Renewable Agriculture and Food Systems

www.cambridge.org/raf

Increasing pasture diet diversity in broiler chickens: insights from DNA metabarcoding

Kantima Thongjued[1], Karina Garcia[1,2], Viktor Halmos[1], Kathleen Pulliam[1], David J. Gonthier[1] and Julian R. Dupuis[1]

[1]Department of Entomology, University of Kentucky, Lexington, KY, USA and [2]Live Ops Sustainability, Handsome Brook Farms, New York, NY, USA

Research Paper

Cite this article: Thongjued K., Garcia K., Halmos V., Pulliam K., Gonthier D.J., and Dupuis J.R. 2025. Increasing pasture diet diversity in broiler chickens: insights from DNA metabarcoding. *Renewable Agriculture and Food Systems*, **40**, e13, 1–13 https://doi.org/10.1017/S1742170525000092

Received: 09 December 2024
Revised: 25 March 2025
Accepted: 31 March 2025

Keywords:
diet analysis; DNA metabarcoding; feed restriction; pasture-raised broilers; pasture utilization; stocking density

Corresponding author:
Kantima Thongjued;
Email: kth279@uky.edu

Abstract

Consumer demand for pastured poultry continues to increase, but production is challenged by high feed costs. Increasing poultry use of pasture resources, including pasture plants and insects, could help offset feed costs, but few studies describe management strategies to increase poultry pasture use. Here, we introduced broiler chickens (broilers hereafter) to cover crop pastures over three growing seasons and assessed the impact of stocking density, sex, short-term feed restriction, and breed on diet diversity. Throughout each experiment, we quantified pasture use by measuring the diet richness of pasture plants and arthropods through DNA metabarcoding of broiler excreta. We found that pastured broilers consumed many cover crop and weed plant families, but the diet richness of insects was unexpectedly low. Lower stocking density increased diet richness across all 3 years of the study. A short-term feed restriction increased diet richness compared to an unrestricted feed treatment. For fast-growing broilers, individuals with greater weight gain consumed the greatest diversity of diet items; however, the opposite pattern was observed for slow-growing broilers. As expected from anecdotal evidence, slow-growing broilers tended to have higher diet richness compared to fast-growing broilers. Despite increased diet richness, stocking density and short-term feed restriction did not increase the feed-use efficiency (weight gain/feed consumed) of broilers. Further, slow-growing broilers had lower feed-use efficiency compared to fast-growing broilers. This study marks the first application of DNA metabarcoding to elucidate the dietary composition of pasture-raised broilers. Future research should expand on optimal rates of access to pasture, supplemental feed, and breed selection to maximize the cost-effectiveness of pastured poultry production.

Introduction

Chicken (*Gallus gallus domesticus*) is the most commonly consumed meat globally, and chicken consumption is growing faster than that of any other meat type (Bennett et al., 2018). In recent years, consumer interest in animal welfare has generated minimum welfare standards and banned battery cages in high-income countries, such as the United States, United Kingdom, and Australia (Sossidou et al., 2015; Scrinis et al., 2017). This has also led to the growth of the 'pasture-raised' poultry market, defined as operations in which poultry have continuous access to pasture and are moved to fresh pasture regularly (Rothrock Jr et al., 2019). In addition to the perceived benefits to animal welfare, pastured poultry may also provide ecosystem services to production systems, including weed and insect pest suppression, and increased soil quality stemming from manure deposition (Clark and Gage, 1996; Sossidou et al., 2011; Elkhoraibi et al., 2017). At the same time, poultry raised in these farms gain access to pastured dietary resources, such as grasses and forbs, plant seeds, and insects (Clark and Gage, 1996; Burbaugh et al., 2010). This setting is generally perceived as an environment that not only improves bird health but produces eggs and meat of higher quality than those from conventional systems (Ponte et al., 2008b; Karsten et al., 2010; Skřivan and Englmaierová, 2014; Hammershøj and Johansen, 2016; Englmaierová et al., 2021).

Chickens are omnivorous. There is some evidence that pastured poultry consumes sufficient pasture resources to have important ecological consequences for pasture plant and arthropod communities. Clark and Gage (1996) found free-range chickens and geese consumed a variety of insects, including pests, which were found in 75% of dissected digestive crops. Additionally, geese were able to reduce weed cover to less than 10%. In our past research, broilers grown for meat were found to have strong top-down effects on pasture plant biomass and plant-dwelling arthropods, in addition to having strong bottom-up effects on ground-dwelling arthropod communities (Garcia et al., 2023). Pastures without broilers had 6 and 10 times more pasture plant biomass than did pastures with low and high broiler density, respectively. Further, pastures without broilers had at least three times more plant-dwelling Coleoptera, Hemiptera, and Hymenoptera relative to pastures with broilers (Garcia et al., 2023), suggesting broilers do exert top-down effects on plant and plant-dwelling arthropod communities. However, these findings may not all be due to the direct consumptive effects of broilers on plant and arthropod communities, as broiler trampling of pasture vegetation may have accounted for significant reductions in pasture plant biomass and plant-dwelling arthropod abundance (Garcia et al., 2023).

Despite significant access to pasture resources such as plants and insects, poultry growth and productivity in pastured poultry systems are dependent on supplemented feed. Surveys of pastured poultry growers suggest that the high cost of supplemented feed is one of the most important challenges to pastured poultry production (Hilimire, 2012; Elkhoraibi et al., 2017). While some research finds no differences in growth and feed-use efficiency between pasture-raised and conventional-confined broiler chickens (Sales, 2014), others suggest that in a well-managed system, pasture foraging can substitute 5%–20% of a grain-based diet (Mattocks, 2002; Burbaugh et al., 2010), suggesting pastured environments could help offset feed costs. Thus, there is a clear need to understand which plants and insects make up poultry diets in pastured systems and which breeds and management practices can improve their feed-use efficiency.

The vast differences in foraging behavior across poultry breeds help explain some of the challenges in increasing pasture use. On one extreme, Junglefowl (*G. gallus*; the ancestor to modern-day chicken) are described as generalist omnivores consuming nuts, berries, leaf matter, and invertebrates (Mench, 2009). Akin to their ancestors, domesticated Junglefowl exhibit contrafreeloading behaviors where individuals preferentially select food that requires effort to obtain, even when identical food items are readily available (Lindqvist et al., 2002, 2006; Ferreira et al., 2021). Some modern-domesticated layer-chicken breeds exhibit these behaviors and are often considered ideal breeds for pasture production (Lindqvist et al., 2002, 2006; Baxter, 2018; Ferreira et al., 2021). On the other extreme, broiler breeds appear to adhere to optimal foraging theory, in which they maximize intake and minimize energy expenditures (Osborne, 1977) by concentrating effort on supplemented grain-based feed. For this reason, it is often hypothesized that broilers do not utilize many of the pasture resources available to them.

Nonetheless, there may be several management strategies that may increase pasture use by poultry, including broilers. For one, reducing the stocking density of poultry per area of pasture should theoretically increase the quantity of pasture plant and arthropod dietary resources available to each bird. Mild or short-term feed restrictions may also improve pasture use by forcing broilers to seek food items in pastures during the period of restriction. In pasture-raised broilers, Ponte et al. (2008a) found that restricting the intake of a cereal-based feed (25%–50%) throughout the finishing period (36 days) led to increased consumption of leguminous pasture plants and thus significantly affected bird weight gain, feed conversion, and meat quality traits (Ponte et al., 2008a, 2008b). Likewise, Englmaierová et al. (2021) found that a cereal diet restriction of 20% in pasture-raised broilers led to significant increases in nutritional value metrics compared to broilers raised in conventional systems (Englmaierová et al., 2021). Additionally, for some fast-growing broiler breeds, the slowing of growth through mild or short-term feed restriction can reduce leg disorders and mortality due to metabolic diseases (Yu and Robinson, 1992; Khurshid et al., 2019; Bordin et al., 2021).

One of the challenges of measuring pasture use by poultry is the difficulty in quantifying which plant and animal species are consumed by poultry. Metabarcoding, a DNA-based method for identifying multiple species in a sample, has revitalized research into animal diets (Leray et al., 2013; Moorhouse-Gann et al., 2018; Paprocki et al., 2024). It involves massively parallel sequencing of short DNA fragments from a mixed sample, such as feces, stomach contents, or regurgitates. By comparing these sequences to a reference database, the specific species present in the sample could be accurately identified. This is especially useful for studying organisms whose prey is hard to identify from remains like stomach contents or scat. Metabarcoding's ability to promisingly identify species has provided new insights into dietary habits in many avian systems (Valentini et al., 2009; Ando et al., 2013; Crisol-Martínez et al., 2016; Cabodevilla et al., 2021, 2024; Snider et al., 2022; Höhn et al., 2024; Paprocki et al., 2024), including poultry systems (Thongjued et al., 2024), and provides high resolution for diverse diets compared to traditional morphological or molecular cloning approaches (Ando et al., 2013; Gong et al., 2019). Notably, metabarcoding studies focusing on omnivorous birds have been relatively rare, making this approach valuable for understanding the complex dietary patterns of these species (Spence et al., 2022; Thongjued et al., 2024).

In this study, we employed DNA metabarcoding-based diet analysis to investigate the dietary habits of pasture-raised broilers in an organic system over a 3-year period. Our research had two primary objectives. First, we aimed to document the full spectrum of diet items consumed by pastured broilers, providing a comprehensive understanding of how these birds utilize available pasture resources. This exploratory approach offers valuable insights into the foraging behavior of broilers in pasture-based systems. Second, we examined the effects of various poultry management strategies —including stocking density, breed selection, sex, and feed restriction—on the diversity of pasture plants and arthropods in the broilers' diet. This aspect of our study was driven by the hypothesis that certain management techniques could increase dietary diversity, potentially leading to improved feeding efficiency and overall bird performance. By investigating these relationships, we sought to identify management practices that could optimize both pasture utilization, broiler productivity, feed use efficiency in small-scale, organic, pasture-raised systems.

Materials and methods

Animal welfare

The research was approved and conducted under the permission of Institutional Animal Care and Use Committee (IACUC): protocol number 2020-3446. This study utilized pasture-raised broilers under practices adherent to organic standards for three consecutive years (2020–2022). All birds were raised on pasture in the fall growing season of each year. This project was part of a larger poultry–crop integration study that followed a spring vegetable, summer cover crop, fall pastured poultry rotational system (Garcia et al., 2023). See Table 1 for a summary of the rotation details.

