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Impact Of Genetics On Behavior Variations In Outdoor Raised Hens

by

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Abstract: Outdoor access is essential for natural behaviors expression and supports animal welfare. Previous studies detected variations in outdoor visits of chickens, but the factors influencing this behavior remain unclear. This study explores behavioral patterns along with phenotypic and genetic parameters of ranging in White Leghorn hens. Range use was recorded in 397 hens using radio frequency identification (RFID) technology over a 26-day period between 18 and 22 weeks of age. All hens were vaccinated against four major poultry diseases, genotyped, and assessed for immune and stress-related indicators, including vaccine antibody responses and heterophil-to-lymphocyte (H/L) ratio. We explored associations between range use, weather conditions, and immune traits, and estimated genetic parameters using heritability and genome-wide association analyses. We found a consistent negative association of average visit duration with frequency based ranging metrics, suggesting behavior as multidimensional. Temperature has weak negative correlation (r = -0.1) while humidity, rainfall and wind force have positive correlations (r = 0.2 – 0.35) with the number of hens outside. Weak and infrequent correlations were observed between range use and immune response for Newcastle disease, while no significant relationships for other vaccines. Moderate heritability estimates for all measured ranging traits (h2 = 0.24 – 0.33), indicate genetic influence but no significant genetic variants were identified through genome wide association study, confirming a polygenic effect. These results suggest that range use in laying hens is partly influenced by weather and genetics, but shows limited association with the immune traits considered. Further studies should explore range use patterns in relation to vaccine response over longer periods and identify quantitative trait loci in larger populations.

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GWAS	:	Genome-Wide Association Studies
NDV	:	Newcastle Disease Virus
AEV	:	Avian Encephalomyelitis Virus
IBV	:	Infectious Bronchitis Virus
APV	:	Avian Polyomavirus Vaccine
RFID	:	Radio Frequency Identification
H/L	:	Heterophil/Lymphocyte
QTL	:	Quantitative Trait Loci

Introduction

1.1. Animal behavior and welfare

Animal welfare is defined as the state of an animal, encompassing its ability to adapt to its environment, its emotional experiences, and the expression of natural behaviors (Mellor et al., 2009). Good welfare exists when the animal has positive experiences through its interactions with the environment, without suffering. Welfare can be significantly compromised if the environment prevents the expression of the animal's natural behaviors (Rollin, 2006). Ensuring animal welfare is a fundamental principle of organic agriculture practices (IFOAM, 2021) as better subjective experiences of animals and natural life ensure better health and enhanced biological functioning (Fraser et al., 1997). In recent years, there has been growing societal demand for higher animal welfare standards, driven by increasing public awareness and reinforced by stricter regulations. Behavior is considered as a promising tool to understand the animal's health and state. Natural behavior refers to the actions an animal exhibits under environmental conditions similar to its natural habitat (Bracke & Hopster, 2006). It serves as a reliable indicator of bird welfare and provides a non-invasive approach to assessing animal well-being (María et al., 2004). European Union regulations underline several species-specific behavioral needs, including outdoor access for animals (EC, 2008). Earlier research in ethology considered behavioral patterns regarding feeding and reproduction as important as they are directly related to productivity (Gonyou, 1994). However, natural behaviors, individual preferences, cognitive abilities, and emotional states are also considered appealing now (Bhanja & Bhadauria, 2018). Animals employ a diverse range of behavioral and physiological responses to regulate their lives. Aggression, boredom, stress, and other abnormal behaviors are typically associated with negative welfare states (Fraser, 2008). Selection of animals during domestication and breeding processes also altered their behavior. Genetic predisposition and early rearing environment can also give rise to problematic behavior in animals (Brantsæter et al., 2018).

1.2. Importance of studying poultry behavior

Poultry eggs and meat are one of the most common food sources worldwide, as well as a key to nutrition. Poultry is also one of the most efficient categories of livestock, as it has expanded enormously during the last few decades. According to FAOSTATS, there were 376 million laying hens in the EU in 2021 which produced 6.5 million tons of eggs in the same year. Globally, egg production has undergone remarkable growth in recent decades. According to estimates from ITAVI (ITAVI, 2022), global egg production has more than doubled since 1990, reaching nearly 76 million tons in 2021. The traditional or conventional cage system for poultry negatively effects animal health, welfare, and behavior (Hemsworth, 2021.). Egg industry is currently facing it as the most challenging welfare issue (María et al., 2004). This rapid growth in poultry production brings increased attention to the conditions under which birds are kept, and with it, a greater responsibility to ensure their welfare. Understanding poultry behavior is central to this effort.

Common natural behaviors for laying hens are dust bathing, perching, foraging and nesting (Appleby et al., 1993), comfort behaviors (e.g. wing flapping) (Nicol, 1989), aggressive behaviors (e.g. cannibalism, feather pecking) (Rodenburg et al., 2008) and social behaviors (e.g. flock and group formation, social hierarchy) (Carvalho et al., 2018). These are innate behaviors, often driven by internal factors and regulated physiologically (Hemsworth & Edwards, 2020).

1.3. Ranging behavior in poultry

Ranging behavior, which refers to the tendency of chickens to explore and utilize outdoor areas, is highly diverse and varied at the flock and individual level. It is considered a natural behavior, allowing birds to perform species-specific activities such as foraging, dust bathing, pecking, and exploring. It is dependent on the environment (season, weather, temperature, and humidity) (Dawkins et al., 2003) . Chickens can access the outdoors either to have exploration opportunities or to avoid uncomfortable stimuli inside the shed (Taylor et al., 2017). There is a variety among studies showing either most laying hens like to visit the outdoors or not (Larsen et al., 2017) (Taylor et al., 2017; Gilani et al., 2014) , and reluctance among animals to go too far from their shed is also reported (Dawkins et al., 2003). This variation in ranging behavior has been linked to individual differences in fearfulness, curiosity, and coping styles, which may influence how hens interact with their environment (Campbell et al., 2016; Kolakshyapati et al., 2020). Encouraging range use through good design of the outdoor area, shelter availability, and rearing practices can contribute to improved welfare outcomes by allowing hens to express their behavioral needs more fully.

1.4. Free range versus cage farming

Laying hen farming in Europe has undergone major transformations over the past sixty years. In the 1960s, in response to increasing demand for poultry products, conventional cage farming became widespread (Boyd, 2001), replacing smaller and more diverse traditional systems, such as aviaries or floor-based farming (Le Bouquin et al., 2013). This transition led to a rapid increase in the number of hens per farm, made possible by the mechanization of processes such as egg collection and the distribution of food and water (Leenstra et al., 2016). Hen’s housing systems can be divided into two categories: cage-based farming (Widowski et al., 2017) and cage-free systems (also known as “alternative” systems), which include barn, free-range, and organic farming (Bonnefous et al., 2022).

Conventional cage systems in which hens are kept in a mechanically operated restricted area are easy to manage, economical, more hygienic and normally have a lower rate of infectious disease outbreak (Rodenburg et al., 2005). However, the space provided is often not enough for animals (Hartcher & Jones, 2017), due to which, restricted behavior and reduced physical activity can cause metabolic disorders, disuse osteoporosis (Whitehead & Fleming, 2000; Widowski et al., 2017), and the animals can go through severe frustration due to a lack of normal behaviors such as nesting and foraging (Duncan, 2020). On the other hand, cage-free systems allow hens to express their full natural behavior, however, this is also greatly dependent on their population density and management of range (Campbell et al., 2017) . Outdoor exposure of chickens benefits them in many ways as compared to staying inside (Sherwin et al., 2013). It is also vital to furnish the range area with essential resources like nesting sites and abundant perching space. It is important to consider that the activities like foraging, ground-scratching and dustbathing are impossible in conventional cages and are limited in the furnished cages (Hartcher & Jones, 2017).

Globally, cage farming remains predominant, however consumer preferences are rapidly shifting towards free range hens. This transition demands for more environmentally responsible and animal-friendly food production, particularly emphasizing outdoor access.

