INSTITUTE OF GENETICS
AND ANIMAL BIOTECHNOLOGY
of the Polish Academy of Sciences

Assessment of welfare and behaviour in two chicken genotypes kept in an extensive production system

Dobrostan i behawior kurcząt dwóch grup genetycznych utrzymywanych w ekstensywnym systemie produkcji

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Summary

Poultry meat production is an important branch of Polish agriculture. In 2022, about 2,5 million tons of chicken meat were produced in Poland, which covers about 20% of the European market and ranks the country first in the EU. The vast majority, as much as 95%, of the meat chicken population is reared in Poland in the intensive production systems. Presently however, increasing consumer interest in meat from alternative production systems has been observed. Low popularity of the extensive systems is caused by the lack of clear and standardized production practices, including management methods for flocks kept with access to free range and the lack of availability of genetic lines of chickens adapted to such production conditions.

This dissertation concerned the relationship between the free range frequency of chickens, their welfare and selected aspects of physiology in two chicken genotypes. The research conducted particularly focused on the relationship between the free range frequency and the phenotypic morphological traits of the chickens as well as the bacterial profile and activity of their gut microbiome, as well as the relationships between weather conditions and the free range frequency use by individual birds were also investigated.

Slow-growing Sasso C44 meat chickens and native Polish, two-way performance breed Green-legged Partridge were used in the experiment. All birds had access to grassy outdoor ranges during the duration of the experiment. The frequency of the range use for particular, individually tagged, chickens was determined based on video recordings.

The first publication of this dissertation analyzed the relationships between meteorological indicators (atmospheric pressure, relative humidity, air temperature, wind direction, wind speed) and the free range use frequency by individual chickens. The analysis of the collected data was carried out using univariate and multivariate linear regression models. Significant correlations between meteorological factors and the time birds spent in free range for both genotypes were found. An increase in relative humidity was associated with less frequent use of the free range by Green-legged Partridge, while higher atmospheric pressure and a southerly wind direction were associated with increased frequency of free range use by Sasso chickens. The study also proved that birds within the same genotype respond differently to the same weather factors.

The second publication focused on the analysis of external morphological traits of chickens (length and height of the comb, percentage of feather pigmentation on the neck and percentage of beak pigmentation) in relation to how often the birds used the free range. Analysis was done separately for each of the two genotypes used in the experiment. For this purpose, linear regression models were used. A significant, positive relationship was shown between selected measurements of morphological parameters for Sasso chickens. Birds with higher levels of feather pigmentation on the neck and beak, as well as birds with longer and wider combs were more frequently observed in the free range. No such correlations were found for Green-legged Partridge chickens. The visual assessment of the above-described traits using the scale developed by the authors was additionally done. In conclusion, this publication proved the relationships between the external morphological characteristics of Sasso chickens and free range use frequency.

The third publication examined the relationship between chicken gut microbiota and free range use frequency. Bacteria species composition was investigated, the enzymatic activity (α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, acetic acid, propionic acid, isobutyric acid, butyric acid and isovaleric acid concentrations) of the chickens' intestinal microflora. Within the genotype, the birds were divided into three groups: birds with low, moderate and high frequency of free range use. Then, separately for each genotype, data on the characteristics of the intestinal microflora and their free range frequency were compiled. Statistical analysis of the data was performed using generalized mixed linear models (GLIMMIX procedure). A higher relative amount of *E. Coli* was found for chickens that spent more time in the free range. Such a relationship was noted in both genotypes. In summary, the results confirmed the relationship between genotype, frequency of free range use, and the properties of the gut microflora of the studied chickens.

In conclusion, in this dissertation, for both genotypes of birds, significant relationships between meteorological indicators and the free-ranging frequency were demonstrated, and the meteorological factors that most influenced the use of free range by the examined birds were identified. The relationship between the external morphological traits of Sasso chickens has been proven - individuals with a higher level of pigmentation of feathers on the neck and beak, as well as birds with a longer and wider comb, used outdoor runs more often. The study showed complex relationships between: genotype, free range frequency, and the properties of the gut microflora of chickens of both genotypes.

Streszczenie

Produkcja mięsa drobiowego jest ważną częścią sektora rolno-spożywczego, a Polska jest jej liderem w Europie. W 2022 roku wg. danych Głównego Urzędu Statystycznego w Polsce wyprodukowano ok. 2,5 miliona ton mięsa z kurcząt, co stanowi ok. 20% rynku Unii Europejskiej. Należy podkreślić, że aż 95% populacji kurcząt mięsnych jest utrzymywana w Polsce w systemie chowu intensywnego. Jednocześnie w ostatnich latach obserwuje się na rynku coraz większe zainteresowanie mięsem pochodzącym z alternatywnych systemów chowu, między innymi ptakami z chowu z dostępem do wolnego wybiegu. Jednakże brak jest ustandaryzowanych praktyk produkcyjnych, w tym metod zarządzania tak utrzymywanymi stadami. Ponadto ograniczona jest także w Polsce dostępność linii genetycznych kurcząt przystosowanych do ekstensywnych warunków chowu.

W niniejszej pracy doktorskiej skoncentrowano się na badaniu behawioru oraz dobrostanu dwóch genotypów kurcząt (Sasso C44, Zielononóżka kuropatwiana). W szczególności badano zależność pomiędzy częstością przebywania kurcząt na wolnym wybiegu, a ich fenotypowymi cechami morfologicznymi oraz profilem bakteryjnym i aktywnością mikrobiomu jelitowego. Analizowano także zależności pomiędzy warunkami pogodowymi, a częstością korzystania z wybiegów przez ptaki.

Badaniami objęto wolno rosnące kurczęta mięsne Sasso C44 oraz kurczęta polskiej rasy zachowawczej Zielononóżka kuropatwiana o dwukierunkowym typie użytkowym. Wszystkie ptaki podczas doświadczenia miały pełny dostęp do wybiegów zielonych. Częstość przebywania na wybiegach przez indywidualnie oznakowane kurczęta ustalono na podstawie nagrań monitoringu z kamer video umieszczonych na wybiegach.

W pierwszej publikacji niniejszej pracy doktorskiej poddano analizie zależność pomiędzy czynnikami meteorologicznymi (ciśnienie atmosferyczne, względna wilgotność powietrza, temperatura powietrza, kierunek wiatru, prędkość wiatru), a częstością przebywania na wybiegach przez poszczególne, indywidualnie zidentyfikowane, kurczęta. Analizę zebranych danych przeprowadzono przy użyciu modeli regresji liniowej jedno i wieloczynnikowej. W przypadku obu genotypów ptaków wykazano istotne zależności pomiędzy czynnikami meteorologicznymi,

a czasem przebywania na wolnym wybiegu przez badane ptaki. Ponadto wykazano różnice w zależnościach pomiędzy poszczególnymi czynnikami pogodowymi, a częstością przebywania na wybiegu pomiędzy kurczętami Sasso i Zielononóżki kuropatwianej. Wzrost względnej wilgotności powietrza był związany z rzadszym korzystaniem z wybiegów przez Zielononóżkę kuropatwianą, z kolei wyższe ciśnienie atmosferyczne i południowy kierunek wiatru miały związek ze zwiększoną częstością przebywania na wybiegach przez kurczęta Sasso. Tym samym, w pracy dowiedziono, że ptaki w obrębie tego samego genotypu inaczej reagują na te same czynniki pogodowe.

W drugiej publikacji skupiono się na analizie zewnętrznych cech morfologicznych kurcząt (długość oraz wysokość grzebienia, procent pigmentacji piór na szyi oraz procent pigmentacji dzioba) w odniesieniu do częstości przebywania przez ptaki na wybiegach zewnętrznych, którą przeprowadzono osobno dla każdego z dwóch genotypów objętych badaniami. W tym celu zastosowano liniowe modele regresji. Wykazano istotną, pozytywną zależność pomiędzy wybranymi pomiarami analizowanych parametrów morfologicznych, a częstością korzystania z wybiegów w przypadku kurcząt Sasso. Osobniki o wyższym poziomie pigmentacji piór na szyi oraz dziobie, a także ptaki z dłuższym i szerszym grzebieniem, częściej korzystały z wybiegów zewnętrznych. W przypadku kurcząt Zielononóżki kuropatwianej nie stwierdzono takich zależności. W publikacji tej wykonano również wizualną ocenę wyżej opisanych cech przy użyciu nowo opracowanej skali ich oceny. Podsumowując, w tej publikacji wykazano związek pomiędzy wybranymi cechami morfologicznymi kurcząt Sasso, a częstością przebywania na wybiegach zewnętrznych przez te ptaki.

W trzeciej publikacji zbadano związek pomiędzy składem, aktywnością enzymatyczną mikroflory jelitowej kurcząt (aktywność α-glukozydazy, β-glukozydazy, α-galaktozydazy, β-galaktozydazy, β-glukuronidazy, stężenie kwasu octowego, propionowego, izomasłowego, masłowego oraz izowalerianowego), a częstością korzystania przez nie z wybiegów zewnętrznych. W obrębie genotypu, ptaki zostały podzielone na trzy grupy: ptaki z niską, umiarkowaną oraz wysoką częstością korzystania z wolnego wybiegu. Następnie, osobno dla każdego genotypu zestawiono dane dotyczące właściwości mikroflory jelitowej oraz częstości przebywania ptaków na wybiegu. Analizę statystyczną danych wykonano przy użyciu uogólnionych mieszanych modeli liniowych (procedura GLIMMIX). U kurcząt, które częściej przebywały na wolnym wybiegu stwierdzono większą względną liczbę *E. Coli*. Taką zależność odnotowano dla obu badanych genotypów - Sasso i Zielononóżki kuropatwianej. Uzyskane wyniki potwierdziły zależność pomiędzy genotypem, częstością przebywania na wolnym wybiegu, a właściwościami mikroflory jelitowej badanych kurcząt.

Podsumowując, w niniejszej dysertacji dla obu genotypów ptaków wykazano istotne zależności pomiędzy czynnikami meteorologicznymi, a częstotliwością korzystania z wolnego wybiegu oraz zidentyfikowano czynniki meteorologiczne, które w największym stopniu wpływały na korzystanie z wolnego wybiegu przez badane ptaki. Udowodniono zależność pomiędzy zewnętrznymi cechami morfologicznymi kurcząt Sasso - osobniki o wyższym poziomie pigmentacji piór na szyi i dziobie, a także ptaki o dłuższym i szerszym grzebieniu, częściej korzystały z wybiegów zewnętrznych. Badanie wykazało złożone zależności pomiędzy: genotypem, częstotliwością przebywania na wolnym wybiegu, a właściwościami mikroflory jelitowej kurcząt obu genotypów.

1. Introduction

Increased public concern for animal welfare and the sustainability of production systems resulted in the increased demand for products from the poultry reared in low-input systems (Erian and Phillips, 2017). The recent studies showed that alternative poultry meat tends to have a growing market share in the EU. Poland remains the largest EU chicken producer, covering 20% of EU chicken production. However, alternative poultry production in Poland is marginal with less than 1% of the country's total broiler production. In comparison, for example in Germany it is 10% and in France it is 12%. Poland covers only 0.3% of the EU alternative chicken meat market value (EU Commission DG AGRI, 2022). One of the important reasons for the low popularity of extensive systems in Poland, except for small market for such products, is the lack of best practices on how to achieve high meat yield, good health and welfare status of alternative to conventional meat chicken genotypes. Proper understanding of management factors, like bird genotype choice or associations between weather conditions, external chicken features, host – microbiome interaction may result in optimizing the use of the outdoor ranges which has many beneficial effects on birds' health and welfare (Marchewka et al., 2020).

It is well known that an extensive chicken rearing system aims for optimizing health and welfare of chickens for example, by setting limits on flock size, stocking densities, environment enrichments or access to free range area (Castellini et al., 2006). It provides space for exercise which enhances chickens to express natural behaviour. The access to pasture and soil on the free range area can influence meat quality traits and modify gut microbiota properties (Binek et al., 2017; Sztandarski et al., 2022). Dal Bosco et al., (2016) proved that bioactive compounds like: minerals, vitamins or polyunsaturated fatty acids (PUFAs) are transferred from plants to the meat of chickens and affect the oxidative processes of the meat. Slow-growing chickens reared outdoors show a better antioxidant status and less thiobarbituric acid reactive substances (TBARs) in blood plasma. The meat of chickens reared with access to ranges also has higher antioxidants and n23 series PUFAs (Dal Bosco et al., 2016).

Gut microbiota in chickens is influenced by their access to pasture and consequently so are the immunology response, energy yield, nutrients metabolism, digestion and behaviour. Studies show that the gut microbiota of chickens which spend time at free range areas is more varied than in chicken reared inside buildings (Bjerrum et al., 2006; Kogut, 2019). Torok et al conducted an experiment where they compared chicken performance with gut microbiota composition in three feeding trials. They proved correlation between cecal microbiota composition and better chicken growth rate. For example, the presence of *Lactobacillus spp*, or *Ruminococcaceae*, improved energy absorption from the diet, better feed conversion ratio (FCR) and consequently chicken growth (Torok et al., 2011). Despite the microbiome's meaningful impact on chicken physiology the exact mechanism remains unclear.

Genetic background can strongly impede the extent of beneficial effects of the free range. A positive result depends largely on the choice of genotypes of chicken that are able to bear outside conditions (Sossidou et al., 2011; Dal Bosco et al., 2016). Farmers should preferably choose chicken genotypes selected for their abilities to cope with the natural environment problems, strong immune system, minimal occurrence of injurious behaviours, good conformation and skeletal development, and a proper growth rate. The genetic selection of birds for better growth led to the development of fast-growing chicken lines which are used in intensive poultry farms. These birds reach the slaughter weight within 6-7 weeks and are adapted for living in extremely regulated inside conditions (farm environment, veterinary protection). They have high feed and nutritional demands. The genetic selection of birds for better growth rate has modified their behaviour, appearance and physical futures (Schütz et al., 2001) reducing kinetic activity, increasing breast muscle size and increasing metabolism rate. Fast growing chickens tend to stay indoors rather than use free range areas. They are more susceptible to infections, weather and environment conditions (Bokkers and Koene, 2003; Dal Bosco et al., 2010; Sossidou et al., 2011).

Extensive poultry systems require chickens that are more resistant to fluctuations of environmental conditions due to a better immune resistance and adaptation to poorer diet. Slow growing chickens are more adapted to these systems. They reach the slaughter weight within 9-12 weeks. In comparison with fast-growing chicken, they show more active behaviour, less heart and muscle abnormalities, fewer tendon degeneration lower mortality and ascites (Bokkers and Koene, 2003). Slow-growing chickens spend more time outdoors than indoors, whereas fast-growing chickens present higher proportion of static behaviours, strongly associated with higher energy balance, growth, and muscle accretion. There are two categories of slow-growing chickens: autochthonous breeds and commercial strains. Most of the autochthonous breeds have higher biodiversity and adaptability to extensive conditions, but may have lower productive performance. Due to these limitations, they are not often used by farmers, but they are a reservoir of precious genes. They might be useful in breeding programs to create new lines of appropriate genetic (crossbred) chicken with better adaptation to extensive systems.

With all its benefits a free range system requires a consideration of influencing factors and related hazards for example: predation, parasite, infections etc.

However, the use of free range areas by chickens is predominantly correlated with weather conditions, which is a major variable for outdoor activity (Sztandarski et al., 2021). Unfortunately, the vast majority of free run areas in commercial production do not satisfactorily protect chickens from adverse weather conditions. Outdoor runs are usually large and open spaces without shelters. To help improve the free range areas design it is important to know the impact of weather conditions on chicken behaviour and motivation to forage.

Based on previous studies in laying hens (Rodriguez-Aurrekoetxea and Estevez, 2016), domestic poultry is likely to differ in its individual levels of free range use. Moreover, not all broiler chickens access the outdoor range when the opportunity is provided, indicating potential individual variation within flocks (Durali et al., 2012; Taylor et al., 2017). Campbell et al. (2016) profiled individual laying hens, differing in their ranging profiles, as outdoor-preferring, moderate-outdoor, and indoor-preferring. This profiling attempt has been performed first time in meat-purpose chickens in our experiment, and it has not been determined, neither for layers nor for broilers previously, whether the ranging profiles are associated with the individual birds' welfare.

The management of free range chicken is very complex due to many factors influencing their behaviour, welfare and performance. An optimal combination of good free range area design, health and genetic background can ensure the best results in terms of environmental sustainability without compromising animal welfare and costs.

2. Hypotheses

Publication 1

- Higher relative humidity or wind speed may limit ranging frequency of individual Green-legged Partridge and Sasso chicken. On the other hand, the temperature within the birds' thermal comfort may promote free range use.
- The use of free range depends also on the individual response of particular chickens on the weather conditions.

Publication 2

- In Green-legged Partridge and Sasso hens comb size, proportion of dark feathers on the neck and beak darkness is positively associated with their ranging frequency.
- There is a correlation between measurements of the comb size, proportion of the dark feathers on the neck, beak darkness and ranging frequency.
- Chickens which are scored higher with regard to the abovementioned external features use free range area more frequently.

Publication 3

- Chickens which are identified as homogenous in terms of ranging profile show similar quantitative microbial composition of the same genus and similar gut microbiota activity, regardless of their genotype.

3. Objectives

Publication 1

- Investigate possible associations between weather parameters and the ranging frequency by individual Green-legged Partridge and Sasso chicken
- Elucidate the effects of weather conditions on chicken free range areas use

Publication 2

- Investigate possible associations between morphological traits: neck plumage, beak darkness, comb size and the ranging frequency of the Green-legged Partridge and Sasso hens
- Confirm potential associations of ranging frequency of Sasso and Green-legged Partridge hens with the above listed external features evaluated by practical scoring based on visual assessment and determination of eye color
- Identify correlations between measurements of the hens comb size, proportion of the dark feathers on the neck, and beak darkness.

Publication 3

- Relate the gut microbiota composition, activity and metabolic products in Green-legged Partridge and Sasso chicken to the three ranging frequency profiles: outdoor-preferring, moderate-preferring and indoor-preferring

4. Material and methods

4.1 Animals and housing

The experiment was conducted in the Mazovian region in Poland in August to September of 2018, in the facilities of the experimental farm of the Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences. Until wk 5 of age, 200 birds were reared only indoors in the experimental facility in one common littered pen (5 m × 10 m) with 17 cm/bird perching space provided, automatic feeders and drinkers, providing feed and water *ad libitum*, and natural light. The climate conditions were controlled automatically and infrared heating lamps were used. At the age of 5 wk, 60 individuals with similar body weight within each breed (on average 2030.6 ± 68.9 g for Sasso and 705.9 ± 8.5 g for Green-legged Partridge), were selected and relocated from their rearing facilities to the experimental house, both at the same farm location. Eight female and 2 male chickens were assigned to each single breed group housed in 12 pens (6/breed) until 10 wk of age. Sixty non-beak-trimmed, mixed-sex Green-legged partridge chicken (n=60) and Sasso chicken (n=60) were used in the experiment. Size of the indoor pens was 2.5 m × 3.5 m, resulting in a stocking density at slaughter age of 1.4 kg/m2 for Green-legged Partridge and 2.7 kg/m2 for Sasso. Sawdust litter as bedding material was used, while in each pen, next to the wall there was a 0.5 m stripe covered with sand. Pens were cleaned according to the need. In each pen, there were two 80-cm long wooden perches at 2 perching levels, one at the height of 15 cm and the second at 40 cm. The perching poles were 50 × 50 mm thick and had rounded edges. Each pen had direct access through the pophole (45 cm high × 50 cm wide) to an individual outdoor range area (3.5 m × 30 m) providing 10.5 m2/chicken. Chickens were provided only natural light through uncovered windows there were not artificial lights. Light hours during experimental period ranged from 12.7 h to 15.7 h/day. All the outdoor ranges had the same vegetation plant coverage, no trees or shelters were present. The grass was mowed 1 week before the onset of the experiment. Each free range area was provided with a semiautomatic bell drinker and a wooden box (1 m × 1 m) filled with sand. Outline of the experimental facilities is presented in Figure 1. The feed was composed of wheat, sunflowers maize, soybean expeller legumes mix, pea, gruel corn, monocalcium phosphate, soybean oil, and calcium carbonate with supplements. Table with feed content is presented in publication (Marchewka et al., 2020). Feed and water were provided *ad libitum.* The feed composition was intended to meet the birds' nutritional requirements (Classen, 2017). No coccidiostats or other medication was used. Chickens were adapted for 48 h to the new experimental situation, before pop holes were opened daily from 7.00 until 19.00 h to allow for individual chickens' recognition; all chickens were fitted with a small, laminated paper mark attached to the chickens' back by fitting two elastic bands around the wings. Ten different colors of the marks were randomly assigned in each pen to the individual.

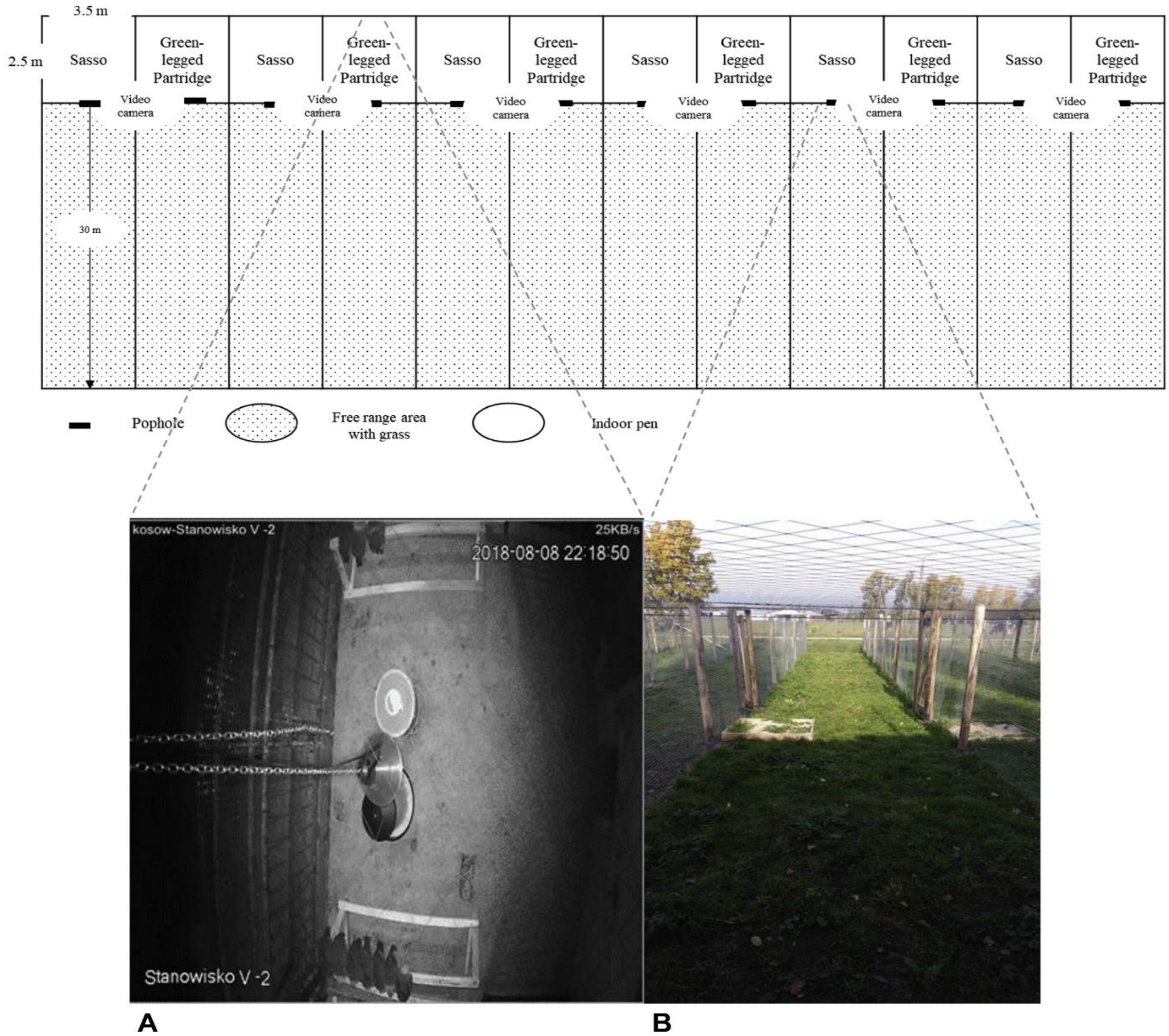

Figure 1. Experimental broiler chicken shed, pens, and range area dimensions with popholes and video cameras location. (A) night-time image of the Green-legged Partridge pen; (B) image of one of the free-ranging areas covered with vegetation and with sand box in the left side of the ranging area.

4.2. Observations of ranging behaviour

For behavioural observations which results are used in publication 1, 2 and 3 six cameras were used (DMIP2401IR-M-IV IP 4 Mpix, BCS company, Warszawa, Poland). The 12 ranges were video recorded simultaneously and continuously using, each completely covering two range areas. The video recordings were automatically saved on the network recorder (BCS-NVR0401-IP 4 channel BC, BCS company, Warszawa, Poland), and from these the birds' behaviours were analyzed by the same trained and experienced person, using the Chickitizer program (Sanchez and Estevez, 1998). The program is specially developed to record data about the location of animals, predefined areas, as it enables graphic mapping of the experimental layout (distribution of compartments). From the recorded videos, three days were chosen per week of experiment (five weeks). On each of those days, at three times of the day (morning—starting at 8:00, noon—starting at 13:00, and evening—starting at 18:00), a three-minute-period with 10 s sampling intervals was set and repeated after 10 min. The observer recorded each of the experimental chickens' absence as "0" or presence as "1" in the range. The possible frequency of outdoor use in the current study was between 0 and 1620. This results from the observation protocol where there were six samplings (one sampling/10 s, making up 1 min) ∗ 3 min ∗ two bouts ∗ three times of day ∗ three days each week ∗ five weeks = max. 1620.

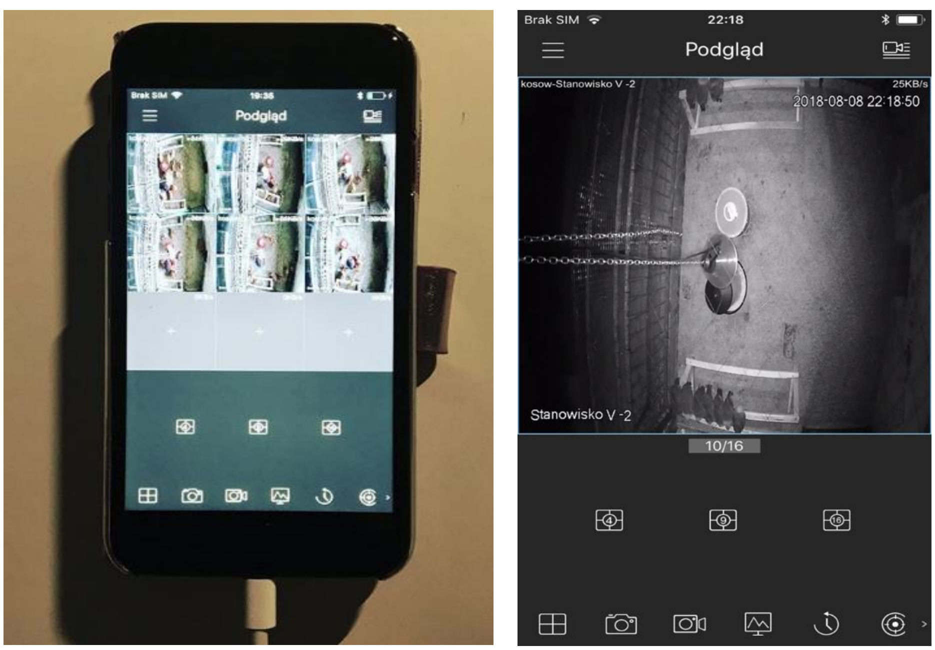

Figure 2. Preview of the application used to video record

4.3 Data weather collection

Weather data were collected once per minute throughout the whole experiment duration. An automatic weather station was used (Davis Instruments Vantage Pro 2 DAV-6152EU, CA). Device was installed at the end of the central ranging area, height of 1 m from the ground. The following parameters were collected: air temperature (°C) and relative humidity (%), wind direction (cardinal directions) and wind speed (m/s), atmospheric pressure (hPa) and the sum of daily precipitation (mm). Data were automatically saved in a Microsoft Excel spreadsheet (2016). For the purposes of statistical analysis, the cardinal directions of the wind were converted to degrees, where degree “0” indicated north wind (N), while interpretation of the increase in the degrees followed the standard compass rose.