Brooding care

Day-old chicks were ordered from commercial hatcheries. In total, 400 birds were used across the experiment including 120 Red Freedom Ranger breed birds that were used in 2020 (Mt. Healthy Hatcheries, Cincinnati, OH) and 160 and 120 Cornish Cross breed birds (hybrid between Cornish and White Rock breeds) that were used in 2021 and 2022 (Murray McMurray Hatchery, Webster City, IA), respectively. Upon arrival to the farm, the chicks were given water to drink and were immediately placed in brooders (CQB20 Chick and Quail Brooder Brower®) in 2020 and 2021, and oval galvanized-steel stock tanks (0.91 m × 0.61 m × 2.43 m (3 ft × 2 ft × 8 ft), 1135.62 liters

Table 1. Summary of vegetable-cover crop-poultry rotation scheme

Season	Rotational plan	Year I (2020)	Year II (2021)	Year III (2022)
Spring	Growing vegetables	Broccoli	Spinach	Lettuce
Summer	Growing cover crop for poultry pasture in fall	Buckwheat Cowpea Teff	Teff Crimson clover Annual rye grass Field borders: Millet Buckwheat	Teff Crimson clover Annual rye grass Field borders: Buckwheat
Fall	Raising pastured broiler	Red Ranger	Cornish Cross	Cornish Cross
Winter	Growing cover crop	Annual rye grass Hairy vetch Crimson clover Tall fescue	Winter barley Hairy vetch Crimson clover	Annual rye grass Hairy vetch Crimson clover

Note: Spring corresponds to mid-March–May, summer corresponds to June–July, fall corresponds to August–October, and winter corresponds to November–February.

(300 gallons, CountyLine) in 2022. The brooder room was equipped with portable air conditioning and heat lamps. Brooder temperatures were controlled to approximately 35 °C (95 °F) during the first week of brooding, and temperatures were reduced by 5 °F or more each week depending on the growth rate of chickens, which varied by breed. Chicks were fed with an organic broiler starter 23% (KOFFI 211, Kentucky Organic Farm and Feed, Inc.) for the first 3 weeks of life and then switched to an organic broiler grower 20% feed for the rest of the experiment (KOFFI 210, Kentucky Organic Farm and Feed, Inc.). The starter and grower feed was composed of corn, soybean, oat, and alfalfa pellets.

Experimental setup

Chicks were brooded until they were covered with adequate feathers to tolerate outdoor temperatures (at 3–4 weeks of age.) and were then moved to the pasture. On pasture, the birds were housed in floorless movable pens ('chicken tractors' hereafter) as shown in Supplementary Fig. S3, constructed in accordance with Skelton et al. (2012). These floorless chicken tractors allowed the birds access to vegetation and insects that were present in the environment. The chicken tractors were made from a looped cattle panel that created a dome-like structure on a 2.43 m × 2.43 m (8 ft × 8 ft) frame. Each tractor was equipped with a door to allow caretakers to do regular checks. Tarps were used to cover each tractor to provide chickens with protection from precipitation and aerial predators, and tarps were manipulated to provide adequate ventilation based on weather conditions. Each tractor was affixed with motion sensor lights to ward off nocturnal predators. Two perimeters of electric fencing were set up to protect chickens from ground predators.

In this study, ~9.75 m × 9.75 m (32 ft × 32 ft) pastures were assigned to two stocking-density treatments using randomized block design. Stocking density was defined by the area of pasture per individual broiler with high density pastures hosting 20 broilers (22 birds in 2021) with ~4.75 m^2 (51.2 ft^2) of pasture per broiler. Low density pastures hosting 10 broilers with 9.51 m^2 (102.4 ft^2) of pasture per broiler. A third treatment (vegetation only) was assigned as a nonchicken control. Each of these three treatments was randomly assigned within four blocks. See Supplementary Fig. S2 for experimental design.

We were able to compare different broiler breeds and sexes across years, though cover crop composition differed between years (Table 1). In 2020, we were only able to obtain straight-run Red Rangers (anticipating a 50:50 male-to-female ratio) due to limited supply during the COVID-19 pandemic. The sex of the birds was determined at 4 weeks by examining their sexual characteristics, such as comb development and overall size. Subsequently, individuals of both sexes were selected in equal numbers and leg banded to serve as experimental subjects. In 2021 and 2022, we ordered only male Cornish Cross birds anticipating a 90:10 male-to-female ratio (the hatchery guarantees only 90% accuracy). Despite the anticipated sex ratio of Cornish Cross birds, sexual size dimorphism became evident at the age of 3 weeks. Consequently, both relatively smaller birds and those of optimal size were allocated to each tractor and leg banded. Chromo Helicase DNA-binding gene amplification was later employed to verify the sex of the birds. Utilizing this technique, 47.36%, 66.67%, and 56.25% of the experimental birds were identified as male in Years 2020, 2021, and 2022, respectively.

The tractors were moved throughout the pasture approximately every other day during the first 2 weeks and every day after that. In 2020, the Red Ranger broilers were maintained on pasture for 9 weeks and were harvested at 12 weeks of age. The Cornish Cross birds were harvested at around 8 weeks of age in 2021 and 6 weeks of age in 2022 (approximately spent 5 and 3 weeks on pasture, respectively). See Supplementary Fig. S4 for tractor moving plan used in this study. To assess pasture use and broiler growth, we collected excreta (see the Section 'Excreta sample collection') samples from all banded birds on a weekly basis. We also weighed all birds per flock and measured the amount of supplemental feed consumed to estimate the feed conversion ratio (FCR) of each flock. As this research was part of a larger integrated vegetable-cover crop-poultry rotation study, each year the pastures was rotated with spring vegetables and summer cover crop establishment (Table 1). Each fall the summer cover crop was utilized as a pasture for poultry, before being rotated back to spring vegetables the following year (Table 1).

Feed restriction experiment

To assess the impact of a short-term feed restriction on broiler diet diversity, we initiated an experiment in 2022. Due to issues with metabolic disease in the Cornish Cross raised in 2021, a short-term feed restriction was initiated across all of the high- and low-density Cornish Cross pastured flocks in 2022. All feeders were raised out of reach of flocks for 4 h (between 12 and 4 pm) but allowed broilers access to supplemental feed for 20 h per day. On the 13th, 17th, and 19th of September 2022, half of the experimental flocks were given full access to supplemental feed for 24 h while the remaining half of the flocks were maintained with the short-term feed restriction. On the 14th, 18th, and 20th of September 2022, the feed access and restriction treatments were reversed such that flocks given full 24-h access were given a short-term feed restriction and vis-a-versa. Pasture use diet diversity was assessed via excreta sampling on these experimental days to assess the impact of a short-term feed restriction.

Excreta sample collection

Chicken excreta samples were collected from experimental birds every week starting in their first week on pasture. To collect excreta samples, individual birds were placed in an 18 gallon-tupperware tub (68.14 liters) lined with clean plastic-coated butcher paper. To allow for proper ventilation while chickens were in the tub, we drilled several holes in the tub and its lid. Sampling occurred generally in the first half of the day with a minimum of 1–2 h of light (and feeding behavior) before sampling commenced. Each bird was placed in the tub and then surveilled for defecation for up to 30 min (surveilled time varied depending on ambient temperature and bird behavior, as described in the IACUC protocol). If the bird had not defecated within 30 min, it was placed back in a tractor. Freshly defecated excreta were immediately collected using sterile, disposable polypropylene spatulas. For each excreta sample, we documented the bird's band number, the tractor's current location within the plot, and the collecting date. Samples were stored in plastic bags on ice and transferred to a −20 °C freezer for long-term storage until DNA extraction.

DNA extraction

Before conducting DNA extraction, each excreta sample (excluding urate and liquid portions of the excreta) was homogenized to ensure sample community uniformity. We homogenized samples in 50 mL sterile tubes with 5 mL of TE buffer and three 3.5 mm diameter glass beads (BioSpec, Cat. No. 11079135) for 3 min at maximum speed (Mini-BeadBeater-96 Homogenizer, Cole-Parmer). From the homogenized excreta (paste-like consistency), we measured 200–220 mg as input for QIAamp PowerFecal Pro DNA Kits (QIAGEN) and followed the manufacturer's instructions. The only adaptation was that we used a bead-beating machine for 10 min at maximum speed (Mini-BeadBeater-96 Homogenizer, Cole-Parmer) for cell lysis instead of a vortex adapter. DNA was quantified using a Thermo Scientific™ NanoDrop™ One C Spectrophotometer and stored at −20 °C. DNA quality and quantity measurements can be found in Supplementary Table S1. In addition, positive controls for successful amplification of Internal Transcribed Spacer (*ITS2*) and Cytochrome c oxidase subunit I (*COI*) were included in every batch of PCR amplification. DNA of positive control samples were extracted from fresh store-bought broccoli (Brassicaceae: *Brassica oleracea*) and flash-frozen mourning cloak butterfly (Lepidoptera: Nymphalidae: *Nymphalis antiopa*) using QIAGEN DNeasy Blood and Tissue kits and QIAGEN DNeasy Plant Pro kits, respectively.

DNA amplification and library preparation

Illumina libraries were constructed using a two-step PCR approach. In the first step, PCRs used locus-specific primers (*COI* for arthropods and ITS2 for plants, following recommendations of Thongjued et al. (2024)) containing overhanging Illumina adapter sequences, and in the second step, individual-specific i5 and i7 Illumina indices were used in a step-out PCR (see Supplementary Table S2 for primer details). PCRs were prepared in 25 μL reactions containing 2 μL (10–100 ng/μL) of DNA template, 0.5 unit of KAPA HiFi DNA polymerase (Kapa Biosystems), 0.5 μL of 10 mM dNTPs, 5 μL of 5X KAPA HiFi Buffer, 1.25 μL of 10 μM each forward and reverse primer, and 14.9 μL PCR grade water. PCR cycling used initial denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, and a final extension of 72 °C for 10 min. For all PCR reactions, positive and negative controls (*B. oleraceae/N. antiopa and* DNA-free water, respectively) were included to verify amplification success and test for DNA contamination, respectively. PCR products were visualized on a 1.5% agarose gel. Twenty microliters of successfully amplified products were purified using Sera-Mag™ Magnetic Speed-Beads™ (GE Healthcare Life Sciences) (Rohland and Reich, 2012), cleaned libraries were quantified using a Qubit dsDNA BR Assay Kits with Qubit4 Fluorometer (Invitrogen, USA), and equal-molar pooling of libraries followed the guide for Illumina 16S Metagenomic Sequencing Library Preparation for the Illumina MiSeq system. Paired-end 300 bp sequencing was conducted on the final pooled library using Illumina MiSeq V3 chemistry at the OncoGenomics Shared Resource Facility of the University of Kentucky Markey Cancer Center.

