For both farms, months in which clover and chicory were present in the pasture, lower concentrations of SFA were observed. The correlation analysis did reveal a negative correlation between n-6:n-3 fatty acid ratio in egg yolks with temperature at the Indiana farm. This reinforces the idea that seasonal changes, including temperature, can induce fatty acid changes in egg yolks. The differences in fatty acid content and composition further demonstrate the regional variation in egg yolk fatty acids.

We acknowledge that there are limitations to this study. Firstly, this study investigates two farms in the Midwest, and there may be variations in nutrient density across different climates and regions that are not reflected in this study. Further, we did not monitor the hens' intake of forage, possibly including insects, berries, or other foraged items, which prevents us from determining the specific forage species driving the nutrient profile of

the eggs. Additionally, the chickens in the Ohio system followed cattle in their rotational grazing, whereas Indiana chickens did not. This difference could influence the nutrients present in Ohio eggs that are not found in Indiana eggs. The original flock started with roughly 300 hens and then was repopulated due to high predation. The exact number of flock size was not recorded throughout the study. Furthermore, during the September collection in Ohio, the breed of the hens used was Comets, while for the remainder of the study, both sets of layer hens were black sex linked. Although variations in breed were only present for one month of the study, we acknowledge that breed differences can influence the nutrient density of eggs. We also acknowledge that the chickens were different ages at the start of the study between locations. In Ohio, the hens were approximately four months old, whereas in Indiana, the hens were approximately one year old. Age differences between locations could play a role in the physical characteristics of the eggs and nutrient deposition into the egg yolks. However, these types of limitations reflect the realities and challenges of the pasture-raised management system, where farmers are unable to control factors like predation, climate, and regional differences, and therefore egg consistency.

Despite these limitations, there are several strengths to this study design. Both farms allowed hens to a ¼ acre (1011 m^2) of unrestricted pasture and a closed hoop house where layer nests and feed were located. The chickens were managed by the Greenacres Foundation and rotated monthly on the same schedule at both farms. Additionally, the feed was consistent between locations, allowing us to attribute nutrient differences to other external sources, such as forage. This controlled approach ensured that the observed variations in egg nutrient density can be more reliably linked to the geographical differences and grazing management practices between farms located in Ohio and Indiana. It is critical to begin to understand how the movement towards pasture-raised egg production impacts the nutrient profile of the consumer product.

5. Conclusions

While our research provides valuable insights into the variability of nutrient density in pasture-raised eggs, it also emphasizes the need for ongoing investigation to fully understand the complexities of these living production systems. This comparative study of Ohio and Indiana pasture-raised layer hen systems reveals that regional variations in forage quality, soil nutrients, and weather patterns significantly influence the nutrient density of eggs. As consumers increasingly opt for pasture-raised eggs and new laws mandate cage-free poultry operations, there is an expectation for egg quality and consistency. However, with the introduction of additional variables, such as soil, forage, and weather conditions, the nutritional quality of eggs can fluctuate. These regional differences in nutrient profiles highlight the challenge in guaranteeing a nutritionally superior product for the consumer. For those paying premium prices for pasture-raised eggs, understanding and ensuring consistent nutritional quality becomes a crucial aspect of supporting both consumer confidence and the broader goals of sustainable and regenerative agriculture. Such research is essential in ensuring these systems deliver on their promises of sustainability produced nutrient-dense food options.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su17073208/s1, Table S1: (a) Soil characteristics of the Ohio System by Month, (b) Soil characteristics of the Indiana System by Month; Table S2: (a) Fatty Acid Profile of the Ohio System Forage by Month, (b) Fatty Acid Profile of the Indiana System Forage by Month; Table S3: (a) Anti-oxidant Profile of the Ohio System Forage by Month, (b) Antioxidant Profile of the Indiana System Forage by Month; Table S4: (a) Proximate Analysis of the Ohio System Forage by Month, (b) Proxi-mate Analysis of the Indiana System Forage by Month; Table S5: (a) Antioxidant Profile of the Ohio System Eggs by Month, (b) Antioxidant Profile of the Indiana System Eggs by

Month; Table S6: (a) Fatty Acid Profile of the Ohio System Eggs by Month, (b) Fatty Acid Profile of the Indiana System Eggs by Month.