Figure 3. Automatic weather station (Davis Instruments Vantage Pro 2 DAV-6152EU, CA) used during experiment

4.4 Measurements (quantitative assessment)

The direct measurements of the external features (Publication 2, Table 1) of each female individual chicken were taken the day before the end of the experiment. There were 3 persons involved in the measurements, each assigned with a different task: 1) identifying (indicated by the color tag) and catching the birds, 2) measuring the comb size using the method described below, and 3) noting the collected information in a spreadsheet and taking a digital picture of the whole body of each bird from the left side. Comb size was measured, using a digital ruler LCD (Kraft&Dele, Koteze, Poland), in the highest (from where the comb met the head to the top of the highest spike) and longest place (from end to end) for each individual bird. From the photos taken, the beak coloring was calculated using ImageJ software (Schneider et al., 2012). Each image of an individual bird was imported to ImageJ software, where the area of the beak was contoured and cropped from the whole image. The cropped-out area was binarized, collapsing the 256 color levels to 2 color levels, while adjusting the grayscale using the automatic thresholding method "AutoLocalThreshold", as a plugin to ImageJ software. This plugin binaries 8-bit image using thresholding method that can deal with unevenly illuminated images. The threshold was computed for each pixel according to the image characterizing within a window of radius r (in pixel units) around it. The segmented phase was always shown as white (255, as the maximum gray level). After thresholding, the dark area was calculated and deduced from the total area of interest providing white area size. The proportion of black to white area measurements ratio was calculated and expressed as a percentage. The same method using ImageJ software was applied to the second identical copy of the individual chicken photo to calculate neck plumage coloring, that is the percentage of dark plumage on the neck, which was defined as the area between the head and the trunk of the bird (Publication 2 Table 1).

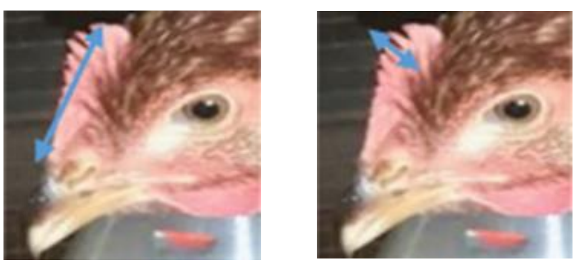

Figure 4. Direct measurement of comb size

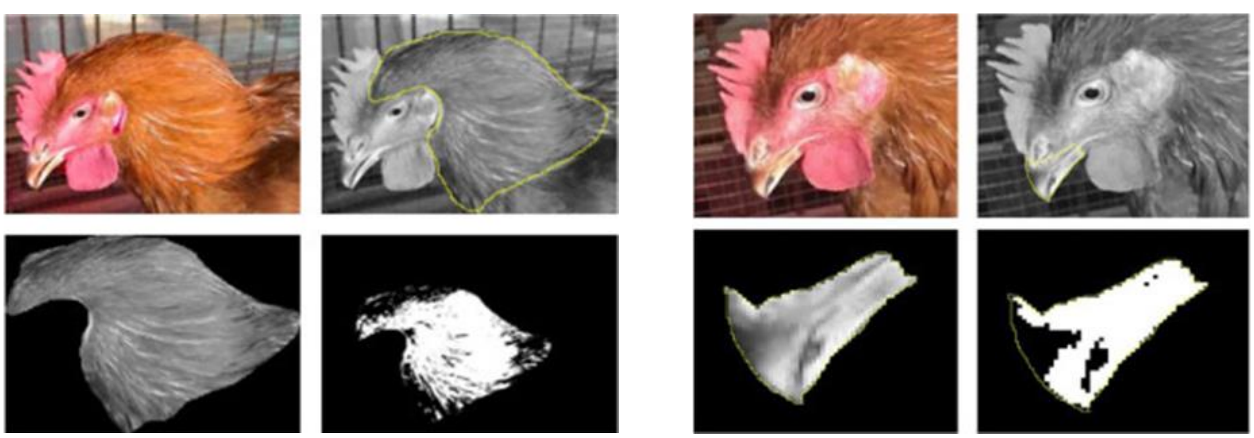

Figure 5. Neck and beak darkness % by ImageJ software

4.5 Scores (qualitative assessment)

After taking the comb measurements and a bird photograph, each female bird was handed into a 2-person team, where one person held the bird and the other, based on visual assessment, scored the bird for 3 external features: comb size, neck plumage darkness, and beak darkness, all on a 3-point scale (1-3) within breed. Definitions and examples for each score of each feature in either of the breeds are presented in (publication 2 in Table 2).

4.6 Sample collection for bacterial composition and activity determination

At 72 day of life, birds from each group (n = 6) were sacrificed by cervical dislocation. Thereafter, the cavity was opened and both ceca were removed. The digesta from both ceca were collected and pooled in one test tube for each bird individually and was then divided into 3 portions to be used for different analysis. The collected digesta was immediately frozen in −80°C.

Figure 6. Digesta sample preparation

4.7 Determination of bacteria relative abundance

The relative abundance of selected bacterial groups in the caeca including *Lactobacillus spp.*, *E. coli*, *Bifidobacterium spp.*, and *Clostridium spp.* was performed using the PCR method. We modified Zhu et al. procedure to isolate bacterial genomic DNA from the cecal digesta (Zhu et al., 2002). Bacterial genomic DNA was extracted from digesta using the QIA amp. Fast DNA Stool Mini Kit (Qiagen, Stockach, Germany) according to the manufacturer's protocol. Then, the yield and purity of the isolated DNA were estimated spectrophotometrically (NanoDrop Technologies, Wilmington, DE).

4.8 Polymerase chain reaction amplification of bacterial 16S rRNA gene

The primers and polymerase chain reaction (PCR) conditions used to amplify the bacterial 16S rRNA gene are presented in publication 3 in Table 1. The universal primer set was used to determine the total bacteria population. The detailed PCR conditions were set-up as previously reported for each respective bacteria group (Michalczuk et al., 2021). The obtained PCR-products were separated by electrophoresis on a 2% agarose gel. PCR products were quantified using ImageJ 1.47v software for densitometry measurements (National Institute of Mental Health, Bethesda, MD), with a density of bands for each bacteria group expressed in relation to the density of the total bacteria primers product. The density of the bands for each of bacteria group was expressed in relation to the density of the total bacteria primer product. Each sample was analyzed in duplicate.

4.9 Bacterial enzyme activity

The activity of the gut microbiota was performed based on the glycolytic activities of 5 bacterial enzymes in the cecal digesta including, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, and β-glucuronidase. Before the analysis, the digesta was thawed at 4°C for 3 h. The activity of the enzymes was determined spectrophotometrically according to Konieczka and Smulikowska, modified from Jurgoński et al. (Jurgoński et al., 2013; Konieczka and Smulikowska, 2018). To determine each specific enzyme: p-nitrophenyl-α-D-glucopyranoside for α-glucosidase, p-nitrophenyl-β-D-glucopyranoside for β-glucosidase, p-nitrophenyl-α-D-galactopyranoside for α-galactosidase, p-nitrophenyl-β-D-galactopyranoside for β-galactosidase, and p-nitrophenyl-β-D-glucuronide for β-glucuronidase were used (Sigma Chemical Co., St. Louis, MO).

4.10 SCFA concentration

The SCFA determination in the cecum digesta was performed according to the procedure described by (Konieczka et al., 2018), using an HP 5890 Series II gas chromatograph (Hewlett

Packard, Waldbronn, Germany) with a flame-ionization detector (FID) and a Supelco Nukol fused silica capillary column (30 m × 0.25 mm internal diameter, film 0.25 mm). Helium was used as the carrier gas. The concentrations of individual SCFAs were estimated with an internal standard (isocaproic acid) using a mixture of standard solutions.

5. Statistical analysis

All statistical analyses described in this thesis were performed in SAS v9.4 (SAS Inst. Inc., Cary, NC).

5.1 Publication 1

Simple and multiple regression models were used. The variable describing either the individual Green-legged Partridge or Sasso chicken range use (sum of the individual bird presences in the outdoor free range during observation periods) was considered as the dependent outcome variable, while weather parameters at the time of the range use observations were considered as the independent variables: air temperature (°C) and relative humidity (%), wind direction (cardinal directions) and wind speed (m/s), atmospheric pressure (hPa) and the sum of daily precipitation (mm). The outcome variable was analyzed for associations with any of the independent variables. The outcome variable was normally distributed across the sample population, thus linear univariate regression was used. Residuals were predicted and checked for normality. Associations with P-value <0.2 were further analyzed in a multivariate linear regression analysis. Models were backward exclusion until all associations reached P-value <0.05. Interactions between independent variables were tested in the final models and were not detected. Residuals were predicted and plotted in normal quantile plots and coefficients of determination (R2) were calculated and used to choose the model that explains the variability of the response data. The likelihood ratio test was used to observe the improvement of the multiple regression models by inclusion and exclusion of independent variables. Akaike's information criterion and Schwarz's Bayesian information criterion were used to compare maximum likelihood of reduced and full models. The selection of the final models was based on the smaller values of the information criterion.

5.2 Publication 2

In each of the simple regression models, the variable describing either the individual Green-legged Partridge or Sasso chicken range use (summed over all observations frequencies of the presences in the outdoor area) was considered as the dependent outcome variable, while each chicken external feature measurement was considered as the independent variable. The outcome variable was analyzed for its association with each independent variable. The outcome variable was normally distributed across the sample population, thus linear univariate regression was used. Furthermore, the residuals were predicted and checked for normality. Residuals were predicted and plotted in normal quantile plots and coefficients of determination (R2) were calculated.

Independent one-way ANOVAs were performed, separately for Sasso and Green-legged Partridges, using the PROC GLIMMIX procedure. Each model included different chicken external features scored as “1”, “2”, or “3” as a fixed factor. However, an independent two-way ANOVA was conducted in the same software package for the model including eye color, as both eye color and breed were added as fixed factors as well as their interaction. Pen was included in the model as the random factor. Least Square Means (LSM) differences were adjusted for multiple comparisons using the posthoc Tukey test.

Spearman correlations were calculated using the PROC CORR script for each breed separately to test the relationships between measured external features.

5.3 Publication 3

The GLIMMIX procedure was used to perform generalized linear mixed models for the microbiome composition, activity and metabolic products using either normal or gamma distribution where appropriate, applying the ranging profile group, breed and their interaction as fixed effects in the model. The pen was included in the model as a random effect. The assumptions of homogeneity of variance and normally distributed residuals were examined visually using the conditional Studentized residuals plots. The results are shown as means with standard errors, and P-values below 0.05 were considered significant, while between 0.05 and 0.06

were considered a significant trend. Tukey's post hoc test was performed to investigate significant differences between test groups.

Birds of both breeds were divided into 3 ranging profiles using rank-frequency distribution (a discrete form of a quantile function in reverse order, giving the size of the element at a given rank) of their range use frequency summed over all the observation periods—that is, between 0 and 1,620 times. All the birds within a breed were assigned a rank based on their individual frequency of outdoor use. Segmentation rank distribution of the birds into 3 ranges was performed: outdoor-preferring ranging profile, with the mean value of 506.1 ± 47.9 total outdoor uses per experiment per bird for Sasso and 502.6 ± 22.5 total outdoor uses per experiment per bird for Green-legged Partridge; moderate-outdoor ranging profile, with the mean value of 219.6 ± 18.8 total outdoor uses per experiment per bird for Sasso and 332.4 ± 13total outdoor uses per experiment per bird for Green-legged Partridge; and indoor-preferring ranging profile, with the mean value of 89.8 ± 11.7 total outdoor uses per experiment per bird for Sasso and 223.9 ± 12.1 total outdoor uses per experiment per bird for Green-legged Partridge. The rank intervals were equal (modified from Campbell et al., 2016).

6. Results

6.1 Results publication 1

The temperature recorded in the building during the experiment ranged from 19°C to 26°C, while relative humidity ranged from 47 to71%. During the day, outside temperature ranged from 12°C to 28°C, outside relative humidity from 46 to 99%, wind speed from 0 to 24 m/s and atmospheric pressure from 1,004 hPa to 1,027 hPa (Figure 1A–1D, publication **1**). The dominating wind direction was western and south - western (Figure 2, publication 1).

Associations Between Weather Parameters and Range Use by Individual Green-Legged Partridge Chickens

The results of the simple and multiple regression models showing associations between range use by individual Green-legged Partridge chickens and weather parameters are presented in (Table 1, publication1**)** together with the mean frequency and standard deviation of the range usage by the birds. Significant associations between the range uses with one weather parameter were identified for 20 birds, while with 2 weather parameters for one bird. For the remaining 39 birds, no significant associations were identified between individual range use and weather parameters. Increased range use of 8 birds was significantly and positively associated with relative humidity, where the proportion of explained variance of the response variable ranged from 10 to 17%. Range use of 3 birds was positively associated with temperature and also for three birds with wind direction expressed in degrees. The proportion of variance of range use explained by the temperature ranged from 12 to 20%, while for wind direction from 9 to 16%. Atmospheric pressure was positively associated with the range use of 3 birds, while one bird used the ranges less often when the atmospheric pressure increased (negative association). In case of 2 birds, an association between higher wind speed and reduced range use was identified. Moreover, the range use of one bird was associated with 2 weather parameters: negatively with relative humidity and positively with the wind direction, where the proportion of explained variance of the response variable by those weather parameters reached 33%.

Associations Between Weather Parameters and Range Use by Individual Sasso Chickens

The results of the simple and multiple regression models showing associations between range use by individual Sasso chicken and weather parameters are presented in (Table 2, publication 1) together with the mean frequency and standard deviation of the range usage by the birds. The significant associations of the range use with one basic weather parameter were identified for 19 birds, with 2 and 3 basic weather parameters each for 2 Sasso birds. No significant associations were identified between individual range use and weather parameters for the remaining 39 birds. Both atmospheric pressure and wind direction were associated with range use of 7 birds. Atmospheric pressure was positively associated with range use (between 9 and 17% of variance explained), while range use was either negatively or positively associated with the wind direction (between 11 and 21% of response variable variance explained). In the case of three Sasso birds, wind speed was negatively associated with the range use frequency. Inconsistent associations between range use and relative humidity were found, as it was negative for one bird and positive for another bird. Moreover, the range use of one bird was associated with two weather parameters: negatively with wind speed and positively with the atmospheric pressure, where the proportion of explained variance reached 23%. In the case of one bird, association with three weather parameters was identified (relative humidity, wind speed and wind direction), which explained 35% of the range use variance.

6.2 Results publication 2

Measurements

The results of the simple regression models showing associations between range use by either Sasso or Green-legged Partridge hens and their external features are presented in (Table 3, publication 2). In Sasso hens, significant and positive associations between the range use frequency and comb length and height as well as neck plumage darkness and beak darkness were identified. The proportion of explained variance of the response variable ranged from 18% for beak darkness up to 33% in case of neck plumage darkness. No significant associations between the range use frequency and external features were identified for Green-legged Partridge hens.

Visual Assessment

Significant effects of external features as assessed by scoring were identified in Sasso hens for neck plumage darkness (P = 0.03) and comb size (P = 0.04), as presented in (Table 4, publication 2). For both features, birds scored the highest used the ranging areas more frequently as compared to birds presenting the lowest score. Moreover, a trend (P = 0.06) for an effect of beak darkness on the range areas use frequency was identified. No significant effect of any of the external features on the range use was identified for Green-legged Partridges.

Eye Color

There was a significant breed by eye color interaction effect on the range use of the hens (F = 4.40; P = 0.04) in the current study (Figure 1**,** publication 2). Sasso hens with gray eye color used the ranges significantly less frequently, as compared to Green-legged Partridges with either brown or gray eyes.

Correlations

Correlations between external features were identified within each breed (Table 5, publication 2). In Sasso hens, all external features were significantly and positively correlated between each other, with the exception that no significant correlation was identified between beak darkness and comb length. The strongest positive correlation (r = 0.85) was identified between comb length and comb height.

Among Green-legged Partridges, fewer and weaker correlations were identified as compared to Sasso hens (Table 5, publication 2). Similarly, to Sasso hens, the strongest positive correlation was identified between comb length and comb height (r = 0.55). Moreover, comb height was significantly and positively correlated with neck plumage darkness and beak darkness (r = 0.39 and 0.33, respectively).

6.3 Results publication 3

Bacteria Composition

Effects of breed, ranging profile and their interaction on the relative abundance of selected bacteria in the ceca are presented in (Table 2, publication 3). An effect of the interaction between breed and ranging profile was identified for the relative abundance of *E. coli* (P = 0.0087) and *Bifidobacterium spp.* (P = 0.0002). The lowest relative abundance of *E. coli* was identified for outdoor-preferring Sasso and Green-legged Partridges and indoor-preferring Sasso birds. The lowest relative abundance of *Bifidobacterium spp*. was found in the intestinal content of indoor-preferring Sasso birds as compared to all other birds in the experiment. The effect of breed was observed in the *Clostridium spp*. relative abundance (P = 0.0493): it was higher in Sasso chickens, as compared to Green-legged Partridges.

No significant differences were identified between ranging profiles of either Sasso or Green-legged Partridges regarding bacterial relative abundance.

Microbial Enzymes Activity

Effects of breed, ranging profile, and their interaction on the microbial enzymes activity are presented in (Table 3, publication 3). No effect of the interaction between breed and ranging profile was observed for any of the investigated enzymes activities. However, there was an effect of the breed on 3 of the enzymes that is, α-glucosidase ($P = 0.013$), β-glucuronidase ($P = 0.008$), and β-galactosidase ($P = 0.04$), where higher activity was observed in Green-legged Partridges, as compared to Sasso chickens.

No significant differences were identified between ranging profiles of either Sasso or Green-legged Partridges regarding microbial enzymes activity.

SCFA

Effects of breed, ranging profile and their interaction on the SCFA concentration are presented in (Table 4, publication 3)

An effect of the interaction between breed and ranging profile was identified only for valerian SCFA ($P = 0.016$). The observed concentration of valerian SCFA was higher for moderate-outdoor Green-legged Partridges, as compared to moderate-outdoor Sasso chickens. An effect of breed on the isovalerian concentration was observed ($P = 0.03$), being higher in Sasso as compared to Green-legged Partridge chickens.

No significant differences were identified between ranging profiles regarding SCFA concentrations.

7. General discussion

This doctoral dissertation was developed to investigate associations between free range use and weather conditions, external morphometric chicken features and gut microbiome properties. The current dissertation sums up three publications and is an outcome of research work in the EU funded project: Optimizing the use of the free range as a key to improve organic chicken production - “FreeBirds” (CORE Organic Cofund, European Union's Horizon 2020).

Extensive systems for animal production are considered as better for animal welfare, health and meat quality, as compared to conventional ones. However, to obtain the satisfying production results various requirements, such as appropriate birds’ genotype, housing, feeding and management need to be met.

The weather conditions have been recognized as a major factor that might influence use of free ranges by birds. Publication 1 investigated the potential weather impact on individual free range use. Better understanding of chicken ranging behaviour could help to improve the management and range facilities design, to ensure optimal ranging opportunities but also optimal productivity and welfare of the birds (Taylor et al., 2017). Many studies showed that ranging behaviour is altered by the time of the day, weather conditions (rainfall, sunlight, temperature, wind speed and direction) and surface of the range (e.g., shrubs, trees, straw huts) (Nielsen et al., 2003; Stamp Dawkins et al., 2004; Jones et al., 2007; Rivera-Ferre et al., 2007; Stadig et al., 2016). However, how such factors impact on ranging behaviour patterns of individual broiler chickens has not been reported. The main reason is that still precise technology is not available that is noninvasive, reliable and flexible enough for long-time tracking an individual chicken's exact location, especially in outdoor runs (Siegford et al., 2016). Focusing on the individual ranging frequency, as compared to flock level behaviour analysis, has recently proven to be very crucial. Newest experiments which tracked individual chicken ranging behaviour show that 75% to 95% of chickens in a flock access the range (Durali et al., 2014; Taylor et al., 2017). Green-legged Partridge and Sasso chicken genotypes have been chosen in the current experiment because it allows us to minimize the risk of birds not using the ranges due to health reasons, for instance locomotion issues.

Associations of birds' free range use with weather indicators were distributed across all observed weather parameters, however different associations were identified for Green-legged Partridges and Sasso. Relative outdoor humidity was important factor behind the frequency of the free range use in Green-legged Partridges. Similarly, in previous studies in layers, such association was negative. Laying hens used the free range more frequently when the relative humidity was low, i.e. on cold days and with no rain (Gilani et al., 2014). Previously, the use of the outdoor area was reduced in wet weather (Mirabito and Lubac, 2001; Hegelund et al., 2005; Gilani et al., 2014). Chickens tend to avoid wetting the feathers, which decreases their thermal comfort feeling and requires higher time investments for preening (Huber-Eicher and WECHSLER, 1998). Sasso birds showed more resilience to this weather parameter and their ranging frequency in wet weather is higher than in Green-legged Partridge. Sasso has been described as better adapted to warm climate than other lines of meat chicken (Yakubu et al., 2018). Also, in more Sasso birds' positive associations with the atmospheric pressure (hPa), as compared to Green-legged Partridges were observed. It is known that birds can perceive changes in atmospheric pressure (Paige, 1995). Higher atmospheric pressure in Poland's climate is usually associated with sunny days and weak wind conditions, which are favored by the chickens, as opposed to wet and windy weather (Nielsen et al., 2003; Jones et al., 2007). In the current study, a detailed explanation as to why Sasso would be more susceptible to atmospheric pressure in relation to ranging behaviour was provided, however further research is still necessary.

In publication 1 identified that the ranging frequency of both genotype birds was associated with wind direction. Moreover, in some cases ranging frequency was associated with the wind speed. Wind, principally strong or gusty, can disturb a bird's vigilance, as the additional stimuli in the background increase. Since windy conditions cause birds to feel more distracted and endangered by predators, they look for shelter or even stay indoors (Nicol, 2015). In the current experiment, the free range use by Sasso chicken was more influenced by wind direction than in Green-legged Partridge. Free ranging frequency increased when the wind blew from the SW, WSW, SSW and W directions. In Poland, such wind directions are linked to mostly mild and warm wind blows, but also characterized by low speed which seemed favorable for birds.

Free range use of Green-legged Partridges was positively associated with outside temperature, while this association was not observed for any of the Sasso birds. Air temperature higher than

26°C is described as unfavorable for the comfort of domestic poultry (Etches et al., 2008; Mignon-Grasteau et al., 2015). During the experiment, the outdoor temperature at the observation time points did not rise above 28°C, however the average air temperature measured at the behavioural trial points was 19.6 ± 0.6°C, which is within known poultry thermal comfort range for birds of that age (Pereira and Nääs, 2008). Higher air temperature is often linked with more sun rays. In broilers outdoor shelter effectively encourages chickens to use the free range area under increasing solar radiation (Stadig et al., 2017). Birds tend to stay indoors if shelters are unavailable and they cannot cool down (Stadig et al., 2017). Therefore, it might be assumed, based on the current results, that the ranging behaviour of the Green-legged Partridge chickens is positively associated with the air temperature. The birds' choice to foray at free range may have been instigated by either positive or negative motivation. For example, chickens may access the outdoor free range to explore and discover a more complex environment than the typical indoor shed condition, but, on the other hand, they can try to avoid negative unfavorable, frightening, or painful stimuli, either in the shed or outdoors (Taylor et al., 2017). In the current experiment associations between free range use and weather conditions were proved. Individual patterns of birds' behaviour and preferences were found. Surprisingly, in both genotypes the proportion of individuals which were susceptible to weather conditions was comparable. Understanding the motivations behind the ranging behaviour, for instance reactions to contrast in climate between indoor and outdoor environment, needs further investigations, with consideration of other types of underlying factors than weather conditions, such as birds' health, indoor housing conditions, stocking density or group size. Farmers have limited possibilities to reduce the impact of weather conditions on chickens reared in extensive systems with free range access, while optimizing their free range use. However, particular construction protecting birds from wind, rain or humidity can be applied on the outdoor areas. Positive effects of enhancing more birds to use the areas away from the house by providing natural and artificial wind barriers and covers have been shown in layers (Zeltner and Hirt, 2003). Furthermore, it has been proven that planting shrubs, trees or other forms of shading improved the use of free ranges by chickens, by protecting them against sun and predators (Stadig et al., 2017). As an argument to convince extensive chicken farmers to implement strategies encouraging their birds to use the free runs more, evidence was presented that birds react individually to the same weather conditions. Therefore, designing free ranging areas such that they accommodate individual preferences, needs, for instance, by including provision of multiple construction and vegetation elements is important.

Results presented in the publication 2 provided some evidence of existing associations between external Sasso chickens morphometric traits with free ranging frequency profiles. Hypothesis concerning the birds' feather pigmentation, was confirmed but only for Sasso chickens. Sasso are selected for performance including good growth rates, but also for suitability to the extensive systems. In birds, the α melano-cyte stimulating hormone (MSH), is as part of the avian melanocortin system, which controls pigment regulation and is directly related to energy homeostasis (Takeuchi et al., 2003). Chickens with black pigment, eumelanin in their phenotype, carry at least one copy of the wild-type PMEL17 allele (Kerje et al., 2004). In the study focusing on chicken behaviour pattern and brain gene expression, Karlsson et al. (2010) identified plumage coloration genotypes PMEL17 to have a pleiotropic effect on societal and explorative behaviour in chickens and that wild type birds (i/i) are more active in socializing and exploring in comparison with white birds homozygous for the mutant allele (I/I) (Karlsson et al., 2010). Animals discover their environment or new stimuli and approach them in order to, for instance, find water or food, making this explorative behaviour pattern essential for survival (Powell et al., 2004; Nicol, 2015). Exploration mechanism is thought to counterbalance fear (Meuser et al., 2021). High levels of fear responses to situations indicated low exploration and foraging of the entire environment (Campbell et al., 2019), which may link to limited free range use of the chickens and low adaptation of the animal to the production system. What is more, melanin-based color traits in birds are noticed by conspecifics to carry a superior underlying genotypic quality and consequently the more intense color patches the better fitness benefits (McGraw, 2008; Guindre-Parker and Love, 2014). No previous study identified associations between pigmentation pattern and more frequent free range use. To further support the underlying mechanism behind the dark pigmentation in Sasso chickens a positive correlation between neck plumage and beak darkness was discovered. If repeated and confirmed by further investigations using molecular genetics methods, neck darkness could be a valuable and practical trait, which could help to select birds in breeding programs suitable for extensive systems with outdoor access. Of course, that would apply only to genetic strains with dark pigmentation present.

Associations between comb measurements, behavioural traits and social structure in chickens (Johnsen et al., 1995; Cornwallis and Birkhead, 2007; Navara et al., 2012) or their fitness and wellbeing traits (Johnsson et al., 2012) have been investigated. In chickens, the major role of the comb is thermoregulation (Van Kampen, 1971). The comb in mature chickens also plays a crucial role in the mating process, as it is used for making reproduction decisions both by female and male birds (Pizzari et al., 2003). Comb size is influenced by hormonal status in both sexes (Eitan et al., 1998; Joseph et al., 2003). In males, it is an indicator of social rank and pecking order (Parker and Ligon, 2003). Sexual maturation also favors the development of comb and wattles (Joseph et al., 2003), so the more sexual developed chickens have bigger combs. In hens, it indicates reproductive potential (Cornwallis and Birkhead, 2007; Wright et al., 2008). Moreover, correlations of comb size with bone mass have been described (Wright et al., 2008). Therefore, a bigger comb is an indicator of better fitness of a chicken. In laying hens, combs have been described to be darker in flocks that used the free range area more intensively, while more fearful chickens had smaller combs (Bestman and Wagenaar, 2014). Farmers assess bright red combs as a useful and practical indicator of current hen health condition. However, the association between comb size and free ranging frequencies, as found for Sasso, remains to the best of the authors' knowledge unexplored. Based on the results in publication 2, the comb size of Sasso could be a potential indicator of their free ranging frequency, albeit the comb size to some degree is affected by reproductive status (Eitan et al., 1998; Joseph et al., 2003). Only in Sasso chickens, the most of the visual traits, found to be associated with free range use and correlated between each other within genotype. Therefore, a visual profile of a female bird of this genotype with a higher frequency of free range use could be suggested. Such correlations have been proved for males of other bird species (Yang et al., 2013). However, there is lack of study providing such information on broiler hens. Findings suggest that not only the individual traits but also the set of the visual characteristics of the Sasso hens can be linked with higher free range use.