Molecular sex determination

The sex of birds was initially speculated based on morphology but was not always accurate. Since chicken cells were defecated and coextracted from excreta samples, we then used the extracted excreta DNA as templates to confirm bird sex with *Chromo Helicase DNA-binding gene* (*CHD*) following Vucicevic et al. (2013). Amplification was carried out in 20 μL reactions containing 10 μL of Phire Hot Start II PCR Master Mix (Thermo Scientific), 1 μL of each 10 μM primer from 2550F/2718R primer set, 2 μL DNA template, and 6 μL of PCR water. The thermal protocol was modified from that of Vucicevic et al. (2013). PCR cycling was initiated at 98 °C for 30 s, followed by 35 cycles of denaturation at 98 °C for 5 s, annealing at 55 °C for 15 s, extension at 72 °C for 15 s, and a final extension step at 72 °C for 60 s (Vucicevic et al., 2013). The PCR product was then visualized on a 2.5% agarose gel. Reactions with unsuccessful amplification were repeated with 0.5 μL of PCR product as a DNA template.

Metabarcoding data processing

Sequence data analysis was performed in QIIME 2 version 2022.11 (Bolyen et al., 2019), and default parameters were used unless otherwise noted. Phred quality scores greater than 20 (indicating at least 99% base-call accuracy) were required during demultiplexing. Raw sequences were quality filtered using the q2-demux plugin followed by denoising, chimeric sequence removing, singleton removing, denoised paired-end read joining, and sequences dereplicating with DADA2 (Callahan et al., 2016). Amplicon sequence variants (ASVs) were inferred using DADA2, which resolves sequences at single-nucleotide resolution. Taxonomic assignments were performed against custom databases which were developed by Thongjued et al 2024, using the q2 feature-classifier plugin (Bokulich et al., 2018).

The accuracy of species identification was manually verified with BLAST 2.15.0. Species determinations were made when a query sequence had ≥98% similarity to a record in the National Center for Biotechnology Information (NCBI nucleotide database).

Query sequences matching at <98% were assigned to higher taxonomic levels (i.e., genus, family, order). In rare circumstances where a sequence matched multiple species, these sequences were cross-referenced with the Barcode of Life Database (BOLD) for secondary verification. This additional step involved checking biogeographical data and examining recorded photographs to ensure accuracy. To clean the dataset, ASVs that could not be identified to at least family level or ASVs with less than a minimum count fraction of 0.01% were removed from the taxonomic table to avoid possible sequence data contamination (as in Crisol-Martínez et al. 2016). Cleaned data sets were then used to assess diet richness for each locus and overall. The frequency of occurrence (FOO) for each diet species was calculated as a percentage using the number of samples that detected a given species divided by the total number of samples tested. For example, if a given species was detected in 10 samples of 100 samples total, then that species would have an FOO of 10%.

Statistical analysis

Statistical analyses were conducted using R Studio version 4.3.0 (R Core Team 2022). The package 'lme4' and 'glmmTMB' were used for linear mixed-effects models (LMMs) and binomial models, respectively. For LMMs, we applied a square root transformation to dependent variables when necessary to meet assumptions of a Gaussian distribution when model residuals were not normal as tested by the Shapiro–Wilk test. We conducted four main modeling analyses to test relationships between management strategies and pastured-poultry diet. First, we used an LMM to assess the impacts of stocking density, sex, weight gain, breed, and the interaction between weight gain and breed on diet richness across all 3 years. We utilized generalized linear mixed effects models (GLMMs) with a binomial distribution to assess the effect of stocking density, weight gain, and sex on the FOO of the four most common pasture plant and weed species (pigweed [*Amaranthus* spp.], ragweed [*Ambrosia* spp.], goosefoot [*Chenopodium album*], and gallant soldier [*Galinsoga parviflora*]). Each model included a unique band number of each broiler, block, and year as random effects.

To assess the effect of short-term feed restriction on diet richness, we subset the dataset to only include year 3 (2022) because this year was the only year that contained a short-term feed restriction. We used LMM to assess the effect of short-term feed restriction treatments and included stocking density, sex, and weight gain as additional factors in the model. Additionally, we assessed the impact of the short-term feed restriction on the FOO of the four most common plant species using GLMM with a binomial distribution. For these feed-restriction models, we used the unique band number of each broiler and block as random effects.

To assess the impact of stocking density and year on the FCR (ratio of supplemented feed consumed to weight gained), we estimated the total amount of feed consumed for each flock (each tractor) and the total weight gained for each flock for all treatments in all years. We then used LMM with stocking density and year as fixed effects in the model and block as a random effect.

We used nonmetric multidimensional scaling (NMDS) to visualize the differences in combined arthropod and plant diet community composition between the treatments (high versus low stocking density, male versus female, Cornish Cross versus Red Ranger, feed restricted versus nonfeed restricted). To accomplish this, we used a presence/absence matrix of plant and arthropod diet items for each sample and created a Jaccard-based distance matrix using 'vegdist' from the 'vegan' package. This distance matrix was then used by the function 'metaMDS' from package 'vegan' (Oksanen et al., 2013) with a parameter of '*k*', which specifies the number of dimensions for the ordination space, set at 2. The metaMDS function reported a stress value around 0.2 which was considered acceptable for the goodness of fit for the ordination for visualization purposes. In order to test for multivariate homogeneity of group dispersion, we used the permutation-based function 'betadisper' from the package 'vegan', followed by an ANOVA on the output to interpret the significance of the F statistic, in which the null hypothesis states that there is no difference in dispersion between groups. The 'adonis2' function from the 'vegan' package was used to perform permutational multivariate analysis of variance (PERMANOVA) to determine whether diet community composition significantly differed across treatments. We set the number of permutations to 9999 for each PERMANOVA.

Results

Sequence descriptive statistics

A total of 258 excreta samples were successfully amplified and created 522 sequencing libraries for ITS2, *COI*, and positive/negative controls, which were sequenced on a single MiSeq lane. The final library generated 17,232,532 raw read pairs and after quality control filtering, a total of 2,489,885 clean read pairs were retained. The majority loss of the filtered reads at this stage was due to chimeric sequences, a well-known phenomenon in metabarcoding libraries (Bjørnsgaard Aas et al., 2017). The number of filtered sequences from each quality control step is shown in Supplementary Table S3, and cleaned sequences were clustered into 3,051 ASVs for ITS2, and 2,796 for *COI*. QIIME outputs for both loci are provided in Supplementary Tables S4 and S5.

After strict filtering criteria, the data from 222 excreta samples across 3 years were included in the final dataset for statistical analysis. Overall, we detected plants, arthropods, and fungi, and ASVs were taxonomically assigned to 12 orders and 16 families of plants, and seven orders and 16 families of arthropods (full taxonomy classification provided in Supplementary Table S6). Fungi were ignored for downstream analyses.

Diet richness

For plant diet composition at the family level, Asteraceae was the most abundant family found in 163 out of 222 analyzed excreta samples (73.42%). Other commonly detected families were Amaranthaceae (66.67%), Poaceae (48.65%), and Chenopodiaceae (42.34%) (Fig. 1A). The most common species found in chicken diet were pigweed (*Amaranthus* spp.; Amaranthaceae), ragweed (*Ambrosia trifida*; Asteraceae), flax (*Linum* sp.; Linaceae), gallant soldier (*G. parviflora*; Asteraceae), and white goosefoot (*C. album;* Chenopodiaceae) (Supplementary Table S6).

For insect diet composition, the most abundant sequences belonged to the family Cecidomyiidae (Diptera; Fig. 1B), Pyroglyphidae (Sarcoptiformes), and Suidasiidae (Sarcoptiformes). For Cecidomyiidae, species identification was unsuccessful for these ASVs given the paucity of sequence records for the family. See Supplementary Table S6 for species identification of other families.

Influence of management strategies on diet richness and chicken performance (3-year analyses)

The LMM revealed significant effects of weight gain, stocking density, and breed on diet richness (Table 2). Across all years, birds

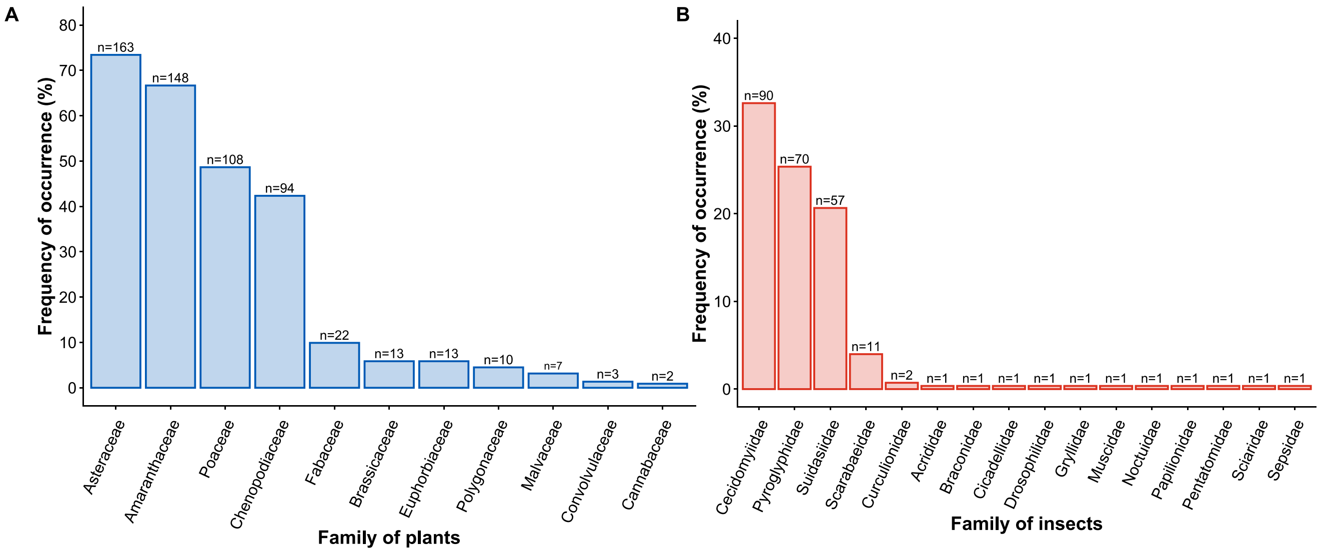

Figure 1. Frequency of occurrence of plant (A) and insect (B) families found in pasture-raised chicken excreta. Sample sizes reflect the number of excreta samples where we detected each family.