1.5. Ranging behavior tracking through radio frequency identification

Studying individual hen behavior in large commercial flocks exhibits significant constraints. Conventional methods like human observation are labor-intensive and often impractical for large-scale studies (Rozempolska-Rucińska et al., 2017; Siegford et al., 2016). Various technologies are used in precision livestock farming to monitor poultry behavior, like image processing, for flock activity analysis, sound analysis for growth, and other biological conditions, and radio frequency identification for tracking location and locomotion of animals (Li et al., 2020). Radio frequency identification technology (RFID) is a method of wireless transmission which uses radio wave frequency to track and tag items. It consists of three components, i.e. a tag, an antenna and a reader. A normal RFID tag has a chip, a circuit to harvest energy and memory (Feiyang et al., 2016). RFID tags could be passive, semi-passive or active depending upon their reliance upon reader power source and communication system. There are RFID tags of low frequency (120-134 kHz) which read in the 10 cm range and have slower speed and that of high frequency around 13 MHz (Finkenzeller, 2010). RFID tags can be banded on chicken's leg back or wings band. Leg tagging is commonly practiced because of minimal interference with animals' natural activities (Siegford et al., 2016). These tags are detected by RFID antennas that are commonly placed at critical points in range, like pop holes or designated entry or exit areas. Recording of each tag by a unique signal generated by RFID chip by antennae allows monitoring of duration and timing of visit (Stadig et al., 2018). However, limitations exist, including interference from environmental factors such as metal structures, which can disrupt signal transmission (Catarinucci et al., 2013). Additionally, tag loss or damage due to pecking or wear over time can reduce data reliability. Moreover, semi-passive or active tags may require battery replacements, adding maintenance costs in large-scale commercial systems (Larsen et al., 2017). RFID offers several advantages over traditional methods, such as visual observation and manual data collection (Ilie-Zudor et al., 2011). Various studies have used RFID-based monitoring for individual hen behavior such as assessing their activity and location (Siegford et al., 2016) finding ranging behavior patterns (Larsen et al., 2017) and other behaviors like perching (Cauchoix et al., 2022). A key advantage is its non-invasive nature, which minimizes stress and discomfort for the animals during the monitoring process. Technological limitations like signal obstruction, high-speed movement causing missed readings, and tag collisions in dense flocks built challenges to the reliability of data. These factors can confound behavior classification and increase the risk of bias in results (Iserbyt et al., 2018). Therefore, while RFID technology enables detailed behavioral tracking, careful experimental design, statistical handling of time-series data, and validation procedures are essential to account for the complexity and variability inherent in large-group poultry systems (Siegford et al., 2016)

1.6. Genetic determinism of ranging behavior

Ranging behavior is a complex trait influenced by both genetic and environmental factors (Pettersson et al., 2016; Buitenhuis et al., 2005). Genotype plays a significant role in determining range use frequency in chickens, as certain breeds exhibit a greater tendency to explore outdoor areas than others." (Ferreira et al., 2024). In identical farming conditions, traditional or heritage chicken breeds tend to explore the outdoor range more than commercial laying hens, which are primarily selected for productivity (Sokołowicz et al., 2020). White Lohmann Selected Leghorn (LSL) hens moved outdoors more frequently and spent more time in outdoor compared to brown Lohmann Traditional (LT) hens, which preferred the grassland area (Mahboub et al., 2004). Ranging behavior is also found to be associated with certain other behavioral traits, such as adaptability and reduced aggression, are known to improve the animals' compatibility with free-range systems (Ferreira et al., 2020). Hens with lesser outdoor preference have elevated fear levels (Campbell et al., 2016) which indicates a high level of stress and secretion of corticosterone and are negatively correlated with performance traits (egg size, quality, sexual maturity, etc.). Eliminating such birds from breeding programs and using fearfulness indicators as selection criteria can improve welfare and productivity (Rozempolska-Rucińska et al., 2017). Understanding the genetic mechanisms underlying this behavior might allow the development of innovative strategies such as selective breeding for enhanced outdoor use or adapting rearing conditions to better match the behavioral tendencies of specific genetic lines. It can help to improve poultry welfare and optimize free-range systems.

1.7. Vaccine response in outdoor hens

Vaccination is the foremost strategy in veterinary medicine to protect against pathogens. Animals are vaccinated to prevent infectious diseases, but individual vaccine responses are highly varied across different chicken lines, highlighting the role of host genetics on vaccine response variability (Simon et al., 2016; Arango et al., 2024; Luo et al., 2013). Genetic variations have been shown to lead to a variability in individual vaccine responses in hens (Pinard van der Laan et al.). In addition to the role of host genetics, previous studies showed that the gut microbiota composition can impact vaccine response levels in chicken (Yitbarek et al., 2019). Since hens raised outdoor harbor a distinct microbiota (Schreuder et al., 2020), it can hypothesize that this different microbiota composition has an effect on the vaccine response.

As outdoor access influences exploration of animals for diet and its microbiota composition (Kers et al., 2018), it can be a factor for differential vaccine response in groups of animals according to their behavior. Beside the differential microbiota, another hypothesis is about greater energy expenditure of highly active hens (possibly accessing more outdoors) which can increase their metabolic demand. This might come at a trade-off with other biological functions like immune responsiveness . However, Hofmann et al., 2020 reviewed that though housing conditions might impact immune system and vaccination response in hens, the results are not simple and unanimous enough to draw the conclusion and more investigation is needed to be done. Humoral response in free-range hens is reported to be lower than caged ones against Newcastle’s virus by Arbona et al., 2011 while another study found higher antibody production against Newcastle and infectious bronchitis virus disease viruses in Asil chicken breed (Rehman et al., 2017). Since vaccine response is crucial for managing animal health, understanding its relationship with outdoor access can help inform better housing or vaccination strategies that support both health and welfare.

1.8. Physiological stress in outdoor hens

Stress occurs when an animal detect changes in environment and get stimulated to regulate it by homeostasis (Odihambo Mumma et al., 2006). One widely used biomarker for assessing physiological stress in poultry is the heterophil-to-lymphocyte (H/L) ratio. Heterophils are a type of white blood cell involved in the bird’s innate immune response and are typically elevated in response to stress, inflammation, or infection (Harmon, 1998). Lymphocytes, on the other hand, are responsible for adaptive immunity and tend to decrease under prolonged stress (Clark, 2015). An elevated H/L ratio is commonly associated with increased stress levels, whereas a lower ratio indicates better coping ability and welfare status (Gross & Siegel, 1983). In the context of ranging behavior, the H/L ratio is used to assess whether hens that use the outdoor area more frequently experience lower chronic stress levels. There is no clear relationship established between outdoor access and H/L ratio because of several cofounding factors present in previous studies like aviary system, pen size, age (Lentfer et al., 2015), weather conditions (Kim et al., 2022) or tonic immobility (Mahboub et al., 2004). However, increased H/L ratio are reported in cage systems specially in poor conditions (Moe et al., 2010). Comparing stress levels in hens across different durations of outdoor access can provide a clearer understanding of their overall welfare

1.9. Objectives of study

The demand for outdoor-reared chickens is rising, driven by societal expectations for improved animal welfare. However, little is known about its real impact on animal health and welfare. The aim of this study is to explore the impact of host genetic variations on animal behavior. Another objective is to establish how behavior and various health-related parameters are associated. The answers to these questions will enable us to identify innovative ways to control free-range chicken farming by measuring behavior.

The main objectives of this study are to:

- Obtain individual data from raw identification data, evaluate the behavior of going outside, which will be used to determine variations in parameters in the population.
- Look for phenotypic correlations with other traits, e.g., vaccine response and heterophil/ lymphocyte ratio as stress indicator
- Link the variations with individual genotypes to measure their heritability and, if distribution in population allows, to search for genetic regions controlling variations by genome-wide association studies.

Materials and methods

2.1. Animals and husbandry

A total of 570 White Leghorn laying hens were raised at the INRAE Nouzilly site (PEAT Experimental Unit, Val de Loire, France, in collaboration with Novogen). Following week 5, animals were selected to reduce the population to 410. This selection was carried out based on their pedigree determined by genotyping to optimize family composition by equilibrating the number of sibs per family, resulting in the selection of animals from 294 hens bred with 56 cocks (with only 27 real sisters). This step was necessary because the eggs furnished by Novogen were not identified individually and 410 was the maximum number of hens that could be handled for this experimentation during 22 weeks. The hens were given outdoor access starting at 10 weeks of age and were monitored until 22 weeks of age. Feed and water were provided ad libitum inside the shed. Daily outdoor access was granted from 8.30 a.m. to 10.30 p.m. The experimental period extended from April 4, 2023, to September 4, 2023. To assess vaccine response, blood samples were collected at multiple time points. At the end of the experiment, spleen samples were collected to evaluate cell-mediated immune responses.