Author Contributions: Conceptualization, J.I.F., C.A.B. and S.S.; methodology, J.I.F., S.S., R.V.D. and J.A.; validation, J.I.F., J.A. and R.V.D.; formal analysis, J.A.; resources, J.I.F.; data curation, S.S., R.V.D. and J.A.; writing—original draft preparation, J.A., R.V.D. and S.S.; writing—review and editing, J.A., R.V.D., S.S. and J.I.F.; visualization, J.A., R.V.D. and J.I.F.; supervision, J.I.F.; project administration, J.I.F. and C.A.B.; funding acquisition, J.I.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by The Greenacres foundation, grant number 016298-00001, and the APC was funded by The Greenacres foundation.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Acknowledgments: We would like to thank Jennifer Mansfield for assisting in the on-farm data collections for both Ohio and Indiana. During the preparation of this manuscript/study, the authors used OpenAI ChatGPT (March 2025 version) for the purposes of proofreading and organization. The authors have reviewed and edited the output and take full responsibility for the content of this publication.

Conflicts of Interest: Author Chad A. Bitler is employed by the Foundation-Greenacres. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors declare that this study received funding from a grant from the Greenacres Foundation. The funder had the following involvement with the study: The funder provided the management practices, eggs, forage, feed, and soil samples from their farm for this study. The funder was not involved in the design of the methods, sample or data analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

Abbreviations

The following abbreviations are used in this manuscript:

SFA	Saturated fatty acid
MUFA	Monounsaturated fatty acid
PUFA	Polyunsaturated fatty acid
BCFAs	Branch chain fatty acid
n-6	Omega-6 fatty acid
n-3	Omega-3 fatty acid
n-6:n-3 ratio	Omega-6 fatty acid to Omega-3 fatty acid ratio
GC-MS	Gas chromatography-mass spectrometer
CLA	Conjugated linoleic acid
AOAC	Association of Official Analytical Chemists
BHT	Butylated hydroxytoluene
ANOVA	Analysis of Variance
ADF	Acid detergent fiber
NDF	Neutral detergent fiber
TDN	Total digestible nutrients
ppm	Parts per million
FW	Fresh weight