Table 2. Summary statistics of the linear mixed effects model

	Est ± SE	*t* value	Pr(>|*t*|)
Weight gain	0.2 ± 0.1	3.7	0.0003***
Breed	2.4 ± 0.6	4.1	<0.0001***
Sex	0.0 ± 0.1	0.2	0.8591
Stocking density	0.2 ± 0.1	2.6	0.0116*
Weight gain × breed	−0.4 ± 0.1	−3.7	0.0003***

Note: Chicken weight gain and farm management factors (including chicken breed, sex, and stocking density) were treated as explanatory variables while diet richness was the response variable in the model. Individual bird identifiers, block, and sample collecting dates were included as random effects. *p < 0.05; **p < 0.01; ***p < 0.001.

in the low-density treatment had higher diet diversity than birds in the high-density treatment (Fig. 2D). Red Ranger birds, in 2020, tended to have a higher diet richness than Cornish Cross birds in 2021 and 2022 (Fig. 2B). There was a significant interaction between weight gain and breed, where diet richness increased with weight gain for Red Rangers but decreased with weight gain for Cornish cross (Fig. 2A; Table 2). Bird sex did not impact diet richness (Fig. 2C; Table 2).

Influence of management on the FOO of individual diet items

For the four most common plant species (pigweed, ragweed, goosefoot, and gallant soldier; 85.1%, 64.4%, 40.1%, and 37.4% FOO consumption, respectively), binomial models revealed varying effects of weight gain, stocking density, and sex (Table 3). For pigweed, the birds in low-density treatment had higher FOOs relative to birds in the high-density treatment. For ragweed, birds with lower weight gain rates had higher FOOs than birds with higher weight gain rates. However, for goosefoot, birds with higher weight gain rates had higher FOOs than birds with lower weight gain rates. For gallant soldier, no variable significantly explained variation in FOO.

Effect of short-term feed restriction on diet richness and on FOO (2022 only)

There was a significant effect of feed restriction on diet richness (Fig. 3), in which the feed-restricted birds had more diverse diets compared to the birds that were fed *ad libitum* (Table 4). For the four most common plant species (pigweed, ragweed, goosefoot, gallant soldier) consumed by pastured broilers, only gallant soldier was consumed more frequently by birds under a feed restriction (Table 5).

Diet community composition

Permutation tests of multivariate homogeneity of group dispersions found heterogeneous dispersion in stocking density ($p = 0.037$) and breed ($p < 0.001$) treatment groups but not sex ($p = 0.533$) or feed restriction ($p = 0.103$) treatment groups. A PERMANOVA analysis found that broiler diet composition differed between high- and low-density treatments (The adonis test result showed $p = 0.04$; for years 2020–2022; Fig. 4C). However, there was no difference in diet composition between different sexes ($p = 0.384$; Fig. 4B) or breeds of birds ($p = 1.000$ Fig. 4A). In 2022, the diet composition of broilers in the short-term feed restriction treatment differed from the unrestricted treatment group ($p = 0.032$; Fig. 4D).

Effect of management on FCR

The FCR was impacted by the year of the experiment ($F_{2,17} = 16$, $p < 0.001$) but not stocking density treatment ($F_{1,17} = 0.5$, $p = 0.497$). Across years, the FCR of broilers in 2020 (slow-growing broilers; Red Ranger), was 68% ($p = 0.0017$) and 91% ($p = 0.0001$) higher than in years 2021 and 2022 (fast-growing broilers; Cornish Cross), respectively (Fig. 5). This suggests the slow-growing broilers in 2020 had lower feed-use efficiency per kg of meat produced relative to fast-growing broilers. There was no difference between the feed conversion ratios of the year 2021 (no-feed restriction) and 2022 (short-term feed restriction; $p = 0.4603$) for fast-growing broilers, suggesting the short-term feed restriction did not improve or reduce feed-use efficiency.

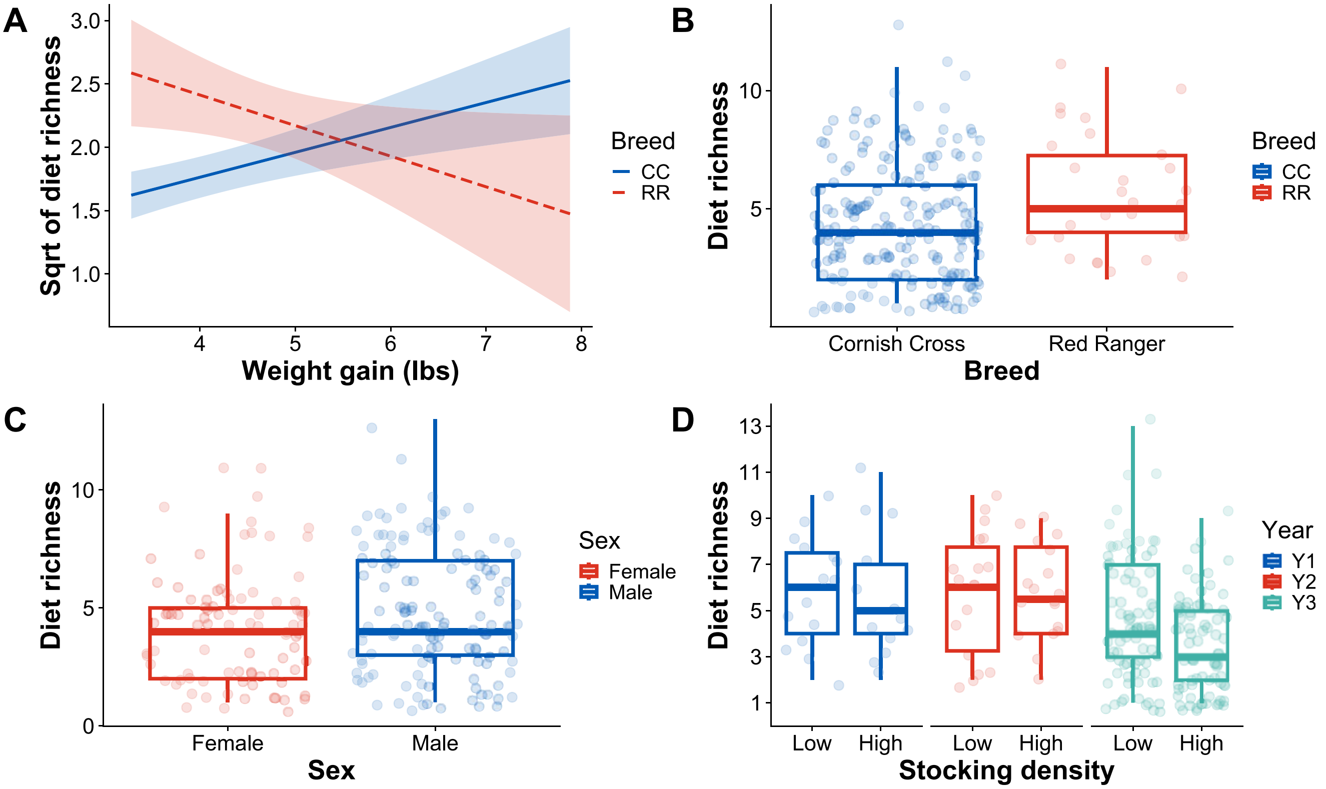

Figure 2. The visualization of linear mixed effects model result. (A) The interaction plot shows the relationship between diet richness (*y*-axis), chicken weight gain (*x*-axis), and breed. CC: Cornish Cross breed (solid blue line), RR: Red Ranger breed (dashed red line). (B) Diet richness (*y*-axis) versus breeds (*x*-axis). (C) Diet richness (*y*-axis) versus sex (*x*-axis) and (D) diet richness (*y*-axis) versus stocking density (low versus high) across three years of experiments.

Discussions

Chickens are omnivorous and have been shown to suppress insect pest populations in organic orchard settings (Clark and Gage, 1996). However, their impact on insect populations in pasture environments is less clear. While it is hypothesized that chickens may negatively impact insect populations through direct consumption or habitat disturbance, the specific mechanisms and dietary preferences of pastured chickens remain poorly understood. To address this knowledge gap, we conducted a study involving broiler chickens introduced to a cover crop pasture. The birds were housed in movable-floorless tractors, enabling them to access fresh vegetation and insects daily. We compared different management strategies,

Table 3. Summary statistics from binomial model analysis

Response variable	Predictor	Est ± SE	*z* value	Pr(>|*z*|)
FOO of pigweed	Weight gain	0.4 ± 0.5	0.7	0.4935
	Stocking density	1.0 ± 0.4	2.5	0.0139*
	Sex	−0.6 ± 0.6	−1	0.3318
FOO of ragweed	Weight gain	−0.7 ± 0.2	−3.2	0.00158**
	Stocking density	0.2 ± 0.4	0.5	0.6184
	Sex	0.7 ± 0.5	1.5	0.1424
FOO of goosefoot	Weight gain	0.4 ± 0.2	2.4	0.01679*
	Stocking density	0.4 ± 0.3	1.4	0.17667
	Sex	−0.2 ± 0.4	−0.6	0.55851
FOO of gallant soldier	Weight gain	−0.2 ± 0.2	−1.2	0.2497
	Stocking density	0.5 ± 0.3	1.7	0.0875
	Sex	0.2 ± 0.4	0.5	0.6525

Note: The presence of the four commonly observed plant species, pigweed, ragweed, goosefoot, and gallant soldier were the response variables in these models while weight gain, stocking density, and sex were the explanatory variables. *p < 0.05; **p < 0.01; ***p < 0.001.

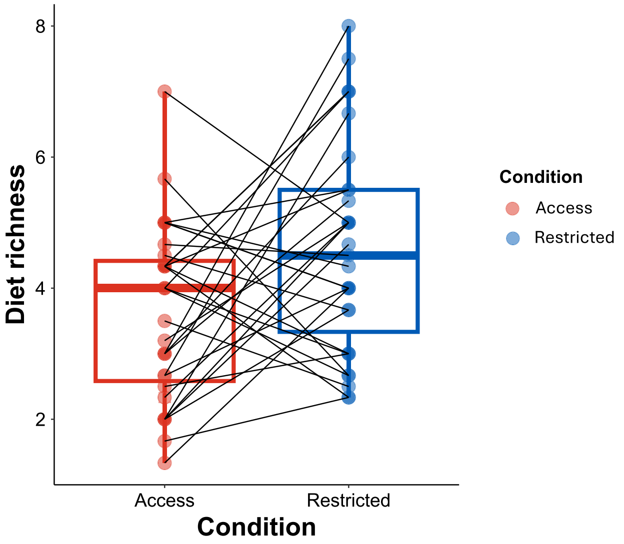

Figure 3. Boxplot of paired observations between feed restriction treatment shows diet richness differed between treatments. Each pair of data points represents an individual bird in two different conditions (at different time points, either experiencing feed restriction or not).