No associations between the measured or visually assessed external features and free ranging frequency were identified for Green-legged Partridges. Probably their very good adaptation to the outside ranging conditions (Siwek et al., 2013) and overall high ranging activity diminished the relation between their free range use and their external features.

In publication number 3 gut microbiota properties were investigated (bacterial species composition, enzyme activity, and SCFA concentration in the ceca) with three free-ranging profiles: outdoor-preferring, moderate-outdoor and indoor-preferring chickens. The ranging profiles were combined according to the frequency of the free range use, separately for both genotypes. Gastrointestinal (GI) tract is inhabited by a large number of different microbes species, collectively termed the microbiota. Complex, dynamic, and metabolically active, these commensal microbes are proved to constantly interact with the host as an important mediator for physiological processes: immune cell development, gut epithelial tissue, homeostasis and digestion. The examination of the bacteria species' relative abundance in the ceca of the experimental birds showed the interaction between the bird's genotype and ranging profile in two cases: *E. coli* and *Bifidobacterium spp*. relative DNA abundance. The lowest relative DNA abundance of *E. coli* was discovered for indoor-preferring Sasso and Green-legged Partridges. The moderate and outdoor-preferring birds characterized a bigger relative abundance of *E. coli*, which can suggest that the main reservoir or source of *E. coli* was found outdoors at the free ranges.

Bacterial composition in eminent way can be influenced by type of consumed forage, plants etc. (Shakouri et al., 2009; Torok et al., 2011; Kers et al., 2018; Chen et al., 2019). In case of outdoor-preferring birds, the consumption of pasture, roughage originating feed sources as a supplement to the indoor accessible cereal-based diet may have had a positive effect on the chickens' microbial profile. Moreover, the lowest relative DNA abundance of *Bifidobacterium spp.* was found in the caecal content of indoor-preferring Sasso birds, as compared to all other birds. *Bifidobacteria* play an important role. They produce lactic and acetic acids and take part in the supporting and stabilization of gastrointestinal barrier. They modulate the local and systemic immune responses, inhibit the pathogenic invasion and promote the bioconversion of unavailable dietary compounds into bioactive usable molecules (Rossi and Amaretti, 2010). Some strains of *Bifidobacterium spp*. have been proved to prevent *E. coli* colonization in the gastrointestinal tract. The main physiology mechanism is via acetic acid synthesis, resulting in the reduction of the luminal pH (Asahara et al., 2004). The inhibitory role of *Bifidobacterium spp*. in indoor-preferring Sasso birds may occur, however, contradictory to low abundance of *E. coli* found in the same birds. Nevertheless, more studies are necessary to explain the mechanisms ruling the abundance of bacteria strains in indoor-preferring Sasso birds. This could help to improve birds' health and optimize their welfare, while potentially promoting free range use.

The genotype of the chickens in this experiment was associated with relative *Clostridium spp*. DNA abundance, which was higher in Sasso chickens as compared to the Green-legged Partridge. In some conditions it may indicate unpleasant microbiome features. Some poultry pathogens belong to *Clostridium spp.* group. For example, *Clostridium perfringens* causes *necrotic enteritis* (Olkowski et al., 2008). On the other hand, dietary supplementation *Clostridium butyricum* has a positive influence on the growth, digestion and immune status of broilers (Li et al., 2021). The effect of the interaction between a genotype and free ranging profile was found within the concentration of valeric acid, where the highest concentration was noticed in moderate-outgoing Green-legged Partridges. Microbiota play a crucial role in the stimulation growth process of gut by producing SCFA (Dunkley et al., 2007), modulating the structure of the intestinal tract (Shakouri et al., 2009), and consequently influencing nutrient digestion and absorption (Choct, 2009). The indigestible carbohydrates, for instance from pasture, can be used and converted into SCFAs by the microbial activity in broilers (Józefiak et al., 2004). SCFA profiles and concentrations are used as biomarkers of gut microbiota development and microbial-host interactions (Liao et al., 2020). The concentration and SCFA profiles depend on the fermentation products formed by gut bacteria. Amount and type available substrates influence bacteria fermentation strategy and finally can alter composition of products (Liao et al., 2020). Valeric acid or glyceride esters, added to the feed mixture, enhance broiler growth, positively affect the morphology of the small intestinal mucosa and decrease probability of *necrotic enteritis* (Onrust et al., 2018). In moderate-outgoing Green-legged Partridges the amount of the pasture matter in the crop was 3 times higher, as compared to moderate-outgoing Sasso, and generally there was significantly more pasture matter identified, as compared to other ranging profiled birds of that genotype. Precise characteristics of consumed forage and measurements of gustatory tract are described in publication: Marchewka et al (2021). Therefore, it can be suspected that the higher concentrations of valeric acid in moderate-outgoing Green-legged Partridges is associated with the pasture matter-rich diet those birds had. However, the direct and precise associations between the diet, intestinal tract health, and gut microbial composition in birds of various genotypes, with outdoor pastures are yet to be discovered. On the other hand, higher isovaleric acid levels were observed in Sasso as compared to Green-legged Partridges, regardless of the ranging profile. Increased production of isovaleric acid, which belongs to the putrefactive SCFA is indicative of unfavorable conditions in the gut, including increased shifts in pathogenic bacteria and increased ammonia production (Koh et al., 2016). Higher concentrations of isovaleric acid in Sasso chickens may indicate a poorer intestinal health resulting in poorer birds' welfare, which require further attention. Finally, the study design, where birds were reared in breed-specific groups, could influence the results to some extent, as other studies reported that birds housed together show less variation of the gut microbiota, known as the cage effect (Meyer et al., 2012; Zhao et al., 2013; Chen et al., 2019), which may have wiped out the ranging profile effect.

In publication 3 differences between the activity of bacterial enzymes in the investigated chicken groups were found. The biggest discrepancy occurred between genotypes. The activity of the 3 investigated bacterial enzymes, including α-glucosidase, β-glucuronidase and β-galactosidase was smaller in Sasso birds, as compared to Green-legged Partridges. Within the intestinal microbiota, species with the potential to improve poultry performance are very important, as they are also involved in cross-relation between the microbiota, gut epithelium, immune system and nervous system, providing resistance to enteric pathogens (Konieczka et al., 2019). Probiotic species (for instance *Bifidobacterium spp)* contribute to an increase in the activity of many bacterial glycolytic enzymes, such as α-galactosidase, which hydrolyses dietary α-galactosides (RFO and other oligosaccharides); β-galactosidase, which is responsible for hydrolysis of β-galactosides; and α- and β-glucosidase, which hydrolyze NSPs (cellulose, β-glucans); (Hübener et al., 2002; Zdunczyk et al., 2014). The hyperactivity of some bacterial enzymes, specially β-glucosidase and β-glucuronidase, may be detrimental to the bird's health (Jin et al., 2000; Konieczka et al., 2018). It is worth to pay attention to these results, since the increased activity of β-glucuronidase may also be indicative of increased proliferation of pathogenic bacteria in the gut, and it is associated with the higher risk of toxic and carcinogenic substances generation from nontoxic glycosides (Beaud et al., 2005).

To sum up, weather conditions, external birds' morphological features and gut microbiome properties were discovered to be linked with free ranging frequency on different levels: genetic group, ranging profile and individual level. Better health of the birds and optimal adaptation of their genotype to the housing systems with outdoor access safeguards their high welfare and also high productivity. Therefore, good understanding of the weather, appearance and host-microbiome relationship remains integral. Furthermore, in the current study, some knowledge gaps have been identified, serving a background for future research on optimizing range use by chickens in extensive production systems.

8. Conclusion

In the presented dissertation significant associations between different weather parameters and the individual use of the free ranges by Green-legged Partridge and Sasso chickens were confirmed.

- The character of the associations between range use frequency by the genotype and particular weather parameters differed between genotypes, with relative humidity occurring most frequently in Green-legged Partridges, while air pressure and wind direction were most common in Sasso.
- There were significant associations between measurements of the morphometric external features and ranging activity only for Sasso chickens. Birds with higher levels of pigmentation used free range more frequently, which may be a practical indication for the birds' free range use.
- Significant associations between external features and ranging activity in Green-legged Partridges was not confirmed.
- In outdoor-preferring birds' consumption of pasture-originating feed sources as a supplement to the indoor accessible cereal-based diet had positive effects on the birds' microbial profile.
- The biggest differences in case of gut microbiota activity and species composition occurred between genotypes and not between ranging profiles of birds.

9. References

Asahara, T., K. Shimizu, K. Nomoto, T. Hamabata, A. Ozawa, and Y. Takeda. 2004. Probiotic bifidobacteria protect mice from lethal infection with Shiga toxin-producing Escherichia coli O157: H7. Infect. Immun. 72:2240–2247.

Beaud, D., P. Tailliez, and J. Anba-Mondoloni. 2005. Genetic characterization of the β-glucuronidase enzyme from a human intestinal bacterium, Ruminococcus gnavus. Microbiology 151:2323–2330.

Bestman, M., and J.-P. Wagenaar. 2014. Health and welfare in Dutch organic laying hens. Animals 4:374–390.

Binek, M., A. A. Cisek, M. R. D. Chrobak-Chmiel, I. Stefańska, and M. Kizerwetter-Świda. 2017. Mikrobiom jelitowy kury domowej - Rozwój i funkcja. Med. Weter. 73:618–625.

Bjerrum, L., R. M. Engberg, T. D. Leser, B. B. Jensen, K. Finster, and K. Pedersen. 2006. Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. Poult. Sci. 85:1151–1164 Available at http://dx.doi.org/10.1093/ps/85.7.1151.

Bokkers, E. A. M., and P. Koene. 2003. Behaviour of fast-and slow growing broilers to 12 weeks of age and the physical consequences. Appl. Anim. Behav. Sci. 81:59–72.

Campbell, D. L., Hinch, G. N., Downing, J. A., & Lee, C. 2016. Fear and coping styles of outdoor-preferring, moderate-outdoor and indoor-preferring free-range laying hens. Applied Animal Behaviour Science, 185, 73-77.

Campbell, D. L. M., E. J. Dickson, and C. Lee. 2019. Application of open field, tonic immobility, and attention bias tests to hens with different ranging patterns. PeerJ 7:e8122.

Castellini, C., S. Bastianoni, C. Granai, A. Dal Bosco, and M. Brunetti. 2006. Sustainability of poultry production using the emergy approach: Comparison of conventional and organic rearing systems. Agric. Ecosyst. Environ. 114:343–350.

Chen, S., H. Xiang, H. Zhang, X. Zhu, D. Wang, J. Wang, T. Yin, L. Liu, M. Kong, H. Li, and X. Zhao. 2019. Rearing system causes changes of behaviour, microbiome, and gene expression of chickens. Poult. Sci. 98:3365–3376.

Choct, M. 2009. Managing gut health through nutrition. Br. Poult. Sci. 50:9–15.

Classen, H. L. 2017. Diet energy and feed intake in chickens. Anim. Feed Sci. Technol. 233:13–21.

Cornwallis, C. K., and T. R. Birkhead. 2007. Experimental evidence that female ornamentation increases the acquisition of sperm and signals fecundity. Proc. R. Soc. B Biol. Sci. 274:583–590.

Dal Bosco, A., C. Mugnai, S. Mattioli, A. Rosati, S. Ruggeri, D. Ranucci, and C. Castellini. 2016. Transfer of bioactive compounds from pasture to meat in organic free-range chickens. Poult. Sci. 95:2464–2471 Available at http://dx.doi.org/10.3382/ps/pev383.

Dal Bosco, A., C. Mugnai, F. Sirri, C. Zamparini, and C. Castellini. 2010. Assessment of a global positioning system to evaluate activities of organic chickens at pasture. J. Appl. Poult. Res. 19:213–218.

Dunkley, K. D., C. S. Dunkley, N. L. Njongmeta, T. R. Callaway, M. E. Hume, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2007. Comparison of in vitro fermentation and molecular microbial profiles of high-fiber feed substrates incubated with chicken cecal inocula. Poult. Sci. 86:801–810.

Durali, T., P. Groves, A. Cowieson, and M. Singh. 2014. Evaluating range usage of commercial free range broilers and its effect on birds performance using radio frequency identification (RFID) techology.Pages 103–106 in 25th Annual Australian Poultry Science Symposium Sydney, Australia.

Eitan, Y., M. Soller, and I. Rozenboim. 1998. Comb size and estrogen levels toward the onset of lay in broiler and layer strain females under ad libitum and restricted feeding. Poult. Sci. 77:1593–1600.

Erian, I., and C. J. C. Phillips. 2017. Public understanding and attitudes towards meat chicken production and relations to consumption. Animals 7:20.

Etches, R. J., T. M. John, and A. M. V. Gibbins. 2008. Behavioural, physiological, neuroendocrine and molecular responses to heat stress. Poult. Prod. hot Clim. 2:48–79.

European Commision DG AGRI https://agriculture.ec.europa.eu/farming/animal-products/poultry_en 4.12.2023

Gilani, A.-M., T. G. Knowles, and C. J. Nicol. 2014. Factors affecting ranging behaviour in young and adult laying hens. Br. Poult. Sci. 55:127–135.

Guindre-Parker, S., and O. P. Love. 2014. Revisiting the condition-dependence of melanin-based plumage. J. Avian Biol. 45:29–33.

Hegelund, L., J. T. Sørensen, J. B. Kjaer, and I. S. Kristensen. 2005. Use of the range area in organic egg production systems: effect of climatic factors, flock size, age and artificial cover. Br. Poult. Sci. 46:1–8.

Hübener, K., W. Vahjen, and O. Simon. 2002. Bacterial responses to different dietary cereal types and xylanase supplementation in the intestine of broiler chicken. Arch. Anim. Nutr. fur Tierernahrung 56:167–187.

Huber-Eicher, B., and B. WECHSLER. 1998. The effect of quality and availability of foraging materials on feather pecking in laying hen chicks. Anim. Behav. 55:861–873.

Jin, L. Z., Y. W. Ho, N. Abdullah, and S. Jalaludin. 2000. Digestive and bacterial enzyme activities in broilers fed diets supplemented with Lactobacillus cultures. Poult. Sci. 79:886–891.

Johnsen, T. S., S. L. Popma, and M. Zuk. 1995. Male courtship displays, ornaments and female mate choice in captive red jungle fowl. Behaviour 132:821–836.

Johnsson, M., I. Gustafson, C.-J. Rubin, A.-S. Sahlqvist, K. B. Jonsson, S. Kerje, O. Ekwall, O. Kämpe, L. Andersson, and P. Jensen. 2012. A sexual ornament in chickens is affected by pleiotropic alleles at HAO1 and BMP2, selected during domestication.

Jones, T., R. Feber, G. Hemery, P. Cook, K. James, C. Lamberth, and M. Dawkins. 2007. Welfare and environmental benefits of integrating commercially viable free-range broiler chickens into newly planted woodland: A UK case study. Agric. Syst. 94:177–188.

Joseph, N. S., F. E. Robinson, R. A. Renema, and K. A. Thorsteinson. 2003. Comb growth during sexual maturation in female broiler breeders. J. Appl. Poult. Res. 12:7–13.

Józefiak, D., A. Rutkowski, and S. A. Martin. 2004. Carbohydrate fermentation in the avian ceca: a review. Anim. Feed Sci. Technol. 113:1–15.

Jurgoński, A., J. Juśkiewicz, and Z. Zduńczyk. 2013. An anthocyanin-rich extract from Kamchatka honeysuckle increases enzymatic activity within the gut and ameliorates abnormal lipid and glucose metabolism in rats. Nutrition 29:898–902.

Van Kampen, M. 1971. Some aspects of thermoregulation in the White Leghorn fowl. Int. J. Biometeorol. 15:244–246.

Karlsson, A.-C., S. Kerje, L. Andersson, and P. Jensen. 2010. Genotype at the PMEL17 locus affects social and explorative behaviour in chickens. Br. Poult. Sci. 51:170–177.

Kerje, S., P. Sharma, U. Gunnarsson, H. Kim, S. Bagchi, R. Fredriksson, K. Schütz, P. Jensen, G. Von Heijne, and R. Okimoto. 2004. The Dominant white, Dun and Smoky color variants in chicken are associated with insertion/deletion polymorphisms in the PMEL17 gene. Genetics 168:1507–1518.

Kers, J. G., F. C. Velkers, E. A. J. Fischer, G. D. A. Hermes, J. A. Stegeman, and H. Smidt. 2018. Host and environmental factors affecting the intestinal microbiota in chickens. Front. Microbiol. 9:1–14.

Kogut, M. H. 2019. The effect of microbiome modulation on the intestinal health of poultry. Anim. Feed Sci. Technol. 250:32–40.

Koh, A., F. De Vadder, P. Kovatcheva-Datchary, and F. Bäckhed. 2016. From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites. Cell 165:1332–1345.

Konieczka, P., J. Czerwiński, J. Jankowiak, K. Ząbek, and S. Smulikowska. 2019. Effects of partial replacement of soybean meal with rapeseed meal, narrow-leaved lupin, DDGS, and probiotic supplementation, on performance and gut microbiota activity and diversity in broilers. Ann. Anim. Sci. 19:1115–1131.

Konieczka, P., K. Nowicka, M. Madar, M. Taciak, and S. Smulikowska. 2018. Effects of pea extrusion and enzyme and probiotic supplementation on performance, microbiota activity and biofilm formation in the broiler gastrointestinal tract. Br. Poult. Sci. 59:654–662 Available at https://doi.org/10.1080/00071668.2018.1507017.

Konieczka, P., and S. Smulikowska. 2018. Viscosity negatively affects the nutritional value of blue lupin seeds for broilers. Animal 12:1144–1153.

Li, W., B. Xu, L. Wang, Q. Sun, W. Deng, F. Wei, H. Ma, C. Fu, G. Wang, and S. Li. 2021. Effects of Clostridium butyricum on Growth Performance, Gut Microbiota and Intestinal Barrier

Function of Broilers. Front. Microbiol. 12:777456.

Liao, X., Y. Shao, G. Sun, Y. Yang, L. Zhang, Y. Guo, X. Luo, and L. Lu. 2020. The relationship among gut microbiota, short-chain fatty acids, and intestinal morphology of growing and healthy broilers. Poult. Sci. 99:5883–5895.

Marchewka, J., P. Sztandarski, Ż. Zdanowska-Sąsiadek, K. Damaziak, F. Wojciechowski, A. B. Riber, and S. Gunnarsson. 2020. Associations between welfare and ranging profile in free-range commercial and heritage meat-purpose chickens (Gallus gallus domesticus). Poult. Sci. 99:4141–4152.

McGraw, K. J. 2008. An update on the honesty of melanin-based color signals in birds. Pigment Cell Melanoma Res. 21:133–138.

Meuser, V., L. Weinhold, S. Hillemacher, and I. Tiemann. 2021. Welfare-related behaviours in chickens: characterization of fear and exploration in local and commercial chicken strains. Animals 11:679.

Meyer, B., A. W. Bessei, W. Vahjen, J. Zentek, and A. Harlander-Matauschek. 2012. Dietary inclusion of feathers affects intestinal microbiota and microbial metabolites in growing leghorn-type chickens. Poult. Sci. 91:1506–1513.

Michalczuk, M., E. Holl, A. Möddel, A. Jóźwik, J. Slósarz, D. Bień, K. Ząbek, and P. Konieczka. 2021. Phytogenic Ingredients from Hops and Organic Acids Improve Selected Indices of Welfare, Health Status Markers, and Bacteria Composition in the Caeca of Broiler Chickens. Animals 11:3249.

Mignon-Grasteau, S., U. Moreri, A. Narcy, X. Rousseau, T. B. Rodenburg, M. Tixier-Boichard, and T. Zerjal. 2015. Robustness to chronic heat stress in laying hens: a meta-analysis. Poult. Sci. 94:586–600.

Mirabito, L., and S. Lubac. 2001. Descriptive study of outdoor run occupation by'Red Label'type chickens. Br. Poult. Sci. 42:S16–S17.

Navara, K. J., E. M. Anderson, and M. L. Edwards. 2012. Comb size and color relate to sperm quality: a test of the phenotype-linked fertility hypothesis. Behav. Ecol. 23:1036–1041.

Nicol, C. J. 2015. The behavioural biology of chickens. CABI.

Nielsen, B. L., M. G. Thomsen, P. S∅ rensen, and J. F. Young. 2003. Feed and strain effects on the use of outdoor areas by broilers. Br. Poult. Sci. 44:161–169.

Olkowski, A. A., C. Wojnarowicz, M. Chirino-Trejo, B. Laarveld, and G. Sawicki. 2008. Sub-clinical necrotic enteritis in broiler chickens: novel etiological consideration based on ultra-structural and molecular changes in the intestinal tissue. Res. Vet. Sci. 85:543–553.

Onrust, L., K. Van Driessche, R. Ducatelle, K. Schwarzer, F. Haesebrouck, and F. Van Immerseel. 2018. Valeric acid glyceride esters in feed promote broiler performance and reduce the incidence of necrotic enteritis. Poult. Sci. 97:2303–2311.

Paige, K. N. 1995. Bats and barometric pressure: conserving limited energy and tracking insects from the roost. Funct. Ecol.:463–467.

Parker, T. H., and J. D. Ligon. 2003. Female mating preferences in red junglefowl: a meta-analysis. Ethol. Ecol. Evol. 15:63–72.

Pereira, D. F., and I. de A. Nääs. 2008. Estimating the thermoneutral zone for broiler breeders using behavioural analysis. Comput. Electron. Agric. 62:2–7.

Pizzari, T., C. K. Cornwallis, H. Løvlie, S. Jakobsson, and T. R. Birkhead. 2003. Sophisticated sperm allocation in male fowl. Nature 426:70–74.

Powell, S. B., M. A. Geyer, D. Gallagher, and M. P. Paulus. 2004. The balance between approach and avoidance behaviours in a novel object exploration paradigm in mice. Behav. Brain Res. 152:341–349.

Rivera-Ferre, M. G., E. A. Lantinga, and R. P. Kwakkel. 2007. Herbage intake and use of outdoor area by organic broilers: effects of vegetation type and shelter addition. NJAS-Wageningen J. Life Sci. 54:279–291.

Rossi, M., and A. Amaretti. 2010. Probiotic properties of bifidobacteria.

Sanchez, C., and I. Estevez. 1998. The Chickitizer software program. Coll. Park. Maryland, USA Univ. Maryl.

Schütz, K. E., B. Forkman, and P. Jensen. 2001. Domestication effects on foraging strategy, social behaviour and different fear responses: a comparison between the red junglefowl (Gallus gallus) and a modern layer strain. Appl. Anim. Behav. Sci. 74:1–14.

Shakouri, M. D., P. A. Iji, L. L. Mikkelsen, and A. J. Cowieson. 2009. Intestinal function and gut microflora of broiler chickens as influenced by cereal grains and microbial enzyme supplementation. J. Anim. Physiol. Anim. Nutr. (Berl). 93:647–658.

Siegford, J. M., J. Berezowski, S. K. Biswas, C. L. Daigle, S. G. Gebhardt-Henrich, C. E. Hernandez, S. Thurner, and M. J. Toscano. 2016. Assessing activity and location of individual laying hens in large groups using modern technology. Animals 6:10.

Siwek, M., D. Wragg, A. Sławińska, M. Malek, O. Hanotte, and J. M. Mwacharo. 2013. Insights into the genetic history of G reen-legged P artridgelike fowl: mt DNA and genome-wide SNP analysis. Anim. Genet. 44:522–532.

Sossidou, E. N., A. Dal Bosco, H. A. Elson, and C. M. G. A. Fontes. 2011. Pasture-based systems for poultry production: Implications and perspectives. Worlds. Poult. Sci. J. 67:47–58.

Stadig, L. M., T. B. Rodenburg, B. Ampe, B. Reubens, and F. A. M. Tuyttens. 2017. Effects of shelter type, early environmental enrichment and weather conditions on free-range behaviour of slow-growing broiler chickens. Animal 11:1046–1053.

Stadig, L. M., T. B. Rodenburg, B. Reubens, J. Aerts, B. Duquenne, and F. A. M. Tuyttens. 2016. Effects of free-range access on production parameters and meat quality, composition and taste in slow-growing broiler chickens. Poult. Sci. 95:2971–2978.

Stamp Dawkins, M., C. A. Donnelly, and T. A. Jones. 2004. Chicken welfare is influenced more by housing conditions than by stocking density. Nature 427:342–344.

Sztandarski, P., J. Marchewka, P. Konieczka, Ż. Zdanowska-Sąsiadek, K. Damaziak, A. B. Riber,

S. Gunnarsson, and J. O. Horbańczuk. 2022. Gut microbiota activity in chickens from two genetic lines and with outdoor-preferring, moderate-preferring, and indoor-preferring ranging profiles. Poult. Sci. 101:102039.

Sztandarski, P., J. Marchewka, F. Wojciechowski, A. B. Riber, S. Gunnarsson, and J. O. Horbańczuk. 2021. Associations between weather conditions and individual range use by commercial and heritage chickens. Poult. Sci. 100:101265.

Taylor, P. S., P. H. Hemsworth, P. J. Groves, S. G. Gebhardt-Henrich, and J.-L. Rault. 2017. Ranging behaviour of commercial free-range broiler chickens 1: Factors related to flock variability. Animals 7:54.

Torok, V. A., R. J. Hughes, L. L. Mikkelsen, R. Perez-Maldonado, K. Balding, R. MacAlpine, N. J. Percy, and K. Ophel-Keller. 2011. Identification and characterization of potential performance-related gut microbiotas in broiler chickens across various feeding trials. Appl. Environ. Microbiol. 77:5868–5878.

Wright, D., S. Kerje, H. Brändström, K. Schütz, A. Kindmark, L. Andersson, P. Jensen, and T. Pizzari. 2008. The genetic architecture of a female sexual ornament. Evol. Int. J. Org. Evol. 62:86–98.

Yakubu, A., E. I. Epko, and O. I. A. Oluremi. 2018. Physiological adaptation of sasso laying hens to the hot-dry tropical conditions. Agric. Conspec. Sci. 83:187–193.

Yang, C., J. Wang, Y. Fang, and Y.-H. Sun. 2013. Is sexual ornamentation an honest signal of male quality in the Chinese grouse (Tetrastes sewerzowi)? PLoS One 8:e82972.

Zdunczyk, Z., J. Jankowski, A. Rutkowski, E. Sosnowska, A. Drazbo, P. Zdunczyk, and J. Juskiewicz. 2014. The composition and enzymatic activity of gut microbiota in laying hens fed diets supplemented with blue lupine seeds. Anim. Feed Sci. Technol. 191:57–66 Available at http://dx.doi.org/10.1016/j.anifeedsci.2014.01.016.

Zeltner, E., and H. Hirt. 2003. Effect of artificial structuring on the use of laying hen runs in a free-range system. Br. Poult. Sci. 44:533–537.

Zhao, L., G. Wang, P. Siegel, C. He, H. Wang, W. Zhao, Z. Zhai, F. Tian, J. Zhao, H. Zhang, Z. Sun, W. Chen, Y. Zhang, and H. Meng. 2013. Quantitative genetic background of the host influences gut microbiomes in chickens. Sci. Rep. 3:1–6.