References

1. Jaeger, S.R.; Harker, F.R.; Ares, G. Consumer insights about sustainable and 'beyond organic' agriculture: A study of biodynamics in the United Kingdom, Australia, Singapore, and Germany. *J. Clean. Prod.* **2023**, *401*, 136744. [CrossRef]
2. Spain, C.V.; Freund, D.; Mohan-Gibbons, H.; Meadow, R.G.; Beacham, L. Are they buying it? United States consumers' changing attitudes toward more humanely raised meat, eggs, and dairy. *Animals* **2018**, *8*, 128. [CrossRef] [PubMed]
3. Spratt, E.; Jordan, J.; Winsten, J.; Huff, P.; Van Schaik, C.; Jewett, J.G.; Filbert, M.; Luhman, J.; Meier, E.; Paine, L. Accelerating regenerative grazing to tackle farm, environmental, and societal challenges in the upper midwest. *J. Soil Water Conserv.* **2021**, *76*, 15A–23A. [CrossRef]
4. Stampa, E.; Schipmann-Schwarze, C.; Hamm, U. Consumer perceptions, preferences, and behavior regarding pasture-raised livestock products: A review. *Food Qual. Prefer.* **2020**, *82*, 103872. [CrossRef]
5. Connor, D.S.; Oppenhein, D. Demand for pasture-raised livestock products: Results from michigan retail surveys. *J. Agribus.* **2008**, *26*, 1–20. [CrossRef]
6. Anderson, K.E. Comparison of fatty acid, cholesterol, and vitamin A and E composition in eggs from hens housed in conventional cage and range production facilities. *Poult. Sci.* **2011**, *90*, 1600–1608. [CrossRef]
7. Kiczorowska, B.; Samolińska, W.; Kwiecień, M.; Winiarska-Mieczan, A.; Rusinek-Prystupa, E.; Ridha, A.; Al-Yasiry, M. Nutritional value and the content of minerals in eggs produced in large-scale, courtyard and organic systems. *J. Elem.* **2015**, *20*, 887–895. [CrossRef]
8. Mugnai, C.; Sossidou, E.N.; Dal Bosco, A.; Ruggeri, S.; Mattioli, S.; Castellini, C. The Effects of Husbandry System on the Grass Intake and Egg Nutritive Characteristics of Laying Hens. *J. Sci. Food Agric.* **2014**, *94*, 459–467. Available online: https://scijournals.onlinelibrary.wiley.com/doi/abs/10.1002/jsfa.6269 (accessed on 26 March 2025).
9. Hammershøj, M.; Johansen, N.F. Review: The effect of grass and herbs in organic egg production on egg fatty acid composition, egg yolk colour and sensory properties. *Livest. Sci.* **2016**, *194*, 37–43. [CrossRef]
10. Hammershøj, M.; Steenfeldt, S. Organic egg production. ii: The quality of organic eggs is influenced by hen genotype, diet and forage material analyzed by physical parameters, functional properties and sensory evaluation. *Anim. Feed Sci. Technol.* **2015**, *208*, 182–197. [CrossRef]
11. Wallace, T.C.; Montenegro-Bethancourt, G.; Rohloff, P.; Jimenez, E.Y.; Proaño, G.V.; McCabe, G.P.; Steiber, A.; Ruosch, A.; Laessig, I.; Ladwig, E.; et al. Comparison of the nutrient composition of eggs produced in the guatemalan highlands during the wet and dry seasons. *Food Sci. Nutr.* **2023**, *11*, 8163–8173. [CrossRef]
12. Michigan Legislature. MCL—Section 287.746. Available online: https://www.legislature.mi.gov/Laws/MCL?objectName=MCL-287-746 (accessed on 20 February 2025).
13. Shell Egg Labeling Guidance | Agricultural Marketing Service. Available online: https://www.ams.usda.gov/grades-standards/shell-egg-labeling-guidance (accessed on 24 February 2025).
14. Sergin, S.; Jambunathan, V.; Garg, E.; Rowntree, J.E.; Fenton, J.I. Fatty acid and antioxidant profile of eggs from pasture-raised hens fed a corn- and soy-free diet and supplemented with grass-fed beef suet and liver. *Foods* **2022**, *11*, 3404. [CrossRef] [PubMed]
15. Sergin, S.; Goeden, T.; Krusinski, L.; Kesamneni, S.; Ali, H.; Bitler, C.A.; Medina-Meza, I.G.; Fenton, J.I. Fatty acid and antioxidant composition of conventional compared to pastured eggs: Characterization of conjugated linoleic acid and branched chain fatty acid isomers in eggs. *ACS Food Sci. Technol.* **2021**, *1*, 260–267. [CrossRef]
16. Eisen, E.J.; Bohren, B.B.; McKean, H.E. The haugh unit as a measure of egg albumen quality1. *Poult. Sci.* **1962**, *41*, 1461–1468. [CrossRef]
17. Bertoncelj, J.; Gašperlin, A.; Korošec, M. Yolk colour of eggs from different housing systems. *MESO Prvi Hrvat. Časopis O Mesu* **2019**, *21*, 378–385. [CrossRef]
18. Official Methods of Analysis, 22nd Edition. 2023. Available online: https://www.aoac.org/official-methods-of-analysis/ (accessed on 14 January 2025).
19. Van Soest, P.J.; Robertson, J.B.; Lewis, B.A. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* **1991**, *74*, 3583–3597. [CrossRef]
20. *Nutrient Requirements of Dairy Cattle: Seventh Revised Edition, 2001*; National Academies Press: Washington, DC, USA, 2001; ISBN 978-0-309-06997-7.
21. Bronkema, S.M.; Rowntree, J.E.; Jain, R.; Schweihofer, J.P.; Bitler, C.A.; Fenton, J.I. A nutritional survey of commercially available grass-finished beef. *Meat Muscle Biol.* **2019**, *3*, 116. [CrossRef]
22. Jenkins, T.C. Lipid metabolism in the rumen. *J. Dairy Sci.* **1993**, *76*, 3851–3863. [CrossRef]
23. Kramer, J.K.G.; Hernandez, M.; Cruz-Hernandez, C.; Kraft, J.; Dugan, M.E.R. Combining results of two GC separations partly achieves determination of all cis and trans 16:1, 18:1, 18:2 and 18:3 except cla isomers of milk fat as demonstrated using ag-ion SPE fractionation. *Lipids* **2008**, *43*, 259–273. [CrossRef]
24. Nimalaratne, C.; Lopes-Lutz, D.; Schieber, A.; Wu, J. Free aromatic amino acids in egg yolk show antioxidant properties. *Food Chem.* **2011**, *129*, 155–161. [CrossRef]