Table 4. Summary statistics of linear mixed effects model obtained from the feed restriction experiment

	Est ± SE	*t* value	Pr(>|*t*|)
Feed restriction	0.2 ± 0.1	2.5	0.0118***
Sex	0.0 ± 0.2	0.1	0.9553
Weight gain	0.2 ± 0.2	0.9	0.3638
Stocking density	0.2 ± 0.1	2.4	0.0172*

Note: The feed restriction treatments (restricted or free access to feed), weight gain, sex, and stocking density were included as predictor variables while diet richness was the response variable in the model. The band numbers of experimental birds, block, and sample collecting dates were included as random effects. The breed was not considered in this model since the feed restriction experiment was performed only with Cornish Cross in the third year of the study (2022). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 5. Summary statistics from binomial model analysis of the feed restriction experiment

Response variable	Predictor	Est ± SE	*z* value	Pr(>|*z*|)
FOO of pigweed	Weight gain	0.9 ± 0.8	1.1	0.2714
	Stocking density	0.8 ± 0.4	2	0.0477*
	Sex	−0.7 ± 0.6	−1.1	0.2621
	Feed restriction	0.2 ± 0.3	0.5	0.6189
FOO of ragweed	Weight gain	1.3 ± 0.8	1.5	0.1210
	Stocking density	0.5 ± 0.4	1.3	0.2110
	Sex	0.4 ± 0.6	0.7	0.5040
	Feed restriction	−0.0 ± 0.4	−0.1	0.9040
FOO of goosefoot	Weight gain	1.3 ± 0.8	1.6	0.1139
	Stocking density	0.4 ± 0.4	1.1	0.2880
	Sex	−0.2 ± 0.6	−0.4	0.6803
	Feed restriction	0.7 ± 0.4	1.7	0.0581
FOO of gallant soldier	Weight gain	−1.2 ± 0.8	−1.6	0.1154
	Stocking density	0.4 ± 0.4	1.1	0.2734
	Sex	0.7 ± 0.6	1.3	0.2066
	Feed restriction	1.0 ± 0.4	2.7	0.0063**

Note: Only excreta samples from 2022 (year 3) were included in this analysis. The model compares the effect of weight gain, stocking density, sex, and feed restriction on the frequency of occurrence of pigweed, ragweed, goosefoot, and gallant soldier. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

including chicken breed, stocking density, and supplemental feed restriction, to assess their influence on diet composition. The primary finding of this study is that pastured broilers consumed a diversity of pasture plants but few insect species. Slower-growing broiler chickens, reduced poultry density, and short-term feed restriction led to increased diet diversity. However, it was observed that slow-growing broilers had a higher FCR. Moreover, our analysis revealed no evidence to suggest that lowered stocking density or short-term feed restriction resulted in a statistical reduction of FCR or improvement of feed-use efficiency. These outcomes not only contribute to the informed management of pastured poultry but also offer valuable insights into the ecological dynamics of pastured poultry systems and integrated agricultural practices. In the following sections, we discuss insight into pasture-raised broiler diet composition through the lens of DNA metabarcoding-based diet analysis, pertinent management implications and recommendations, and propose avenues for future research.

Plant and arthropod diet composition

Poultry in this study consumed a diverse diet of plants but few arthropod species (Fig. 1). The plant species identified as diet items were those we expected to find, including those purposefully planted as part of the cover crop and common farm weed species. Interestingly, common farm weeds (e.g., pigweed, ragweed, gallant soldier, goosefoot) were consumed more substantially than the planted cover crop species (e.g., teff, clover, cowpea). It has been shown that poultry preference for forage species is dependent on the plant species, the nutritional content, height, and stage of growth of the plant, as well as the nutritional needs of the bird, starvation status, and its foraging instincts (Wood, 1956; Salatin, 2004; Horsted, 2006; Singh and Cowieson, 2013; Meng et al., 2016). In our pasture setting, teff and cowpea thrived and could be over 30 cm in height when the birds were introduced to the pasture; thus, thicker/woodier stems may have made these species less preferred by the poultry, similar to the results from Horsted (2006) who found preference for shorter forage species.

Garcia et al. (2023) assessed arthropod diversity and cover crop biomass in the same experimental setup as this study for the years 2020 and 2021. Pitfall traps were used to collect ground-dwelling arthropods, and sweep-net sampling was conducted to collect plant-dwelling arthropods in plots previously occupied by chickens. Interestingly, the introduction of broilers to the pasture resulted in a decrease in the abundance of plant-dwelling arthropods. This reduction could be the result of a combination of direct consumption of insects as well as trampling of plant matter and destruction of insect habitats (see Supplementary Fig. S1). However, our finding that broilers consume a low arthropod diet diversity (discussed below) and a higher diversity of pasture plants corroborates the hypothesis that poultry impact plant-dwelling arthropods through the elimination of pasture plants and not through direct consumption (Garcia et al. 2023).

Forage plant species have been shown to make up anywhere from 5% to 20% of pastured poultry diets depending on the type and age of poultry, and the quality of forage (Mattocks, 2002). However, observations from the higher end of that range are the result of foraging on crop species such as clover and alfalfa (Mattocks, 2002), and most studies comparing poultry performance and pasture intake have been done in free-range areas consisting of grass pasture or monocropping pasture systems (Bassler, 2005; Antell and Ciszuk, 2006; Horsted, 2006; Ponte et al., 2008a, 2008b; Singh and Cowieson, 2013). The consumption of many plant species on pastures also highlights the importance of broilers in weed management, but the explicit study of plant species preferences would elucidate this usefulness.

Unlike plant diet items, arthropod diet items were relatively rare and lacked diversity. Only 18 arthropod species/ASVs were identified in all 3 years of the study, and the majority of those were singleton detections from a single excreta sample. Overall, the families detected here do match our expectations of arthropod diversity in this ecosystem. Garcia et al. (2023) used pitfall traps and sweep net samples to assess arthropod diversity in this setting and found similar family representation as detected here. Although Garcia et al. (2023) did not conduct species-level morphological identifications as part of their study, we did superficially identify the same morphospecies in their samples as we identified molecularly here (Thongjued, Garcia, unpublished).

The most prevalent arthropod detected in the diet of these pastured poultry were gall midges of the family Cecidomyiidae. This family is highly diverse, with >6,600 described species (Dorchin et al., 2019),

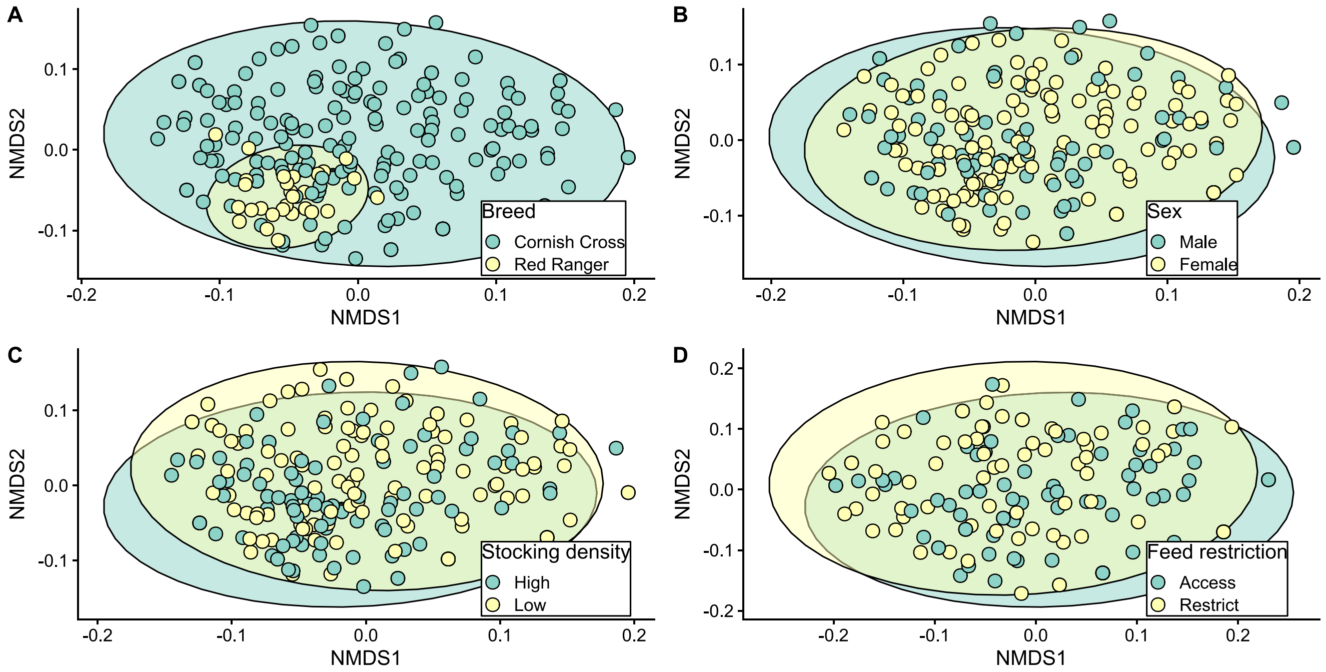

Figure 4. Non-metric multidimensional scaling plots show combined plant and arthropod diet community composition comparing between the different treatment of (A) chicken breed, (B) sex, (C) stocking density, and (D) feed restriction. Adonis test confirmed that feed restriction and stocking density influenced diet community composition ($p = 0.032$ and 0.04, respectively) and ellipses represent a 95% confidence interval of treatment-specific centroids (not-shown). Stress was 0.229 for A, B, and C and 0.194 for D. Number of reduced dimensions was $k = 2$ for all ordinations.