2.2. Genotyping

All animals were genotyped using a 57K SNP chip. DNA extractions and genotyping were done at Labogena (Labogena, Palaiseau, France). The initial dataset consisted of 571 samples with 55,189 variants. The genotype data were provided in PLINK format (.bim, .bed, and .fam) (Purcell et al., 2007). The first step of data processing involved the removal of samples with sex discrepancies, reducing the number of animals to 557. Subsequently, quality control was performed using PLINK (Purcell et al., 2007) with the following parameters: minor allele frequency (MAF) ≥ 0.05, call rate $\leq 5\%$ missing genotypes (--geno 0.05), and individual call rate $\leq 5\%$ missing genotypes (--mind 0.05), alongside a Hardy-Weinberg equilibrium test (HWE) threshold of 10*e-6. Samples deviating by more than three standard deviations from the expected heterozygosity were also excluded. After these steps, 552 samples remained for further analysis, with 34,348 variants passing the quality control filters.

2.3. Sample collection

Vaccination and sample collection were performed as part of a previously conducted experiment. The hens were vaccinated against Infectious Bronchitis Virus (IBV), Avian Encephalomyelitis Virus (AEV), Avian Polyomavirus Vaccine (APV) , and Newcastle Disease Virus (NDV) at various time points up to 22 weeks of age. For vaccine responses to NDV, IBV, AEV, and APV vaccinations, blood samples were taken at weeks 5, 8, 10, 12, 14, 16, 18, and 22 from venous occipital sinus and processed for humoral immune response analysis using commercial ELISA kits (Innovative Diagnostics (ID Screen® Newcastle Disease Indirect Conventional Vaccines, ID Screen® Infectious Bronchitis Indirect 2.0, ID Screen® AEV Indirect, and ID Screen® Avian Metapneumovirus Indirect),. A cellular immune response to NDV was also assessed at week 22 using an ELISpot (enzyme-linked immunospot) assay. Hematological parameters, including the heterophil-to-lymphocyte (H/L) ratio, were determined by blood cell counting by flow cytome

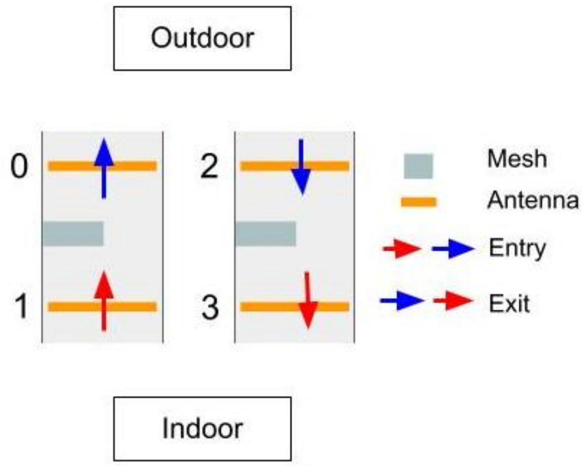

Figure 1. Illustration of two hatches consisting of RFID antennas and zig-zag passage. Arrows showing the direction of entry and exit from range.

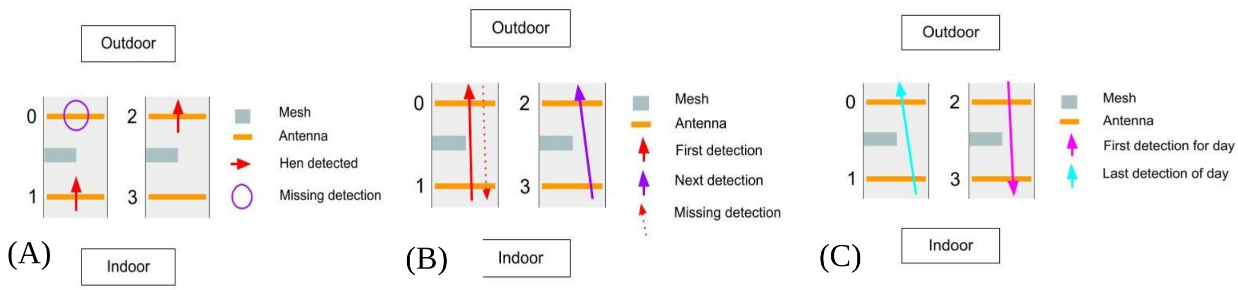

Figure 2. Illustration of various type of erroneous detections in data; (A) aberrant flag when a hen has first detection in one tunnel and very next detection in second tunnel; (B) Two consecutive entries without any exit detection means that an exit is missing; (C) if first detection in the day was an exit or last detection was an entry

2.4. RFID antennas and tracking

Two tunnel-like hatches were constructed at the shed's openings to allow outdoor access. Each tunnel was equipped with two RFID antennas, as illustrated in Figure 1. A visit to the outdoor area was defined when a hen passed both antennas in the outbound direction (entry into the range area), and the visit ended when the hen crossed both antennas again in the inbound direction (returning to the shed). The entry and exit times were recorded for each visit and each individual hen.

To minimize signal loss due to dirt accumulation on the detectors during the day, personnel cleaned the antennas several times daily. To ensure proper tracking, the tunnels were designed to require hens to follow a zigzag path. Additionally, the ceiling of the tunnel was lowered to encourage hens to walk directly over the detectors, and not to fly, ensuring accurate signal detection. To avoid these physical modifications from discouraging the hens or limiting their willingness to exit the shed, all obstacles were created with wire mesh structures. This allowed hens to maintain visual contact with the outdoor area, encouraging natural movement toward the range

2.5. Data collection

RFID detection was started on July 25, 2023 (Week 17), but due to an interruption in the data recording process, we considered the data from August 7, 2023, to September 4, 2023. 20, 21, and 31 of August were also skipped from the analysis due to an interruption in recording and an incomplete dataset. For each detection at an antenna, the animal's unique RFID identifier, time of detection, antenna number, and date were recorded. When a hen entered or exited the tunnel, it was detected by two antennas, one after the other. For calculating the visit duration, we took the time from the first antenna when entering and the time from the second antenna when exiting. Entry and exit pathways are clearly illustrated in Figure 1. Wrong detections were marked as flags and removed from the data. Wrong entries included: (i) if a hen has first detection in one tunnel and very next detection in second tunnel, which is physically not possible; (ii) if there were two consecutive entries or two consecutive exits; or (iii) if the first detection of the day was an exit or last detection was an entry. The errors detected are graphically presented in Figure 2. All of these detections were removed from the data. Hence were generated the daily visit files consisting of date, animal ID, entry and exit time, and visit duration. All daily visit files were merged to a single file for data analysis.

2.6. Statistical analysis

All statistical analysis detailed below was done in R studio V. 4.4.1 (R core team, 2024). The daily visit file initially contained 46,738 observations. After removing erroneous detections which are described in section 2.5, 18,899 valid observations remained. These were used for subsequent analysis and the generation of individual-level data. Normality of data was checked using Shapiro-wilk (Shapiro & Wilk, 1965) and Anderson darling test (Anderson & Darling, 1954) and plotting histograms.

2.6.1. Correlation between daily hen visits and weather parameters

Meteorological data for Tours was sourced from the French public data platform https://www.data.gouv.fr/. As hens visits did not meet the assumptions of normality, non-parametric Spearman's rank correlation coefficients (Spearman, 1961) were computed to evaluate the strength and direction of monotonic associations between climatic factors and the number of outdoor visits by hens.

2.6.2. Individual hen profiling and clustering based on ranging variables

Six ranging behavior traits for each individual hen were derived from daily visit files. These traits were: the number of days spent outside, the total number of visits, the total duration outside, the average duration outside per day, the average number of visits per day, and the average duration per visit. A Principal component analysis (PCA) was performed using the *FactoMineR* package (Husson et al., 2020) to identify the main axes of variation in ranging behavior among hens.

Hens were grouped based on the total number of days they were detected outside and the time of day of their activity. For the first grouping that was based on ranging frequency, hens were categorized into three groups—low, moderate, and high visitors—based on the first and third quartiles of the number of days spent outside. For the second grouping based on chronotype, a morning-to-evening visit ratio was calculated by dividing the total number of morning visits (04:00–13:59) by the total number of evening visits (14:00–23:59) for each hen. Hens with a ratio ≥ 1.5 were classified as morning hens, those with a ratio ≤ 0.5 as evening hens, and those with ratios between 0.5 and 1.5 as neutral. A Kruskal-Wallis test (Kruskal & and Wallis, 1952) was conducted to assess significant differences among the three groups for the six behavioral variables.