10. Appendices

10.1 Percentage contribution of authors to each publication

I hereby give the percentage of each author’s contribution to the publication:

Sztandarski, P., Marchewka, J., Wojciechowski, F., Riber, A. B., Gunnarsson, S., & Horbańczuk, J. O. (2021). Associations between weather conditions and individual range use by commercial and heritage chickens. *Poultry Science, 100(8), 101265.*

Lp.	**Imię i nazwisko** ***(name and surname)***	**Opis udziału autora** ***(Description of the author's participation)***	**% udział w opracowaniu publikacji** ***(participation in the publication in %)***	**Jednostka** ***(Institution)***
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Sztandarski, P., Marchewka, J., Wojciechowski, F., Riber, A. B., Gunnarsson, S., & Horbańczuk, J. O. (2021). Associations between neck plumage and beak darkness, as well as comb size measurements and scores with ranging frequency of Sasso and Green-legged Partridge chickens. *Poultry Science, 100(9), 101340.*

Lp.	**Imię i nazwisko** ***(name and surname)***	**Opis udziału autora** ***(Description of the author's participation)***	**% udział w opracowaniu publikacji** ***(participation in the publication in %)***	**Jednostka** ***(Institution)***
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Sztandarski, P., Marchewka, J., Konieczka, P., Zdanowska-Sąsiadek, Ż., Damaziak, K., Riber, A. B. & Horbańczuk, J. O. (2022). Gut microbiota activity in chickens from two genetic lines and with outdoor-preferring, moderate-preferring, and indoor-preferring ranging profiles. *Poultry Science, 101(10), 102039*.

Lp.	**Imię i nazwisko** ***(name and surname)***	**Opis udziału autora** ***(Description of the author's participation)***	**% udział w opracowaniu publikacji** ***(participation in the publication in %)***	**Jednostka** ***(Institution)***
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10.2 Publications

ANIMAL WELL-BEING AND BEHAVIOR

Associations between weather conditions and individual range use by commercial and heritage chickens

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ABSTRACT Ranging area use by domestic poultry is not always optimal and differences in it exist on the levels of breed, flock and individual bird. Outdoor shelters are usually not protective for all weather parameters and may not fulfil a protective role to all birds within the flock all time, if individuals are sensitive to different weather conditions. The aim of this study was to investigate associations between different weather parameters and the use of the range by individual Green-legged Partridge and Sasso C44 chickens. In August 2018, 60 birds per genetic strain were housed in groups of 10 from wks 5 to 10, under conditions exceeding minimal EU requirements of organic meat chicken production. Birds in each pen had access to an outdoor range that was video-recorded during the experiment to obtain frequencies of individual birds' use of the ranges. Weather data were collected each minute throughout the whole experiment by an automatic weather station. In each pen, birds tagged individually with a laminated color tag, had access to an outdoor range that was video-recorded during the experiment. Frequencies of individual birds' use of the ranges were manually obtained from the recordings. Univariate and multivariate linear regression models were used to investigate the associations between the variables. The results showed significant associations between weather parameters and range use for one third of Green-legged Partridge and Sasso chickens (n = 21 in both breeds). Between breeds, range use associations with different weather parameters were identified. Negative associations with relative humidity occurred most frequently in Green-legged Partridges (n = 8; R^2 from 0.1 to 0.17), while positive associations with atmospheric pressure (n = 7; R^2 from 0.09 to 0.17) were most common in Sasso chickens. Further investigations into the reasons behind individual sensitivity of meat-purpose chickens to specific weather conditions would increase the understanding of their preferences and needs, which over time will improve animal welfare.

Key words: weather, organic, broiler, range, behavior

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INTRODUCTION

The behavior, welfare and productivity of broiler chickens are influenced by the genetic makeup and environmental factors (Zuidhof et al., 2014). In conventional broiler production systems, birds are reared in strictly controlled indoor conditions (Lima and Nääs, 2005). Increased public concerns of animal welfare in those systems (Marchewka et al., 2013), including decreased ability of the poultry to express natural behaviors, has directed consumers' attention to meat from poultry reared in low-input systems, known as optimizing the management and use of internal production inputs and minimizing the use of production inputs (Biala et al., 2007; FAO, 2007; Erian and Phillips, 2017). In some systems, as for instance in the European organic systems, birds are provided with ranging area (EU, 2007, 2008).

Previous studies have shown that the ranging area use by broiler chickens is not always optimal and that differences exist not only on the flock or breed level, but also between individual birds in the same flock, even if equal opportunity of access to the range is provided (Dawkins et al., 2003; Taylor et al., 2017). Basic outdoor environmental factors which are likely to influence animal comfort are air temperature, relative humidity and speed of air movement (Dec et al., 2018). While rearing

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birds in an environment which promotes better comfort and therefore assures good animal welfare (Silva et al., 2003), the comfort zone related to weather parameters for free-range broilers has not yet been investigated (Santos et al., 2014).

In modern commercial free-range chicken production systems, outdoor ranges may have large open spaces and very little shelter. In order to promote the use of the ranging area by the birds, the facilities should protect the birds from adverse weather conditions by providing for instance sun shade or wind protection. However, available shelters may not be protective under all weather conditions, they may not fulfil their protective role in the particular geographical and climate zone or they may not be available to a sufficient number of birds in a flock at once when needed (Stadig et al., 2017). Better understanding of the motivation behind the ranging choices birds make, could help to improve the facilities provided to them.

One of the still unknown aspects is whether on an individual level birds' ranging activity is associated with the prevailing conditions. Most of the broiler studies to that, that have associated weather parameters with range use, have averaged across the breed or treatment group (Stadig et al., 2017; Taylor et al., 2017). However, individual birds in a flock reach outdoor areas at various time points that would have associated weather conditions specific to that particular moment. If individual birds react differently to the weather conditions, it would make an important argument in the discussion regarding design of ranging areas and need for the simultaneous use of the various weather protecting elements, such as: shrubs, trees, wind, and sun panels on the same range.

Only one study to date has focused on the within-flock variability, where individual ranging behavior of free-range broiler chickens was recorded using the radiofrequency identification system. However, in this study the weather variables predicted the total numbers of chickens in the flock that accessed the range (Taylor et al., 2017). Moreover, in the above-mentioned study, the weather parameters were collected every 10 min in the summer and twice a day in the winter, which did not allow exact matching of range access with instantly changing weather parameters such as wind speed. Therefore, to our knowledge no previous studies have investigated range use of individually identified birds within a broiler chicken flock across the production cycle and matched with the weather parameters collected in the exact same time points and location.

The aim of this study was to investigate associations between weather parameters and the frequency of the range area use by individual Green-legged Partridge and Sasso chickens. The Green-legged Partridge is an old native Polish breed characterized by green-colored shanks, which is well adapted to the local environmental conditions (Siwek et al., 2013). This breed is especially adequate for maintenance in extensive, outdoor access production systems, as characterized by good health and low prevalence of welfare issues (Marchewka et al., 2020). The average body weight of Green-legged Partridge roosters is around 2.5 kg and hens around 1.7 kg, which is achieved at about 5 mo of age. The slower growing chicken hybrid Sasso is widely and successfully used in the commercial production across the globe (Hendrix Genetics BV and Sasso, France). It is well skilled to forage on outdoor ranges and has been especially well adapted to various environmental conditions, from the European continental climate, as in the Label Rouge production system, to the African hot climate (Getiso et al., 2017). Sasso birds reach a slaughter weight of 2.3 to 2.8 kg at about 2 mo of age, while their meat is characterized by a very good taste and quality (Getiso et al., 2017). We hypothesized that higher relative humidity or wind speed may limit range use of individual Green-legged Partridge and Sasso chickens, while temperature increase, within the birds thermal comfort ranges may promote it.

MATERIALS AND METHODS

The experiment took place from the August 21 until September 22, 2018 in the Mazovian region in Poland, at the facilities of the experimental farm of the Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences.

Animals, Housing, and Management

Sixty mixed-sex, non-beak trimmed birds, of each of 2 breeds (total n = 120 birds), Green-legged Partridge and Sasso line C44 (for consistency, both Sasso and Green-legged Partridge will be referred to as "breed," although Sasso is a hybrid) were used in the experiment. Before wk 5 of age, birds were not allowed outdoor access. At the age of 5 wk, 120 birds were categorized as healthy by the veterinarian assigned to care for the animals in the experimental facility. Individuals with similar body weight within each breed (on average 2030.6 ± 68.9 g for Sasso and 705.9 ± 8.5 g for Green-legged Partridge), were selected and relocated from their rearing facilities, located at the same breeding station as the experimental house. Eight female and 2 male chickens were assigned to each single breed group housed in 12 pens until 10 wk of age. No birds died during the experiment. The size of the indoor pens was 2.5 m × 3.5 m, resulting in a stocking density at slaughter age of 1.4 kg/m^2 for Green-legged Partridge and 2.7 kg/m^2 for Sasso. Sawdust litter was added on top of the floor, while next to the wall there was a 0.5 m strip covered with sand. New litter was supplied weekly and pens were partly cleaned according to the need. In each pen, there were two 80-cm long wooden perches with 2 perching levels, one at the height of 15 cm and the second at 40 cm. The perching poles were 50 × 50 mm thick and had rounded edges. Each pen had direct access to an individual outdoor range (3.5 m × 30 m), through the pophole (45 cm high × 50 cm wide), providing 10.5 m^2/chicken. All the outdoor ranges had equal vegetation coverage regarding botanical composition and height but no trees or shelters were present. The grass was mowed 1 wk before the onset of the experiment. Each ranging area was provided a semiautomatic bell drinker and a wooden box (1 m × 1 m) filled with sand. The schematic figure representing the experimental facilities was presented in Marchewka et al. (2020).

After relocation, the birds were habituated for 48 h to the new housing and social situation before popholes were opened daily from 7.00 until 19.00 h. To allow for individual bird identification, all birds were fitted with a laminated paper mark (9 cm high × 7 cm wide) attached to the birds' back by fitting 2 elastic bands around its wings. Ten different colors of the marks were assigned in each pen randomly to the individual birds. Birds were equipped with their color mark during the entire experiment, and they were inspected twice a day to assure their health and welfare and control for any unpredicted events. Commercial pelleted feed (Agro-Handel Mirsk, Poland) was used to nourish the birds. The feed was composed of wheat, maize, sunflower expeller, pea, soybean expeller legumes mix, gruel corn, monocalcium phosphate, soybean oil, calcium carbonate (components proportions protected by the local manufacturer) with supplements (Marchewka et al., 2020). The dietary composition of the feed was designed to meet slow-growing birds' nutritional requirements under the organic production circumstances at the age between 5 and 10 wk of age. It contained 20% of protein, 5% of fat, 6% of fiber, 6.5% of ash, 1.05% of calcium, 0.82% of lysine, 0.65% of phosphorus, 0.34% of methionine and 0.16% of sodium. No coccidiostats or other medication was used. Feed and water were available ad libitum.

Birds were provided only natural light through uncovered windows as the room had no artificial lights. Light hours during the experimental period ranged from 12.7 h to 15.7 h/day. There was natural ventilation in the building. Indoor climate parameters were automatically and continuously collected by an add-on device of the main weather measuring device (Davis Instruments Vantage Pro 2 DAV-6152EU, CA) placed in the middle of the chicken rearing house on a height of 1 m.

Data Weather Collection

Weather data were collected once per minute throughout the whole experiment. An automatic weather station used for this purpose (Davis Instruments Vantage Pro 2 DAV-6152EU, CA) was installed at the end of the central ranging area, height of 1 m from the ground. The following parameters were collected: air temperature (°C) and relative humidity (%), wind direction (cardinal directions) and wind speed (m/s), atmospheric pressure (hPa) and the sum of daily precipitation (mm). These data were automatically saved in a Microsoft Excel spreadsheet (2016). For the purposes of statistical analysis, the cardinal directions of the wind were converted to degrees, where degree "0" indicated north wind (N), while interpretation of the increase in the degrees followed the standard compass rose.

Observations of Ranging Behavior

Ranging behavior of the birds was recorded using video cameras. The 12 outdoor pens were video-recorded simultaneously and continuously using 6 cameras (BCS company Poland-DMIP2401IR-M-IV IP 4 Mpix), each completely covering 2 ranging areas. The films were automatically saved on the network recorder (BCS-NVR0401-IP 4 channel BC). Video material was analyzed and bird location was recorded by the same trained and experienced person, using the Chickitizer program (Sanchez and Estevez, 1998). It is a computer application in which the presence of animals in predefined areas can be recorded with a single mouse click. The data from this application can easily be transferred to a calculation spreadsheet. From the recorded videos, 3 d were chosen per week of experiment (5 wk). On each of those days, 3 times of the day (morning: starting at 8:00, noon: starting at 13:00, and evening: starting at 18:00) a 3-min-period with 10 s sampling intervals was set and repeated after 10 min. In short, the observation protocol consisted of 6 samplings (1 sampling/10 s, making up to 1 min) * 3 min * 2 bouts * 3 times of day * 3 d each week * 5 wk. The observer recorded each of the experimental birds' absence as "0" or presence as "1 " in the outdoor area. Therefore, the frequency of individual outdoor use in the current study was between 0 and 1,620.

Statistical Analysis

In the simple and multiple regression models, the variable describing either the individual Green-legged Partridge or Sasso chicken range use (sum of the individual bird presences in the outdoor area during observation periods) was considered as the dependent outcome variable, while weather parameters at the time of the range use observations were considered as the independent variables: air temperature (°C) and relative humidity (%), wind direction (cardinal directions) and wind speed (m/s), atmospheric pressure (hPa) and the sum of daily precipitation (mm). The outcome variable was analyzed for associations with any of the independent variables. The outcome variable was normally distributed across the sample population, thus linear univariate regression was used. Residuals were predicted and checked for normality. Associations with P-value <0.2 were further analyzed in a multivariate linear regression analysis. Models were backward exclusion until all associations reached P-value <0.05. Interactions between independent variables were tested in the final models and were not detected. Residuals were predicted and plotted in normal quantile plots and coefficients of determination (R^2) were calculated and used to choose the model that explains the variability of the response data. The likelihood ratio test was used to observe the improvement of the multiple regression models by inclusion and exclusion of independent variables. Akaike's information criterion and Schwarz's Bayesian information criterion were used to compare maximum likelihood of reduced

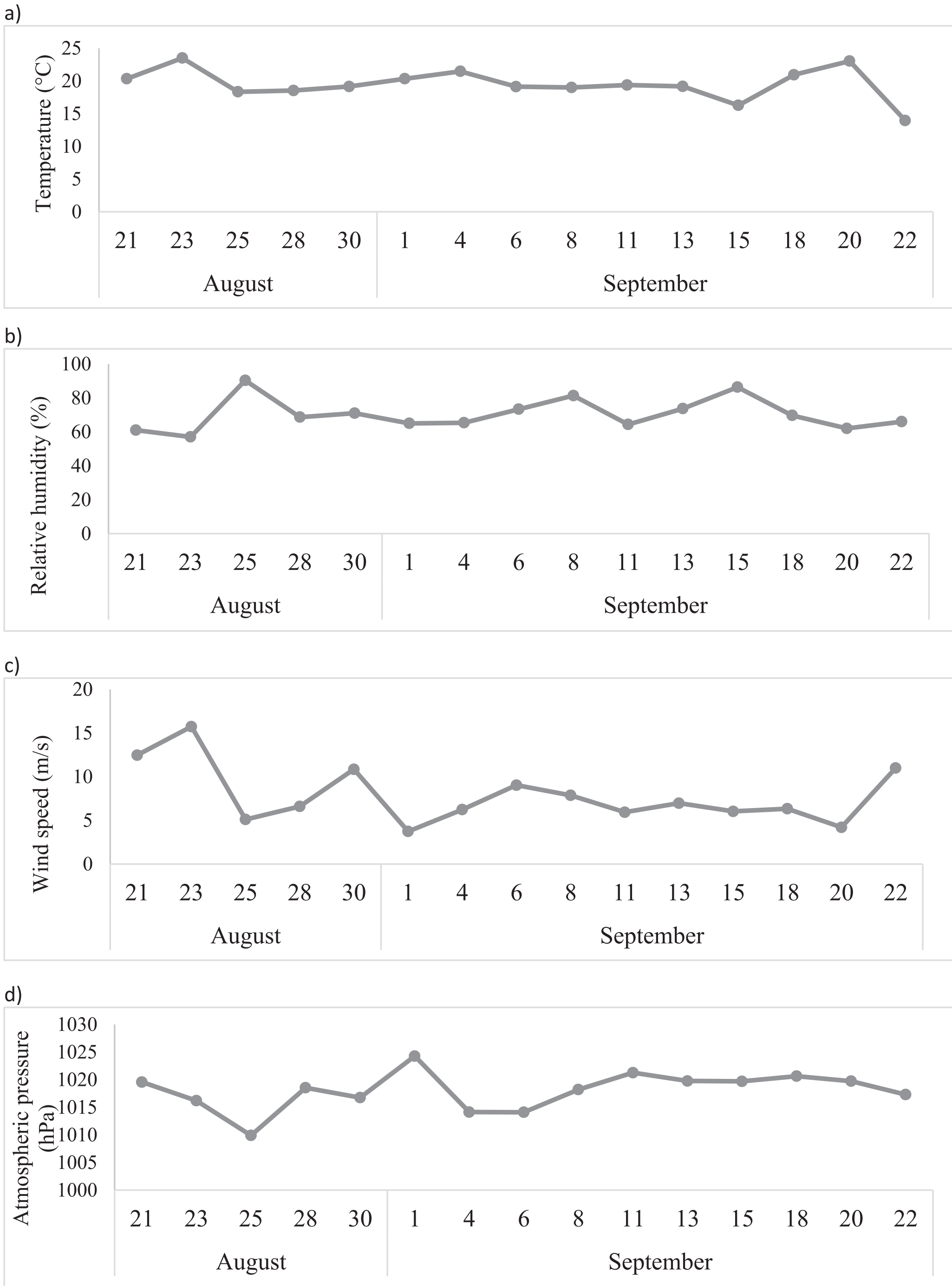

Figure 1. Outdoor weather parameters recorded during the behavioral observation periods and averaged per observation day; (A) temperature; (B) humidity; (C) wind speed; (D) atmospheric pressure.

and full models. The selection of the final models was based on the smaller values of the information criterion.

RESULTS

The temperature recorded in the building during the experiment ranged from 19°C to 26°C, while relative humidity ranged from 47 to71%. During the day, outside temperature ranged from 12°C to 28°C, outside relative humidity from 46 to 99%, wind speed from 0 to 24 m/s and atmospheric pressure from 1,004 hPa to 1,027 hPa (Figure 1A–1D). The dominating wind direction was western and south - western (Figure 2).

Associations Between Weather Parameters and Range Use by Individual Green-Legged Partridge Chickens

The results of the simple and multiple regression models showing associations between range use by individual Green-legged Partridge chickens and weather parameters are presented in Table 1 together with the mean

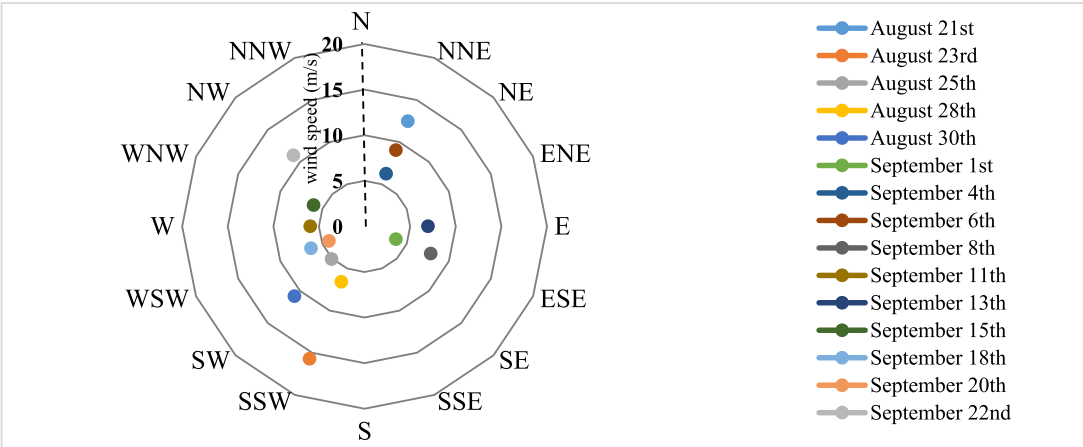

Figure 2. Wind direction recorded during the behavioral observation periods per observation day presented in cardinal directions. Each day is marked on the graph with a different color and for each day, the average wind speed is indicated. *Collected data of cardinal directions were first converted to degrees, where degree "0" indicated north wind (N), interpreted following the standard compass rose and averaged for each day, afterwards reconverted to the cardinal directions.

frequency and standard deviation of the range usage by the birds. Significant associations between the range uses with one weather parameter were identified for 20 birds, while with 2 weather parameters for one bird. For the remaining 39 birds, no significant associations were identified between individual range use and weather parameters.

Increased range use of 8 birds was significantly and positively associated with relative humidity, where the proportion of explained variance of the response variable ranged from 10 to 17%. Range use of 3 birds was positively associated with temperature and also for three birds with wind direction expressed in degrees. The proportion of variance of range use explained by the temperature ranged from 12 to 20%, while for wind direction from 9 to 16%. Atmospheric pressure was positively associated with the range use of 3 birds, while one bird used the ranges less often when the atmospheric pressure increased (negative association). In case of 2 birds, an association between higher wind speed and reduced range use was identified. Moreover, the range use of one bird was associated with 2 weather

Table 1. Associations between free range use by individual Green-legged Partridge chickens and different weather parameters.

									Individual free range use	
Weather parameter	R^2	Parameter estimate (r)	SE	T Value	Pr > |t|	95% Confidence	Limits	Pen	Mean/ day	SE
Simple linear regression models (n = 20)										
Atmospheric pressure (hPa)										
	0.13	0.98	0.41	2.40	0.02	0.16	1.80	1	9.29	1.49
	0.11	0.68	0.30	2.28	0.03	0.08	1.28	2	4.33	1.08
	0.11	0.95	0.41	2.30	0.03	0.12	1.78	3	12.49	1.50
	0.09	−0.53	0.26	−2.05	0.05	−1.06	−0.01	5	3.44	0.93
Relative humidity (%)										
	0.15	−0.19	0.07	−2.66	0.01	−0.33	−0.04	1	7.22	1.21
	0.12	−0.14	0.06	−2.41	0.02	−0.26	−0.02	2	2.93	1.01
	0.10	−0.16	0.08	−2.13	0.04	−0.31	−0.01	2	6.98	1.27
	0.15	−0.18	0.07	−2.67	0.01	−0.31	−0.04	3	7.49	1.17
	0.17	−0.19	0.07	−2.91	0.01	−0.33	−0.06	3	9.44	1.17
	0.12	−0.17	0.07	−2.36	0.02	−0.32	−0.02	4	8.00	1.45
	0.12	−0.20	0.08	−2.32	0.03	−0.37	−0.03	4	5.51	1.23
	0.16	−0.24	0.09	−2.75	0.01	−0.42	−0.06	6	7.22	1.21
Temperature (°C)										
	0.20	1.19	0.38	3.17	0.00	0.43	1.95	1	11.13	1.64
	0.13	0.86	0.35	2.46	0.02	0.16	1.57	3	7.64	1.44
	0.12	0.99	0.41	2.40	0.02	0.16	1.82	6	13.56	1.72
Wind direction (°)										
	0.16	0.03	0.01	2.86	0.01	0.01	0.06	1	7.13	1.41
	0.09	0.02	0.01	2.15	0.04	0.00	0.04	2	5.00	1.14
	0.15	0.03	0.01	2.71	0.01	0.01	0.06	5	6.36	1.28
Wind speed (m/s)										
	0.12	−0.54	0.22	−2.41	0.02	−0.99	−0.09	5	4.80	0.95
	0.15	−0.62	0.23	−2.70	0.01	−1.09	−0.16	5	5.78	1.01
Multiple regression model (n = 1)										
Relative humidity (%)	0.33	−0.31	0.08	−4.03	0.00	−0.46	−0.15	1	9.60	1.23
Wind direction (°)		0.03	0.01	2.28	0.03	0.00	0.05			
No model selected (n = 39)	Not applicable							1-6	7.83	0.46

Table 2. Associations between free range use by individual Sasso chickens and different weather parameters.

									Individual free range use	
Weather parameter	R^2	Parameter estimate (r)	SE	t Value	Pr > |t|	95% Confidence	limits	Pen	Mean/day	SE
Simple linear regression models (n = 19)										
Atmospheric pressure (hPa)										
	0.09	0.93	0.46	2.05	0.05	0.01	1.85	8	8.36	1.64
	0.10	0.97	0.45	2.15	0.04	0.06	1.89	9	11.09	1.65
	0.19	0.49	0.19	2.43	0.02	0.07	0.90	10	1.21	0.88
	0.15	0.81	0.38	2.11	0.04	0.02	1.60	10	4.00	1.66
	0.16	0.71	0.33	2.17	0.04	0.04	1.39	10	6.43	1.44
	0.17	1.18	0.40	2.94	0.01	0.37	1.99	11	7.09	1.51
	0.11	1.01	0.44	2.32	0.03	0.13	1.90	11	9.82	1.60
Relative humidity (%)										
	0.14	−0.23	0.09	−2.57	0.01	−0.42	−0.05	8	12.16	1.60
	0.09	0.05	0.02	2.08	0.04	0.01	0.09	12	0.56	0.39
Temperature (°C)	Not applicable									
Wind direction (°)										
	0.11	−0.03	0.01	−2.31	0.03	−0.06	−0.01	8	6.84	1.51
	0.16	0.04	0.01	2.80	0.01	0.01	0.07	8	7.18	1.65
	0.21	0.04	0.01	3.38	0.01	0.02	0.06	8	6.36	1.38
	0.13	−0.02	0.01	−2.54	0.02	−0.03	−0.01	9	2.69	0.86
	0.12	0.03	0.01	2.44	0.02	0.01	0.05	9	10.13	1.43
	0.11	0.04	0.02	2.27	0.03	0.01	0.07	12	12.73	1.89
	0.20	0.03	0.01	3.27	0.01	0.01	0.06	12	7.76	1.31
Wind speed (m/s)										
	0.09	−0.71	0.35	−2.03	0.05	−1.40	−0.01	8	8.38	1.54
	0.11	−0.64	0.28	−2.31	0.03	−1.20	−0.08	11	7.40	1.25
	0.11	−0.51	0.23	−2.23	0.03	−0.97	−0.05	11	4.44	1.03
Multiple regression model (n = 2)										
Wind speed (m/s)	0.23	−0.82	0.34	−2.43	0.02	−1.51	−0.14	9	7.87	1.61
Atmospheric pressure (hPa)		0.91	0.42	2.16	0.04	0.06	1.75			
Relative humidity (%)	0.35	−0.25	0.09	−2.56	0.01	−0.45	−0.05	8	13.58	1.75
Wind speed (m/s)		−1.22	0.38	−3.24	0.00	−1.99	−0.46			
Wind direction (°)		0.04	0.03	3.05	0.00	0.01	0.04			
No model selected (n = 39)	Not applicable							7-12	5.12	0.69

parameters: negatively with relative humidity and positively with the wind direction, where the proportion of explained variance of the response variable by those weather parameters reached 33%.

Associations Between Weather Parameters and Range Use by Individual Sasso Chickens

The results of the simple and multiple regression models showing associations between range use by individual Sasso chicken and weather parameters are presented in Table 2. together with the mean frequency and standard deviation of the range usage by the birds. The significant associations of the range use with one basic weather parameter were identified for 19 birds, with 2 and 3 basic weather parameters each for 2 Sasso birds. No significant associations were identified between individual range use and weather parameters for the remaining 39 birds.

Both atmospheric pressure and wind direction were associated with range use of 7 birds. Atmospheric pressure was positively associated with range use (between 9 and 17% of variance explained), while range use was either negatively or positively associated with the wind direction (between 11 and 21% of response variable variance explained). In the case of three Sasso birds, wind speed was negatively associated with the range use frequency. Inconsistent associations between range use and relative humidity were found, as it was negative for one bird and positive for another bird. Moreover, the range use of one bird was associated with two weather parameters: negatively with wind speed and positively with the atmospheric pressure, where the proportion of explained variance reached 23%. In the case of one bird, association with three weather parameters was identified (relative humidity, wind speed and wind direction), which explained 35% of the range use variance.