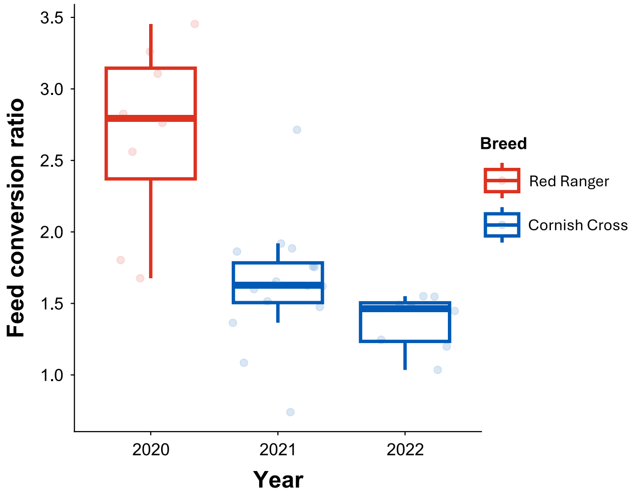

Figure 5. Box plot shows feed conversion ratio (FCR) comparing between years of experiment. The Red Ranger birds were used for year 2020 and the Cornish Cross birds were used in year 2021 (feed un-restricted) and 2022 (short-term feed restriction).

but given their small size and difficult identification, there are very few Cecidomyiid DNA barcodes available on NCBI/BOLD that are identified to species. Thus, we were limited to family-level identification for all 79 detections of the family in our data. We hypothesize that most of this consumption was indirect feeding on larvae inside their galls on various cover crops and weed plants. Gall midges are very common in agricultural settings, both in cover crops, e.g., clover, teff, rye grass (Felt, 1911; Macdougall, 1913; Agee and Holdaway, 1963), and common farm weed species, e.g., dandelion, ragweed, plantain, goosefoot, and ironweed (Felt, 1911; Gagne, 1975; Gagné and Jaschhof, 2004; Dorchin et al., 2019). Other commonly detected arthropods included various species of mites (Class Arachnida) and scarab beetles (Coleoptera: Scarabaeidae). Mite diversity in our data was dominated by two species of dust mites and the scaly grain mite (*Suidasia nesbitti*), and thus, were likely also the result of indirect feeding on mite-infested feed, a recognized problem in poultry production (Ta-Phaisach et al., 2023). Scarab beetles are common in agricultural settings in this region, and morpho-species identification of scarabs collected by Garcia et al. (2023) indicate six species. Interestingly, other prevalent arthropods which we expected to represent major components of chicken diets were generally only detected as singletons, e.g., various pest caterpillars (*Spodoptera* armyworms), orthopterans (*Melanoplus* grasshoppers), and hemipterans (Cicadellidae; *Forcipata loca* and Pentatomidae; *Euschistus servus*). We also only detected one occurrence of the common house fly *Musca domestica*, which was the dominant arthropod species found by Garcia et al. (2023), representing 21% of all specimens collected (11,069 of 52,692 specimens).

This discrepancy between plant and arthropod consumption in our data may be explained in several ways. First, it has been shown that when young birds shift from cereal-based feed to natural forage, a transition time of 6–7 weeks is required for physical adaptation of the digestive system (including crop, gizzard, and small intestine) to forage diet (Horsted and Hermansen, 2007). Here, the broilers were placed on pasture for 3–8 weeks, which might not be long enough for the birds to physically adapt to new food resources. Foraging activity in pasture settings is also positively correlated with the age of broilers and early exposure to the pasture/range area (Almeida, 2012; Ferreira et al., 2022), and thus young birds with no exposure to older individuals (from which they might learn more effective foraging behavior) may decrease foraging effectiveness. Finally, given that both feed and forage plants were readily available without excessive effort, optimal foraging theory (the least effort hypothesis) would predict individuals to maximize intake and minimize energy expenditure (Osborne, 1977). Thus, arthropod diet items, which require chasing, scratching, and digging may fall outside the scope of 'low effort' food. Additionally, domesticated poultry are less likely to engage in the behavior of contrafreeloading than junglefowl (the ancestor of the modern chicken) (Lindqvist et al., 2006), which is an observed behavior where individuals will preferentially select food that requires effort to obtain, when given the choice between that and identical food that is readily available (Lindqvist et al., 2002, 2006; Ferreira et al., 2021). Within domesticated varieties, layers are also more prone to contrafreeload than broilers (Lindqvist et al., 2002, 2006; Baxter, 2018; Ferreira et al., 2021), which has been proposed to be a byproduct of artificial selection for increased production traits (Lindqvist et al., 2006). In the breeds used here, the Red Rangers did have higher diet richness (Table 2; Fig. 2B) and were behaviorally more active than the Cornish Cross broilers, supporting previous findings. Thus, the use of broilers (Cornish Cross) and hybrid breeds (Red Ranger) in our study may predispose these birds to low-energy investment feeding strategies and food items.

Management implications

Alternative poultry production systems are gaining popularity in the United States as they are perceived as improving animal welfare and as a more sustainable option than conventional mass production systems. Producers who integrate poultry into their production systems can potentially increase profits through product diversification, reduced farm input, and ecosystem services that the poultry may provide. Historically, studies examining these alternative production systems have focused on economic and environmental benefits and production such as breeds of the birds (genetics) and forage access (Salatin, 1993; Castellini et al., 2002; Fanatico et al., 2005, 2009). However, methods to increase forage utilization are not well-informed, and quantitative diet data for chickens on pasture remain scarce.

Short-term feed restriction can potentially be used to increase pasture use and diet diversity without limiting feed-use efficiency or increasing resource use rate per bird. We showed that a 4-h feed restriction increased fast-growing broiler consumption of pasture diet items (in 2022). Further, of the 3 years of the study, the short-term feed restriction had the greatest feed use efficiency. This is consistent with many studies that have found that feed restriction increases foraging behavior. Girard et al. (2017) found that 'skip-a-day-fed' pullets exhibited less restless behavior and foraged more, as compared to precision-fed pullets, in which underweight individual birds were provided small individual meals, multiple times per day (Girard et al., 2017). Dixon et al (2014) found that feed-restricted birds worked harder to reach foraging areas, took less time to reach those areas, and spent more time foraging in those areas than birds with greater access to food (Dixon et al., 2014). Short-term feed restriction may also have benefits to metabolic health of fast-growing broilers. In 2021, the Cornish Cross birds were provided with unrestricted access to feed, resulting in excessive consumption and some birds had metabolic health challenges including ascites and heart failure-like symptoms (Julian, 2005; Olkowski et al., 2008). However, these issues were mitigated in 2022 by implementing a brief period of feed restriction during the finishing phase. Thus, at least for fast-growing breeds, employing feed restriction appears to increase forage use. Again, a more explicit study with a wider variety of breeds will expand on this management recommendation.

The birds in low-density treatments across 3 years of experiment consumed a higher diversity of diet items from the pasture. This more diverse diet may be due to less competition among individuals in accessing food resources. In addition, low stocking density may reduce stress and the likelihood of aggressive behaviors (e.g., threatening, chasing, pecking, fighting) that can prevent individuals from foraging (Phillips and Heins, 2021). Sanchez-Casanova et al., (2019, 2021) conducted outdoor access experiments with two stocking densities and found that Cornish Cross birds that were given outdoor access with low stocking density foraged more, were significantly heavier, and had a lower FCR than those reared in the high-density group (Sanchez-Casanova et al., 2019, 2021). Thus, increasing pasture availability for higher stocking density may be an option to increase pasture use without jeopardizing feed-use efficiency. This could be achieved through increased rotational grazing (number of pen movements) in confined pull pens or via increasing access to total pasture in a 'free-range' pastured system. In our study, broilers were confined to a pen that was moved throughout the pasture; however, many producers allow broilers to free-range throughout the pasture during the day and only confine broilers to pens at night for protection from predators. Future studies could evaluate the effectiveness of broiler foraging in free-range and confined pens, as well as across a greater variation in the density of birds per pasture area.

Slow-growing broilers are marketed as excellent pasture-raised options with improved foraging (Fanatico et al., 2009; Ussery, 2011; Spencer, 2013; Fisher, 2016; Tufarelli et al., 2018); however, they have higher FCR than fast-growing breeds. We used Cornish Cross and Red Ranger breeds as representatives of fast- and slow-growing breeds, with weeks to marketable weight of 6–8 weeks and 10–12 weeks, respectively. Overall, Red Rangers had higher diet richness than Cornish Cross, which supports the previous assumption that slow-growing broiler breeds are better adapted to pasture conditions as they are more active, particularly in foraging. Ultimately, however, we found the slow-growing Red Ranger broilers had higher FCR, and thus may be less cost-effective as compared to fast-growing broilers. It should be noted that the pasture composition differed between years (Table 1), and therefore, the differences observed in diet richness and FCR between breeds may not be due to breed alone. Nonetheless, economic feasibility studies comparing pasture-raised slow-growing and fast-growing broilers have concluded that slow-growing broilers take longer to achieve marketable weight, higher costs per pound of production, lower marketable weights, and higher break-even prices ($/lb) relative to fast-growing broilers (Painter et al., 2015). This may explain why fast-growing broilers are the most commonly used breed for free-range/pasture-raised broiler production across the United States (Fanatico et al., 2005; Conner, 2010; Pitesky et al., 2019), even though they may have health issues caused by the rapid growth (Julian, 2005; Fanatico et al., 2009). Further, in some environments with high humidity and temperatures, slow-growing broilers may be a more ethical consideration, given their more robust capability for growth and survival under these conditions. Given the diversity of breeds used in conventional and alternative poultry operations, this is an area that is ripe for future research and comprehensive evaluation of breed foraging characteristics may highlight other breeds that are more cost-effective via optimal use of pastured environments.

Conclusions

Alternative production systems such as free-range, pasture-raised, and organic have increased in popularity in the United Stated in recent years. Studying the functional ecology and relationships among organisms in this complex system play a key role to inform proper management. In this study, we used DNA metabarcoding diet analysis for the first time to study arthropod and plant diet composition in pasture-raised poultry. In addition to characterizing poultry diet in this system, we assessed the effects of poultry management techniques on diet diversity to identify practices for optimizing pasture utilization, feed use efficiency, and broiler productivity. We found that pastured broilers consumed many cover crop and weed plant families, but the diet richness of insect food items was surprisingly low. The most dominant insect food item was gall midges in the family Cecidomyiidae, which we hypothesize were consumed via galls in/on cover crops. Stocking density, feed restriction, and chicken breed affected diet richness, indicating an influence of those factors on forage utilization/foraging activities in pasture-raised broilers. These results support our hypothesis that certain management techniques can enhance dietary diversity and might be used to potentially offset feed costs. This study serves as a foundation for future investigations into the complex relationships between pasture ecology, poultry behavior, and management practices in alternative production systems. By advancing our understanding of these dynamics, we contribute to the development of more sustainable and efficient pasture-raised poultry productions.