2.6.3. Association analysis of ranging behavior with vaccine response and physiological stress

For the NDV, IBV, and APV vaccines, the correlation between humoral vaccine response and six behavioral variables was assessed for each week (5, 8, 10, 12, 14, 18, and 22) using *Spearman* correlation. For AEV, the vaccine response at week 22 was evaluated in relation to behavioral traits. A chi-square test of independence was conducted to assess the association between vaccine response status (positive or negative) and the different hen group categories. Additionally, the correlation between NDV specific cell-mediated immune response at week 22 and behavioral traits was also examined. Phenotyping of chicken blood cells was done by flow cytometry. Associations between heterophil-to-lymphocyte (H/L) ratio and ranging variables were calculated by the Spearman correlation method (Spearman, 1961). A chi-square test of independence (Pearson,1900) was conducted to assess the association between high or low H/L ratio (categorized based on median value) and the different hen group categories.

2.6.4. Genetic determinism of ranging traits

To assess the genetic contribution to variation in these traits, heritability analyses were conducted using the *BGLR* (Bayesian Generalized Linear Regression) package in R (Paulino & Campos, 2014). We first computed the genomic relationship matrix (GRM) using SNP genotype data. The model applied was mixed linear model to estimate the additive genetic variance and residual variance for each phenotype:

$$Y = \mu + Xb + Za + e$$

Where Y represents the vector of phenotypic observations (including both behavioral and immune response traits), μ is the overall mean, X is the incidence matrix for fixed effects, and b is the vector of fixed effects such as batch or environmental factors. The matrix Z relates observations to the random additive genetic effects denoted by a, while e represents the vector of residual errors.

The random effects were assumed to follow a normal distribution such that

$$a \sim N(0, G \ \sigma_a^2)$$

$$e \sim N(0, I\sigma_a^2)$$

G is the genomic relationship matrix, σ_a^2 is additive genetic variance, I is identity matrix, σ_e^2 is residual variance.

BGLR package fits a genomic prediction model with a reproducing kernel Hilbert space (RKHS) approach. Markov Chain Monte Carlo (MCMC) sampling was run for 700,000 iterations. After discarding 140,000 iterations as burn-in and posterior estimates were calculated as the mean of the remaining samples. The results of the analysis were reported as posterior means and standard deviations for each parameter of interest. Similar analyses were done in the case of categorical traits by changing response_type to “ordinal” instead or “gaussian”. The heritability was computed using additive genetic variance and residual variance based on using the following formula.

$$h^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_e^2}$$

Genetic correlations between traits were estimated by pairwise comparison between two traits. between two traits, divided by the square root of the product of their respective genetic variances, thereby quantifying the extent to which genetic effects are shared between traits. Genetic correlations between ranging variables and vaccine response and inter-correlation between ranging variables were also calculated using BGLR.

Genome-wide association studies (GWAS) were conducted using the *GCTA* software (version 1.94.1) (Yang et al., 2011) to investigate genetic associations with behavioral traits. Among the continuous traits evaluated, only the average duration outside per day exhibited a normal distribution consistent with a bell-shaped curve. Therefore, this trait was analyzed using the GCTA-mlma (mixed linear model-based association) method, as well as the GCTA-loco (leave-one-chromosome-out) approach. For binary traits, GWAS was performed using the fastGWA-GLMM module within GCTA, which implements a generalized linear mixed model (GLMM). Significance thresholds for SNP associations were adjusted using the False Discovery Rate (FDR) approach based on the Benjamini-Hochberg procedure (Benjamini, & Hochberg, 1995) . To ensure a balance between statistical rigor and the ability to detect potential signals, FDR-adjusted p-values at both 0.05 and 0.10 significance levels were evaluated.

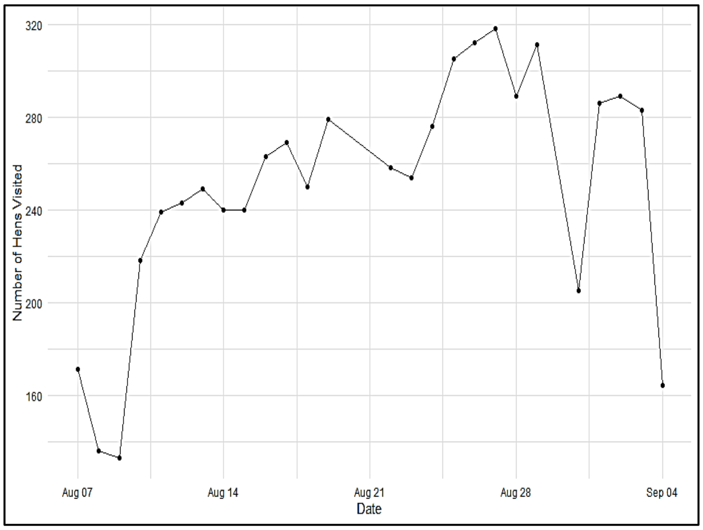

Figure 3: Graphical representation of the number of hens visiting outdoor each day from 07-08-2023 to 04-09-2023. 20, 21 and 31 august are removed due to interruptions in data recording.

Table 2: Grouping of hens on the basis of ranging intensity

Number of days	Number of hens	Visiting group
< 12	88	Less
12 - 22	203	Moderate
>22	106	High

Table 3: Grouping of hens on the basis of chronotype

Duration	Number of hens
Morning	96
Evening	66
Neutral	235

Table 1: Description and statistics of all ranging variables measured from daily visit files

Quantitative ranging variables	Definition	Mean	Standard deviation	Unit
Total duration outside	Total duration spent outside by hen in total 26 days	2880.71	1482.392	Minutes
Average duration per day	Total duration outside divided by number of days outside for a hen	172.71	55.04043	Minutes
Total visits	Total number of visits done by hen in total 26 days	48	33.32496	Number of visits
Average visits per day	Total visits divided by days outside	3	1.144536	Number of visits
Average visit duration	Total duration outside divided by total visits for a hen	71.790	34.74113	Minutes
Days outside	Total days in which hen was detected outside at least once.	16	6.459026	Number of days

Results

3.1. Correlation Between Ranging Behavior and Weather Conditions

Average temperature outside was 20.99°C (minimum: 5°C; maximum: 40.99°C). Weather indexes and number of visiting hens per day are shown in figure 4. Weather indexes show diverse associations with the number of visiting hens per day (figure 5). All correlations were statistically significant. Temperature has a weak negative correlation, while rainfall, humidity, and wind force have weak to moderate positive correlation with the number of visiting hens. Cloud cover did not have any correlation.

3.2. Descriptive Statistics of Ranging Variables.

Daily visits of hens from 07-08-2023 to 04-09-2023 are shown in figure 3. The daily number of hens ranges from the highest, 318 on 07-08-2023, to the lowest, 133 on 09-08-2023. There are unusually fewer hens on the 9th and 31st of August and the 4th of September.

Descriptive statistics for quantitative ranging variables are presented in the table 1. The statistics are averaged for 397 hens over period of 26 days. Over the observation period, hens spent an average of 2880.71 minutes (SD = 1482.39) outside, with a daily average duration of 172.71 minutes (SD = 55.04). The total number of visits averaged 47.6 (SD = 33.32), corresponding to approximately 2.69 visits per day (SD = 1.14). The average duration per visit was 71.79 minutes (SD = 34.74). Hens spent outside an average of 16.32 days (SD = 6.46) during the study period.

3.3. Individual Profiling and Behavioral Typologies

For grouping based on ranging frequency, hens with fewer than 12 days were classified as the low visit group (n = 88), those with 12 to 22 days formed the moderate group (n = 203), and those with more than 22 days constituted the high visit group (n = 106). Second, hens were classified by their temporal preference of outdoor activity, reflecting their chronotype. Based on peak activity times, 96 hens were most active in the morning, 66 preferred the evening, and the remaining 235 showed no clear preference, labeled as neutral. Number of hens in each group are represented in table 2 & 3. These groupings were later used to explore associations with immune traits and behavioral phenotypes.