DISCUSSION

A free-range systems provide animals with the choice when, where, and how they spend the time. Monitoring these choices can permit an understanding of what free-range broiler chickens want, which is an integral part of defining and safeguarding welfare (Dawkins, 2004). The current study was developed to answer a question on how range use of individual chickens is associated with the weather conditions. Following the undertaken approach of the individual bird' range use analysis, the birds' behavior was matched precisely in time with the weather parameters collected each minute. If individual birds in the same flock react differently to the weather conditions, it would make an important argument in the discussion regarding the need for the simultaneous use of various weather protecting elements on the range to promote range use.

Better understanding of chicken ranging behavior could help to improve management and range design, to ensure optimal ranging opportunities but also optimal productivity and welfare of the birds (Taylor et al., 2017). Previous studies indicated that broiler chicken ranging behavior is affected by the time of the day, weather variables (rainfall, direct sunlight, temperature, and wind speed) and resources on the range (e.g., trees and straw huts) (Dawkins et al., 2003; Nielsen et al., 2003; Jones et al., 2007; Rivera-Ferre et al. 2007; Stadig et al., 2017). However, how such parameters affect ranging patterns of individual broiler chickens has not been reported. The primary reason is that still limited technology is available that is noninvasive, reliable and feasible enough for long-term tracking an individual chicken's precise location, especially in outdoor conditions of a commercial farm (Siegford et al., 2016). However, focus on the individual ranging chickens, as compared to flock level behavior analysis, has recently proven to be very important. Recent investigations using methods of monitoring individual broiler chicken ranging behavior suggested that 75 to 95% of chickens in a flock accessed the range (Durali et al., 2014; Taylor et al., 2017), as compared to the 3% to 27% of birds in a flock accessing the range as noted during scan observations at the flock level (Rodriguez-Aurrekoetxea et al., 2014; Fanatico et al., 2016).

The choice of Green-legged Partridge or Sasso chicken breeds in the current experiment allowed us to minimize the risk of birds not using the ranges due to poor health reasons, for instance mobility issues. Results from the present study confirmed low occurrence of such health issues (see Marchewka et al., 2020).

The proportion of variance explained by the weather parameters in range use ranged between 9% and 35%. Even though such levels of variance explained may not be considered important in predictive type of studies, they may be considered as a meaningful part of variance in associative studies such as the current one (Pedhazur, 1997).

Associations of birds' range use with weather conditions were distributed across all recorded weather parameters, however differently for Green-legged Partridges and Sasso. The weather parameter that the range use of Green-legged Partridge birds was most often associated with was relative humidity outdoor. The association was negative, which is in agreement with previous studies in layers, where more laying hens ranged away from the shed when the relative humidity level was low, i.e. on cooler days and with no rainfall (Gilani et al., 2014), while use of the outdoor areas was reduced in wet weather (Mirabito and Lubac, 2001; Hegelund et al., 2005; Gilani et al., 2014). Broilers tend to avoid wetting the feathers, which decrease their thermal comfort and requires higher time investments in preening (Huber-Eicher and Wechsler, 1998).

Such negative association of ranging with relative humidity was identified only for one Sasso bird, indicating higher resilience of those birds to this condition. Sasso has been described as having the genetic potential of tropically adapted birds (Yakubu et al., 2018), where the hot season in the tropics is characterized by periods of high temperatures and high relative humidity, which can be compared to some extent to the weather conditions in August and September of 2018 in Poland, especially in the mornings. The large combs of Sasso birds were suggested to be an adaptive feature that might function as a biological heat exchanger (Yakubu et al., 2018), facilitating evaporative cooling of the brain; a feature to maintain thermal homeostasis when birds are exposed to high environmental temperature and relative humidity (Gerken et al., 2006).

Ranging behavior of seven Sasso birds was positively associated with the atmospheric pressure, as compared to three Green-legged Partridges. It is well known that birds can sense changes in barometric pressure (Paige, 1995). Higher atmospheric pressure in moderate warm climate, as in Poland, is usually associated with no precipitation and weak wind conditions, which are preferred by the chickens, as opposed to humid and windy weather (Nielsen et al., 2003; Jones et al., 2007). Nevertheless, the exact explanation as to why Sasso would be more sensitive to atmospheric pressure in relation to ranging behavior remains unclear.

In the current experiment, we identified that ranging behavior of ten birds was associated with wind direction, out of which seven birds were Sasso and three were Green-legged Partridges. Moreover, in five other birds, three Sasso and two Green-legged Partridges, ranging was associated with the wind speed. Wind is a complex atmospheric phenomenon, which affects animals in many dimensions. In flying birds, the light and often variable winds enable migrant birds to fly with little risk of drift from its preferred heading or track (Van Doren et al., 2016). Wind, especially strong or gusty, can distract bird's vigilance, as the amount of stimuli in the background increases, which can cause birds to feel more endangered by predators and look for shelter or even stay indoors (Nicol, 2015). In our experiment, the range use of the Green-legged Partridges increased when the wind blew from the SSW, SW, WSW and W directions. In the Polish climate, such wind directions are related to mostly mild and warm air blows, but also characterized by low speed which seemed favorable by the Green-legged Partridges

Range use of three Green-legged Partridges was positively associated with air temperature, while surprisingly this association was not observed for any of the Sasso birds. Air temperature higher than 26°C has been described as unfavorable for the activity and comfort of domestic poultry (Etches et al., 2008; Mignon-Grasteau et al., 2015). During the current experiment, the outdoor air temperature at the observation time points did not exceed maximum of 28°C degrees, however the average air temperature measured at the behavioral observation points was 19.6 $\pm$ 0.6°C, which is within known poultry thermal comfort range for birds of that age (Pereira and Naas, 2008). Higher air temperature is often associated with more sunshine. In broilers outdoor shelter effectively encouraged chickens to use the range area under increasing solar radiation (Stadig et al., 2017). If no shelter would be present and birds' attempts to seek shady areas to cool down were unsuccessful, it may result in birds remaining indoors (Stadig et al., 2017). Therefore, we may assume, based on the current results, that ranging behavior of the three Green-legged Partridge chickens positively associated with the air temperature indicated that individuals may have variable thermal preferences, probably as long as the upper level of the thermal comfort zone is not exceeded. Moreover, as stated above, Sasso may overall be more adapted to higher air temperatures, due to their genetic makeup (Gerken et al., 2006).

For three birds in the current study multiple regression models were identified, which could provide some preliminary indications regarding weather parameters, which combined are assuring the thermal comfort of ranging chickens. Range use of one Green-legged Partridge chicken was negatively associated with relative humidity and positively with the wind direction. In Sasso, one model included wind speed and atmospheric pressure, while another one included relative humidity, wind speed and wind direction. Those models explained between 23% up to 35% of the variance in response variable. Wind is air pressure converted into movement of air. When air slows down, its pressure increases (Tweel and Turner, 2014). Even though those two measurements in the current study were not correlated when selecting the dependent variables to be tested by regression analysis, interpretation of this multiple regression model should be done with caution.

The birds' choice to venture outside may have been instigated by either positive or negative motivation. For instance, chickens may access the outdoor range to explore a more complex environment than the typical indoor shed environment, but, on the other hand, they may try to avoid negative uncomfortable, frightening, or painful stimuli, either in the shed or outdoors (Taylor et al., 2017). The ranging behavior of the two third of the birds in this experiment was not associated, either negatively or positively with the collected weather parameters. Surprisingly, in both breeds this proportion of individuals was the same. Interestingly, these individuals used the ranging areas on a similar frequency level as the remaining birds in the experiment. Understanding the motivations behind the ranging behavior of this group of birds, for instance reactions to contrast in climate between indoor and outdoor environment, requires further investigations, with consideration of other types of underlying factors than weather conditions, such as birds health status, indoor housing environment, birds stocking density or group size.

Producers have only limited possibilities to reduce the impact of weather conditions on chickens reared in low-input systems with range access, while optimizing their range use. However, certain structures such as wind, rain or humidity protections can be applied on the outdoor areas. Positive effects of motivating more birds to use the areas away from the house by providing natural and artificial wind protections and covers have been shown in layers (Zeltner and Hirt, 2003). Furthermore, it has been found that planting shrubs, trees or using other forms of shading improved the use of ranges by chickens, by protecting them against sun and predators (Stadig et al., 2017). As a step to convince free-range meat-purpose chicken producers to implement strategies encouraging their birds to use the outdoor areas more, we have presented evidence that birds react individually to the same weather conditions. Therefore, we suggest designing ranging areas such that they accommodate individual preferences/needs, for example, by including provision of multiple construction and vegetation elements.

In conclusion, we found significant associations between different weather parameters and the individual use of the ranges for approximately one third of Green-legged Partridge and Sasso chickens. Between breeds, the associations to the particular weather parameters were different, with relative humidity occurring most frequently in Green-legged Partridges, while air pressure and wind direction were most common in Sasso. Further investigations into the reason behind increased sensitivity of some commercial and heritage meat-purpose chickens to particular weather conditions would be beneficial for a better understanding of their needs, which over time will improve animal welfare.

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DISCLOSURES

The authors have no conflicts of interest to disclose.

REFERENCES

Biala, K., J. M. Terres, P. Pointereau, and M. L. Paracchini. 2007. Low input farming systems: an opportunity to develop sustainable agriculture. Proceedings of the JRC Summer University Ranco. 2−5.

Dawkins, M. S. 2004. Using behaviour to assess animal welfare. Anim. Welf. 13:3–7.

Dawkins, M. S., P. A. Cook, M. J. Whittingham, K. A. Mansell, and A. E. Harper. 2003. What makes free-range broiler chickens’ range? In situ measurement of habitat preference. Anim. Behav. 66:151–160.

Dec, E., B. Babiarz, and R. Sekret. 2018. Analysis of temperature, air humidity and wind conditions for the needs of outdoor thermal comfort. 10th Conference on Interdisciplinary Problems in Environmental Protection and Engineering EKO-DOK 2018, Polanica-Zdrój, Poland; E3S Web of Conferences, Volume 44, id.00028.

Durali, T., P. Groves, A. Cowieson, and M. Singh. 2014. Evaluating range usage of commercial free-range broilers and its effect on bird's performance using radio frequency identification (RFID) techology. Page 103 in Proceedings of the 25th Annual Australian Poultry Science Symposium, Sydney, Australia, 16−19 February 2014.

Erian, I., and C. J. Phillips. 2017. Public understanding and attitudes towards meat chicken production and relations to consumption. Animals 7:20.

Etches, R. J., T. M. John, and A. M. Gibbins. 2008. Behavioural, physiological, neuroendocrine and molecular responses to heat stress. Pages 48−79 in Poultry Production in Hot Climates. 2nd ed. N. J. Daghir, ed. CAB International, Wallingford, UK.

EU. 2007. Council Directive 834/2007 on organic production and labelling of organic products. Off. J. Eur. Communities L. 189:1–23.

EU. 2008. Commission regulation (EC) no 889/2008 of 5 September 2008 laying down detailed rules for the implementation of council regulation (EC) no 834/2007 on organic production and labelling of organic products with regard to organic production, labelling and co. Off. J. Eur. Union L. 250:1–84.

Fanatico, A. C., J. A. Mench, G. S. Archer, Y. Liang, V. B. B. Gunsaulis, C. M. Owens, and A. M. Donoghue. 2016. Effect of outdoor structural enrichments on the performance, use of range area, and behavior of organic meat chickens. Poult. Sci. 95:1980–1988.

FAO. 2007. Family farming knowledge platform. Fact sheet: low input farming systems. Accessed March 2021. http://www.fao.org/family-farming/detail/en/c/1115210/.

Gerken, M., R. Afnan, and J. Dörl. 2006. Adaptive behaviour in chickens in relation to thermoregulation. Arch. Gefl. Ügelk. 70:199–207.

Getiso, A., B. Bekele, B. Zeleke, D. Gebriel, A. Tadesse, E. Abreham, and H. Jema. 2017. Production performance of Sasso (distributed by ethio-chicken private poultry farms) and Bovans brown chickens breed under village production system in three agro-ecologies of Southern Nations, Nationalities, and Peoples Regional State (SNNPR), Ethiopia. Int. J. Livest. Prod. 8:145–157.

Gilani, A. M., T. G. Knowles, and C. J. Nicol. 2014. Factors affecting ranging behaviour in young and adult laying hens. Br. Poult. Sci. 55:127–135.

Hegelund, L., J. T. Sørensen, J. B. Kjær, and I. S. Kristensen. 2005. Use of the range area in organic egg production systems: effect of climatic factors, flock size, age and artificial cover. Br. Poult. Sci. 46:1–8.

Huber-Eicher, B., and B. Wechsler. 1998. The effect of quality and availability of foraging materialson feather pecking in laying hen chicks. Appl. Anim. Behav. Sci. 55:861–873.

Jones, T., R. Feber, G. Hemery, P. Cook, K. James, C. Lamberth, and M. Dawkins. 2007. Welfare and environmental benefits of integrating commercially viable free-range broiler chickens into newly planted woodland: a UK case study. Agric. Syst. 94:177–188.

Lima, A., and I. Nääs. 2005. Evaluating two systems of poultry production: conventional and free-range. Br. J. Poult. Sci. 7:215–220.

Marchewka, J., T. T. N. Watanabe, V. Ferrante, and I. Estevez. 2013. Welfare assessment in broiler farms: transect walks versus individual scoring. Poult. Sci. 92:2588–2599.

Marchewka, J., P. Sztandarski, Ż. Zdanowska-Sąsiadek, K. Damaziak, F. Wojciechowski, A. B. Riber, and S. Gunnarsson. 2020. Associations between welfare and ranging profile in free-range commercial and heritage meat-purpose chickens (Gallus gallus domesticus). Poult. Sci. 99:4141–4152.

Mignon-Grasteau, S., U. Moreri, A. Narcy, X. Rousseau, T. B. Rodenburg, M. Tixier-Boichard, and T. Zerjal. 2015. Robustness to chronic heat stress in laying hens: a meta-analysis. Poult. Sci. 94:586–600.

Mirabito, L., and S. Lubac. 2001. Descriptive study of outdoor run occupation by'Red Label'type chickens. Br. Poult. Sci. 42:16–17.

Nielsen, B. L., M. G. Thomsen, P. Sorensen, and J. F. Young. 2003. Feed and strain effects on the use of outdoor areas by broilers. Br. Poult. Sci. 44:161–169.

Nicol, C. 2015. The Behavioural Biology of Chickens. CABI, Wallingford, UK.

Paige, K. N. 1995. Bats and barometric pressure: conserving limited energy and tracking insects from the roost. Funct. Ecol. 463–467.

Pedhazur, E. J. 1997. Multiple Regression in Behavioral Research. 3rd. ed. Harcourt Brace College Publishers, Fort Worth, TX.

Pereira, D. F., and I. Naas. 2008. Estimating the thermoneutral zone for broiler breeders using behavioral analysis. Comput. Electron. Agr. 62:2–7.

Rivera-Ferre, M. G., E. A. Lantinga, and R. P. Kwakkel. 2007. Herbage intake and use of outdoor area by organic broilers: effects of vegetation type and shelter addition. NJAS-Wagening. J. Life Sci. 54:279–291.

Rodriguez-Aurrekoetxea, A., E. H. Leone, and I. Estevez. 2014. Environmental complexity and use of space in slow growing free-range chickens. Appl. Anim. Behav. Sci. 161:86–94.

Sanchez, C., and I. Estevez. 1998. The Chickitizer Software Program. University of Maryland, College Park, MD.

Santos, M. J. B., P. Heliton, C. B. V. Rabello, S. Edney, T. Thaysa, P. A. Santos, W. B. Morril, and N. M Duarte. 2014. Performance of free-range chickens reared in production modules enriched with shade net and perches. Rev. Bras. Cienc. Avic. 16:19–27.

Siegford, J. M., J. Berezowski, S. K. Biswas, C. L. Daigle, S. G. Gebhardt-Henrich, C. E. Hernandez, S. Thurner, and M. J. Toscano. 2016. Assessing activity and location of individual laying hens in large groups using modern technology. Animals 6:10.

Silva da, M. AN., P. H. Filho, M. F. Rosário, A. A. Domingos Coelho, V. J. M. Savino, A. A. Franco Garcia, I. J. Oliveira da Silva, and J. F. Machado Menten. 2003. Influência do sistema de criação sobre o desempenho, a condição fisiológica e o comportamento de linhagens de frangos para corte. Rev. Bras. Cienc. Avic. 32:208–213.

Siwek, M., D. Wragg, A. Sławińska, M. Malek, O. Hanotte, and J. M. Mwacharo. 2013. Insights into the genetic history of Green-legged Partridge Like fowl: mt DNA and genome-wide SNP analysis. Anim. Genet. 44:522–532.

Stadig, L., T. Rodenburg, B. Ampe, B. Reubens, and F. Tuyttens. 2017. Effects of shelter type, early environmental enrichment and weather conditions on free-range behaviour of slow-growing broiler chickens. Animal 11:1046–1053.

Taylor, P., P. Hemsworth, P. Groves, S. Gebhardt-Henrich, and J. L. Rault. 2017. Ranging behaviour of commercial free-range broiler chickens 1: factors related to flock variability. Animals 7:54.

Tweel, A., and R. Turner. 2014. Contribution of tropical cyclones to the sediment budget for coastal wetlands in Louisiana, USA. Landsc. Ecol. 20:273–287.

Van Doren, B. M., K. G. Horton, P. M. Stepanian, D. S. Mizrahi, and A. Farnsworth. 2016. Wind drift explains the reoriented morning flights of songbirds. Behav. Ecol. 27:1122–1131.

Yakubu, A., E. Ekpo, and O. Oluremi. 2018. Physiological adaptation of Sasso laying hens to the Hot-Dry tropical conditions, agric. Conspec. Sci. 83:187–193.

Zeltner, E., and H. Hirt. 2003. Effect of artificial structuring on the use of laying hen runs in a free-range system. Br. Poult. Sci. 44:533–537.

Zuidhof, M. J., B. L. Schneider, V. L. Carney, D. R. Korver, and F. E. Robinson. 2014. Growth, efficiency, and yield of commercial broilers from 1957, 1978, and 2005. Poult. Sci. 93:2970–2982.

Associations between neck plumage and beak darkness, as well as comb size measurements and scores with ranging frequency of Sasso and Green-legged Partridge chickens

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ABSTRACT Despite the intensive genetic selection in modern poultry, variability of domestic fowl phenotypes has remained, especially in breeds adapted to local conditions. The relevance of this variability to the chicken outdoor ranging activities remains unknown. The aim of this study was to investigate if external features were associated with the ranging frequency of the 48 female chickens from each of the 2 breeds: Sasso and Green-legged Partridge. In each of 6 single-breed pens, 8 hens and 2 roosters were housed under conditions of EU requirements for organic meat chicken production, including free access to an outdoor range, from wk 5 to 10 of age. The birds were video-recorded during the experiment to obtain frequencies of individual birds' use of the ranges. Comb size (length and height) was measured using a digital ruler, while the sizes of the dark area of neck plumage and beak were processed and analyzed using ImageJ software. The same traits were scored using direct visual assessment by a trained observer on a scale of 1-3. In addition, the eye color of the bird was recorded. Statistical analysis was conducted independently for each breed using regression models, ANOVAs and Spearman correlations. Significant positive associations between neck plumage ($P < 0.01$), beak darkness ($P = 0.03$) measurements, comb length ($P < 0.01$) and comb height ($P < 0.01$) and frequency of range use were identified for Sasso. Sasso hens scored with darkest neck plumage ($P = 0.03$) and biggest comb size ($P = 0.04$) ranged the most, while their external features were significantly and positively correlated between each other, except beak darkness and comb length. No significant associations between ranging and external features were found in Green-legged Partridge birds, except that their comb height was significantly and positively correlated with neck plumage and beak darkness ($r = 0.39$ and 0.33, respectively). In some genetic strains, better understanding of the associations between chickens' external features with ranging behavior could contribute to improve selection programs and bird welfare, assuring production of breeding stock suitable for outdoor conditions.

Key words: external traits, organic, phenotype, broiler, ranging behavior

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INTRODUCTION

In wild animals, phenotype traits are prominent characteristics of an individual that are essential for its survival due to for example, aposematism (Ruxton et al., 2018), species recognition (Santana et al., 2012), and sexual selection (Andersson, 1994). Farm animals have been genetically selected for productivity. The strong selection pressure has affected their phenotypes (Johnsson et al., 2012), and resulted in animals within the same breed or genetic strain being largely homogenous (FAO - Food and Agriculture Organization, 2000). However, some variability of external features in the production animals' phenotypes has remained.

In conventional broiler production systems, birds are reared in strictly controlled indoor conditions (Lima and Nääs, 2005). Increased public concern of animal welfare in those systems (Marchewka et al., 2013), including decreased ability of the birds to express natural behaviors, has increased consumers' demand for meat from poultry reared in less intense systems (Erian and Phillips, 2017). Those systems are characterized by longer production cycles, where the chickens from slow-growing

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breeds or hybrids may develop sexual dimorphism, including adult comb size, plumage and beak coloration. Moreover, in some less intensive systems, for example, organic systems in EU, birds are provided with ranging area (EU, 2007, 2008). Use of ranging area by broiler chickens is not always optimal, and differences exist not only on the flock or breed level, but also between individual birds in the same flock, even if equal opportunity of access to the range is provided (Dawkins et al., 2003; Taylor et al., 2017).

Domestic chickens are particularly interesting for studies testing the links between phenotype and behavior because, as although maintained in captivity, they have retained many of the behavioral characteristics and social structure of their Asian ancestor, red jungle fowl (*Gallus gallus*) (Navara et al., 2012). The majority of studies have focused on roosters, as they exhibit dramatic and conspicuous sexual signals (Sheldon, 1994) and hens are known to choose males based on a composite assessment of multiple secondary sexual characteristics, including bright red combs and wattles, hackle color, and mating behaviors, such as waltzing (Zuk et al., 1990; Johnsen et al., 1995). However, a variability in phenotypic external features can be observed in hens as well. Although it has not been investigated as frequent as in males, the feather distribution and color of the beak or eyes have been shown to be linked to ranging propensity, particularly in hens of some breeds (Al-Atiyat et al., 2017). Nevertheless, such associations have previously not been investigated, neither in Sasso nor in Green-legged Partridge birds.

One of variable phenotypic external features is iris color, often referred to in animals, including chicken, as eye color (Nelson, 1947). The iris of the eye primarily controls the amount of light that enters the eye, by varying the size of the pupil opening. However, variation in iris color is caused by either presence or absence of different types of pigmentation such as melanin, pteridines, and purines, as well as superficial blood vessels and/or eye structure, irrespective of pigmentation (Waldvogel, 1990). Phenotypic eye color has been suggested as an indicator of genetic predisposition toward certain behaviors, where dark-eyed subjects would tend to display behaviors requiring sensitivity, speed, and reactive responses, while with ones with light-colored eyes, behaviors requiring hesitation, inhibition, and self-paced responses, both in humans and in animals (Elias et al., 2008). Furthermore, it has been proposed that eye coloration in various species may be related to social ranks, aggression, mate recognition, and sexual selection (e.g., Volpato et al., 2003, Amat et al., 2013). Chicken eye color is largely determined by genetics, but age, diet, and disease can affect it as well (Nelson, 1947). However, to our knowledge no studies have investigated the link of the eye color with the behavior of chickens in free-range systems.

Some chicken behaviors have been found to be associated with external features like plumage coloration (Volpato et al., 2003; Keeling et al. 2004; Nätt et al. 2007). Individuals with the dark colored wild-type i/i PMEL17 gene version showed higher level of vocal-based social reinstatement behavior under open-field conditions than white colored I/I gene birds (Nätt et al. 2007), which was suggested to be associated with prelaying anxiety (Freire et al. 1997), suggesting that I/I females are more motivated to find a nesting place or they are more uncomfortable in the prelaying phase. Keeling et al. (2004) observed that wild type coloration birds victimization to feather pecking increased in flocks with increased numbers of wild-type homozygous (i/i) relative to white homozygous mutant (I/I) individuals. Thus, results have already demonstrated that PMEL17 genotype responsible for plumage coloration affects several behavioral patterns but further studies are needed to explore a wider spectrum, including ranging behavior.

As an alternative to dominance establishment by aggression, some studies have found that chickens use comb sizes as a signal of status or fighting ability in the formation of hierarchies, avoiding costly and stressful contests (Cloutier et al., 1996; Pagel and Dawkins, 1997; Campo et al., 2009). In broilers reared in conventional production systems, the comb is involved in heat regulation, and therefore may also assist in survival in crowded intensive production conditions (van Kampen, 1971). However, in broiler chickens selected for extensive production systems, including those with range access, survival in crowded conditions is not a prioritized selection trait. Therefore, it is of interest how the comb size is associated to a prioritized trait in the rearing systems with outdoor access, which is range use.

Indigenous or free-range chickens have variable plumage and biometrical traits representing genes of adaptation to their own environment (Al-Atiyat et al., 2017). Free-range chicken breeds are often classified as gene reservoirs reflecting unique adaptation to their agro-ecological environments (Horst, 1989). The adaptive genes of chickens to the use of free-range can be either precisely measured or visually recognized and scored. Time consuming and manpower demanding measurements of such traits could be included in selection programs for improvement of bird welfare, as it could assure production of breeding stock adapted for the outdoor environmental conditions. Qualitative and subjective scoring are additional approaches to assess animal visual traits. Such indicators can be collected on a large scale and incorporated into livestock breeding schemes to enhance animal welfare and overall resilience (Marchant-Forde, 2015). Moreover, practical on-farm scoring of external features could help producers identifying individuals, which potentially do not use the outdoor ranges to the extent expected, such that corrective appropriate flock management strategies can be implemented.

The current study aimed to investigate, if neck plumage and beak darkness, as well as comb size were associated with the ranging frequency of the hens from 2 breeds: Sasso hybrid and heritage Green-legged Partridge chickens. We hypothesized that in Sasso and in Green-legged Partridge hens comb size, proportion of dark feathers on the neck and beak darkness will be positively associated to their range use. We furthermore aimed to confirm potential associations of ranging frequency of Sasso and Green-legged Partridge hens with the above listed external features evaluated by practical scoring based on visual assessment and determination of eye color. We hypothesized more range use in birds of both breeds scored highest with regard to the external features. If the visual traits proved to be associated to the birds' range use, the correlations between measured external features in each breed would allow identifying the set of the visual characteristics of the birds frequently using the range. Therefore, we aimed to identify correlations between measurements of the hens comb size, proportion of the dark feathers on the neck, and beak darkness.

MATERIALS AND METHODS

The experiment took place in the Mazovian region of Poland, at the experimental farm of Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences, in August and September of 2018. The experimental procedures followed standard production methods in the EU organic broiler chicken production system. No invasive manipulations requiring local ethical commission permission were applied to the birds in the current study.

Animals, Housing and Management

Forty-eight, nonbeak trimmed chickens, of each of 2 breeds (total n = 96 birds), Green-legged Partridge and Sasso line C44 were used in the experiment. Green-legged Partridge chickens are the indigenous Polish breed of heritage chicken (Siwek et al., 2013), available only in the partridge color-variety, in which the hens are buff-brown. The slow growing, multicolored broiler chicken hybrid Sasso is widely and successfully used in commercial production across the globe (Hendrix Genetics BV and Sasso, Boxmeer, The Netherlands) and has been especially well adapted to European continental climate.