Supplementary material. The supplementary material for this article can be found at http://doi.org/10.1017/S1742170525000092.

Acknowledgments. We are grateful to Poultry Research Specialist, Michael J. Ford for providing chicken samples to use as positive controls in sex determination. We wish to thank Ariana Spina for assistance with DNA extraction and Nat Colten for building chicken tractors, and Nate Haan, Holly Myers, Colby Grant, Alexis Gauger, Elaine Losekamp, Brianna Bazile (Gonthier/Hann lab members) for comments on an early draft of this manuscript, and two anonymous reviewers for their input.

Author contribution. Conceived and designed the experiments: J.R.D. and D.J.G.; performed the experiments: K.T., K.G., V.H., K.F.P.; analyzed the data: K.T., K.G., D.J.G.; contributed reagents/materials/analysis tools: D.J.G. and J.R.D.; wrote the article: K.T., K.G., K.F.P., V.H., J.R.D., and D.J.G.

Funding statement. This research was supported by USDA-NIFA HATCH grants to J.R.D. (Project KY008091) and D.G. (KY008079) and by USDA OREI Grant #2019-51300-30244 awarded to D.G. Sequencing was conducted at the OncoGenomics Shared Resource Facility of the University of Kentucky Markey Cancer Center (P30CA177558).

Competing interests. The authors declare none.

References

Agee, H.R. and Holdaway, F. (1963) 'The morphology and histology of red clover florets injured by the clover seed midge, dasyneura leguminicola (Diptera: Cecidomyiidae)', *Annals of the Entomological Society of America*, **56**, pp. 561–564.

Almeida, G.F. (2012) 'Use of forage and plant supplements in organic and free-range broiler systems: implications for production and parasite infections', *PhD thesis*. University of Aarhus, Denmark.

Ando, H., Setsuko, S., Horikoshi, K., Suzuki, H., Umehara, S., Inoue-Murayama, M. and Isagi, Y. (2013) 'Diet analysis by next-generation sequencing indicates the frequent consumption of introduced plants by the critically endangered red-headed wood pigeon (*Columba janthina nitens*) in oceanic island habitats', *Ecology and Evolution*, **3**, pp.4057–4069.

Antell, S. and Ciszuk, P. (2006) 'Forage consumption of laying hens-the crop content as an indicator of feed intake and AME content of ingested forage', *European Poultry Science*, **70**, 154–160.

Bassler, A.W. (2005) *Organic broilers in floorless pens on pasture', PhD thesis* Swedish University of Agricultural Sciences Uppsala, Sweden.

Baxter, M. (2018) *Evaluating the use and effectiveness of environmental enrichments in intensive broiler housing.* United Kingdom: Queen's University Belfast.

Bennett, C.E., Thomas, R., Williams, M., Zalasiewicz, J., Edgeworth, M., Miller, H., Coles, B., Foster, A., Burton, E.J. and Marume, U. (2018) 'The broiler chicken as a signal of a human reconfigured biosphere', *Royal Society Open Science*, **5**, pp.180325.

Bjørnsgaard Aas, A., Davey, M.L. and Kauserud, H. (2017) 'ITS all right mama: investigating the formation of chimeric sequences in the ITS 2 region by DNA metabarcoding analyses of fungal mock communities of different complexities', *Molecular Ecology Resources*, **17**, pp. 730–741.

Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R., Huttley, G.A. and Gregory Caporaso, J. (2018) 'Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin', *Microbiome*, **6**, pp. 1–17.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M. and Asnicar, F. (2019) 'Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2', *Nature Biotechnology*, **37**, pp. 852–857.

Bordin, T., Pilotto, F., Pesenatto, D., de Mendonça, B.S., Daroit, L., Rodrigues, L.B., Dos Santos, E.D. and Dickel, E.L. (2021) 'Performance of broiler chicken submitted to a quantitative feed restriction program', *Tropical Animal Health and Production*, **53**, pp. 1–5.

Burbaugh, B., Toro, E. and Gernat, A. (2010) *Introduction to pasture-raised poultry: maximizing foraging behavior.* Animal Science Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida Document No. AN237

Cabodevilla, X., Mougeot, F., Bota, G., Mañosa, S., Cuscó, F., Martínez-García, J., Arroyo, B. and Madeira, M.J. (2021) 'Metabarcoding insights into the diet and trophic diversity of six declining farmland birds', *Scientific Reports*, **11**, pp. 21131.

Cabodevilla, X., Ortiz-Santaliestra, M.E., Fernández-Tizón, M., Zurdo, J., Madeira, M.J., Giralt, D., Sardà-Palomera, F., Fernández-Benéitez, M.J. and Mougeot, F. (2024) 'Dietary DNA metabarcoding reveals a trophic niche partitioning among sympatric Iberian sandgrouses and bustards', *Science of The Total Environment*, **933**, pp. 172989.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A. and Holmes, S.P. (2016) 'DADA2: high-resolution sample inference from Illumina amplicon data', *Nature Methods*, **13**, pp. 581–583.

Castellini, C., Bosco, A.D., Mugnai, C. and Bernardini, M. (2002) 'Performance and behaviour of chickens with different growing rate reared according to the organic system', *Italian Journal of Animal Science*, **1**, pp. 290–300.

Clark, M.S. and Gage, S.H. (1996) 'Effects of free-range chickens and geese on insect pests and weeds in an agroecosystem', *American Journal of Alternative Agriculture*, **11**, pp. 39–47.

Conner, B. (2010) *Pastured poultry budgets: slow-growing broiler organic comparisons*. ATTRA-Sustainable Agriculture, National Center for Appropriate Technology. https://ucanr.edu/sites/default/files/2017-12/275179.pdf. [Retrieved August 16, 2024]

Crisol-Martínez, E., Moreno-Moyano, L.T., Wormington, K.R., Brown, P.H. and Stanley, D. (2016) 'Using next-generation sequencing to contrast the diet and explore pest-reduction services of sympatric bird species in macadamia orchards in Australia', *PLoS One*, **11**, pp. e0150159.

Dixon, L.M., Brocklehurst, S., Sandilands, V., Bateson, M., Tolkamp, B.J. and D'Eath, R.B. (2014) 'Measuring motivation for appetitive behaviour: food-restricted broiler breeder chickens cross a water barrier to forage in an area of wood shavings without food', *PLoS One*, **9**, pp. e102322.

Dorchin, N., Harris, K.M. and Stireman III, J.O. (2019) 'Phylogeny of the gall midges (Diptera, Cecidomyiidae, Cecidomyiinae): systematics, evolution of feeding modes and diversification rates', *Molecular Phylogenetics and Evolution*, **140**, pp. 106602.

Elkhoraibi, C., Pitesky, M., Dailey, N. and Niemeier, D. (2017) 'Operational challenges and opportunities in pastured poultry operations in the United States', *Poultry Science*, **96**, pp. 1648–1650.

Englmaierová, M., Skřivan, M., Taubner, T., Skřivanová, V. and Čermák, L. (2021) 'Effect of housing system and feed restriction on meat quality of medium-growing chickens', *Poultry Science*, **100**, pp. 101223.

Fanatico, A., Owens, C. and Emmert, J.L. (2009) 'Organic poultry production in the United States: broilers', *Journal of Applied Poultry Research*, **18**, pp. 355–366.

Fanatico, A., Polson, S. and Born, H. (2005) *Poultry genetics for pastured production.* Publication of ATTRA, the National Sustainable Agriculture Information Service 1–8.

Felt, A. (1911) 'Hosts and galls of American gall midges', *Journal of Economic Entomology*, **4**, pp. 451.

Ferreira, V.H.B., Simoni, A., Germain, K., Leterrier, C., Lansade, L., Collin, A., Mignon-Grasteau, S., Le Bihan-Duval, E., Guettier, E. and Leruste, H. (2021) 'Working for food is related to range use in free-range broiler chickens', *Scientific Reports*, **11**, pp. 6253.

Ferreira, V.H.B., Simoni, A., Germain, K., Leterrier, C., Lansade, L., Collin, A., Mignon-Grasteau, S., Le Bihan-Duval, E., Guettier, E. and Leruste, H. (2022) 'Foraging behavior shows individual-consistency over time, and predicts range use in slow-growing free-range male broiler chickens', *Frontiers in Veterinary Science*, **9**, pp. 814054.

Fisher, T. (2016) *Specialty poultry production: impact of genotype, feed strategies, alternative feedstuffs, and dietary enzymes on the growth performance and carcass characteristics of heritage breed chickens*. United States: University of Kentucky.

Gagne, R.J. (1975) 'The gall midges of ragweed, Ambrosia, with descriptions of two new species (Diptera: Cecidomyiidae)', *Proceedings of the Entomological Society of Washington*, **77**, pp. 50–55.

Gagné, R.J. and Jaschhof, M. (2004) *A catalog of the Cecidomyiidae (Diptera) of the world.* Citeseer.

Garcia, K., Halmos, V., Thongjued, K., Dupuis, J.R. and Gonthier, D.J. (2023) 'Net effects of pasture-raised poultry on arthropod communities driven by top-down and bottom-up forces in a mixed-cover crop system', *Frontiers in Sustainable Food Systems*, **7**, pp. 1162753.

Girard, M.T.E., Zuidhof, M.J. and Bench, C.J. (2017) 'Feeding, foraging, and feather pecking behaviours in precision-fed and skip-a-day-fed broiler breeder pullets', *Applied Animal Behaviour Science*, **188**, pp. 42–49.

Gong, M., Ning, Y., Han, M., Zhao, C., Tian, J., Li, L., Xiao, H. and Liu, G. (2019) 'A comparison of next-generation sequencing with clone sequencing in the diet analysis of Asian great bustard', *Conservation Genetics Resources*, **11**, pp. 15–17.

Hammershøj, M. and Johansen, N.F. (2016) 'The effect of grass and herbs in organic egg production on egg fatty acid composition, egg yolk colour and sensory properties', *Livestock Science*, **194**, pp. 37–43.

Hilimire, K. (2012) 'The grass is greener: farmers' experiences with pastured poultry', *Renewable Agriculture and Food Systems*, **27**, pp. 173–179.

Höhn, D., Masello, J.F., Kümmel, M.N., Griep, S., Goesmann, A. and Quillfeldt, P. (2024) 'Nestling diet of two sympatric insectivorous passerines in different habitats—a metabarcoding study', *Birds*, **5**, pp. 67–89.

Horsted, K. (2006) *Increased foraging in organic layers. PhD thesis*, University of Aarhus, Denmark.