3.4. Principal Component Analysis

Principal component analysis revealed the first two components accounted for 59 % and 27.5 % of total variance. Together they account for 86.5% of the cumulative variance, which is a substantial proportion of total variance. This indicates that significant variability of data can be represented in a two-dimensional space. PCA- variable plot (figure 6-A) shows all six ranging variables are well represented on either dimension 1 or 2. Total visits, average visits per day and total days outside are well represented and oriented towards dimension 1, while being closely aligned to each other. Average visit duration and average duration outside are more oriented towards dimension 2 than

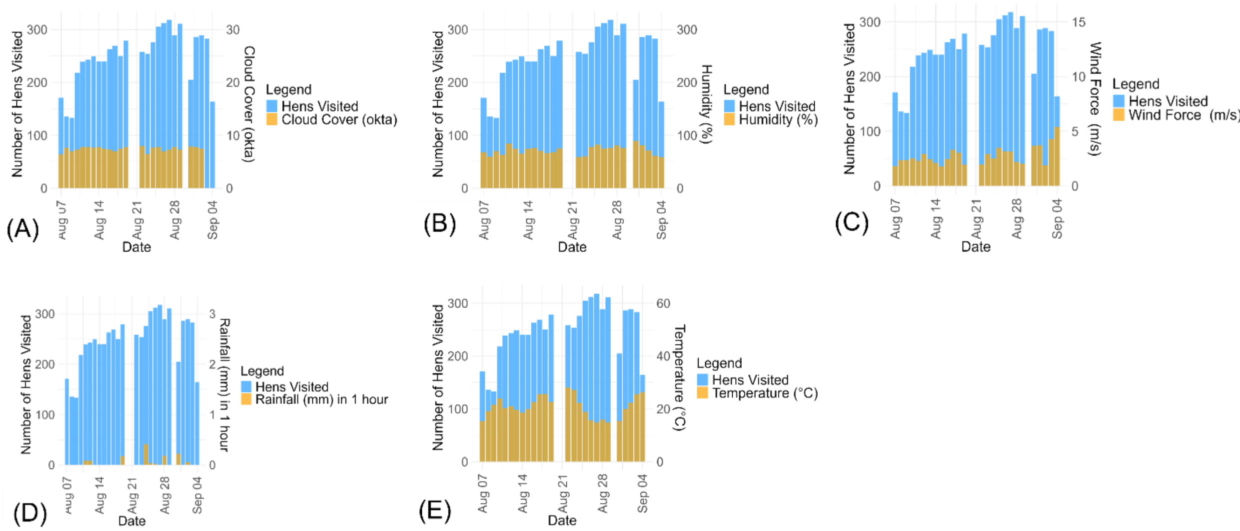

Figure 4: Grouped bar charts comparing number of visiting hens with weather metrics across 26 days.

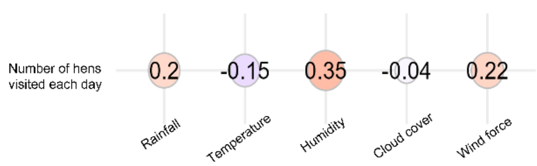

Figure 5: Correlation between number of visiting hens and weather metrics.

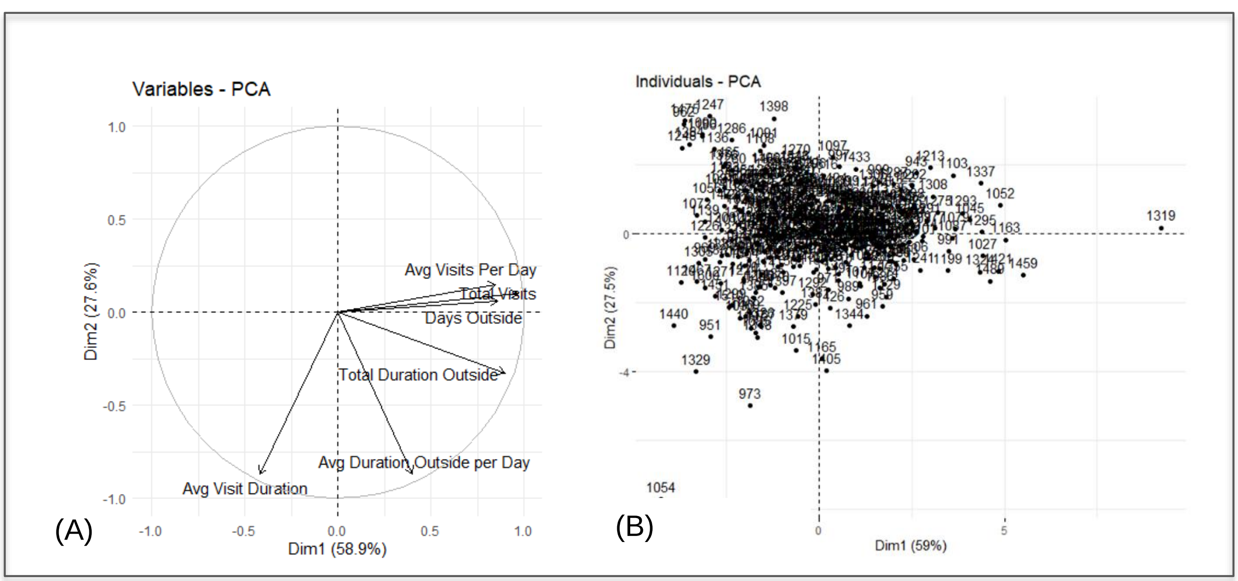

Figure 6: PCA presenting the orientation and representation of (a) ranging variables and (b) individuals along axis 1 and 2.

Dim 1. Total Minutes Outside shares a similar directional alignment with Average Minutes Outside, although with a slightly stronger association with Dim1 than Dim2. Individual PCA plot presented as figure 6-B, shows that majority of hens are located within a dense central region, forming a main cluster that reflects broadly similar behavioral profiles.

However, within this main cluster, there is a noticeable scattered pattern of individuals indicating subtle variability among hens. Additionally, a Few hens, such as individuals 1054 and 1319, are clearly separated from the central cloud, representing behavioral outliers with markedly distinct profiles.

3.5. Inter-correlation of ranging variables

All correlation coefficients are presented in Figure 7(A&B). Total duration outside, total number of visits, average visits per day, and number of days spent outside exhibit strong positive correlations with one another, with correlation coefficients ranging from 0.63 to 0.92. In contrast, average duration outside per day shows weak to moderate correlations with the other variables, with coefficients ranging from 0.19 to 0.62. Average visit duration demonstrates a moderate positive correlation ($r = 0.46$) with average duration outside per day, and moderate negative correlations with average visits per day, number of days outside, and total visits, with coefficients ranging from -0.37 to -0.59.

3.6. Immunological Response and Behavior

3.6.1. Association Between Vaccine Antibody Titers and Ranging

All correlation results between vaccine responses and ranging behavior traits are presented in Figure 8 & 9-A. For Newcastle Disease Virus (NDV), weak correlations were found for days spent outside and total number of visits at weeks 14, 16, and 22, and for average visits per day at weeks 16 and 18. Average visit duration exhibited weak negative correlations with NDV response at weeks 10, 14, 16, and 22. No significant associations were identified between Infectious Bronchitis Virus (IBV) vaccine response levels and ranging behavior traits, except for weak correlations with average visit duration and average visits per day at week 8, and with total visits at week 22. A weak positive correlation was observed between Avian Encephalomyelitis Virus (AEV) vaccine response at week 22 and average visits per day. For Avian Pneumovirus (APV), a few weak positive correlations were detected during the earlier weeks (12 and 14), but correlations were negligible or absent in the later weeks. Regarding NDV-specific cell-mediated immune responses, weak positive correlations were observed between days outside, total duration outside, and total visits with both net activity and spot-forming unit (SFU) levels. In contrast, average visit duration showed weak negative correlations with these immune parameters. Average visits per day exhibited a positive correlation with net activity only.

Chi square test of independence did not show any significant association of vaccine response status (positive / negative) with ranging frequency categories (low/moderate/high) as well as with Hen chronotypes (morning/evening/neutral).