Until wk 5 of age, 200 birds were reared only indoors in the experimental facility in one common littered pen (5 m × 10 m) with 17 cm/bird perching space provided, automatic feeders and drinkers, providing feed and water ad libitum, and natural light. The climate conditions were controlled automatically and infrared heating lamps were used. At the age of 5 wk, 60 individuals with similar body weight within each breed (on average 2030.6 ± 68.9 g for Sasso and 705.9 ± 8.5 g for Green-legged Partridge), were selected and relocated from their rearing facilities to the experimental house, both at the same farm location. Eight female and 2 male chickens were assigned to each single breed group housed in 12 pens (6/breed) until 10 wk of age. In the current study, only hens were investigated. The size of the indoor pens was 2.5 m × 3.5 m, resulting in a stocking density at slaughter age of 1.4 kg/m^2 for Green-legged Partridge and 2.7 kg/m^2 for Sasso. Birds were housed on sawdust litter, while in each pen, next to the wall, there was a 0.5 m strip covered with sand. Pens were cleaned according to need. Each pen contained two 80 cm long wooden perches with 2 perching levels, one at the height of 15 cm and the second at 40 cm. The perching poles were 50 × 50 mm thick and had rounded edges. Each pen had direct access through the pophole (H × W: 45 cm × 50 cm) to an individual outdoor range (3.5 m × 30 m) providing 10.5 m^2/chicken. All the outdoor ranges had equal vegetation coverage regarding botanical composition, while no trees or shelters were present. The grass was mowed 1 wk before the onset of the experiment. Each ranging area was provided a semiautomatic bell drinker and a wooden box (1 m × 1 m) filled with sand. Additional information about the experimental facilities can be found in Marchewka et al. (2020).

The birds were habituated for 48 h to the new housing after relocation from the rearing facilities to the experimental house. After the habituation period, the popholes were opened (daily from 7.00 until 19.00 h). To allow for individual birds' recognition, all birds were fitted with a laminated paper mark of the size of 9 cm high and 7 cm wide attached to the birds' back by fitting 2 elastic bands around its wings. Ten different colors of the marks were assigned in each pen randomly to the individual birds. Birds were equipped with their color mark during the entire experiment. Birds were inspected twice a day. Commercial pelleted feed (Marchewka et al., 2020) and water were available ad libitum. No coccidiostats or other medications were used. No birds died during the experiment.

Birds were provided only natural light through uncovered windows. Light hours during the experimental period ranged from 12.7 h to 15.7 h/d. There was natural ventilation in the building.

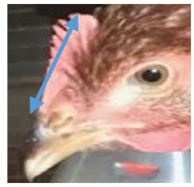
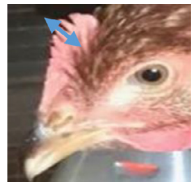
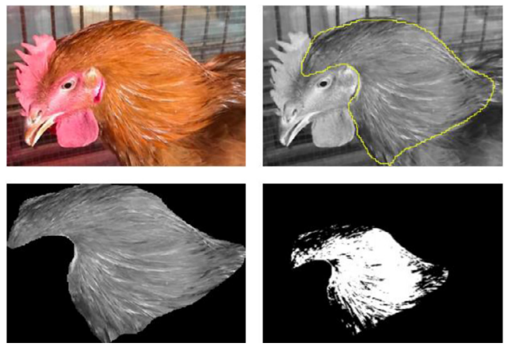
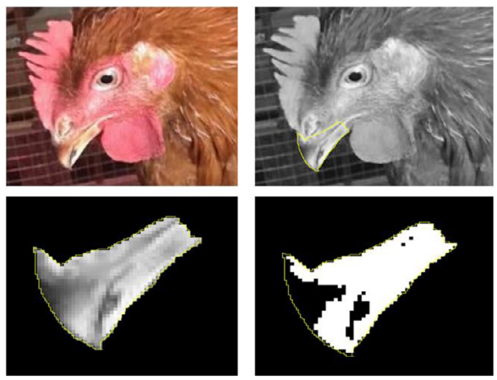
Observations of Ranging Behavior

Ranging behavior of the birds was recorded using video cameras. The 12 outdoor pens were video-recorded simultaneously and continuously using 6 cameras (BCS-DMIP2401IR-M-IV IP 4 Mpix), each covering 2 ranging areas. The films were automatically saved on the network recorder (BCS-NVR0401-IP 4 channel BC). Video material was analyzed and bird behaviors were recorded by the same trained and experienced person, using scan sampling and the Chickitizer program (Sanchez and Estevez, 1998). From the recorded videos, 3 d were chosen per week of experiment (5 wk). On each of those days, 3 times of the day (morning [starting at 8:00], noon [starting at 13:00], and evening [starting at 18:00]), a 3-min period with 10-s sampling intervals was set and repeated after 10 min. The observer recorded each of the experimental birds' absence as "0" or presence as "1″ in the outdoor area.

Measurements (Quantitative Assessment)

The direct measurements of the external features (Table 1) of each individual bird were taken the day

Table 1. Methods and pictorial examples of measurements of the comb length and height, neck plumage, and beak darkness conducted both in Sasso and Green-legged Partridge chickens.

Measurement	Method	Example
Comb length (mm)	Digital ruller	
Comb height (mm)		
Neck plumage darkness (%)	Photograph and ImageJ software	
Beak darkness (%)		

before the end of the experiment. There were 3 persons involved in the measurements, each assigned with a different task: 1) identifying (indicated by the color tag) and catching the birds, 2) measuring the comb size using the method described below, and 3) noting the collected information in a spreadsheet and taking a digital picture of the whole body of each bird from the left side. Comb size was measured, using a digital ruler LCD (Kraft&-Dele, Koteze, Poland), in the highest (from where the comb met the head to the top of the highest spike) and longest place (from end to end) for each individual bird. From the photos taken, the beak coloring was calculated using ImageJ software (Schneider et al., 2012). Each image of an individual bird was imported to ImageJ software, where the area of the beak was contoured and cropped from the whole image. The cropped-out area was binarized, collapsing the 256 color levels to 2 color levels, while adjusting the grayscale using the automatic thresholding method “AutoLocalThreshold”, as a plugin to ImageJ software. This plugin binarised 8-bit image using thresholding method that can deal with unevenly illuminated images. The threshold was computed for each pixel according to the image characterizing within a window of radius r (in pixel units) around it. The segmented phase was always shown as white (255, as the maximum gray level). After thresholding, the dark area was calculated and deduced from the total area of interest providing white area size. The proportion of black to white area measurements ratio was calculated and expressed as a percentage. The same method using ImageJ software was applied to the second identical copy of the individual chicken photo to calculate neck plumage coloring, that is, the percentage of dark plumage on the neck, which was defined as the area between the head and the trunk of the bird (Table 1).

Scores (Qualitative Assessment)

After taking the comb measurements and a bird photograph, each bird was handed into a 2-person team, where one person held the bird and the other, based on visual assessment, scored the bird for 3 external features: comb size, neck plumage darkness, and beak darkness, all on a 3-point scale (1-3) within breed. Definitions and examples for each score of each feature in either of the breeds are presented in Table 2.

Statistical Analysis

All the statistical analyses were conducted using software package SAS version 9.4 (SAS, Cary, NC). In each of the simple regression models, the variable describing either the individual Green-legged Partridge or Sasso chicken range use (summed over all observations frequencies of the presences in the outdoor area) was considered as the dependent outcome variable, while each chicken external feature measurement was considered as the independent variable. The outcome variable was analyzed for its association with each independent variable. The outcome variable was normally distributed across the sample population, thus linear univariate regression was used. Furthermore, the residuals were predicted and checked for normality. Residuals were predicted and plotted in normal quantile plots and coefficients of determination (R^2) were calculated.

Independent one-way ANOVAs were performed, separately for Sasso and Green-legged Partridges, using the PROC GLIMMIX procedure. Each model included different chicken external feature scored as "1", "2", or "3" as a fixed factor. However, an independent two-way ANOVA was conducted in the same software package for the model including eye color, as both eye color and breed were added as fixed factors as well as their interaction. Pen was included in the model as the random factor. Least Square Means (**LSM**) differences were adjusted for multiple comparisons using the posthoc Tukey test.

Spearman correlations were calculated using the PROC CORR script for each breed separately to test the relationships between measured external features.

RESULTS

Measurements

The results of the simple regression models showing associations between range use by either Sasso or Green-legged Partridge hens and their external features are presented in Table 3. In Sasso hens, significant and positive associations between the range use frequency and comb length and height as well as neck plumage darkness and beak darkness were identified. The proportion of explained variance of the response variable ranged from 18% for beak darkness up to 33% in case of neck plumage darkness. No significant associations between the range use frequency and external features were identified for Green-legged Partridge hens.

Visual Assessment

Significant effects of external features as assessed by scoring were identified in Sasso hens for neck plumage darkness ($P = 0.03$) and comb size ($P = 0.04$), as presented in Table 4. For both features, birds scored the highest used the ranging areas more frequently as compared to birds presenting the lowest score. Moreover, a trend ($P = 0.06$) for an effect of beak darkness on the range areas use frequency was identified. No significant effect of any of the external features on the range use was identified for Green-legged Partridges.

Eye Color

There was a significant breed by eye color interaction effect on the range use of the hens ($F = 4.40$; $P = 0.04$) in the current study (Figure 1). Sasso hens with gray eye color used the ranges significantly less frequently, as compared to Green-legged Partridges with either brown or gray eyes.

Correlations

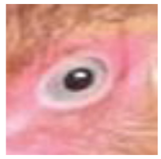
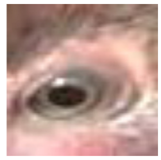
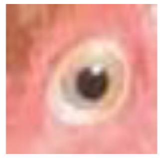
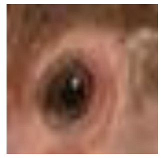
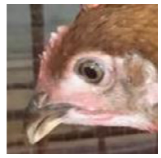
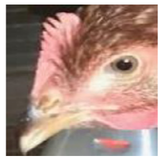
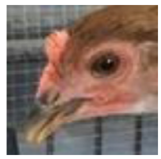
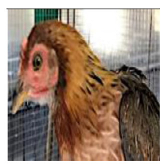
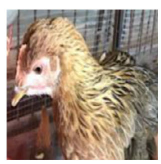
Correlations between external features were identified within each breed (Table 5). In Sasso hens, all external features were significantly and positively correlated between each other, with the exception that no significant correlation was identified between beak darkness and comb length. The strongest positive correlation ($r = 0.85$) was identified between comb length and comb height.

Among Green-legged Partridges, fewer and weaker correlations were identified as compared to Sasso hens (Table 5). Similarly to Sasso hens, the strongest positive correlation was identified between comb length and comb height ($r = 0.55$). Moreover, comb height was significantly and positively correlated with neck plumage darkness and beak darkness ($r = 0.39$ and 0.33, respectively).

DISCUSSION

The current study aimed to investigate, if neck plumage and beak darkness, as well as comb size measurements were associated with the ranging frequency of the female chickens of 2 breeds: Sasso hybrid and heritage Green-legged Partridge. Moreover, we aimed to test if

Table 2. Definitions and pictorial examples for each score of visually assessed external features: eye color, comb size, neck plumage, and beak darkness in Sasso and Green-legged Partridge chickens.

External feature		Breed	
Eye color	Definition	Sasso	Green-legged Partridge
Grey			
Brown			
Comb size score			
1	Very small comb, not much raised from the head		
2	Medium size comb, raised from the head, the height of the tallest spike was not larger than the distance from the eye to the middle of the comb base		
3	Large, marked comb, raised from the head, the height of the tallest spike was larger than the distance from the eye to the middle of the comb base		
Neck plumage coloration score			
1	No or very few dark feathers present (0−5 dark feathers)		
2	Some dark feathers present (6−10 dark feathers)		
3	Dark feathers present (more than 10 dark feathers)		

(continued)

Table 2 (*Continued*)

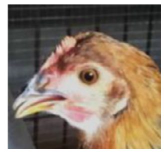
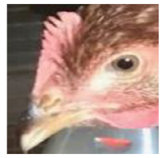
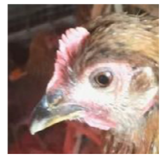
External feature		Breed	
Eye color	Definition	Sasso	Green-legged Partridge
Beak coloration score			
1	No or very small dark area on the beak (<10%)		
2	From 10% up to 50% of the beak area was dark		
3	Majority of the beak was dark (>50%)		

Table 3. Associations between range use and comb length, comb height, neck plumage darkness, and beak darkness measurements in Sasso and Green-legged Partridge, respectively.

		Ranging activity						
Breed	External feature (measurement)	R^2	Parameter estimate (r)	SE	t value	Pr > |t|	95% confidence Limits	
Sasso								
	Comb length (mm)	0.27	13.35	4.52	2.95	0.0069	4.02	22.69
	Comb height (mm)	0.28	21.31	7.05	3.02	0.0059	6.77	35.86
	Neck plumage darkness (%)	0.33	12.76	3.72	3.43	0.0022	5.08	20.44
	Beak darkness (%)	0.18	3.03	1.33	2.28	0.032	0.28	5.77
Green-legged Partridge								
	Comb length (mm)	0.02	3.00	3.53	0.85	0.3989	-4.10	10.12
	Comb height (mm)	0.001	1.09	5.20	0.21	0.8352	-9.38	11.56
	Neck plumage darkness (%)	0.0006	0.15	0.88	0.17	0.8658	-1.63	1.93
	Beak darkness (%)	0.0085	1.00	1.59	0.63	0.5335	-2.21	4.20

such potential associations could be identified by more practical visual assessment, suitable under production conditions, of the comb size and darkness of neck plumage and beak. We also investigated the eye color and breed effect on the range use frequency of the birds. This is important, as increased use of the range area has been found to be positively associated with welfare of the ranging chickens (Taylor et al., 2020). Furthermore, birds that more often used outdoor areas had potentially greater access to its vegetation, providing a larger variety of food sources. In the current study, the analysis was conducted separately for each of the breeds, due to the differences between them in their body sizes and coloration patterns, except for the eye color, which is possible to compare between breeds qualitative trait.

Significant effect of the interaction between eye color and breed on ranging frequency was identified. In chicken, 5 main iris colors: gold, red, brown, black, and pink (albino) can be distinguished (Nelson, 1947). In some colored breeds like Barred Plymouth Rock, green-gray irises are common, for simplicity called gray, and they were correlated with quantity of black feathers in this breed (Slinger and McIlraith, 1944). In the current study, only 2 eye colors, gray and brown, were observed in both breeds. Variation in eye color depends to a large extent on the pigmentation and blood supply to a number of structures within the eye (Crawford, 1990). Wild birds may have intraspecific eye color variability, which seems to be due to the developmental stage of the individual, its breeding status, and/or sexual dimorphism (Negro et al., 2017). Furthermore, eye coloration may be related to visual needs, as the pigments involved capture different light wavelengths (Oliphant et al., 1992). Nonetheless, the origin and functions of eye colors are still poorly understood (Davidson et al. 2017). In this study, Sasso birds with gray eye color used the ranges significantly less frequently, as compared to Green-legged Partridges with either brown or gray eyes. In the

Table 4. Dependency of ranging activity on the scores (1-3) of comb size, neck plumage darkness, and beak darkness of Sasso and Green-legged Partridges, respectively.

Breed	External feature (score)	Ranging activity (mean ± SEM)				
		Score 1	Score 2	Score 3	F value	*P* value
Sasso						
	Comb size	180.8 ± 40.9^{b}	222.7 ± 53.3^{ab}	382.0 ± 55.7^{a}	3.6	0.0435
	Neck plumage darkness	200.8 ± 34.2^{b}	259.2 ± 62.5^{ab}	475.3 ± 64.3^{a}	4.14	0.0291
	Beak darkness	190.5 ± 36.3	288.1 ± 54.0	462.0 ± 116.0	3.15	0.0619
Green-legged Partridge						
	Comb size	344.4±23.3	386.5±31.0	302.3±42.3	0.96	0.3909
	Neck plumage darkness	377.1±41.1	348.9±33.0	357.8±25.3	0.17	0.8434
	Beak darkness	not present	324.75±66.9	364.3±19.2	0.35	0.5572

Different letters (a, b) next to mean ± SEM values in the same row indicate statistically significant differences ($P < 0.05$).

past, it was a standard procedure to eliminate chickens with gray eyes from the production, to avoid potential risk of introducing pathological lymphomatosis (Nelson, 1947). Nevertheless, chicken can have gray eyes unrelated to any pathology, as was the case for the birds in the current study (Marchewka et al., 2020), while lymphomatosis cannot be accurately diagnosed on the basis of color alone (Nelson and Thorp 1943). Further research into iris color and its associations with chicken health, welfare and productivity would be valuable.

We confirmed the stated hypothesis concerning the birds' feather coloration, but only for Sasso chickens. Sasso have been selected for performance including efficient growth rates, but also for suitability to the ranging systems. Moreover, they are described by the producer as "colored chickens," where it is characteristic for this hybrid to have some degree of dark feathering. In chicken, the α melano-cyte stimulating hormone (**MSH**), as part of the avian melanocortin system, controlled pigment regulation and was directly related to energy homeostasis by regulating food intake (Takeuchi et al., 2003). Chickens expressing any black pigment, eumelanin, carry at least one copy of the wild-type PMEL17 allele (Kerje et al., 2004). Interestingly, in a study focusing on chicken behavior and brain gene expression, Karlsson et al. (2010) identified plumage color genotypes PMEL17 to have a pleiotropic effect on

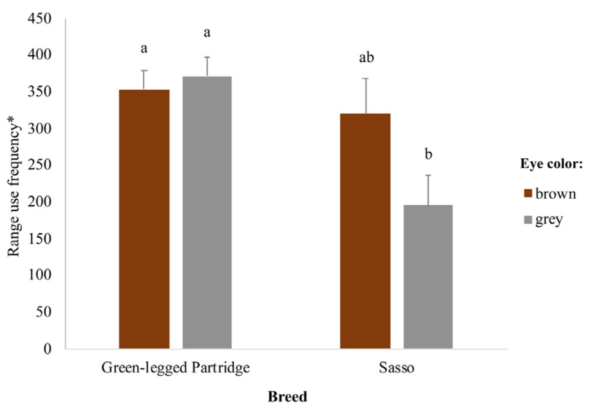


Figure 1. Range use frequency of birds with different eye colors (brown and gray) across breeds (Sasso and Green-legged Partridge). Different letters indicate significant differences in the model including the interaction between eye color and breed.

social and explorative behavior in chickens, where wild type birds (i/i) were more active in socializing and exploring, as compared to white chickens homozygous for the mutant allele (I/I). Animals explore their environment or novel stimuli and approach them in order to, for example, find food or water, making this explorative behavior essential for survival (Powell et al., 2004; Nicol, 2015). Exploration is thought to counterbalance fear (Meuser et al., 2021). High fear responses to objects indicated low exploration and foraging of the entire environment (Campbell et al., 2019), indicating reduced range use of the chickens and low adaptation of the animal to the husbandry system. Furthermore, melanin-based color traits in birds often act as honest signals of their quality, as signalers with larger or more intense color patches are perceived by conspecifics as bearers of a superior underlying genotypic quality and as a consequence achieve higher fitness benefits than others displaying smaller or less intense color patches (McGraw, 2008; Guindre-Parker and Love, 2014). To our knowledge, no previous study identified such associations between pigmentation and more frequent range use. To further support underlying mechanism behind the dark pigmentation in Sasso chickens, we identified a positive correlation between neck plumage and beak darkness. If confirmed by further investigations using molecular genetics methods, neck darkness score could be a valuable and practical trait, which could help to select birds in breeding programs suitable for rearing systems with outdoor access, although only in genetic strains with dark pigmentation present.

Relationships between comb size, behavioral characteristics and social structure in chickens (Johnsen et al. 1995; Cornwallis and Birkhead, 2007; Navara et al. 2012) or their fitness traits (Johnsson et al., 2012) have previously been investigated. In chickens, the primary role of the comb is in heat regulation (Van Kampern, 1971). The comb in adult chickens also plays an important role in reproduction, as it is used for making mating decisions both by male and female birds (Pizzari et al., 2003). Comb size is affected by hormonal status in both hens and cockerels (Eitan et al., 1998; Joseph et al., 2003). In roosters, it is an indicator of social rank, with females actively soliciting matings from males with larger combs (Zuk et al., 1990; Parker and Ligon, 2003). Sexual

Table 5. Correlations between comb length, comb height, neck plumage darkness, and beak darkness measurements presented for Sasso and Green-legged Partridge, respectively.

Breed		Neck plumage darkness (%)	Beak darkness (%)	Comb height (mm)	Comb length (mm)
Sasso					
	Neck plumage darkness (%)	1	0.41*	0.44*	0.43*
	Beak darkness (%)		1	0.43*	0.25
	Comb height (mm)			1	0.85**
	Comb length (mm)				1
Green-legged Partridge					
	Neck plumage darkness (%)	1	0.01	0.39*	0.07
	Beak darkness (%)		1	0.33*	0.22
	Comb height (mm)			1	0.55**
	Comb length (mm)				1

*$P < 0.01$.
**$P > 0.001$.

maturation also promotes the development of comb and wattles on chicken (Joseph et al., 2003), so the more developed chickens have larger combs. In hens, it is indicative of greater reproductive potential (Cornwallis and Birkhead, 2007; Wright et al., 2008). Furthermore, correlations of comb size with bone mass have been identified (Wright et al., 2008). Therefore, a larger comb is an indicator of higher fitness of a chicken. In laying hens, combs have been found to be darker in flocks that used the range area more intensively, while more fearful flocks had lighter combs (Bestmaan and Wagenaar, 2014). Since several diseases and health problems can cause a paler comb, as well as a smaller comb, farmers regard bright red combs as a practical indicator of current hen health. However, the association between comb size and ranging frequencies, as found for Sasso in the present study, remain to the best of the authors' knowledge unexplored. Based on the current results, comb size of Sasso could serve as an indicator of their ranging frequency, although comb size to some degree is affected by reproductive status (Eitan et al., 1998; Joseph et al., 2003).

Only in Sasso, the majority of the visual traits, found to be associated to the range use, also correlated between each other within breed. Therefore, a visual profile of a female bird of this breed with a higher range use could be suggested. Such correlations have been identified for males of other bird species (Yang et al., 2013). However, we are not aware of any studies providing such information in broiler hens. Our findings allow us to suggest not only the individual traits but also the set of the visual characteristics of the Sasso hens with higher range use.

No significant associations between any of the measured or visually assessed external features and ranging activity were identified for Green-legged Partridges. Considering the very good adaptation to the ranging conditions of Green-legged Partridges (Siwek et al., 2013), it is possible that their overall high ranging activity diminished differentiation of range use based on their external features. Therefore, even though the correlations were also identified between external features in Green-legged Partridges, no similar set of traits of the birds, which frequently use the range, could be identified in this breed.

To conclude, we found significant associations between measurements of the external features and ranging activity only for Sasso chickens. Visual assessment of those features, a more practical way of evaluating birds' phenotype than measurements, confirmed the findings obtained by measurements for comb size and neck plumage darkness and tended to do so for beak darkness of Sasso. However, no significant associations between external features and ranging activity were found in Green-legged Partridges. Better understanding of the associations between chickens' external features with their ranging behavior could profitably be included in selection programs and contribute to improvement of bird welfare, as it could assure production of breeding stock adapted for the outdoor environmental conditions.

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DISCLOSURES

All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version. This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue. The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

REFERENCES

Al-Atiyat, R. M., R. S. Aljumaah, A. M. Abudabos, M. N. Alotybi, R. M. Harron, A. S. Algawaan, and H. S. Aljooan. 2017. Differentiation of free-ranging chicken using discriminant analysis of phenotypic traits. Rev. Bras. de Zootec. 46:791–799.

Amat, F., K. C. Wollenberg, and M. Vences. 2013. Correlates of eye colour and pattern in mantellid frogs. Salamandra 49:7–17.

Andersson, M. 1994. Sexual Selection. Vol. 72. Princeton University Press, Princeton, NJ.

Bestmaan, M., and J. P. Wagenaar. 2014. Health and welfare in Dutch organic laying hens. Animals 4:374–390.

Campbell, D. L., E. J. Dickson, and C. Lee. 2019. Application of open field, tonic immobility, and attention bias tests to hens with different ranging patterns. Peer J. 7:e8122.

Campo, J. L., M. T. Prieto, and S. García Dávila. 2009. Relationships between fluctuating asymmetry and sexual maturity, social aggressiveness and comb size in chickens. Arch. für Geflugelkunde. 73:193–200.

Cloutier, S., J. P. Beaugrand, and P. C. Lague. 1996. The role of individual differences and patterns of resolution in the formation of dominance orders in domestic hen triads. Behav. Process. 38:227–239.

Cornwallis, C. K., and T. R Birkhead. 2007. Experimental evidence that female ornamentation increases the acquisition of sperm and signals fecundity. Proc. Biol. Sci. 274:583–590.

Crawford, R. D. 1990. Poultry Breeding and Genetics. No. 04; SF492. C7.

Davidson, G., A. Thorton, and N. S. Clayton. 2017. Evolution of iris color in relation to cavity nesting and parental care in passerine birds. Biol. Lett. 13:20160783.

Dawkins, M. S., P. A. Cook, M. J. Whittingham, K. A. Mansell, and A. E. Harper. 2003. What makes free-range broiler chickens' range? In situ measurement of habitat preference. Anim. Behav. 66:151–160.

Elias, V. L., C. L. Nicolas, and C. I. Abramson. 2008. Eye color as an indicator of behavior: revisiting Worthy and Scott. Psychol. Rep. 102:759–778.

Eitan, Y., M. Soller, and I. Rozenboim. 1998. Comb size and estrogen levels toward the onset of lay in broiler and layer strain females under ad libitum and restricted feeding. Poult. Sci. 77:1593–1600.

Erian, I., and C. J. Phillips. 2017. Public understanding and attitudes towards meat chicken production and relations to consumption. Animals 7:20.

EU. 2007. Council Directive 834/2007 on organic production and labelling of organic products. Off. J. Eur. Commun. L. 189:1–23.

EU. 2008. Commission regulation (EC) no 889/2008 of 5 September 2008 laying down detailed rules for the implementation of council regulation (EC) no 834/2007 on organic production and labelling of organic products with regard to organic production, labelling and co. Off. J. Eur. Union L 250:1–84.

FAO - Food and Agriculture Organization, B. D. Scherf. 2000. World Watch List for Domestic Animal Diversity. 3rd ed. Food and Agriculture Organization, Rome, Italy.

Freire, R., M. C. Appleby, and B. O. Hughes. 1997. Assessment of prelaying motivation in the domestic hen. Anim. Behav. 54:313–319.

Guindre-Parker, S., and O. P. Love. 2014. Revisiting the condition-dependence of melanin-based plumage. J. Avian Biol. 45:29–33.

Horst, P. 1989. Native fowl as reservoir for genomes and major genes with direct and indirect effects on the adaptability and their potential for tropically oriented breeding plans. Arch. Anim. Breed. 53:93–101.

Johnsen, T. S., S. L. Popma, and M. Zuk. 1995. Male courtship displays, ornaments and female mate choice in captive red jungle fowl. Behaviour 132:821–836.

Johnsson, M., I. Gustafson, C. J. Rubin, A. S. Sahlqvist, K. B. Jonsson, S. Kerje, and D. Wright. 2012. A sexual ornament in chickens is affected by pleiotropic alleles at HAO1 and BMP2, selected during domestication. PLoS Genet. 8:e1002914.

Joseph, N. S., F. E. Robinson, R. A. Renema, and K. A. Thorsteinson. 2003. Comb growth during sexual maturation in female broiler breeders. J. Appl. Poult. Res. 12:7–13.

Van Kampen, M. 1971. Some aspects of thermoregulation in the white leghorn fowl. Int. J. Biometeorol. 15:244–246.

Karlsson, A. C., S. Kerje, I. Andersson, and P. Jensen. 2010. Genotype at the PMEL17 locus affects social and explorative behaviour in chickens. Br. Poult. Sci. 51:170–177.

Keeling, L., L. Andersson, K. E. Schütz, S. Kerje, R. Fredriksson, Ö. Carlborg, and P. Jensen. 2004. Feather-pecking and victim pigmentation. Nature 431:645–646.

Kerje, S., P. Sharma, U. Gunnarsson, H. Kim, S. Bagchi, R. Fredriksson, and L. Andersson. 2004. The dominant white, dun and smoky color variants in chicken are associated with insertion/deletion polymorphisms in the PMEL17 gene. Genetics 168:1507–1518.

Lima, A., and I. Nääs. 2005. Evaluating two systems of poultry production: conventional and free-range. Br. J. Poult. Sci. 7:215–220.

Marchant-Forde, J. N. 2015. The science of animal behavior and welfare: challenges, opportunities, and global perspective. Front. Vet. Sci. 2:16.