Horsted, K. and Hermansen, J.E. (2007) 'Whole wheat versus mixed layer diet as supplementary feed to layers foraging a sequence of different forage crops', *Animal*, **1**, pp. 575–585.

Julian, R.J. (2005) 'Production and growth related disorders and other metabolic diseases of poultry–a review', *The Veterinary Journal*, **169**, pp. 350–369.

Karsten, H., Patterson, P., Stout, R. and Crews, G. (2010) 'Vitamins A, E and fatty acid composition of the eggs of caged hens and pastured hens', *Renewable Agriculture and Food Systems*, **25**, pp. 45–54.

Khurshid, A., Khan, A., Banday, M., Ganai, A., Khan, H., Choudhary, A., Adil, S., Manzoor, A., Afzal, I. and Untoo, M. (2019) 'Effect of feed restriction on performance of broiler chicken', *Journal of Entomology Zoology Studies*, **7**, pp. 1054–1056.

Leray, M., Yang, J.Y., Meyer, C.P., Mills, S.C., Agudelo, N., Ranwez, V., Boehm, J.T. and Machida, R.J. (2013) 'A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents', *Frontiers in Zoology*, **10**, pp. 1–14.

Lindqvist, C., Schütz, K. and Jensen, P. (2002) 'Red jungle fowl have more contrafreeloading than white leghorn layers: effect of food deprivation and consequences for information gain', *Behaviour*, **139**, pp. 1195–1209.

Lindqvist, C., Zimmerman, P. and Jensen, P. (2006) 'A note on contrafreeloading in broilers compared to layer chicks', *Applied Animal Behaviour Science*, **101**, pp. 161–166.

Macdougall, R. (1913) 'The Red Clover Gall Gnat, *Amblyspatha ormerodi*, sp. nov., Kieffer', *Journal of the Board of Agriculture and Fisheries*, **20**, pp. 225–230.

Mattocks, J. (2002) *Pastured-raised poultry nutrition*. Prepared for Heifer International.

Mench, J. (2009) 'Behaviour of fowl and other domesticated birds', in Jensen, P. (ed.) *The ethology of domestic animals*. Cambridge, MA: CAB International, pp. 121–136.

Meng, L., Mao, P., Guo, Q. and Tian, X. (2016) 'Evaluation of meat and egg traits of Beijing-you chickens rotationally grazing on chicory pasture in a chestnut forest', *Brazilian Journal of Poultry Science*, **18**, pp. 1–6.

Moorhouse-Gann, R.J., Dunn, J.C., De Vere, N., Goder, M., Cole, N., Hipperson, H. and Symondson, W.O. (2018) 'New universal ITS2 primers for high-resolution herbivory analyses using DNA metabarcoding in both tropical and temperate zones', *Scientific Reports*, **8**, pp. 8542.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'hara, R., Simpson, G.L., Solymos, P., Stevens, M.H.H. and Wagner, H. (2013) *Package 'vegan'*. Community ecology package, version 2**:** 1-295.

Olkowski, A., Wojnarowicz, C., Nain, S., Ling, B., Alcorn, J. and Laarveld, B. (2008) 'A study on pathogenesis of sudden death syndrome in broiler chickens', *Research in Veterinary Science*, **85**, pp. 131–140.

Osborne, S.R. (1977) 'The free food (contrafreeloading) phenomenon: a review and analysis', *Animal Learning & Behavior*, **5**, pp. 221–235.

Painter, K.M., Myhre, E.A., Bary, A.I., Cogger, C.G. and Jemmett, W. (2015) *Break-even analysis of small-scale production of pastured organic poultry*. United States: University of Idaho.

Paprocki, N., Blair, S., Conway, C.J., Adams, J., Nerkowski, S., Kidd, J. and Waits, L. (2024) 'Comparison of seven DNA metabarcoding sampling methods to assess diet in a large avian predator', *Environmental DNA*, **6**, pp. e70000.

Phillips, H.N. and Heins, B.J. (2021) 'Effects of outdoor stocking density on growth, feather damage and behavior of slow-growing free-range broilers', *Animals*, **11**, pp. 688.

Pitesky, M., Thorngren, A. and Niemeier, D. (2019) 'Feeding and lighting practices on small-scale extensive pastured poultry commercial farms in the United States', *Poultry Science*, **98**, pp. 785–788.

Ponte, P., Prates, J., Crespo, J., Crespo, D., Mourão, J.L., Alves, S., Bessa, R., Chaveiro-Soares, M., Gama, L. and Ferreira, L. (2008a) 'Restricting the intake of a cereal-based feed in free-range-pastured poultry: effects on performance and meat quality', *Poultry Science*, **87**, pp. 2032–2042.

Ponte, P., Rosado, C., Crespo, J., Crespo, D., Mourão, J.L., Chaveiro-Soares, M., Bras, J., Mendes, I., Gama, L. and Prates, J. (2008b) 'Pasture intake improves the performance and meat sensory attributes of free-range broilers', *Poultry Science*, **87**, pp. 71–79.

Rohland, N. and Reich, D. (2012) 'Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture', *Genome Research*, **22**, pp. 939–946.

Rothrock Jr, M.J., Gibson, K.E., Micciche, A.C. and Ricke, S.C. (2019) 'Pastured poultry production in the United States: strategies to balance system sustainability and environmental impact', *Frontiers in Sustainable Food Systems*, **3**, pp. 74.

Salatin, J. (1993) *Pastured poultry profits*. Swoope, VA: Polyface, Inc.

Salatin, J. (2004) *Holy cows and hog heaven*. Swoope, VA: Polyface, Inc.

Sales, J. (2014) 'Effects of access to pasture on performance, carcass composition, and meat quality in broilers: a meta-analysis', *Poultry Science*, **93**, pp. 1523–1533.

Sanchez-Casanova, R., Sarmiento-Franco, L. and Phillips, C. (2021) 'The effects of outdoor access and stocking density on the performance of broilers reared under tropical conditions', *British Poultry Science*, **62**, pp. 632–637.

Sanchez-Casanova, R., Sarmiento-Franco, L., Segura-Correa, J. and Phillips, C.J. (2019) 'Effects of outdoor access and indoor stocking density on behaviour and stress in broilers in the subhumid tropics', *Animals*, **9**, pp. 1016.

Scrinis, G., Parker, C. and Carey, R. (2017) 'The caged chicken or the free-range egg? The regulatory and market dynamics of layer-hen welfare in the UK, Australia and the USA', *Journal of Agricultural and Environmental Ethics*, **30**, pp. 783–808.

Singh, M. and Cowieson, A. (2013) 'Range use and pasture consumption in free-range poultry production', *Animal Production Science*, **53**, pp. 1202–1208.

Skelton, S., **Jacob, J. and Pescatore, T.** (2012) 'Making a Hoop Pen for Pasture Poultry'. Bulletin ASC-189. http://www2.ca.uky.edu/agcomm/pubs/ASC/ASC189/ASC189.pdf. [Retrieved April 20, 2024]

Skřivan, M. and Englmaierová, M. (2014) 'The deposition of carotenoids and α-tocopherol in hen eggs produced under a combination of sequential feeding and grazing', *Animal Feed Science and Technology*, **190**, pp. 79–86.

Snider, A.M., **Bonisoli-Alquati, A.**, **Pérez-Umphrey, A.A.**, **Stouffer, P.C. and Taylor, S.S.** (2022) 'Metabarcoding of stomach contents and fecal samples provide similar insights about Seaside Sparrow diet', *The Condor*, **124**, pp. duab060.

Sossidou, E., **DAL BOSCO, A.**, **Castellini, C. and Grashorn, M.** (2015) 'Effects of pasture management on poultry welfare and meat quality in organic poultry production systems', *World's Poultry Science Journal*, **71**, pp. 375–384.

Sossidou, E., **Dal Bosco, A.**, **Elson, H. and Fontes, C.** (2011) 'Pasture-based systems for poultry production: implications and perspectives', *World's Poultry Science Journal*, **67**, pp. 47–58.

Spence, A.R., **Wilson Rankin, E.E. and Tingley, M.W.** (2022) 'DNA metabarcoding reveals broadly overlapping diets in three sympatric North American hummingbirds', *The Auk*, **139**, pp. ukab074.

Spencer, T. (2013) *Pastured poultry nutrition and forages*. ATTRA-Sustainable Agriculture, National Center for Appropriate Technology. https://ucanr.edu/sites/default/files/2017-12/275179.pdf.

Ta-Phaisach, N., **Konvipasruang, P.**, **Sringarm, K.**, **Chiu, C.-I.**, **Attasopa, K. and Chanbang, Y.** (2023) 'Mites (Acari) infesting stored animal feed products in northern Thailand', *Systematic and Applied Acarology*, **28**, pp. 1579–1596.

Thongjued, K., **Garcia, K.**, **Scott, D.**, **Gonthier, D.J. and Dupuis, J.R.** (2024) 'DNA metabarcoding diet analysis in a generalist omnivore: feeding trials reveal the efficacy of extraction kits and a multi-locus approach for identifying diverse diets', *Integrative Zoology*, **5**, pp. 790–806.

Tufarelli, V., **Ragni, M. and Laudadio, V.** (2018) 'Feeding forage in poultry: a promising alternative for the future of production systems', *Agriculture*, **8**, p. 81.

Ussery, H. (2011) *The small-scale poultry flock: an all-natural approach to raising chickens and other fowl for home and market growers*, Chelsea Green Publishing.

Valentini, A., **Miquel, C.**, **Nawaz, M.A.**, **Bellemain, E.**, **Coissac, E.**, **Pompanon, F.**, **Gielly, L.**, **Cruaud, C.**, **Nascetti, G. and Wincker, P.** (2009) 'New perspectives in diet analysis based on DNA barcoding and parallel pyrosequencing: the trnL approach', *Molecular Ecology Resources*, **9**, pp. 51–60.

Vucicevic, M., **Stevanov-Pavlovic, M.**, **Stevanovic, J.**, **Bosnjak, J.**, **Gajic, B.**, **Aleksic, N. and Stanimirovic, Z.** (2013) 'Sex determination in 58 bird species and evaluation of CHD gene as a universal molecular marker in bird sexing', *Zoo Biology*, **32**, pp. 269–276.

Wood, G.M. (1956) 'Consumption of forage by chickens', *Poultry Science*, **35**, pp. 1083–1089.

Yu, M. and Robinson, F. (1992) 'The application of short-term feed restriction to broiler chicken production: a review', *Journal of Applied Poultry Research*, **1**, pp. 147–153.