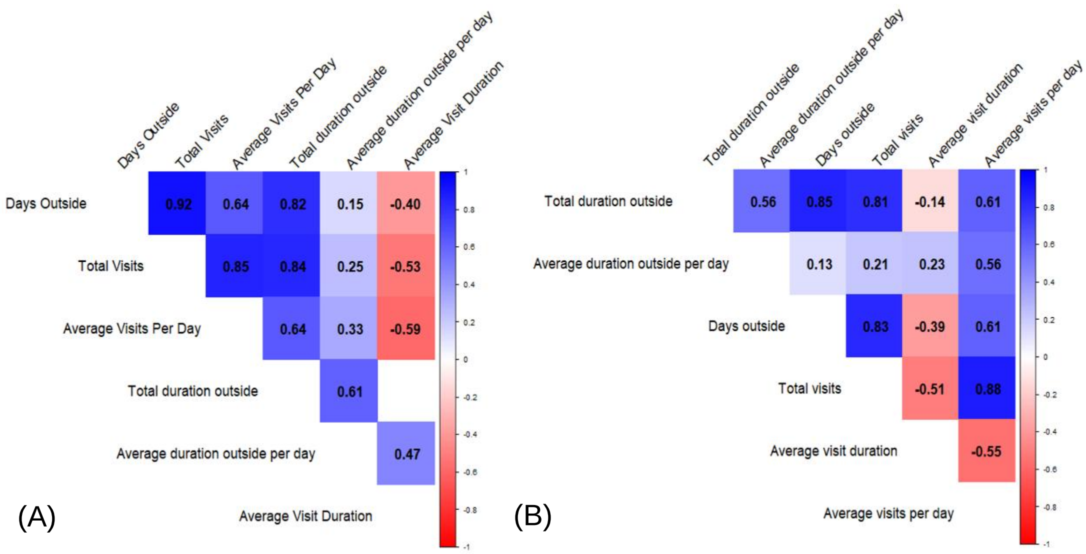

Figure 7: Intercorrelation of ranging variables (A) phenotypic correlations (B) genetic correlations

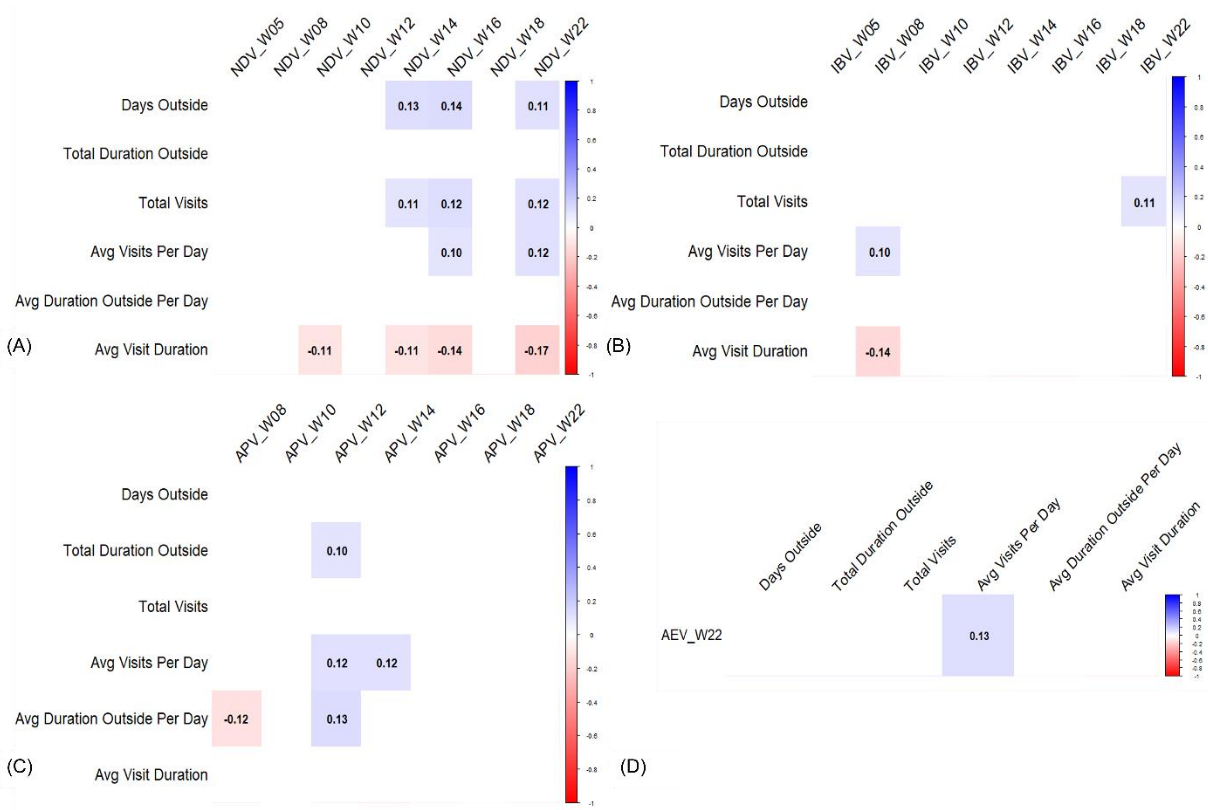

Figure 8: Correlation between humoral vaccine response (A) NDV; (B) IBV; (C) APV; (d) AEV week wise and ranging variables. All correlation given are significant which left out spaces show no significant correlation

3.6.2. Association Between Heterophil/Lymphocyte Ratio and Ranging Traits

Among the ranging traits, days outside and total number of visits outside demonstrated statistically significant positive correlations with the H/L ratio. Specifically, the number of days a hen spent outside showed a weak positive correlation ($r = 0.117$, $p = 0.034$). Likewise, total visits outside exhibited a weak positive correlation ($r = 0.133$, $p = 0.016$), while other ranging behavior measures, including total minutes outside, average minutes outside per day, average visit duration, and average visits per day, did not show statistically significant associations with the H/L ratio (all $p > 0.05$). Correlation between heterophil lymphocytes ratio and ranging variable is presented in figure 9-B. Chi square test of independence did not show any significant association of vaccine response status (positive/negative) with ranging frequency categories (low/moderate/high) as well as with hen chronotypes (morning/evening/neutral). However, figure 10 indicate that high visiting hens have high H/L ratio as compared to low visiting hens.

3.7. Heritability of ranging behavior

Heritability for quantitative ranging traits came out in the moderate range (0.24-0.32) with a statistically significant p-value. Total duration outside ($h^2 = 0.30$; SD = 0.06), Average duration outside ($h^2 = 0.27$; SD=0.06), Days outside ($h^2 = 0.32$; SD = 0.07), total visits ($h^2 = 0.26$; SD = 0.05), average visits duration ($h^2 = 0.32$; SD = 0.07) and average visits per day ($h^2 = 0.24$; SD = 0.05). For categorical traits, ranging frequency ($h^2 = 0.33$; SD = 0.07) and chronotype ($h^2 = 0.27$; SD = 0.06) have moderate range or heritability value. All these statistics are significant. Heritability statistics along with genetic and residual variance and p-value are presented in Table 4 & 5.

3.8. Genetic Correlations

3.8.1. Intercorrelation of ranging variables.

Genetic inter-correlation results of ranging variables strongly align with their phenotypic correlations mentioned before. Total duration outside, total number of visits, average visits per day, and number of days spent outside exhibit moderate to strong positive correlations with one another, with correlation coefficients ranging from 0.54 to 0.88. In contrast, average duration outside per day shows weak to moderate correlations with the other variables, with coefficients ranging from 0.13 to 0.56. Average visit duration demonstrates a weak positive correlation ($r = 0.23$) with average duration outside per day, and moderate negative correlations with average visits per day, number of days outside, and total visits, with coefficients ranging from -0.14 to -0.55. All correlation coefficients are presented in Figure 6-b.

No significant genetic correlation was found between ranging behavior traits and vaccine response for IBV, APV and AEV vaccine. Only for NDV week-22, average visit duration shows negative correlation (-0.33; $p = 0.02$). Overall results show that vaccine response does not have any significant genetic correlation with ranging behavior.

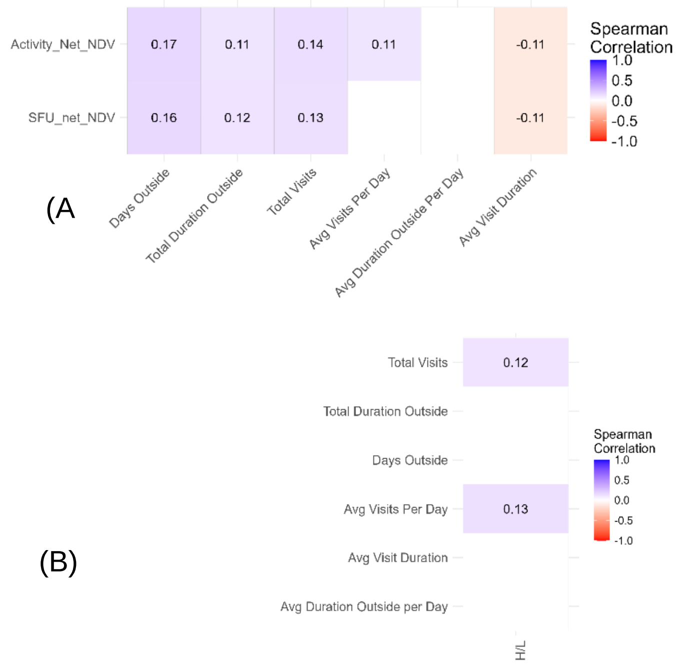

Figure 9: Correlation between ranging variables and NDV specific cell mediated response

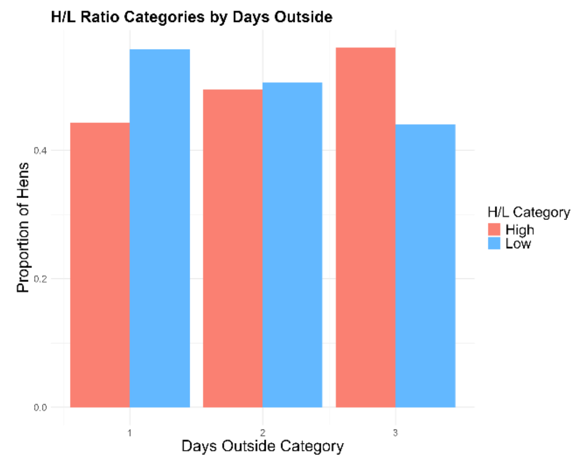

Figure 10: Proportion of hens with high (red) and low (blue) H/L ratio in each category of ranging frequency group.