Marchewka, J., P. Sztandarski, Ż. Zdanowska-Sąsiadek, K. Damaziak, F. Wojciechowski, A. B. Riber, and S. Gunnarsson. 2020. Associations between welfare and ranging profile in free-range commercial and heritage meat-purpose chickens (Gallus gallus domesticus). Poult. Sci. 99:4141–4152.

Marchewka, J., T. T. N. Watanabe, V. Ferrante, and I. Estevez. 2013. Welfare assessment in broiler farms: transect walks versus individual scoring. Poult. Sci. J. 92:2588–2599.

McGraw, K. J. 2008. An update on the honesty of melanin-based color signals in birds. Pigment Cell Melanoma Res. 21:133–138.

Meuser, V., L. Weinhold, S. Hillemacher, and I. Tiemann. 2021. Welfare-related behaviors in chickens: characterization of fear and exploration in local and commercial chicken strains. Animals 11:679.

Nätt, D., S. Kerje, L. Andersson, and P. Jensen. 2007. Plumage color and feather pecking—behavioral differences associated with PMEL17 genotypes in chicken (Gallus gallus). Behav. Genet. 37:399–407.

Navara, K. J., E. M. Anderson, and M. L. Edwards. 2012. Comb size and color relate to sperm quality: a test of the phenotype-linked fertility hypothesis. Behav. Ecol. 23:1036–1041.

Negro, J. J., M. Carmen Blázquez, and I. Galván. 2017. Intraspecific eye color variability in birds and mammals: a recent evolutionary event exclusive to humans and domestic animals. Front Zool. 14:53.

Nelson, N. M. 1947. Normal eye color in the chicken. Poult. Sci. J. 26:61–66.

Nelson, N. M., and F. Thorp Jr.. 1943. Ocular lymphomatosis with special reference to chromatism of the irides. Am J Vet res 4:294–304.

Nicol, C. J. 2015. The Behavioural Biology of Chickens. CABI, Wallingford, UK.

Oliphant, L. W., J. Hudon, and J. T. Bagnara. 1992. Pigment cell refugia in homeotherms. The unique evolutionary position of the iris. Pigment Cell Res. 5:367–371.

Pagel, M., and M. S. Dawkins. 1997. Peck orders and group size in laying hens: futures contracts' for non-aggression. Behav. Process. 40:13–25.

Parker, T. H., and J. D. Ligon. 2003. Female mating preferences in red jungle fowl: a meta-analysis. Ethol. Ecol. Evol. 15:63–72.

Pizzari, T., C. K. Cornwallis, H. Løvlie, S. Jakobsson, and T. R. Birkhead. 2003. Sophisticated sperm allocation in male fowl. Nature. 426:70–74.

Powell, S. B., M. A. Geyer, D. Gallagher, and M. P Paulus. 2004. The balance between approach and avoidance behaviors in a novel object exploration paradigm in mice. Behav. Brain Res. 152:341–349.

Ruxton, G. D., W. L. Allen, T. N. Sherratt, and M. P. Speed. 2018. Avoiding Attack: The Evolutionary Ecology of Crypsis, Aposematism, and Mimicry. Oxford University Press, Oxford, UK.

Sanchez, C., and I. Estevez. 1998. The Chickitizer Software Program. University of Maryland, College Park, MD.

Santana, S. E., J. Lynch Alfaro, and M. E. Alfaro. 2012. Adaptive evolution of facial colour patterns in Neotropical primates. Proc. Biol. Sc. 279:2204–2211.

Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9:671–675.

Sheldon, B. C. 1994. Male phenotype, fertility, and the pursuit of extra-pair copulations by female birds. Proc. R. Soc. B. Biol. Sci. 257:25–30.

Siwek, M., D. Wragg, A. Sławińska, M. Malek, O. Hanotte, and J. M. Mwacharo. 2013. Insights into the genetic history of Green-legged Partridge like fowl: mt DNA and genome-wide SNP analysis. Anim. Genet. 44:522–532.

Slinger, S. J., and J. J. MacIlraith. 1944. The correlation between green-grey irises and black feathers in barred Plymouth Rock pullets. Poult. Sci. 23:533–537.

Takeuchi, S., S. Takahashi, R. Okimoto, H. B. Schiöth, and T. Boswell. 2003. Avian melanocortin system: α-MSH may act as an autocrine/paracrine hormone: a minireview. Ann. N. Y. Acad. Sci. 994:366–372.

Taylor, P. S., P. H. Hemsworth, P. J. Groves, S. G. Gebhardt-Henrich, and J. L. Rault. 2020. Frequent range visits further from the shed relate positively to free-range broiler chicken. Anim. Welf. 14:138–149.

Taylor, P., P. Hemsworth, P. Groves, S. Gebhardt-Henrich, and J. L. Rault. 2017. Ranging behaviour of commercial free-range broiler chickens 1: factors related to flock variability. Animals 7:54.

Volpato, G. L, A. C. Luchiari, C. R. A. Duarte, R. E. Barreto, and G. C. Ramanzini. 2003. Eye color as an indicator of social rank in the fish Nile tilapia. Braz. J. Med. Biol. Res. 36:1659–1663.

Waldvogel, J. A. 1990. The bird's eye view. Am. Sci. 78:342–353.

Wright, D., S. Kerje, H. Brändström, K. Schütz, A. Kindmark, L. Andersson, and T. Pizzari. 2008. The genetic architecture of a female sexual ornament. Evolution 62:86–98.

Yang, C., J. Wang, Y. Fang, and Y.-H. Sun. 2013. Is sexual ornamentation an honest signal of male quality in the Chinese Grouse (*Tetrastes sewerzowi*)? PLoS One 8:e82972.

Zuk, M., R. Thornhill, J. D. Ligon, K. Johnson, S. Austad, S. H. Ligon, and C. Costin. 1990. The role of male ornaments and courtship behavior in female mate choice of red jungle fowl. Am. Nat. 136:459–473.

Gut microbiota activity in chickens from two genetic lines and with outdoor-preferring, moderate-preferring, and indoor-preferring ranging profiles

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ABSTRACT Despite the existing research into the gut microbiome of meat chickens, the associations between gut microbiome composition, its activity and chicken outdoor ranging frequency remain unexplored. The aim of this study was to determine the gut microbiota composition, activity and metabolic products in chickens of 2 different lines and 3 ranging profiles. Sixty non-beak trimmed birds, either Sasso or Green-legged Partridge were housed with access to outdoor ranges from wk. 5 to 10 of age. Outdoor ranges were video recorded to obtain frequencies of the birds' range use. The information about relative abundance of selected bacterial groups in the ceca including *Lactobacillus* spp., *E. coli, Bifidobacterium* spp., and *Clostridium* spp. was obtained with the PCR method. Gut microbiota activity was assessed based on the glycolytic activity of bacterial enzymes including, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, and β-glucuronidase as well as based on the concentration of short-chain fatty acids (**SCFA**) in the caecal digesta. Statistical analysis was conducted by generalized linear mixed models, applying the breed and ranging profile as fixed effects and pen as a random factor. The lowest relative abundance of *Bifidobacterium* spp. was found in the cecal content of indoor-preferring Sasso birds (0.01 ± 0.001), as compared to all other birds in the experiment (ranging from 0.03 ± 0.01 to 0.11 ± 0.07; $P = 0.0002$). The lowest relative abundance of *E. coli* was identified for all outdoor-preferring birds and indoor- preferring Sasso birds (0.01 ± 0.001; $P = 0.0087$). Cecal activity of: α-glucosidase, β-glucuronidase and β-galactosidase was higher in Green-legged Partridges, as compared to Sasso ($P = 0.013$; $P = 0.008$; $P = 0.004$). Valeric acid concentrations were higher in moderate Green-legged Partridges than in Sasso of the same ranging profile (2.03 ± 0.16 vs. 1.5 ± 0.17; 0.016). The majority of the current results confirmed an effect of genotype and ranging profile on the various analyzed parameters. In outdoor-preferring birds, the consumption of pasture originating feed sources as a supplement to the indoor accessible cereal-based diet likely caused the positive effects on the birds' microbial profile.

Key words: free range, broiler, organic, microbiota activity

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INTRODUCTION

The chicken microbiome, defined as the entire environment of symbiotic, commensal, and pathogenic microorganisms present in the gastrointestinal tract (**GIT**), is important mainly for the digestion processes (Kogut, 2019). This is especially important in meat purpose chickens in commercial production, where higher efficiency is the main economic aim of the production.

There are more than 900 species of bacteria in the chicken gut microbiome (Binek et al., 2017). Not all off its characteristics and its functions have been well understood yet, but on the basis of the current knowledge, the composition of microorganisms inhabiting the chicken gastrointestinal tract is associated with gut morphology (Forder et al., 2007), health and immunity (Pan

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and Yu, 2014) or even behavior (van der Eijk et al., 2020) of the birds. For example, selected bacteria of the genus *Clostridium* (i.e., *Clostridium perfringens*) negatively affect the health of chickens. They might cause necrotic enteritis, and disrupt the proper functioning of the digestive system (Gharib-Naseri et al., 2019). On the other hand, bacteria of the genus *Lactobacillus* (i.e., *Lactobacillus acidophilus*) have a positive effect on bird health and performance supporting digestion and immune processes in the digestive system of chickens (Brisbin et al., 2011).

It has been found that the form, type and chemical composition of feed are closely linked to the gut microbiota activity. In the housing systems with outdoor access, the feed is not only available indoors, but the birds also find edible items on the ranges, like for instance insects, grass, herbs and stones, providing greater variety in feed forms and sources. Fermentable substances like: non-starch polysaccharides (**NSP**), starch and proteins that escape digestion and absorption in the upper part of the gut cause changes in the gut microbiota activity which is manifested in specific changes in SCFA concentrations, high β-glucuronidase activity and increased *E. coli*presence. Such high fermentation activity may be considered as detrimental to birds' health and performance (Konieczka et al., 2018). Production systems with outdoor access were found also to be associated with higher abundance of *Clostridium* spp. or *Lactobacillus* spp. in the chicken gut due to contact with soil and natural vegetation (Bjerrum et al., 2006; Hubert et al., 2019). Access to the range may alter the composition of the gut microbiota even due to weather factors like natural light or rain (Thaiss et al., 2016). The duration of time spent at the range may be as well important.

The relationships between the housing environment and the chicken microbiome composition and activity are also not fully explored, especially considering the various chicken production systems and genetic strains of birds, but research confirms that such relationships exist (Hubert et al., 2019; Kers et al., 2019; Ocejo et al., 2019). It is known that poultry housing environment influences microbiota diversity and structure (Kers et al., 2018). For instance, the presence of increased levels of ammonia in conventional poultry housing systems can permute infections caused by *E. coli* spread from the birds GIT to the environment (Landman et al., 2013).

The level of development of the GIT and its content have been found to be potential indicators of the chickens' ranging profiles developed for classifying the frequency with which birds used the outdoor ranges (Marchewka et al., 2020) and forage consumption (Marchewka et al., 2021). Therefore, as the outdoor range use differs among individual birds, the composition and the gut microbiota activity may also potentially differ. Further investigations are necessary to confirm this.

Gut microflora activity and composition may be influenced by genetic factors. In a previous study we discovered significant differences between 2 genotypes of birds: slow-growing Sasso chicken and native Polish chicken Green-legged Partridge, mainly in digestive tract measurements for example, small intestine length, ceca length, colon length, and villus area. However, no previous studies have investigated microbiome activity in relation to ranging levels in slow-growing Sasso chickens and Green-legged Partridge chickens.

Understanding the associations of the ranging profiles of birds with different genetic background on their microbiota composition and activity is important to ensure the optimal health and welfare of the birds reared in housing systems with access to the outdoor ranges. This is particularly important nowadays as we can notice increased public concerns of animal welfare and higher attention of consumers toward meat from poultry reared in low-input systems.

The aim of this study was to determine the gut microbiota composition, activity and metabolic products in 2 genotypes of chickens, each with the three ranging profiles: outdoor-preferring, moderate-preferring and indoor-preferring (Marchewka et al., 2020). We hypothesized that the chickens which were identified as homogeneous in terms of ranging profile would show similar quantitative microbial composition of the same genus and similar gut microbiota activity, regardless of the breed.

MATERIALS AND METHODS

The experiment took place in the Mazovian region of Poland, at the experimental farm of the Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences, in August and September of 2018. No Ethical Committee approval was required, as the current study was performed with no invasive experimental procedures applied to the animals during their life. In this experiment, mimicking the real on-farm production cycle, we did not perform any procedures exceeding standard husbandry procedures.

Animals, Housing and Management

One hundred twenty non-beak trimmed mixed sex birds of each of 2 breeds (total n = 120 birds), Green-legged Partridge - indigenous Polish breed of heritage chicken and Sasso line C44 were used in the experiment. Sasso C44 is a commercially available, colored slow-growing hybrid of broilers (Hendrix Genetics BV, The Netherlands). Sasso birds are well skilled to forage on the outdoor ranges, having high resistance to low temperatures and diseases, while the meat is characterized by a very good taste and quality (Getiso et al., 2017). Sasso birds reach their slaughter weight of 2.3 to 2.8 kg at about 2 months of age. Until wk 5 of age, 120 birds were reared in the experimental facility without outdoor access in 2 pens, divided by the breed into 2 groups (1 group per pen) of 60 birds. At the age of 5 wk, all individuals were relocated from the rearing facility to the experimental house, both at the same location. Eight female and 2 male chickens were assigned to each single-breed group housed in 12 pens until 10 wk of age. In each pen, 6 birds (5 females and 1 male) with similar body weight within each breed (on average 2,030.6 ± 68.9 g for Sasso and 705.9 ± 8.5 g for Green-legged Partridge) were selected as focal animals. To make the recognition of individuals possible all birds were fitted with a laminated paper mark of the size of 9 cm high and 7 cm wide attached to the birds' back by 2 elastic bands around its wings. Ten different colors of the marks were assigned in each pen randomly to the individual birds. Birds were wearing color mark during the entire experiment. They were inspected twice a day. No birds died during the experiment.

The outline of the experimental facilities has previously been presented in Marchewka et al. (2020) and Sztandarski et al. (2021). In short, the size of the indoor pens was 2.5 m × 3.5 m, resultingin a stocking density at slaughter age of 1.4 kg/m^2 for Green-legged Partridge and 2.7 kg/m^{2}for Sasso. Birds were housed on the sawdust litter, while in each pen, next to the wall there was a 0.5 m stripe covered with sand. Pens were cleaned when needed. In each pen, there were two 80-cm long wooden perches at 2 perching levels, one at the height of 15 cm and the second at 40 cm. The perching poles were 50 × 50 mm thick and had rounded edges. Each pen had direct access through the pophole (45 cm high × 50 cm wide) to an individual outdoor range (3.5 m × 30 m) providing 10.5 m^2/chicken. All the outdoor ranges had the same vegetation coverage regarding botanical composition, no trees or shelters were present. The grass was mowed 1 wk. before the onset of the experiment. Each free-range area was provided with a semiautomatic bell drinker and a wooden box (1 m × 1 m) filled with sand.

The birds were habituated for 48 h to the new housing and social situation. Popholes were opened daily from 7.00 until 19.00 h. Commercial pelleted feed was used to nourish the birds. Feed and water were available ad libitum. The feed was composed of wheat, maize, sunflower expeller, pea, soybean expeller legumes mix, gruel corn, monocalcium phosphate, soybean oil, and calcium carbonate with supplements (Marchewka et al., 2020). The feed composition was intended to meet the birds' nutritional requirements (Classen, 2017). No coccidiostats or other medication was used.

Birds were provided only natural light through uncovered windows. Light hours during the experimental period ranged from 12.7 h to 15.7 h/day. There was natural ventilation in the building. Indoor climate parameters were continuously collected by a device of the weather measuring device (Davis Instruments Vantage Pro 2 DAV-6152EU, CA) placed in the middle of the chicken rearing house at height of 1 m.

Observations of Ranging Behavior

The behavioral data collection of range use in the current study has previously been described (Marchewka et al., 2020). Range use of the birds was recorded using video cameras. The 12 outdoor pens were video-recorded simultaneously and continuously using 6 cameras (BCS-DMIP2401IR-M-IV IP 4 Mpix), each covering 2 free-range areas. The cameras were attached to the wall of the experimental facility at a height of 3 m from the ground. The video material was recorded with the network recorder BCS-NVR0401-IP 4 channel BC. After that it was analyzed by one trained and experienced person, using the Chickitizer program (Sanchez and Estevez, 1998). From the recorded videos, 3 days were chosen per week of the experiment (5 wk.). On each of those days, 3 times of the day (at 8:00, at 13:00, at 18:00) a 3-min-period with 10 s sampling intervals was set and repeated after 10 min. The observer registered the absence or presence each of the experimental birds' in the outdoor area.

Sample Collection for Bacterial Composition and Activity Determination

At 72 d of life, birds from each group (n = 6) were sacrificed by cervical dislocation. Thereafter, the cavity was opened and both ceca were removed. The digesta from both ceca were collected and pooled in one test tube for each bird individually and was then divided into 3 portions to be used for different analysis. The collected digesta was immediately frozen in −80°C.

Determination of Bacteria Relative Abundance

The relative abundance of selected bacterial groups in the caeca including *Lactobacillus* spp., *E. coli, Bifidobacterium* spp., and *Clostridium* spp. was performed using the PCR method. We modified Zhu et al. procedure to isolate bacterial genomic DNA from the cecal digesta (Zhu et al., 2002). Briefly; bacterial genomic DNA was extracted from digesta using the QIA amp. Fast DNA Stool Mini Kit (Qiagen, Stockach, Germany) according to the manufacturer's protocol. Then, the yield and purity of the isolated DNA were estimated spectrophotometrically (Nanodrop, NanoDrop Technologies, Wilmington, DE).

Polymerase Chain Reaction Amplification of Bacterial 16S rRNA Gene

The primers and polymerase chain reaction (**PCR**) conditions used to amplify the bacterial 16S rRNA gene are shown in Table 1. The universal primer set was used to determine the total bacteria population. The detailed PCR conditions were set-up as previously reported for each respective bacteria group (Michalczuk et al., 2021). The obtained PCR-products were separated by electrophoresis on a 2% agarose gel. PCR products were quantified using ImageJ 1.47v software for densitometry measurements (National Institute of Mental Health, Bethesda, MD), with a density of bands for each bacteria group expressed in relation to the density of the total bacteria primers product. The density of the bands for

Table 1. Sequences of primers used for amplification of bacterial 16S rRNA gene.

Bacterial group	Primers	Sequence 5'-3'	Base pair
Total bacteria	Forward	CGTGCCAGCCGCGGTAATACG	611
	Reverse	GGGTTGCGCTCTTGCGGGACTTAACCCAACAT	
Lactobacillus spp.	Forward	CATCCAGTGCAAACCTAAGAG	286
	Reverse	GATCCGCTTGCCTTCGCA	
Escherichia coli	Forward	GGGAGTAAAGTTAATACCTTTGCTC	585
	Reverse	TTCCCGAAGGCACATTCT	
Clostridium spp.	Forward	AAAGGAAGATTAATACCGCATAA	722
	Reverse	ATCTTGCGACCGTACTCCCC	
Bifidobacterium spp.	Forward	CGGGTGCTTCCCACTTTCATG	1417
	Reverse	GATTCTGGCTCAGGATGAACG	

each of bacteria group was expressed in relation to the density of the total bacteria primer product. Each sample was analyzed in duplicate.

Bacterial Enzyme Activity

The activity of the gut microbiota was assessed based on the glycolytic activities of 5 bacterial enzymes in the cecal digesta including, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, and β-glucuronidase. Before the analysis, the digesta was thawed at 4°C for 3 h. The activity of the enzymes was determined spectrophotometrically according to Konieczka and Smulikowska, modified from Jurgoński et al. (Jurgoński et al., 2013; Konieczka and Smulikowska, 2018). To determine each specific enzyme we used: p-nitrophenyl-α-D-glucopyranoside for α-glucosidase, p-nitrophenyl-β-D-glucopyranoside for β-glucosidase, p-nitrophenyl-α-D-galactopyranoside for α-galactosidase, p-nitrophenyl-β-D-galactopyranoside for β-galactosidase, and p-nitrophenyl-β-D-glucuronide for β-glucuronidase (Sigma Chemical Co., St. Louis, MO).

SCFA Concentration

The SCFA determination in the cecum digesta was performed according to the procedure described previously (Konieczka et al., 2018), using an HP 5890 Series II gas chromatograph (Hewlett Packard, Waldbronn, Germany) with a flame-ionization detector (**FID**) and a Supelco Nukol fused silica capillary column (30 m × 0.25 mm internal diameter, film 0.25 mm). Helium was employed as the carrier gas. The concentrations of individual SCFAs were estimated with an internal standard (isocaproic acid) using a mixture of standard solutions.

Statistical Analysis

Birds of both breeds were divided into 3 ranging profiles using rank-frequency distribution (a discrete form of a quantile function in reverse order, giving the size of the element at a given rank) of their range use frequency summed over all the observation periods—that is, between 0 and 1,620 times. All the birds within a breed were assigned a rank based on their individual frequency of outdoor use. We segmented the rank distribution of the birds into 3 ranges: outdoor-preferring ranging profile, with the mean value of 506.1 ± 47.9 total outdoor uses per experiment per bird for Sasso and 502.6 ± 22.5 total outdoor uses per experiment per bird for Green-legged Partridge; moderate-outdoor ranging profile, with the mean value of 219.6 ± 18.8 total outdoor uses per experiment per bird for Sasso and 332.4 ± 13total outdoor uses per experiment per bird for Green-legged Partridge; and indoor-preferring ranging profile, with the mean value of 89.8 ± 11.7 total outdoor uses per experiment per bird for Sasso and 223.9 ± 12.1 total outdoor uses per experiment per bird for Green-legged Partridge. The rank intervals were equal (modified from Campbell et al., 2016).

Statistical analyses were performed with SAS 9.4. The GLIMMIX procedure was used to perform generalized linear mixed models for the microbiome composition, activity and metabolic products using either normal or gamma distribution where appropriate, applying the ranging profile group, breed and their interaction as fixed effects in the model. The pen was included in the model as a random effect. The assumptions of homogeneity of variance and normally distributed residuals were examined visually using the conditional Studentized residuals plots. The results are shown as means with standard errors, and P-values below 0.05 were considered significant, while between 0.05 and 0.06 were considered a significant trend. Tukey's post hoc test was performed to investigate significant differences between test groups.

RESULTS

Bacteria Composition

Effects of breed, ranging profile and their interaction on the relative abundance of selected bacteria in the ceca are presented in Table 2.

An effect of the interaction between breed and ranging profile was identified for the relative abundance of *E. coli* ($P = 0.0087$) and *Bifidobacterium* spp. ($P = 0.0002$). The lowest relative abundance of *E. coli* was identified for outdoor-preferring Sasso and Green-legged Partridges and indoor-preferring Sasso birds. The lowest relative abundance of *Bifidobacterium* spp. was found in the intestinal content of indoor-preferring Sasso birds as compared to all other birds in the experiment. The effect of breed was observed in the *Clostridium* spp. relative abundance

Table 2. Effects of breed, ranging profile, and their interaction on the relative abundance of selective bacteria in the caeca.

Factors		DNA abundance			
		Clostridium spp.	*Lactobacillus* spp.	Bifidobacterium spp.	*E. coli*
Breed					
	Sasso (n = 36)	0.32±0.03^{A}	0.17±0.03	0.06±0.01	0.01±0.01^{B}
	Green-legged Partridge (n = 36)	0.24±0.03^{B}	0.14±0.02	0.07±0.02	0.04±0.01^{A}
Ranging profile					
	Indoor-preferring	0.24±0.03	0.14±0.03	0.06±0.03	0.02±0.01^{BA}
	Moderate-preferring	0.30±0.04	0.13±0.02	0.05±0.02	0.04±0.02^{A}
	Outdoor-preferring	0.30±0.03	0.19±0.03	0.08±0.02	0.01±0.00^{B}
Breed*ranging profile					
	Sasso*indoor-preferring	0.23±0.05	0.15±0.05	0.01±0.00^{B}	0.01±0.00^{C}
	Sasso*moderate-preferring	0.39±0.05	0.14±0.04	0.08±0.03^{A}	0.03±0.02^{BAC}
	Sasso*outdoor-preferring	0.33±0.05	0.21±0.04	0.07±0.03^{A}	0.01±0.00^{C}
	Green-legged Partridge*indoor-preferring	0.25±0.05	0.12±0.03	0.11±0.07^{A}	0.04±0.02^{BA}
	Green-legged Partridge*moderate-preferring	0.22±0.05	0.13±0.02	0.03±0.01^{BA}	0.05±0.03^{A}
	Green-legged Partridge*outdoor-preferring	0.26±0.05	0.18±0.04	0.09±0.03^{A}	0.01±0.00^{C}
		P-value			
Breed		0.0493	0.5018	0.1952	0.0074
Ranging profile		0.3109	0.3333	0.1715	0.0016
Breed*ranging profile		0.1501	0.9822	0.0002	0.0087

$^{A\text{-}C}$Different letters within factor indicate significant differences (If the P value is < 0.05).

($P = 0.0493$): it was higher in Sasso chickens, as compared to Green-legged Partridges.

No significant differences were identified between ranging profiles of either Sasso or Green-legged Partridges regarding bacterial relative abundance.

Microbial Enzymes Activity

Effects of breed, ranging profile, and their interaction on the microbial enzymes activity are presented in Table 3.

No effect of the interaction between breed and ranging profile was observed for any of the investigated enzymes activities. However, there was an effect of the breed on 3 of the enzymes that is, α-glucosidase ($P = 0.013$), β-glucuronidase ($P = 0.008$), and β-galactosidase ($P = 0.04$), where higher activity was observed in Green-legged Partridges, as compared to Sasso chickens.

No significant differences were identified between ranging profiles of either Sasso or Green-legged Partridges regarding microbial enzymes activity.

SCFA

Effects of breed, ranging profile and their interaction on the SCFA concentration are presented in Table 4.

An effect of the interaction between breed and ranging profile was identified only for valerian SCFA ($P = 0.016$). The observed concentration of valerian SCFA was higher for moderate-outdoor Green-legged Partridges, as compared to moderate-outdoor Sasso chickens. An effect of breed on the isovalerian concentration was observed ($P = 0.03$), being higher in Sasso as compared to Green-legged Partridge chickens.

No significant differences were identified between ranging profiles regarding SCFA concentrations.

DISCUSSION

Birds reared with access to the pasture consume material found outdoors, such as plants, insects, and stones. In our previous study we found that the frequency of range use by the chicken was associated not only with the ingested material, but also with the development of the bird gut and those associations differed between Green-legged Partridges and Sasso birds (Marchewka et al., 2021). However, it has not until now been investigated whether the relationship between outdoor range use and chicken gut microbiota exists.

The aim of this study was to investigate microbiota: selected main bacterial species presence, microbial enzymes activity, and SCFA concentration in the ceca (the main site of fermentation) of chickens with 2 different genotypes and 3 free-ranging profiles: outdoor-preferring, moderate-outdoor and indoor-preferring (Marchewka et al., 2020). The birds were divided into ranging profiles within each breed based on the frequency of the range use. Both breeds were well adapted to the rearing systems with outdoor access (Marchewka et al., 2020). Nevertheless, differences in the range use exist on the individual level, even if equal opportunity of outdoor access is provided (Rodriguez-Aurrekoetxea and Estevez, 2016).

The chicken intestinal microbiome contains several taxa. Non-pathogenic *Campylobacter* spp. or *E. coli* may be present in concentrations up to 10^7 colony-forming units per gram (cfu/g) in the chicken intestine (Stern et al., 1995). Bacteria present in the GIT of chickens at lower concentrations are *E. coli*. Broiler chickens, especially in conventional housing systems are frequently infected with *E. coli*, which often results in disease and high economic losses, yet healthy poultry birds possess an innate resistance to infections (Moharrery and Mahzonieh, 2005). Certain strains of *E. coli* may, however, causes opportunistic secondary infections in poultry birds (Gross, 1990).

Table 3. Effects of breed, ranging profile, and their interaction on the microbial enzymes activity.