25. Chen, Y.-S.; Aluwi, N.A.; Saunders, S.R.; Ganjyal, G.M.; Medina-Meza, I.G. Metabolic fingerprinting unveils quinoa oil as a source of bioactive phytochemicals. *Food Chem.* **2019**, *286*, 592–599. [CrossRef]
26. Biehler, E.; Mayer, F.; Hoffmann, L.; Krause, E.; Bohn, T. Comparison of 3 spectrophotometric methods for carotenoid determination in frequently consumed fruits and vegetables. *J. Food Sci.* **2010**, *75*, C55–C61. [CrossRef] [PubMed]
27. Determinations of Total Carotenoids and Chlorophylls a and b of Leaf Extracts in Different Solvents | Biochemical Society Transactions | Portland Press. Available online: https://portlandpress.com/biochemsoctrans/article-abstract/11/5/591/57549/Determinations-of-total-carotenoids-and?redirectedFrom=fulltext (accessed on 27 March 2025).
28. US Department of Commerce. N. New 1991–2020 Climate Normals. Available online: https://www.weather.gov/tbw/newnormals (accessed on 25 February 2025).
29. Krusinski, L.; Maciel, I.C.d.F.; Sergin, S.; Goeden, T.; Ali, H.; Kesamneni, S.; Jambunathan, V.; Cassida, K.A.; Singh, S.; Medina-Meza, I.G.; et al. Evaluation of fatty acid and antioxidant variation in a complex pasture system as compared to standard cattle feed in the Great Lakes region. *Front. Sustain. Food Syst.* **2022**, *6*, 945080. [CrossRef]
30. Kim, D.-H.; Lee, Y.-K.; Kim, S.-H.; Lee, K.-W. The impact of temperature and humidity on the performance and physiology of laying hens. *Anim. Open Access J.* **2020**, *11*, 56. [CrossRef]
31. Chen, Y.; Yu, S.; Zhang, L.; Xiao, M.; An, L. Effects and mechanisms investigation of heat stress on egg yolk quality in Huaixiang chickens. *Anim. Open Access J.* **2023**, *13*, 3513. [CrossRef] [PubMed]
32. Kim, D.-H.; Song, J.-Y.; Park, J.; Kwon, B.-Y.; Lee, K.-W. The effect of low temperature on laying performance and physiological stress responses in laying hens. *Anim. Open Access J.* **2023**, *13*, 3824. [CrossRef]
33. National Research Council. Subcommittee on Environmental Stress. In *Effect of Environment on Nutrient Requirements of Domestic Animals*; National Academies Press: Washington, DC, USA, 1981.
34. Allahverdi, A.; Feizi, A.; Takhtfooladi, H.; Nikpiran, H. Effects of heat stress on acid-base imbalance, plasma calcium concentration, egg production and egg quality in commercial layers. *Glob. Vet.* **2013**, *10*, 203–207. [CrossRef]
35. Ebeid, T.A.; Suzuki, T.; Sugiyama, T. High ambient temperature influences eggshell quality and calbindin-d28k localization of eggshell gland and all intestinal segments of laying hens. *Poult. Sci.* **2012**, *91*, 2282–2287. [CrossRef]
36. Niu, Y.; Zhang, Q.; Wang, J.; Li, Y.; Wang, X.; Bao, Y. Vitamin E synthesis and response in plants. *Front. Plant Sci.* **2022**, *13*, 994058. [CrossRef]
37. Mathur, S.; Agrawal, D.; Jajoo, A. Photosynthesis: Response to high temperature stress. *J. Photochem. Photobiol. B* **2014**, *137*, 116–126. [CrossRef]
38. Alig, B.N.; Malheiros, R.D.; Anderson, K.E. Evaluation of physical egg quality parameters of commercial brown laying hens housed in five production systems. *Anim. Open Access J.* **2023**, *13*, 716. [CrossRef]
39. Tůmová, E.; Gous, R.M. Interaction of hen production type, age, and temperature on laying pattern and egg quality. *Poult. Sci.* **2012**, *91*, 1269–1275. [CrossRef] [PubMed]
40. Dewangan, S.K.; Shrivastava, S.; Kumari, L.; Minj, P.; Kumari, J.; Sahu, R. The effects of soil ph on soil health and environmental sustainability: A review. *JETIR* **2023**, *10*, 611–616.
41. Xia, Y.; Feng, J.; Zhang, H.; Xiong, D.; Kong, L.; Seviour, R.; Kong, Y. Effects of soil ph on the growth, soil nutrient composition, and rhizosphere microbiome of Ageratina adenophora. *PeerJ* **2024**, *12*, e17231. [CrossRef] [PubMed]
42. Maharjan, B.; Das, S.; Nielsen, R.; Hergert, G.W. Maize yields from manure and mineral fertilizers in the 100-year-old knorr–holden plot. *Agron. J.* **2021**, *113*, 5383–5397. [CrossRef]
43. Berkhoff, J.; Alvarado-Gilis, C.; Keim, J.P.; Alcalde, J.A.; Vargas-Bello-Pérez, E.; Gandarillas, M. Consumer preferences and sensory characteristics of eggs from family farms. *Poult. Sci.* **2020**, *99*, 6239–6246. [CrossRef]
44. Ortiz, D.; Lawson, T.; Jarrett, R.; Ring, A.; Scoles, K.L.; Hoverman, L.; Rocheford, E.; Karcher, D.M.; Rocheford, T. Biofortified orange corn increases xanthophyll density and yolk pigmentation in egg yolks from laying hens. *Poult. Sci.* **2021**, *100*, 101117. [CrossRef]
45. Van Den Brand, H.; Parmentier, H.K.; Kemp, B. Effects of housing system (outdoor vs cages) and age of laying hens on egg characteristics. *Br. Poult. Sci.* **2004**, *45*, 745–752. [CrossRef]
46. Nimalaratne, C.; Wu, J. Hen egg as an antioxidant food commodity: A review. *Nutrients* **2015**, *7*, 8274–8293. [CrossRef]
47. Martínez-Sánchez, V.; Pérez-Gálvez, A. Chapter 12—Microalgal Carotenoids for Food and Feed Applications. In *Handbook of Food and Feed from Microalgae*; Jacob-Lopes, E., Queiroz, M.I., Maroneze, M.M., Zepka, L.Q., Eds.; Academic Press: Cambridge, MA, USA, 2023; pp. 133–145. ISBN 978-0-323-99196-4.
48. Mendonça, C.X.; Almeida, C.R.M.; Mori, A.V.; Watanabe, C. Effect of dietary vitamin A on egg yolk retinol and tocopherol levels. *J. Appl. Poult. Res.* **2002**, *11*, 373–378. [CrossRef]
49. Frigg, M.; Broz, J. Relationships between vitamin A and vitamin E in the chick. *Int. J. Vitam. Nutr. Res. Int. Z. Vitam.-Ernahrungsforschung J. Int. Vitaminol. Nutr.* **1984**, *54*, 125–133.
50. Sklan, D.; Donoghue, S. Vitamin e response to high dietary vitamin A in the chick. *J. Nutr.* **1982**, *112*, 759–765. [CrossRef] [PubMed]