Table 4: Heritability of quantitative ranging variables

Traits	Genetic variance	Residual variance	Heritability	Standard deviation	p-value
Total duration outside	712574.7	1605270	0.307	0.069	4.88e-06
Average duration outside per day	875.4276	2342.491	0.272	0.062	6.00e-06
Days outside	14.22389	29.31563	0.326	0.070	1.92e-06
Total visits	305.2102	868.5301	0.260	0.057	3.51e-06
Average visit duration	408.943	845.8281	0.325	0.071	2.92e-06
Average visits per day	0.3395536	1.046924	0.244	0.055	4.70e-06

Table 5: Heritability of categorical ranging variables

Traits	Genetic variance	Residual variance	Heritability	Standard deviation	p-value
Ranging frequency	0.508	1	0.337	0.076	5.448e-06
Chronotype	0.379	1	0.274	0.062	5.591e-06

3.9. Genome wide association studies

Genome-wide association studies (GWAS) were conducted using the GCTA software for two binary traits and one continuous trait. No genetic variants reached genome-wide significance for any of the three traits. For the continuous trait, average duration spent outdoors per day, a peak of variant association was observed on chromosome 1; however, it did not surpass the significance threshold. A similar pattern was noted for the binary trait chronotype, with a peak on chromosome 4 also falling below the threshold. For the trait ranging frequency, no distinct peaks were observed, even below the threshold level. Manhattan plots illustrating the GWAS results for all three traits are presented in Figure 11 (A-C).

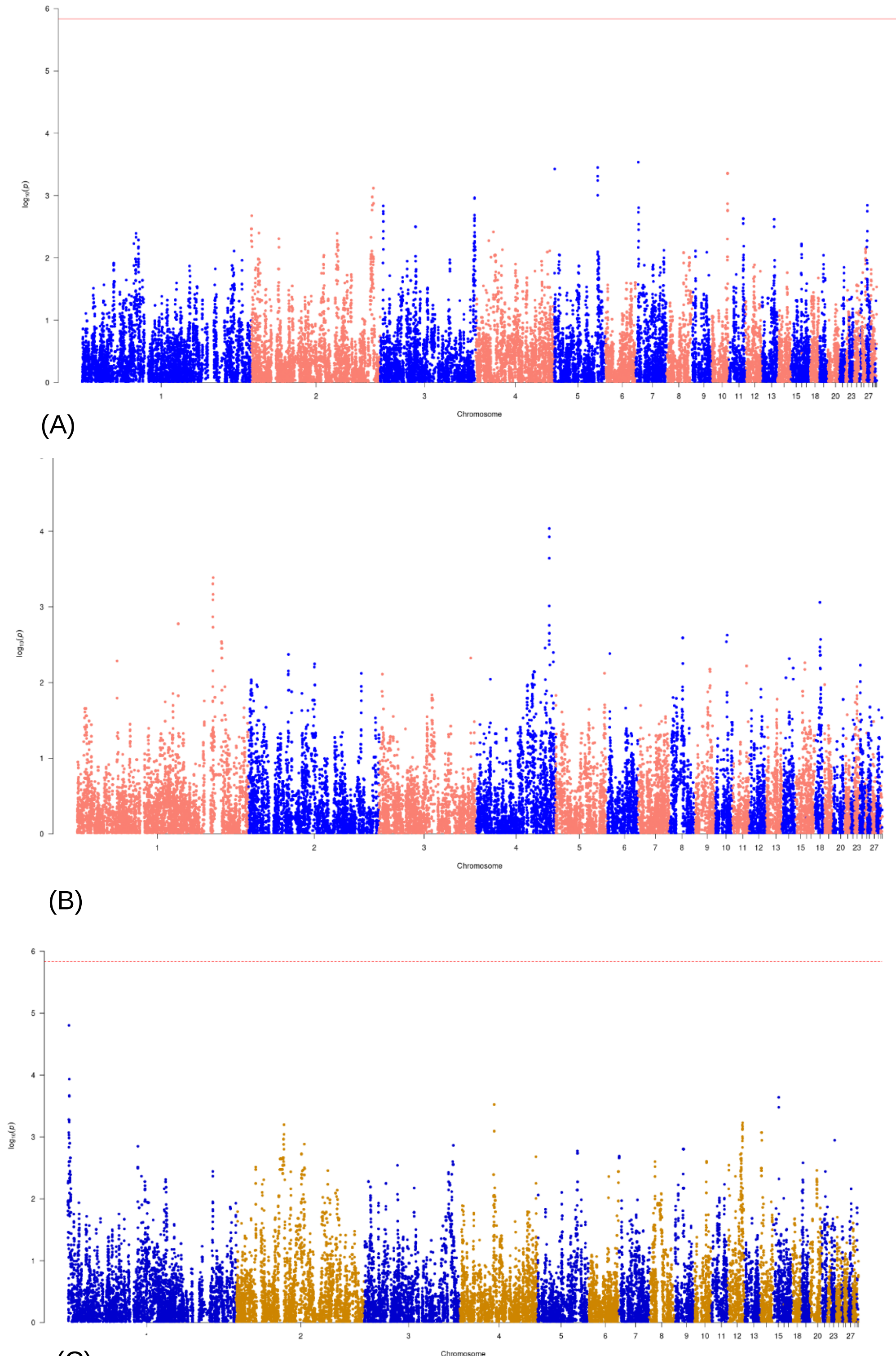

Figure 11: Manhattan plot showing -log10(p-value) of genetic variants across chromosomes, used to identify significant associations in a genome-wide association study (GWAS) for binary traits (A) ranging frequency; (B) chronotype and (C) continuous trait average duration outside per day. Each point represents a SNP, colored by chromosome.

**Red line shows threshold of significance calculated by Benjamini-Hochberg procedure*

Discussion

Despite increasing interest in the welfare and productivity of free-range poultry systems, the drivers of individual ranging behavior remain largely unexplored. This study explored the ranging behavior of white Leghorn hens and investigated potential genetic influences on outdoor access, alongside determining immune parameter correlations with individual behavior. We found significant variability in the ranging behavior in the population of 397 hens over 26 days and moderate genetic influence on it; however, association with immune response is found to be minimal.

The experimental setup, which included the tunnel-like hatches with RFID antennas and physical modifications such as the zigzag path, lowered ceiling, and regular cleaning of antennas was crucial in directing hens to pass accurately over the detectors, minimizing missed or false detections. However, interruptions in data recording on specific days, as well as the exclusion of erroneous detections led to a significant reduction in the number of valid observations. It certainly enhanced data accuracy and reliability; it may have left some behaviors unobserved, potentially biasing activity patterns. Moreover, the physical design of the tunnels, while necessary for detection accuracy, could have influenced natural hen behavior by slightly constraining their movement or altering their willingness to use the outdoor area. These factors must be considered during interpretation of results.