Factors		Cecal digesta enzymes activity				
		α - GLUCOSIDASE 400 nm	β - GLUCOSIDASE 400 nm	α - GALACTOSIDASE 400 nm	β - GLUCURONIDASE 400 nm	β - GALACTOSIDASE 420 nm
Breed						
	Sasso (n = 36)	1.26±0.06[B]	0.78±0.04	1.82±0.03	0.87±0.05[B]	5.53±0.29[B]
	Green-legged Partridge (n = 36)	1.47±0.05[A]	0.85±0.04	1.82±0.01	1.16±0.06[A]	6.77±0.19[A]
Ranging profile						
	Indoor-prefering	1.34±0.07	0.83±0.05	1.86±0.05	0.94±0.07	6.00±0.35
	Moderate-prefering	1.34±0.09	0.77±0.05	1.80±0.03	1.01±0.08	6.04±0.36
	Outdoor-prefering	1.41±0.06	0.85±0.04	1.81±0.01	1.08±0.07	6.36±0.28
Breed*ranging profile						
	Sasso*indoor-prefering	1.31±0.08	0.84±0.06	1.87±0.06	0.93±0.08	5.88±0.41
	Sasso*moderate-prefering	1.09±0.12	0.67±0.04	1.76±0.06	0.82±0.11	5.08±0.58
	Sasso*outdoor-prefering	1.35±0.12	0.79±0.08	1.79±0.02	0.81±0.03	5.30±0.59
	Green-legged Partridge*indoor-prefering	1.46±0.13	0.76±0.09	1.84±0.01	0.98±0.10	6.50±0.62
	Green-legged Partridge*moderate-prefering	1.52±0.11	0.84±0.07	1.82±0.01	1.14±0.11	6.71±0.36
	Green-legged Partridge*outdoor-prefering	1.44±0.06	0.88±0.04	1.82±0.01	1.22±0.09	6.88±0.22
				P-value		
Breed		0.013	0.229	0.535	0.008	0.004
Ranging profile		0.504	0.363	0.382	0.937	0.805
Breed*ranging profile		0.175	0.143	0.603	0.338	0.579

[A-C]Different letters within factor indicate significant differences (If the *P* value is < 0.05).

Table 4. Effects of breed, ranging profile, and their interaction on the short chain fatty acids (SCFA) concentration.

Factors		SCFA (μmol/g)						
		Acetic acid	Propionic acid	Isobutyric acid	Butter acid	Isovaleric acid	Valeric acid	Total SCFA
Breed								
	Sasso (n = 36)	62.13±2.52	20.63±1.04	2.02±0.13	9.90±0.87	1.84±0.11[A]	1.72±0.08	98.24±3.77
	Green-legged Partridge (n = 36)	67.80±2.49	21.67±0.97	2.00±0.12	9.34±0.44	1.63±0.12[B]	1.86±0.08	104.29±3.78
Ranging profile								
	Indoor	60.77±2.89	19.05±1.14	2.02±0.19	9.01±0.76	1.70±0.12	1.70±0.10	94.25±4.28
	Moderate	64.00±3.11	22.23±1.35	2.00±0.16	9.75±1.11	1.85±0.19	1.82±0.13	101.65±4.94
	Outdoor	69.42±3.11	21.96±1.12	2.01±0.10	10.04±0.66	1.67±0.12	1.83±0.08	106.93±4.45
Breed*ranging profile								
	Sasso*indoor	60.58±2.94	18.97±1.30	2.11±0.23	8.83±0.84	1.83±0.13	1.73±0.11[BA]	94.04±4.37
	Sasso*moderate	56.08±1.69	21.72±2.20	1.78±0.14	10.47±2.53	1.76±0.24	1.50±0.17[B]	93.31±5.71
	Sasso*outdoor	72.26±7.75	22.92±2.32	2.08±0.19	11.54±1.69	1.96±0.30	1.94±0.16[BA]	112.7±10.55
	Green-legged Partridge*indoor-prefering	61.58±9.76	19.38±2.63	1.64±0.17	9.79±2.01	1.16±0.13	1.57±0.13[BA]	95.11±14.38
	Green-legged Partridge*moderate-prefering	69.49±4.61	22.59±1.78	2.15±0.24	9.26±0.81	1.91±0.28	2.03±0.16[A]	107.42±7.10
	Green-legged Partridge*outdoor-prefering	68.00±2.80	21.49±1.25	1.97±0.13	9.29±0.46	1.53±0.10	1.78±0.09[BA]	104.05±4.23
					P-value			
Breed		0.303	0.985	0.615	0.449	0.030	0.526	0.659
Ranging profile		0.079	0.196	0.729	0.718	0.159	0.356	0.140
Breed*ranging profile		0.076	0.778	0.123	0.484	0.072	0.016	0.194

[A-C]Different letters within factor indicate significant differences (If the *P* value is < 0.05).

The analysis of the bacteria species relative abundance in the ceca of the birds in the current study showed the presence of the interaction between the genotype and ranging profile in 2 cases: *E. coli* and *Bifidobacterium* spp. relative abundance. The lowest relative abundance of *E. coli* was identified for outdoor-preferring Sasso and Green-legged Partridges and indoor Sasso birds. The lower abundance of *E. coli* identified in indoor-preferring Sasso chickens in the present study could suggest that the main reservoir of *E. coli* was found outdoors at the free ranges. In the case of the low *E. coli* abundance in outdoor-preferring birds, regardless of the genetic background, it can be suspected that it was associated with their frequent presence outside. Cereals commonly used in chicken diet are not only the source of valuable nutrients, but also contain antinutritional factors such as non-starch polysaccharides (**NSPs**), which reduce digestion and the level of peptides that exert beneficial effects on gut physiology, including the microbiome (Shakouri et al., 2009; Torok et al., 2011; Kers et al., 2018; Chen et al., 2019). In outdoor-preferring birds the consumption of pasture originating feed sources as a supplement to the indoor accessible cereal-based diet may have had a positive effect on the birds' microbial profile.

Moreover, the lowest relative abundance of *Bifidobacterium* spp. was found in the caecal content of indoor-preferring Sasso birds, as compared to all other birds in the experiment. *Bifidobacteria* produce lactic and acetic acids in large amounts and take part in the stabilization of the gastrointestinal barrier, modulation of the local and systemic immune responses, inhibition of the pathogenic invasion and promotion of the bioconversion of unavailable dietary compounds into bioactive healthy molecules (Rossi and Amaretti, 2010). Some strains of *Bifidobacterium* spp. have been found to prevent *E. coli* colonization in the mouse GIT, where the main mechanism of this action was via acetic acid synthesis by *Bifidobacterium* spp. strains, resulting in the reduction of the luminal pH (Asahara et al., 2004). This potential inhibitory role of *Bifidobacterium* spp. in indoor-preferring Sasso birds is, however, contradictory to low abundance of *E. coli* found in the same birds. Nevertheless, studies are needed to explain the mechanisms ruling the abundance of bacteria strains in indoor-preferring Sasso birds, which could help to improve those birds' health and optimize their welfare, while potentially promoting range use.

The genotype of the chickens in this study affected *Clostridium spp.* relative abundance, being higher in Sasso chickens as compared to the Green-legged Partridge. In some circumstances it may indicate unfavorable microbiome features in Sasso chickens, as some poultry pathogens belong to the larger *Clostridium* spp. group. For instance *Clostridium perfringens* may cause necrotic enteritis (Olkowski et al., 2008). On the other hand, dietary supplementation *Clostridium butyricum* had positive effects on the growth, immune response, gut microbiota, and intestinal barrier function of broilers (Li et al., 2021).

The significant effect of the interaction between a genotype and ranging profile was found only on the concentration of one SCFA, where the highest concentration of valeric acid was observed in moderate-outgoing Green-legged Partridges. Microbial communities perform an important role in the growth and gut health by producing SCFA (Dunkley et al., 2007), modulating the morphological structure of the intestinal tract (Shakouri et al., 2009), and consequently influencing nutrient digestion and absorption (Choct, 2009). The indigestible carbohydrates, in which pasture diet is rich, in the gut can be used and converted into SCFAs by the microbial communities in broilers (Józefiak et al., 2004). Their concentrations are used as biomarkers of microbiota development and microbial-host interactions (Liao et al., 2020). The concentration and types of fermentation products formed by gut bacteria depend on the relative amounts of each substrate available, bacteria species and fermentation strategy of bacteria involved in the fermentation process (Liao et al., 2020). For example, chicken diet components like cereal type influenced the fermentation process and had an impact on SCFA presence and concentration (Józefiak et al., 2004). Valeric acid glyceride esters, added to the feed, promoted broiler performance, positively affected the morphology of the small intestinal mucosa and reduced the incidence of necrotic enteritis (Onrust et al., 2018). Previously, in moderate-outgoing Green-legged Partridges the weight of the pasture matter in the crop was 3 times higher, as compared to moderate-outgoing Sasso, and there was significantly more pasture matter identified, as compared to other ranging profiled birds of that breed (Marchewka et al., 2021). Hence, it can be suspected that the higher concentrations of valeric acid in moderate-outgoing Green-legged Partridges were associated with the pasture matter-rich diet those birds had, supporting the favorable microbiota composition. However, the direct associations between the diet, intestinal tract health, and gut microbial composition in birds of various genetic backgrounds allowed access to the outdoor pastures are yet to be discovered.

The activity of some investigated bacterial enzymes has been shown to differ between genotypes, primarily based on the type of ingested feed as demonstrated in poultry nutritional studies (Hübener et al., 2002; Shakouri et al., 2009; Zdunczyk et al., 2014; Konieczka and Smulikowska, 2018; Chen et al., 2019; Konieczka et al., 2020). In the present study the activity levels of the 3 investigated bacterial enzymes, including α-glucosidase, β-glucuronidase and β-galactosidase were decreased in Sasso birds, as compared to Green-legged Partridges. Within the commensal intestinal microbiota, species with the potential to improve poultry performance are particularly important, as they are also involved in cross-relation between the microbiota, gut epithelium and immune system, providing resistance to enteric pathogens (Konieczka et al., 2019). Those probiotic species contribute to an increase in the activity of many bacterial glycolytic enzymes, such as α-galactosidase, which hydrolyses dietary α-galactosides (RFO and other oligosaccharides); β-galactosidase, which contributes to the hydrolysis of β-galactosides; and α- and β-glucosidase, which contribute to the hydrolysis of NSPs (cellulose, β-glucans; Hübener et al., 2002; Zdunczyk et al., 2014). The enhanced activity of some bacterial enzymes, particularly β-glucosidase and β-glucuronidase, may be detrimental to the bird's health (Jin et al., 2000; Konieczka et al., 2018). It is worth to pay attention to the current results, since the increased activity of β-glucuronidase may also be indicative of increased proliferation of pathogenic bacteria in the gut, and it is associated with the higher risk of toxic and carcinogenic substances generation from nontoxic glycosides (Beaud et al., 2005).

Higher isovaleric acid levels were observed in Sasso as compared to Green-legged Partridges, regardless of the ranging profile. The genetic background of the host has been recognized previously as a factor that might influence gut microbiota composition (Schokker et al., 2015; Han et al., 2016). Increased production of isovaleric acid, which belong to the putrefactive SCFA are indicative of unfavorable conditions in the gut, including increased shifts in pathogenic bacteria and increased ammonia production (Koh et al., 2016). When comparing 2 breeds used in the current study, Sasso growth rates are much higher due to intensive genetic selection on this trait, as compared to Green-legged Partridges. The average slaughter body weight of roosters is around 2.5 kg and hens around 1.7 kg, which is achieved at about 5 mo of age (Krawczyk, 2009; Siwek et al., 2013). In comparison, Sasso birds reach a slaughter weight of 2.3 to 2.8 kg at about 2 mo of age (Getiso et al., 2017). In broilers (Arbor Acres male broilers), the concentration of isovalerate has previously been identified as increasing with the age of the birds (Liao et al., 2020). Therefore, the identified effects of the genetic background on the isovaleric acid concentrations could reflect the higher growth rates characteristic to Sasso birds. Higher concentrations of isovaleric acid in Sasso chickens may also indicate a poorer intestinal health resulting in poorer birds' welfare, which require further attention. Finally, the study design, where birds were reared in breed-specific groups, could influence the results to some extent, as other studies reported that birds housed together show less variation of the gut microbiota, known as the cage effect (Meyer et al., 2012; Zhao et al., 2013; Chen et al., 2019), which may have wiped out the ranging profile effect.

Gut microbiome profile and diversity are closely linked to ensuring the health of the poultry used for meat production. Microbiome functions include protection against pathogens, nutrients production, and host immune system maturation (Stanley et al., 2014). Better health of the birds and optimal adaptation of their genotype to the housing systems with outdoor access safeguards their high welfare but also high productivity (Aruwa et al., 2021). Therefore, good understanding of the host-microbiome relationship remains integral. In the current study, some important knowledge gaps have been identified. In outdoor-preferring birds' consumption of pasture originating feed sources as a supplement to the indoor accessible cereal-based diet may have positive effects on the birds' microbial profile. However, there is not much known yet about the potential protective role of providing outdoor access to the birds in order to reduce *E. coli* levels in the gut and avoid secondary infections. Finally, the full interactions between the diet and intestinal health in birds of various genetic backgrounds with access to the outdoor pastures are yet to be discovered.

CONCLUSIONS

Our hypothesis that the chickens which have been identified as homogeneous in terms of ranging profile will show similar quantitative microbial composition of the same genus and similar gut microbiota activity regardless of the breed was partially confirmed. The lowest relative abundance of *E. coli* was identified for outdoor-preferring Sasso and outdoor-preferring Green-legged Partridges. Therefore, in outdoor-preferring birds, consumption of pasture originating feed sources as a supplement to the indoor accessible cereal-based diet may have positive effects on the bird's microbial profile. Furthermore, we found significant effects of the genotype on the various parameters analyzed. Nevertheless, direct links between the diet, and gut microbial composition and intestinal health in birds of various genetic backgrounds that had access to the outdoor pastures are yet to be discovered.

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DISCLOSURES

The authors do not have conflict of interest to report.

REFERENCES

Aruwa, C. E., C. Pillay, M. M. Nyaga, and S. Sabiu. 2021. Poultry gut health−microbiome functions, environmental impacts, microbiome engineering and advancements in characterization technologies. J. Anim. Sci. Biotechnol. 12:1–15.

Asahara, T., K. Shimizu, K. Nomoto, T. Hamabata, A. Ozawa, and Y. Takeda. 2004. Probiotic bifidobacteria protect mice from lethal infection with Shiga toxin-producing Escherichia coli O157: H7. Infect. Immun. 72:2240–2247.

Beaud, D., P. Tailliez, and J. Aba-Mondoloni. 2005. Genetic characterization of the β-glucuronidase enzyme from a human intestinal bacterium Ruminococcus gnavus. Microbiology 151:2323–2330.

Binek, M., A. A. Cisek, M. R. D. Chrobak-Chmiel, I. Stefańska, and M. Kizerwetter-Świda. 2017. Mikrobiom jelitowy kury domowej - Rozwój i funkcja. Med. Weter. 73:618–625.

Bjerrum, L., R. M. Engberg, T. D. Leser, B. B. Jensen, K. Finster, and K. Pedersen. 2006. Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. Poult. Sci. 85:1151–1164.

Brisbin, J. T., J. Gong, S. Orouji, J. Esufali, A. I. Mallick, P. Parvizi, P. E. Shewen, and S. Sharif. 2011. Oral treatment of chickens with lactobacilli influences elicitation of immune responses. Clin. Vaccine Immunol. 18:1447–1455.

Campbell, D. L. M., G. N. Hinch, J. A. Downing, and C. Lee. 2016. Fear and coping styles of outdoor-preferring, moderate-outdoor and indoor-preferring free-range laying hens. Appl. Anim. Behav. Sci. 185:73–77.

Chen, S., H. Xiang, H. Zhang, X. Zhu, D. Wang, J. Wang, T. Yin, L. Liu, M. Kong, H. Li, and X. Zhao. 2019. Rearing system causes changes of behavior, microbiome, and gene expression of chickens. Poult. Sci. 98:3365–3376.

Choct, M. 2009. Managing gut health through nutrition. Br. Poult. Sci. 50:9–15.

Classen, H. L. 2017. Diet energy and feed intake in chickens. Anim. Feed Sci. Technol. 233:13–21.

Dunkley, K. D., C. S. Dunkley, N. L. Njongmeta, T. R. Callaway, M. E. Hume, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2007. Comparison of in vitro fermentation and molecular microbial profiles of high-fiber feed substrates incubated with chicken cecal inocula. Poult. Sci. 86:801–810.

Forder, R. E. A., G. S. Howarth, D. R. Tivey, and R. J. Hughes. 2007. Bacterial modulation of small intestinal goblet cells and mucin composition during early posthatch development of poultry. Poult. Sci. 86:2396–2403.

Getiso, A., B. Bekele, B. Zeleke, D. Gebriel, A. Tadesse, E. Abreham, and H. Jemal. 2017. Production performance of Sasso (distributed by ethio-chicken private poultry farms) and Bovans brown chickens breed under village production system in three agro-ecologies of Southern Nations, Nationalities, and Peoples Regional State (SNNPR). Ethiopia. Int. J. Livest. Prod. 8:145–157.

Gharib-Naseri, K., S. K. Kheravii, C. Keerqin, N. Morgan, R. A. Swick, M. Choct, and S.-B. Wu. 2019. Two different Clostridium perfringens strains produce different levels of necrotic enteritis in broiler chickens. Poult. Sci. 98:6422–6432.

Gross, W. B. 1990. Factors affecting the development of respiratory disease complex in chickens. Avian Dis 34:607–610.

Han, Z., T. Willer, C. Pielsticker, L. Gerzova, I. Rychlik, and S. Rautenschlein. 2016. Differences in host breed and diet influence colonization by Campylobacter jejuni and induction of local immune responses in chicken. Gut Pathog. 8:1–14.

Hübener, K., W. Vahjen, and O. Simon. 2002. Bacterial responses to different dietary cereal types and xylanase supplementation in the intestine of broiler chicken. Arch. Anim. Nutr. Tierernahrung 56:167–187.

Hubert, S. M., M. Al-Ajeeli, C. A. Bailey, and G. Athrey. 2019. The role of housing environment and dietary protein source on the gut microbiota of chicken. Animals 9:1–16.

Jin, L. Z., Y. W. Ho, N. Abdullah, and S. Jalaludin. 2000. Digestive and bacterial enzyme activities in broilers fed diets supplemented with Lactobacillus cultures. Poult. Sci. 79:886–891.

Józefiak, D., A. Rutkowski, and S. A. Martin. 2004. Carbohydrate fermentation in the avian ceca: a review. Anim. Feed Sci. Technol. 113:1–15.

Jurgoński, A., J. Juśkiewicz, and Z. Zduńczyk. 2013. An anthocyanin-rich extract from Kamchatka honeysuckle increases enzymatic activity within the gut and ameliorates abnormal lipid and glucose metabolism in rats. Nutrition 29:898–902.

Kers, J. G., F. C. Velkers, E. A. J. Fischer, G. D. A. Hermes, D. M. Lamot, J. A. Stegeman, and H. Smidt. 2019. Take care of the environment: housing conditions affect the interplay of nutritional interventions and intestinal microbiota in broiler chickens. Anim. Microbiome 1:1–14.

Kers, J. G., F. C. Velkers, E. A. J. Fischer, G. D. A. Hermes, J. A. Stegeman, and H. Smidt. 2018. Host and environmental factors affecting the intestinal microbiota in chickens. Front. Microbiol. 9:1–14.

Kogut, M. H. 2019. The effect of microbiome modulation on the intestinal health of poultry. Anim. Feed Sci. Technol. 250:32–40.

Koh, A., F. De Vadder, P. Kovatcheva-Datchary, and F. Bäckhed. 2016. From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites. Cell 165:1332–1345.

Konieczka, P., J. Czerwiński, J. Jankowiak, K. Ząbek, and S. Smulikowska. 2019. Effects of partial replacement of soybean meal with rapeseed meal, narrow-leaved lupin, DDGS, and probiotic supplementation, on performance and gut microbiota activity and diversity in broilers. Ann. Anim. Sci. 19:1115–1131.

Konieczka, P., S. A. Kaczmarek, M. Hejdysz, M. Kinsner, D. Szkopek, and S. Smulikowska. 2020. Effects of faba bean extrusion and phytase supplementation on performance, phosphorus and nitrogen retention, and gut microbiota activity in broilers. J. Sci. Food Agric. 100:4217–4225.

Konieczka, P., K. Nowicka, M. Madar, M. Taciak, and S. Smulikowska. 2018. Effects of pea extrusion and enzyme and probiotic supplementation on performance, microbiota activity and biofilm formation in the broiler gastrointestinal tract. Br. Poult. Sci. 59:654–662.

Konieczka, P., and S. Smulikowska. 2018. Viscosity negatively affects the nutritional value of blue lupin seeds for broilers. Animal 12:1144–1153.

Krawczyk, J. 2009. Quality of eggs from Polish native Greenleg Partridge chicken-hens maintained in organic vs. backyard production systems. Anim. Sci. Pap. Rep. 27:227–235.

Landman, W. J. M., A. Heuvelink, and J. H. H. Van Eck. 2013. Reproduction of the Escherichia coli peritonitis syndrome in laying hens. Avian Pathol. 42:157–162.

Li, W., B. Xu, L. Wang, Q. Sun, W. Deng, F. Wei, H. Ma, C. Fu, G. Wang, and S. Li. 2021. Effects of clostridium butyricum on growth performance, gut microbiota and intestinal barrier function of broilers. Front. Microbiol. 12:777456.

Liao, X., Y. Shao, G. Sun, Y. Yang, L. Zhang, Y. Guo, X. Luo, and L. Lu. 2020. The relationship among gut microbiota, short-chain fatty acids, and intestinal morphology of growing and healthy broilers. Poult. Sci. 99:5883–5895.

Marchewka, J., P. Sztandarski, Ż. Zdanowska-Sąsiadek, K. Damaziak, F. Wojciechowski, A. B. Riber, and S. Gunnarsson. 2020. Associations between welfare and ranging profile in free-range commercial and heritage meat-purpose chickens (Gallus gallus domesticus). Poult. Sci. 99:4141–4152.

Marchewka, J., P. Sztandarski, Ż. Zdanowska-Sąsiadek, D. Adamek-Urbańska, K. Damaziak, F. Wojciechowski, A. B. Riber, and S. Gunnarsson. 2021. Gastrointestinal tract morphometrics and content of commercial and indigenous chicken breeds with differing ranging profiles. Animals 11:1881.

Meyer, B., A. W. Bessei, W. Vahjen, J. Zentek, and A. Harlander-Matauschek. 2012. Dietary inclusion of feathers affects intestinal microbiota and microbial metabolites in growing leghorn-type chickens. Poult. Sci. 91:1506–1513.

Michalczuk, M., E. Holl, A. Möddel, A. Jóźwik, J. Slósarz, D. Bień, K. Ząbek, and P. Konieczka. 2021. Phytogenic ingredients from hops and organic acids improve selected indices of welfare, health status markers, and bacteria composition in the caeca of broiler chickens. Animals 11:3249.

Moharrery, A., and M. Mahzonieh. 2005. Effect of malic acid on visceral characteristics and coliform counts in small intestine in the broiler and layer chickens. Int. J. Poult. Sci. 4:761–764.

Ocejo, M., B. Oporto, and A. Hurtado. 2019. 16S rRNA amplicon sequencing characterization of caecal microbiome composition of broilers and free-range slow-growing chickens throughout their productive lifespan. Sci. Rep. 9:1–14.

Olkowski, A. A., C. Wojnarowicz, M. Chirino-Trejo, B. Laarveld, and G. Sawicki. 2008. Sub-clinical necrotic enteritis in broiler chickens: novel etiological consideration based on ultra-structural and molecular changes in the intestinal tissue. Res. Vet. Sci. 85:543–553.

Onrust, L., K. Van Driessche, R. Ducatelle, K. Schwarzer, F. Haesebrouck, and F. Van Immerseel. 2018. Valeric acid glyceride esters in feed promote broiler performance and reduce the incidence of necrotic enteritis. Poult. Sci. 97:2303–2311.

Pan, D., and Z. Yu. 2014. Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes 5:108–119.

Rodriguez-Aurrekoetxea, A., and I. Estevez. 2016. Use of space and its impact on the welfare of laying hens in a commercial free-range system. Poult. Sci. 95:2503–2513.

Rossi, M., and A. Amaretti. 2010. Probiotic properties of bifidobacteria.

Sanchez, C., and I. Estevez. 1998. The Chickitizer software program. Coll. Park. Maryland, Univ. Maryland.

Schokker, D., G. Veninga, S. A. Vastenhouw, A. Bossers, F. M. de Bree, L. M. T. E. Kaal-Lansbergen, J. M. J. Rebel, and M. A. Smits. 2015. Early life microbial colonization of the gut and intestinal development differ between genetically divergent broiler lines. BMC Genomics 16:1–13.

Shakouri, M. D., P. A. Iji, L. L. Mikkelsen, and A. J. Cowieson. 2009. Intestinal function and gut microflora of broiler chickens as influenced by cereal grains and microbial enzyme supplementation. J. Anim. Physiol. Anim. Nutr. (Berl). 93:647–658.

Siwek, M., D. Wragg, A. Sławińska, M. Malek, O. Hanotte, and J. M. Mwacharo. 2013. Insights into the genetic history of G reen-legged P artridgelike fowl: mt DNA and genome-wide SNP analysis. Anim. Genet. 44:522–532.

Stanley, D., R. J. Hughes, and R. J. Moore. 2014. Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. Appl. Microbiol. Biotechnol. 98:4301–4310.

Stern, N. J., M. R. S. Clavero, J. S. Bailey, N. A. Cox, and M. C. Robach. 1995. Campylobacter spp. in broilers on the farm and after transport. Poult. Sci. 74:937–941.

Sztandarski, P. 2021. Marchewka J., Wojciechowski F., Riber A.B., Grunnarsson S. and Horbańczuk J.O.,. 2021. Associations between weather conditions and individual range use by commercial and heritage chickens. Poultry Science 100, doi:10.1016/j.psj.2021.101265.

Thaiss, C. A., M. Levy, T. Korem, L. Dohnalová, H. Shapiro, D. A. Jaitin, E. David, D. R. Winter, M. Gury-BenAri, E. Tatirovsky, T. Tuganbaev, S. Federici, N. Zmora, D. Zeevi, M. Dori-Bachash, M. Pevsner-Fischer, E. Kartvelishvily, A. Brandis, A. Harmelin, O. Shibolet, Z. Halpern, K. Honda, I. Amit, E. Segal, and E. Elinav. 2016. Microbiota diurnal rhythmicity programs host transcriptome oscillations. Cell 167:1495–1510 e12.

Torok, V. A., R. J. Hughes, L. L. Mikkelsen, R. Perez-Maldonado, K. Balding, R. MacAlpine, N. J. Percy, and K. Ophel-Keller. 2011. Identification and characterization of potential performance-related gut microbiotas in broiler chickens across various feeding trials. Appl. Environ. Microbiol. 77:5868–5878.

van der Eijk, J. A. J., T. B. Rodenburg, H. de Vries, J. B. Kjaer, H. Smidt, M. Naguib, B. Kemp, and A. Lammers. 2020. Early-life microbiota transplantation affects behavioural responses, serotonin and immune characteristics in chicken lines divergently selected on feather pecking. Sci. Rep. 10:1–13.

Zdunczyk, Z., J. Jankowski, A. Rutkowski, E. Sosnowska, A. Drazbo, P. Zdunczyk, and J. Juskiewicz. 2014. The composition and enzymatic activity of gut microbiota in laying hens fed diets supplemented with blue lupine seeds. Anim. Feed Sci. Technol. 191:57–66.

Zhao, L., G. Wang, P. Siegel, C. He, H. Wang, W. Zhao, Z. Zhai, F. Tian, J. Zhao, H. Zhang, Z. Sun, W. Chen, Y. Zhang, and H. Meng. 2013. Quantitative genetic background of the host influences gut microbiomes in chickens. Sci. Rep. 3:1–6.

Zhu, X. Y., T. Zhong, Y. Pandya, and R. D. Joerger. 2002. 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. Appl. Environ. Microbiol. 68:124–137.