51. Iqbal, Y.; Ponnampalam, E.N.; Cottrell, J.J.; Suleria, H.A.R.; Dunshea, F.R. Extraction and characterization of polyphenols from non-conventional edible plants and their antioxidant activities. *Food Res. Int.* **2022**, *157*, 111205. [CrossRef] [PubMed]
52. Gonzalez-Becerra, K.; Barron-Cabrera, E.; Muñoz-Valle, J.F.; Torres-Castillo, N.; Rivera-Valdes, J.J.; Rodriguez-Echevarria, R.; Martinez-Lopez, E. A balanced dietary ratio of n-6:n-3 polyunsaturated fatty acids exerts an effect on total fatty acid profile in RBCs and inflammatory markers in subjects with obesity. *Healthcare* **2023**, *11*, 2333. [CrossRef]
53. Simopoulos, A.P. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed. Pharmacother. Biomed. Pharmacother.* **2002**, *56*, 365–379. [CrossRef]
54. Kop-Bozbay, C.; Akdag, A.; Bozkurt-Kiraz, A.; Gore, M.; Kurt, O.; Ocak, N. Laying performance, egg quality characteristics, and egg yolk fatty acids profile in layer hens housed with free access to chicory- and/or white clover-vegetated or non-vegetated areas. *Anim. Open Access J.* **2021**, *11*, 1708. [CrossRef]