4.1 Ranging behavior patterns

Hens exhibited substantial individual variations in accessing the outdoors, as reflected by wide standard deviations of almost all quantitative ranging variables presented in table 1. This behavioral distinction is also evident from 106 hens having a very high number of days (>22) outside (table 2), making them 27 % of the population. It indicates that certain hens are consistently better adapted or motivated to outdoor environments. Campbell et al., (2016) also categorize ranging hens in three groups based on low, moderate, and high range-preferring hens and show that fear and copying styles affects their range-accessing ability. Fear, as an internal emotional state, promotes behaviors like avoidance or hesitation, while coping style reflects the tendency to adapt to stress. Similarly, 162 out of 397 hens (40.8%) are marking most of their visits at a specific time duration of the day. This could indicate the specificity of circadian rhythms at the individual level as it is demonstrated previously in case of feather pecking (Bessei et al., 2023) and egg laying behavior (Becot et al., 2021). Figure 3 illustrates a gradual increase in the number of hens visiting the range each day over time, indicating their habituation and growing familiarity with the outdoor environment. Similarly, Gilani et al., (2014) observed a rising percentage of hens utilizing the range as time progressed. It was estimated that on average 62.7 % of hens (249/397) visit the outdoors, which is highly related to the finding of Larsen et al., (2017) in which over 60% of hens in two different flocks are ranging outside on all days.

Principal Component Analysis (PCA) revealed that ranging behavior can be structured into two dimensions : (i) a frequency-based dimension (Dimension 1), characterized by strong and positive correlations between frequency-based metrics (total visits, average visits per day, and days outside); and (ii) a duration-based dimension (Dimension 2), comprising average visit duration and average minutes outside. Average visit duration is negatively correlated with all frequency-based metrics (figure 7) which shows that hens tend to adopt either being frequent visitors or long stayers. This inverse relationship also reflects their difference in exploration and energy expenditures. Long-staying hens may have less sensitivity to environmental changes, while frequent visitors might be highly responsive to external stimuli or have lower confidence. (Newberry et al., 2001) talk about difference in perching behavior of domestic fowl and relates it with balance between predation risk and energy expenditure for an animal. These insights can be helpful in defining management strategies in personalized welfare in free-range systems. By recognizing ranging patterns of hens, long-staying hens may benefit from enriched outer space with more exploring sites, while frequent visitors can be provided with secure areas like shaded verandas or visual barriers to lengthen their visits; however benefits of ranging behavior should by explored by more studies to establish its relationship with animal welfare and health. However, as these behavior measurements are solely based on quantitative measurements without observing what activities hens actually performed during visits limits the interpretation. Moreover, a 26-day observation period is moderate but not too long to conclude the stability of these patterns. (Larsen et al., 2017) and (Rodriguez-Aurrekoetxea & Estevez, 2016) used several months time period and found consistent ranging behavior over time with few hens change their range use in response to familiarity to environment

4.2. Weather-ranging correlation

Weather appeared to have limited influence on visits, as shown in figure 5. Among the weather metrics assessed, humidity shows the strongest association with visits ($r = 0.35$) followed by wind force ($r = 0.22$) and rainfall ($r = 0.20$). Inversely, temperature has a weak negative correlation with the number of visiting hens ($r = -0.15$). These findings suggest that high temperature can slightly reduce ranging behavior. Figure 4 also supports this assumption, particularly as the temperature decrease after August 21 coincides with an increase in the number of visiting hens. As we also assumed before that hens are becoming more familiar with the outdoor area over time. It is therefore likely that both habituation and favorable environmental conditions acted together to promote higher range use during this period." Reluctance of hens to range outdoors during higher temperatures is also evident from studies (Bari et al., 2020; Lara & Rostagno, 2013). As temperatures exceed 25°C, behaviors like panting and wing spreading are displayed frequently, reducing ranging behavior (Wasti et al., 2020; Cartoni Mancinelli et al., 2023). However, as weather data was analyzed on a daily level, short-term fluctuations like sudden rain or wind can affect hen behavior more sharply. In addition, during this study, weather metrics are treated as uniform across the area, but micro variations like the availability of shaded or exposed zones could affect ranging decisions.

4.3. Association of ranging behavior with immune and stress response

The correlation between outdoor access and vaccine response parameters shows a subtle association between both type of traits. Out of all variables, the most consistent findings are the weak positive correlation of average visits per day with NDV (week 16 & 18), APV (week 12 & 14), IBV (week 8), AEV (week 22) and NDV specific cell mediated response (week 22) as illustrated in figure 8 & 9-a. However, it also evident that only NDV vaccine response is showing modest correlations at week 14, 16, and 22 while response for other three vaccines, IBV, APV and AEV is showing very scattered and weak. The consistent negative correlation between average visit duration and NDV immune responses across multiple time points indicates that hens which spend longer time per visit outside may exhibit lower humoral and cell-mediated responses to NDV. Interestingly, Arbona et al., (2011) found that caged hens have a better vaccine response against Newcastle disease (NDV) than free-range hens and interpreted that free range hens might experience significant environmental stressors that suppress their humoral response function.

The H/L ratio is a commonly used indicator of physiological stress in birds. It reflects the balance between heterophils (involved in stress and inflammation) and lymphocytes (associated with adapted immunity and recovery). A higher H/L ratio generally indicates a greater physiologically stressed state of the bird (Lentfer et al., 2015). In this study, a weak positive association between frequency-based ranging variables (days outside, total visits, and average visits per day) and H/L ratio is found (0.11-0.14), as shown in figure 9-b, suggesting that frequency of going outside is related to mild physiological stress or immune activation. This finding is completely aligned with a previous study showing that heterophils are increased in the outdoors in summer, particularly due to heat stress (Sanchez-Casanova et al., 2019; Arbona et al., 2011). However, it might be related to being more active rather than being outside, as we don't know if hens staying indoors were in an active or resting state. Figure 10 also visually supports the above assumption, as it shows that hens in the high visitor category have a higher H/L ratio than less visited hens; however, the statistical difference was not significant between these categories.

4.4. Genetic determinism of ranging behavior

Another aim of the study was to assess the genetic basis of behavior variation in ranging behavior. We found moderate heritability across quantitative and categorical traits. As shown in table 4, heritability estimates for quantitative traits ranged from 0.244 to 0.326, with the highest values seen for days outside ($h^2 = 0.326$) and average visit duration ($h^2 = 0.325$). These findings clearly suggest that ranging behavior has a heritable component and is not completely dependent on environmental factors. Previously, Rodenburg et al., (2003) estimated heritability for feather pecking and open field response, which ranged from 0.01 to 0.12 and 0.20 to 0.49, respectively. Farkas et al., (2022) studied nesting behavior in laying in a similar fashion, associating it with genotypes and egg production while having significant differences between all genotypes and laying of eggs and visiting behavior in nests at various locations. They explain that two different aspects of ranging, as explained by our variables, which are how often and how long hens will range, are also genetically determined partially. Heritability of exploratory behavior was estimated by Dingemanse et al., (2002) in wild tits in a moderate range (0.22). Categorical traits, ranging from frequency and chronotype, are also showing moderate heritability, 0.33 and 0.24 respectively. These estimates suggest that ranging behavior can be targeted in genetic selection programs as a selectable trait however, careful consideration is needed to ensure that increased outdoor activity truly reflects improved animal welfare, rather than being associated with unintended factors such as heightened stress. Further investigation is required to confirm whether selecting for this behavior genuinely benefits the animals' welfare and health.

Genome-wide association study (GWAS) was conducted on two binary traits (outing frequency and chronotype) as well as on one continuous trait (average time spent outdoors per day). They revealed no genetic variants reaching the genomic significance threshold. This suggests that no single polymorphism has a major effect on these behaviors, reinforcing a polygenic architecture in which numerous loci of modest effect collectively contribute to the observed variability. Johnsson et al., (2018) identified 24 QTLs affecting social behavior in chickens, highlighting the polygenic nature of these traits. A genome-wide association study in F2-cross of laying hens concludes that behavioral traits are found to be controlled by numerous genes, and no single SNP showed sufficient association to be considered for selection (Lutz et al., 2017). Although our variants remain below statistical significance, genomic regions of interest can be explored further via larger populations. Overall, these results suggest that ranging behaviors in hens present a high genetic complexity, requiring complementary approaches to identify mechanisms.

Conclusion

This study underscores the individual variability in outdoor access preferences among White Leghorn hens and identifies moderate heritability for these traits, with minimal correlation to immune response and physiological stress indicators. Traits related to outdoor access show subtle associations with heterophil-to-lymphocyte (H/L) ratios and vaccine responses. Heritability estimates confirm that both the frequency and duration of ranging behavior are partially genetically determined, however, genome-wide association study (GWAS) findings did not reveal strong individual genetic markers, instead suggesting a polygenic architecture and highlighting the need for larger-scale studies to identify potential quantitative trait loci (QTLs) linked to ranging behavior. Future research should further explore the relationship between outdoor access and immune function in laying hens using larger cohorts.

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